DEVELOPMENT AND APPLICATION OF A NON-TARGETED SCREENING WORKFLOW FOR CHEMICAL EXPOSURE ASSESSMENT IN HUMAN BIOMONITORING AND RELATED STUDIES

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Doctoral Dissertation
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RAZVOJ IN UPORABA NETARČNE ANALIZE ZA OCENO IZPOSTAVLJENOSTI KEMIKALIJAM V HUMANEM BIOMONITORINGU IN SORODNIH ŠTUDIJAH

Doktorska disertacija

Supervisor: Assoc. Prof. Dr. Tina Kosjek

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Mami, ati, Blaž in Vesna,
hvala za vse.

Abstract

Human exposure to environmental stressors is widespread. Highly dynamic, these chemical, physical and social factors, collectively termed as the exposome, interact with internal factors such as genetics, sex, gut microbiome and general health status, and dictate susceptibility and risk for an onset of diseases.

Human exposure to the chemicals is addressed through human biomonitoring which follows the biomarkers of exposure in various biological specimens and determines the distribution and the levels of these chemicals of concern within studied population. This is typically achieved by targeting preselected chemicals of concern, however by doing so, the exposure assessment is limited only to this narrow number of chemicals. Due to an ever-increasing number of potentially concerning chemicals, a new and more comprehensive approach to complement the traditional ones is required. In this respect, the non-targeted screening aims at identifying all known, suspected and unknown exposures, however the appropriate methodology is yet unavailable.

Within the scope of the dissertation, a novel non-targeted workflow was developed. It included development of the sample preparation procedure, chemical analysis and data processing protocol. The workflow was validated and applied to a cohort of Slovenian children, in which 74 urinary biomarkers of exposure were tentatively identified. Some of the identified biomarkers of exposure were known endocrine disrupting chemicals, so to cross-confirm the results of the non-targeted and suspect screening, a targeted analysis of bisphenols, parabens and triclosan was conducted. The analysis quantified the levels and showed that Slovenian children were widely exposed to these chemicals, however their levels were low and comparable to those determined elsewhere. The analysis also provided insight into determinants of exposure and susceptibility to adverse health effects. Targeted identification of these biomarkers of exposure confirmed and validated the results obtained by non-targeted screening.

We tested the applicability of the non-targeted data processing protocol also on a different setting: biodegradation of cytostatic drug imatinib. We identified 8 transformation products, out of which 6 are new to science and we detected one of them in real wastewater samples. As the results show, non-targeted exposomic analysis can be conducted quickly with data processing currently being the main limiting factor. Nonetheless, it can be efficiently applied to routine analysis and human biomonitoring schemes and other fields of research. The capabilities of such global scale analysis were demonstrated through analysis of the exposure of children, which along with the traditional targeted approach showed that the children were exposed to a large number of chemicals, some of which are restricted from use in the European Union. As the toxicity of chemical mixtures can be enhanced due to their synergistic action, the exposure is concerning and can contribute to the development of chronic diseases later in life. With the development of non-targeted screening in human biomonitoring, the capabilities of the approach will be extended in the future and the coverage of chemicals will be even further increased. This will lead to efficient and fast detection of newly emerging chemicals and with this, to reducing the health risks associated with the exposures as they appear.

Povzetek

Ljudje smo izpostavljeni velikemu številu okoljskih dejavnikov. Ti kemijski, fizikalni in socialni dejavniki so dinamični in lahko sodelujejo z notranjimi faktorji, kot so na primer genetska zasnova, črevesni mikrobiom in splošno zdravstveno stanje. Odnosi med temi dejavniki vplivajo na dovzetnost in tveganje za razvoj bolezni.

Izpostavljenost kemikalijam v preiskovani populaciji lahko spremljamo skozi koncept humanega biomonitoringa. Ta poteka preko sledenja koncentracijam in porazdelitvi vnaprej izbranih biomarkerjev izpostavljenosti. Čeprav ta način ponuja dober vpogled v izpostavljenost, pa omejuje tarčno sledenje samo na izbrane kemikalije. Netarčna analiza na drugi strani omogoča identifikacijo znanih, pričakovanih in nepoznanih kemikalij. Četudi tak pristop predstavlja veliko prednost, pa za ta namen še ni ustrezne metodološke podpore.

V sklopu doktorske disertacije smo razvili netarčni protokol za spremljanje izpostavljenosti kemikalijam, ki vključuje pripravo vzorca, kemijsko analizo in obdelavo podatkov. Protokol smo validirali in uporabili za analizo izpostavljenosti skupine slovenskih otrok, pri čemer smo identificirali 74 biomarkerjev izpostavljenosti v urinu otrok. Nekateri od biomarkerjev so bili iz skupin endokrinih motilcev, bisfenolov, parabenov in smo jih, skupaj s triklosanom, v urinu določili tudi s tarčno analizo. Ugotovili smo, da so slovenski otroci izpostavljeni velikemu številu endokrinih motilcev, ampak so ti najdeni v nizkih koncentracijah, ki so primerljive s podatki iz drugih držav. Hkratna določitev biomarkerjev z netarčno in tarčno analizo je služila za navzkrižno potrditev ter validacijo razvitega netarčnega protokola. Učinkovitost slednjega smo testirali tudi v raziskavi na drugem znanstvenem področju. Raziskovali smo biorazgradnjo citostatika imatiniba in pri tem identificirali osem transformacijskih produktov, od katerih jih je šest doslej še nepoznanih. Enega od teh smo določili tudi v realnem vzorcu odpadne vode.

Kot kažejo rezultati, sta v sklopu netarčne analize priprava vzorca in instrumentalna analiza hitra in učinkovita postopka, medtem ko sta glavna omejujoča faktorja računalniško zahtevna obdelava podatkov in identifikacija spojin. Kljub temu rezultati kažejo velik potencial za uporabo netarčnega pristopa v rutinskih analizah, tako v humanem biomonitoringu kot na drugih področjih. Zmogljivosti takšnega analiznega pristopa dokazuje uspešno izvedena netarčna analiza izpostavljenosti otrok. Rezultati le-te kažejo na hkratno izpostavljenost velikemu številu kemikalij, od teh so tri v Evropski uniji celo prepovedane. Izpostavljenost populacije zmesem kemikalij je zaradi potencialnih hkratnih učinkov zmesi, ki lahko prispevajo k razvoju kroničnih obolenj, čedalje bolj zaskrbljujoča. S hitrim razvojem na področju netarčnih analiznih pristopov se bodo v prihodnosti sposobnost obdelave podatkov in identifikacije kemikalij še bistveno povečale, kar bo omogočilo hitrejše določanje novih kemikalij ter s tem tudi bolj učinkovito zmanjševanje tveganja za razvoj obolenj, ki so posledica izpostavljenosti.

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Abbreviations

ADBI . . . Musk ambrette
APCI . . . Atmospheric pressure chemical ionization
APPI . . . Atmospheric pressure photoionization
BFR . . . Brominated flame retardants
BoE . . . Biomarker of exposure
BP3 . . . Benzophenone 3
BP8 . . . Benzophenone 8
BPA . . . Bisphenol A
BPF . . . Bisphenol F
BPS . . . Bisphenol S
BTH . . . Benzothiazole
BTR . . . Benzotriazole
BuP . . . Butyl paraben
BzP . . . Benzyl paraben
CCS . . . Collision cross section
CI . . . Capillary electrophoresis
CY . . . Cypermethrin
CYP . . . Cytochrome P450
DAP . . . Dialkyl phosphate
DBP . . . Dibutyl phthalate
DDA . . . data-dependent acquisition
DDE . . . Dichlorodiphenyldichloroethylene
DDT . . . Dichlorodiphenyltrichloroethane
DEHA . . . Di-(2-ethylhexyl)adipate
DEHP . . . Diethylhexyl phthalate
DEHTP . . . Di-(2-ethylhexyl)terepthtalate
DEP . . . Diethyl phthalate
DIA . . . Data-independent acquisition
DINCH . . . 1.2-cyclohexane dicarboxylic acid diisononyl ester
DiNP . . . Diisononyl phthalate
EC . . . European Commission
EEA . . . European Environmental Agency
EI . . . Electron impact

ESI . . . Electrospray ionization
EtP . . . Ethyl paraben
EU . . . European Union
EWAS . . . Environment-wide Association Studies
FA . . . Fluorene
FAB . . . Fast atom bombardment
FT-ICR . . . Fourier-Transform ion cyclotron resonance
GC . . . Gas chromatography
HBM . . . Human biomonitoring
HEALS . . . Health and Environment-wide Associations based on Large-Population Surveys
HELIX . . . Human Early-Life Exposome Project
HILIC . . . Hydrophilic interaction chromatography
HP . . . 1-hydroxypyrene
HPLC . . . High-performance liquid chromatography
HRMS . . . High-resolution mass spectrometry
IMS . . . Ion-mobility spectrometry
LLE . . . Liquid-liquid extraction
LTQ-Orbitrap . . . Linear ion-trap/orbitrap
MBC . . . 4-methylbenzylidene camphor
MCI . . . Methylchloroisothiazolinone
MDI . . . Methylene diphenyl diisocyanate
MeP . . . Methyl paraben
MI . . . Methylisothiazolinone
MK . . . Musk ketone
MS . . . Mass spectrometry
MX . . . Musk xylene
NDI . . . Naphthalene diisocyanate
NEP . . . *N*-ethylpyrrolidone
NMP . . . *N*-methylpyrrolidone
NMR . . . Nuclear magnetic resonance
NP . . . Napthalene
NT . . . Non-targeted
OCP . . . Organochlorine pesticide
PAH . . . Polycyclic aromatic hydrocarbons
PBDE . . . Polybrominated diphenyl ethers
PCB . . . Perchlorinated biphenyl
PCDD . . . Dibenzo-p-dioxins
PCDFs . . . Polychlorinated dibenzofurans
PER . . . Permethrin
PFC . . . Perfluorinated chemicals

PFOA . . . Perfluorooctanoic acid
PFOS . . . Perflouorooctyl suphate
PH . . . Phenanthrene
PYR . . . Pyrethroids
QC . . . Quality control
Q-Orbitrap . . . Quadrupole-Orbitrap
Q-TOF . . . Quadrupole time-of-flight
REACH . . . Regulation concerning the Registration, Evaluation, Authorization and Restriction of Chemicals
SPE . . . Solid-phase extraction
SS . . . Suspect screening
TCC . . . Triclocarabam
TCS . . . Triclosan
TDI . . . Toluene diisocyanate
TEHTM . . . Tri-(2-ethylhexyl)trimellitate
TOF . . . Time of flight
UDP . . . Uridine diphosphate
UGT . . . Uridine diphosphate glucuronosyltransferase

Chapter 1

Introduction

1.1 Human Biomonitoring in Pursuit of the Chemical Exposome

Human population is exposed to a large number of man-made products and environmental chemicals which occur due to human activity. The chemicals are present in our food, drink, air and water. To describe the chemical space to which humans are exposed, the term **exposome** has emerged [1]. The concept of exposome was developed to emphasize complete environmental exposure of human population. It complements genetic and epigenetic factors and provides comprehensive assessment of lifelong exposure and assesses its contribution to disease risk [2][3]. The exposome includes two main domains: internal and external, where external is subdivided into specific and general external exposome (Figure 1). Internal exposome is individual and includes factors such as age, microbiome and physiology. Specific external factors include environmental exposure, occupational exposure and diet along with physical and biological exposure [4][5]. General external factors are broader and include home location, socioeconomic status and education level. All of the domains are interconnected and particular exposure factors are difficult to assign to only one domain [6][7].

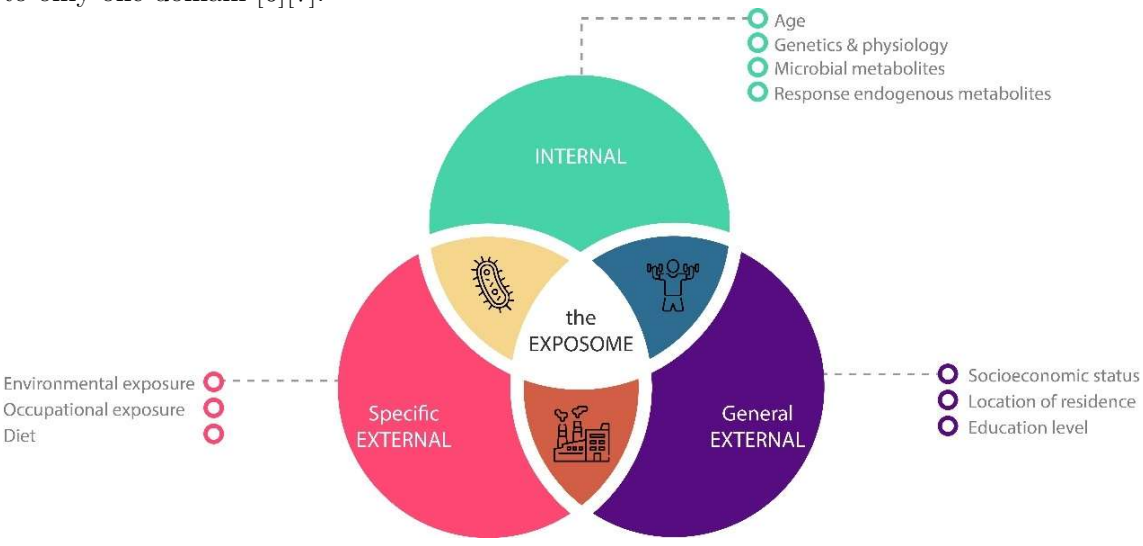

Figure 1: Domains of the exposome are highly interlinked.

The exposome includes non-chemical stressors, such as noise, stress, diet and socioeconomic status, along with chemical stressors. Chemical stressors constitute the **chemical exposome** and include all the environmental chemicals individuals are exposed to [8], and touch all three domains of the exposome. Large variety of environmental chemicals constituting the chemical exposome exhibit biological effects, which can lead to changes and disruption in certain biochemical processes in human body, and with that lead to an onset of a disease. Toxicological effects of chemicals are usually assessed acutely and in high doses. However, the exposure to environmental chemicals is constant and in low levels, therefore the question of the low-level chronic exposure effects has to be answered and furthermore, the synergistic effect or activity of the mixtures of environmental chemicals is still largely unknown and therefore highly concerning.

The exposome is highly dynamic, it changes with time, age and location [9]. However, several critical life stages have been identified, such as infancy, when *in-utero* and early age exposure can have significant effect on individual's future health. Elderly people, on the other hand, are subjected to larger internal doses of persistent and bioaccumulative chemicals, along with having outworn cell repair mechanisms. In adulthood there is a higher chance of occupational exposure. Along with that, previous exposures can influence the effects of new or present exposures and change an individual's susceptibility for adverse health effects [6]. Therefore, it is greatly important to assess the human exposome and monitor the exposure to chemicals [10]. A common strategy for identification and monitoring of biological disturbances in environmental health is to directly target human population with epidemiological studies within human biomonitoring (HBM) [11].

HBM provides aggregated data of the exposures by targeting the chemicals of concern (CECs) and their metabolites in biological matrices [12]. Conceptually, HBM can reflect temporal trends of exposure to environmental compounds as well as measure the efficiency of policy regulations [13]. Within the scope of HBM, concentrations of chemicals in selected matrices are evaluated and compared to reference values, like HBM I and HBM II levels, which are health-related biological exposure limit values [14]. If the concentration of a chemical in the selected matrix is below HBM I value, then there is no health risk assumed. When the level of chemical is between HBM I and HBM II value, the health risk cannot be excluded. If the level of a chemical exceeds HBM II value, then an increased risk of adverse health effects is possible [15]. Currently, several classes of organic and inorganic environmental chemicals are deemed of concern and therefore measured in human samples [16]–[19]. Some chemical classes of organic chemicals of concern are presented in Table 1.

Table 1: Examples of organic CECs.

Category	**Class**	**Examples**
POPs	BFRs	PBDE, BDE-99, HBCDD
	Dioxins	2,3,7,8-TCDD
	Furans	2,3,7,8-TCDF
	OCPs	DDE, DDT, HCB, HCH
	PCBs	PCB 28, PCB 52, PCB 101
	PFAS	PFOA, PFOS
Plastics related chemicals	Bisphenols	BPA, BPF, BPS
	Phthalates	DEHP, DEP, DBP, DiNP
	Phthalate alternatives	DINCH, TEHTM, DEHA, DEHTP

	Diisocyanates	MDI, TDI, NDI, HDI
PCPs	Parabens	MeP, EtP, PrP, BuP, BzP
	Preservatives	TCS, TCC, MCI, MI
	UV-filters	BP8, BP3, MBC
	Fragrances	MX, MK, ADBI, ATII, HHCB, AHTN
VOCs	VOCs	acrylamide, benzene, toluene
Pesticides	OPPs	DAP
	Pyr	CY, PER
Other	PAHs	HP, FA, FE, NP, PH
	Aprotic solvents	NMP, NEP
	BTHs, BTRs	BTR, OHBTR, BTH, OHBTH, SHBTH

1.1.1 HBM in the European Union

HBM concept has been used on a national and international level worldwide. In the EU, the activities to harmonize and include HBM as a monitoring programme for regulatory organs have been in progress.

In 2007, REACH (Regulation concerning the Registration, Evaluation, Authorization and Restriction of Chemicals) was established in the EU to derive information about exposure patterns and time trends on human health and environment by identification of intrinsic properties of chemical substances (Regulation (EC) No 1907/2006 of the European Parliament and of the Council of 18 December 2006 concerning the Registration, Evaluation, Authorization and Restriction of Chemicals (REACH), establishing a European Chemicals Agency, vol. 1907/2006, 2006). It aims to emphasize the responsibility of industry to manage risks connected to produced chemicals and to provide safety information on the substances.

In 2012, European Commission (EC) funded the project COPHES (Consortium to Perform Human Biomonitoring on a European Scale), which developed harmonized protocols for HBM. It aimed to improve the comparability of HBM data to be used on a European scale [20]. In 2013, Health and Environment-wide Associations based on Large-Population Surveys (HEALS) started under the EC 7th Framework Programme (FP7) and ran for 5 years. The objective of HEALS was the refinement of an integrated methodology and application of computational and analytical tools to elucidate human exposome for Environment-Wide Association Studies (EWAS), paving the way of a EU-wide assessment of individual exposure to environmental stressors and predicting health outcomes [21]. In 2016, a new project DEMOCOPHES (Demonstration of as Study to Coordinate and Perform Human Biomonitoring on a European Scale) started. The project aimed to perform the first EU-wide pilot study in order to test harmonized protocols established in COPHES project [20]. Seventeen European countries produced data on the distribution of biomarkers and data related to lifestyle of study populations, which was comparable on the European scale.

After first pilot study with harmonized protocols for HBM had been concluded, in 2015, EC launched the call for European Biomonitoring Initiative, HBM4EU, co-founded under the EU Research and Innovation Programme Horizon 2020 [22]. HBM4EU is a framework to establish patterns of exposure in European population and to establish geographic variation on chemical burden of European citizens. It aims to provide the evidence of actual exposure of citizens to chemicals and their possible health effects and to support policy making. It is a collaboration between 28 EU countries, European Environmental Agency

(EEA) and EC. It has been running since 2017 and will end in 2022. The results of HBM4EU are expected to improve and contribute to EU chemical policies to minimize negative impacts of chemicals on health of European citizens [23][24]. To provide access to HBM data, the Information Platform for Chemical Monitoring (IpCheM) has been developed. It consists of four modules reflecting chemical monitoring categories: environmental monitoring, human biomonitoring, food and feed, product and indoor air. It is a reference point of EC for accessing, searching and retrieving of chemical occurrence data and it has been developed to fill the knowledge gap on Europe's chemical burden [25].

In addition to the abovementioned projects, EU has funded several other projects, like EXPOsOMICS, which aimed for development of new approaches to the assessment of the exposure of humans to high priority environmental pollutants by the means of characterizing the internal and the external exposome [26]. Also, well recognized was the Human Early-Life Exposome Project (HELIX) as a collaboration of 13 European partner institutions, which aimed to characterize exposomes of children, as they progress through life. It was based on 6 birth cohort studies of mothers and children living in Lithuania, Norway, Greece, France, Spain and United Kingdom [6].

Several EU countries, including the Czech Republic, the Flanders region of Belgium, France, Germany, Sweden and Slovenia, have well established HBM programmes on a national level. In Slovenia, HBM is coordinated by the Slovenian Ministry of Health, Chemical Office of the Republic of Slovenia, and is published within Slovenian legislation in Article 51a of the Chemical Act (Official Gazette no. 110/03) and is thus being performed by health and other public institutes [27].

1.1.2 Expansion of the traditional HBM

Traditionally, HBM is performed in a targeted manner by measuring the concentration of preselected targeted chemical or its metabolites (collectively termed as biomarkers of exposure – BoEs) in a certain biological matrix, such as urine or blood. Measurements performed this way act as the main component of exposure assessment and link the exposure to health outcomes *via* following levels and distribution of BoEs within the selected population [28]. Targeted methods are accurate and highly specific for selected BoEs in the selected matrix and provide in-depth assessment of levels of BoEs. However, with an ever-increasing number of environmental chemicals [19], [29], there is an ever-increasing need for the analytical methods including ever-increasing number of BoEs. Based on that, there has been a need to expand current HBM methodology in order to offer wider overview of the human exposome. In contrast to traditional, targeted approach intended for a number of preselected BoEs, the non-targeted (NT) approach theoretically includes all exposures of potential health significance, and can cover exposure from the exogenous sources, such as environmental pollutants, diet and PPCPs and endogenous sources, such as microbiome metabolites and hormonal responses to external stressors. NT approach is agnostic in nature and it is not based on any previous hypotheses; on the contrary, it is a hypothesis-generating approach, and complements traditional targeted HBM approaches by:

- Enabling detection of chemicals not included in targeted HBM and providing data on unexpected exposures;
- Identifying new chemicals with potentially detrimental health effects, which were previously not known;
- Identifying previously unknown chemicals as they occur in the environment and in human samples, rather than that being done retrospectively;
- With detection of not only BoEs but also native metabolites NTS can help to elucidate potential biological responses to exposure.

Within that, the approaches of analysis differ based on the levels of knowledge regarding the investigated analytes. In this respect, suspect screening is based on a list of suspects or compounds of concern, and entries on this list are screened through the analytical data. In the non-targeted screening, no prior suspects are known and the investigator aims to identify compounds of potential significance without any prior knowledge [30].

1.2 Non-Targeted Screening of Human Samples

Non-targeted screening is already firmly established in the field of metabolomics, where the focus are endogenous metabolites, and acts as a basis for analysis of the chemical exposome. Compared to non-targeted metabolomics, the search for BoEs requires a high degree of adjustment to be fit-for-purpose. First, the chemical space of possible BoEs is much larger than the chemical space of endogenous metabolites, as the number of synthesizable chemicals is limited only by their chemistry. Second, BoEs are present in biological matrices in very low levels, often overlapping with high-abundance endogenous metabolites. Third, once xenobiotic chemicals enter the body, they can undergo a number of biotransformations and produce a large number of chemically distinct metabolites that might be already known or yet completely unknown. Due to these challenges, methodology for non-targeted screening of human exposome is currently underdeveloped and lacking [13].

1.2.1 The metabolism of xenobiotics

When introduced to the body, xenobiotics can undergo phase I and II metabolism. Phase I includes oxidation, reduction and hydrolysis reactions. Oxidation and reduction reactions are mediated mainly by cytochrome P450 (CYP) system, while hydrolysis can be a nonspecific side reaction of hydrolase class of enzymes, such as esterase, protease and peptidase. Phase I functionalizes the parent molecule in order to be able to increase its polarity and water solubility in phase II. Phase II involves binding (also termed *conjugation*) of highly polar groups, such as glucuronic acid, sulfuric acid and glutathione to oxygen- and nitrogen-containing functional groups of the parent molecules, which renders it more polar and hence easier to excrete via urine [31]. Conjugation with glucuronic acid, which is the most common conjugate, is mediated by uridine diphosphate (UDP) glucuronosyltransferase (UGT). Non-metabolized parent compounds and all of their metabolites can be followed as BoEs, however in practice, deconjugated compounds are usually followed. To produce conjugate-free compounds, enzymatic hydrolysis with β-glucuronidase/sulfatase is performed. For example, Figure 2 shows metabolic reactions and corresponding enzymes for phase I and phase II metabolism of one of known endocrine disruptors, butyl paraben (butyl 4-hydroxybenzoate).

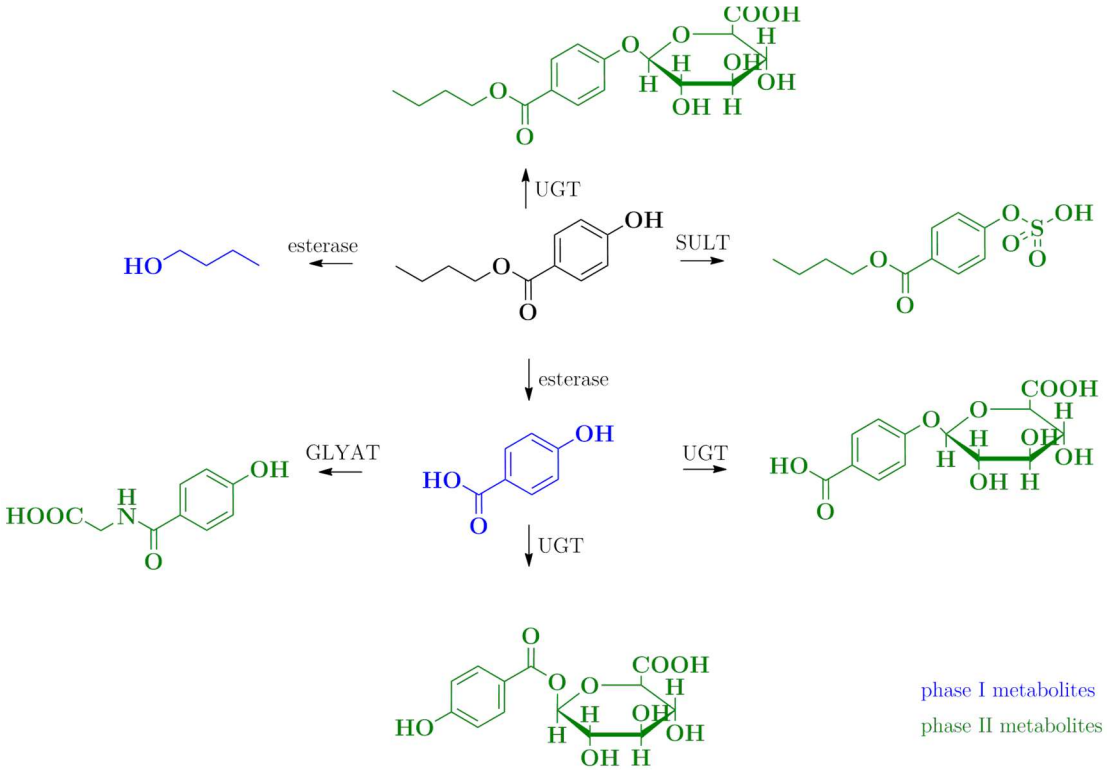

Figure 2: Metabolism of a preservative butyl 4-hydroxybenzoate. Phase I involves hydrolysis by hepatic esterase, while phase II involves glucuronidation, sulfation and glycine conjugation. UGT – uridine triphosphate glucuronosyltransferase, SULT – sulfotransferase, GLYAT – glycine-*N*-acyltransferase [32].

1.2.2 Sample types used in HBM

To determine the environmental burden, levels of BoE can be investigated in different human matrices according to the study question [33]–[35]. The selection of an appropriate matrix is based on the nature of BoE in question (distribution, bioaccumulation, metabolic half-life, elimination pathway) and invasiveness of the sampling. In HBM, blood and urine are the matrices most commonly used [36].

1.2.2.1 Blood

Blood has the advantage that the levels of chemicals are in equilibrium with every organ in the human body and the concentration of biomarkers of exposure (BoE) is not influenced by collected volume, like in urine [36]–[38]. Blood can be used whole or processed to produce either plasma or serum. To obtain plasma, anticoagulant agents are introduced to blood collected by venipuncture, and cells separated by centrifugation. Different anticoagulant agents are used, for example ethylenediamineacetic acid (EDTA) and its salts, citrate and heparin. Each anticoagulant might interfere with analysis of the selected analyte, so these effects must be carefully studied before the analysis. Blood serum is on the other hand prepared by letting the blood to coagulate naturally by exposing it to room temperature for 30 – 60 minutes. In the process, fibrinogen is removed from blood by conversion into a fibrin clot with platelets. Additionally, varying amounts of other proteins are bound in the clot. When the clot is formed, the sample is centrifuged and supernatant collected. For serum, the important variables are storage containers, time of clot removal, centrifugation speed and temperature. The protein content is lower in serum compared to plasma, however

the duration of the clotting process can increase the enzymatic conversion or degradation processes and changes in the concentration of BoEs [34] [39]. Whether either, serum or plasma, is better, is still largely discussed in the scientific community [38]. From a chemical perspective, the limiting of additives that might interfere with the detection of BoEs is desirable. However, as there is a large number of variable factors which are analyte-specific, appropriate sample type must be determined for each analysis separately. Collection of blood-derived samples involves venipuncture and is therefore more invasive than urine collection and is in that view less suitable for specific populations such as infants and children.

1.2.2.2 Urine

Urine is a sample which is readily obtained in large quantities and is non-invasive [40]. The often-higher concentration of parent compounds and metabolites as compared to plasma and serum samples is also an important advantage [41], [42]. Urine is renally filtered and in healthy subjects contains a lower concentration of proteins than blood-derived samples and it is therefore less complex as a sample preparation procedure [43]. The largest proportion of urine is water, and in addition to it, urine also contains a multitude of metabolites, human cells (erythrocytes, leucocytes, urothelial cells, epithelial cells), bacteria, fungi, spermatozoa and non-cellular components (mucous filaments, cylindrical components, urates and inorganic and organic crystals) [44].

Urine samples should be ideally stored at a lowest possible temperature (usually - 80 °C) immediately after collection if not promptly analyzed. Sample alterations can occur due to endogenous or bacterial enzymatic reactions and non-biogenic reactions, such as oxidation.

An important variable which has to be considered prior to analyzing data obtained from urine analysis is variation in urine volume. Fluctuations in urine volume result in dilution of metabolite concentrations [45]. Volume is dependent upon hydration status of the subject. To surpass this problem several normalization methods have been used.

Creatinine normalization is based on independence of creatinine amount on urine volume. Daily excretion of creatinine is constant in healthy individuals and is therefore inversely proportional to urine volume. However, various uncontrollable factors such as age, gender, physical activity, food intake and others can influence its concentration and its validity has been extensively debated and due to considerable drawbacks, it is gradually getting replaced by other normalization methods.

Another commonly used method is normalization to specific gravity (SG), which is less prone to bias connected to creatinine adjustment. To adjust for SG, SG is determined by measurement of urine refractive index. Adjustment to SG proceeds according to Equation 1 [46],

$$c_{adjusted} = c_{measured} \times \frac{SG_s - 1}{SG_i - 1} \quad (1)$$

where $c_{adjusted}$ is the resulting SG adjusted urinary level, $c_{measured}$ is the raw concentration measured in the urine, SG_s is standard SG, which is an average for females and males separately, and SG_i is SG of the sample being adjusted. SG adjustment, however, is not suitable for diseased individuals, as urine density is affected by total mass of the solutes, and when analyzing a urine sample of diseased subjects who excrete larger amounts of certain compounds, such as glucose in diabetes mellitus patients, normalization to specific density can be biased. In addition to creatinine and SG adjustment, osmolality-based

method can be used and it is based upon measuring solute concentration, which is calculated as a difference in melting point or vapor pressure of urine sample and reference. Urine samples can be collected at different time periods. The time of collection largely influences the levels of BoEs in urine, particularly for short-lived chemicals [47]. Spot urine is a type of sample that is collected at a random time during the day and represents a snapshot picture of individual's exposure. The drawback is that the excretion maximum of a compound might be completely missed, leading to assuming no exposure. To avoid this random effect, urine can be collected as a first morning void. Morning void presents exposure from a longer period of time, as urination is scarcer during the night. Best option is 24-h pooled urine, where total volume of urine in 24 hours is collected, therefore the missing of excretion is avoided. However, a 24-h sample is difficult to obtain in large scale monitoring, as it is of higher burden to participants, which might discourage participation and is therefore not often used. But even though a 24-h sample is the optimal from the three sampling strategies, it might be subjected to missing the exposure, due to interday variation of exposure, leading to under- or overestimation of exposure.

Due to high water content, urine contains BoE to chemicals, which are short-lived and are rapidly metabolized within the body and do not bioaccumulate, such as pesticides, bisphenols, phthalates and similar. In the case of exposure to more non-polar compounds, such as persistent organic contaminants (POPs), such as PCBs, PCDDs, polychlorinated dibenzofurans (PCDFs) and chlorinated pesticides (see Table 1), matrices with higher level of lipids are required, such as blood or adipose tissue.

1.2.2.3 Other sample types

Along with commonly used sample types, such as blood and urine, other sample types have been employed for the determination of a variety of BoE as presented in Table 1 [36][48][49].

Table 2: Some sample matrices used in HBM with their advantages and drawbacks.

Matrix	**Advantages**	**Drawbacks**
Nails	Non-invasive	Not suitable for polar compounds
	Covering large time period	
Breast milk	Non-invasive	Limited to lactating women,
	Suitable for non-polar compounds	Not suitable for polar compounds
Saliva	Easy to collect	Excretion dependent on many factors
	Non-invasive	Prone to contamination
Meconium	Covering long time period	Limited to newborns
Hair	Non-invasive	Prone to contamination
	Covering larger time period	Dependent on hair treatment
Deciduous teeth	Non-invasive	Limited amount
	Covering larger time period	Limited to children

*NOPs – nonpersistent organic contaminants

1.2.3 Sample preparation

In view of the essential aim of the NTS, i.e. to identify, in an ideal case, all of the compounds, the sample should be kept as intact as possible to avoid the loss of any constituents via sample preparation. However, to achieve the enrichment of BoE, to reduce matrix complexity and to achieve compatibility with an analytical system, the sample needs to be processed before the instrumental analysis [41], [50]. In the case of blood-derived samples, serum and plasma, proteins need to be removed. This is achieved by the addition of organic solvent, such as acetonitrile. Solids are separated using centrifugation or filtration, resulting in a protein-free liquid phase, which can be further processed. In the case of healthy subjects, urine does not contain a significant amount of proteins, thus the deproteinization step is not required, but salts need to be removed to achieve the compatibility with mass spectrometer.

As described in Section 1.2.1, BoEs undergo phase I and II metabolism. Therefore, to obtain conjugate-free compounds, hydrolysis of phase II metabolites is often performed. This can be achieved using acid or base hydrolysis, or enzymatic hydrolysis (Figure 3). During acid or base hydrolysis, the pH of sample is significantly changed and the process is less specific, as this can affect all hydrolysis-labile chemical bonds. In the case of enzymatic hydrolysis, a specific enzyme is added, namely β-glucuronidase/arylsulfatase and the sample incubated for a set period of time [41]. Variants of this enzyme can be obtained from Helix Pomatia, Abalone or recombinantly expressed from *Escherichia coli*. Even though enzymes are highly specific, non-specific activity, such as lipase, can cause side reactions, lowering the levels of certain compounds and producing new chemical species [51].

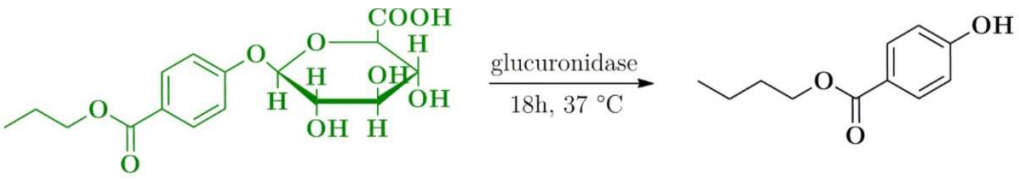

Figure 3: Enzymatic hydrolysis of butyl 4-hydroxybenzoate glucuronide.

To achieve sample purification and enrichment, extraction techniques including liquid-liquid extraction (LLE) and solid-phase extraction (SPE) have been most commonly used for blood and urine samples [52].

Well-established LLE is based on partitioning of the analytes in two immiscible solvents, and is therefore optimal for the separation of low-polar analytes from the polar sample matrix and vice-versa. Ethyl acetate, dichloromethane, hexane and their mixtures are typically used solvents in LLE. Efficiency of the extraction depends on pH, pKa and solubility of the analyte, volume of extraction solvent and number of extraction repetition (Equation 2), where q_{1n} is quantity of a compound in solvent 1 after n-repetitions, q is a starting quantity in solvent 1,

$$q_{1n} = q\left(\frac{V_1}{K_p V_2 + V_1}\right)^n \tag{2}$$

V_1 and V_2 are volumes of solvents 1 and 2, K_p is a ratio of solubilities in solvent 2 versus solvent 1 and n is the number of repetitions.

Although well-established and used for a long time, it is time-consuming and uses a large amount of solvents, with limited selectivity.

SPE is based on partitioning of analytes from a liquid phase to a solid phase, from which they are eluted with a solvent of different polarity as a sample matrix. SPE is available in various formats; as a bulk sorbent, which can be added directly to the sample and then separated, as extraction cartridges of different sizes or in 96-well plate format, which is suitable for lower volumes, as used in analysis of biological samples. Due to the possibility of 96-well plate format and shorter time of preparation, it offers high-throughput and rapid preparation of a high number of samples. Even though coming with several advantages, the price of SPE is significantly higher compared to the price of LLE.

A combination of an array of sorbents, solvents and formats offers higher selectivity and flexibility compared to LLE (Table 3).

Table 3: Overview of SPE sorption mechanisms and available sorbent types.

Retention mechanism	**Type of analytes**	**Sorbents**	**Examples**
Polar	Polar	Silica, modified silica: diol, cyanopropyl, aminopropyl	Strata Si (Phenomenex) Isolute-Si (Biotage)
Non-polar	Neutral, highly non-polar	Modified silica: C6, C8, C18,	Strata C18 (Phenomenex) HyperSep™ C18 (Thermo Fischer), Isolute C18 (Biotage)
Ion Exchange	Charged	Modified silica & functionalised polymers: SO_3^-, COO^-, R_4N^+	Oasis MCX (Waters) Oasis MAX (Waters) Strata X-CW (Phenomenex) Isolute SXC (Biotage)
Mixed Mode	Polar, non-polar & charged	Functionalized polymers: polystyrene divinylbenzene, *N*-pyrrolidone-divinylbenezene, acrylamidopropane sulfonic acid, ethyleneglycoldimetacrilate	Oasis HLB (Waters), Strata X (Phenomenex)

Sorbent variation aids in increasing the selectivity towards the specific analyte, as for example highly non-polar C18 preferentially retain highly non-polar compounds, while acid and base functionalized sorbents retain base or acid functionalized chemicals [53]. However, in non-targeted screening, such selectivity is to be avoided. To evade bias towards a certain group of compounds, mixed-mode sorbents with wide polarity range can be effectively used. In addition to SPE sorbents, other products have been developed, such as Oasis Ostro plate, which offers simultaneous deproteinization and phospholipid removal for serum and plasma samples [54]. Molecularly imprinted polymers, in which specific molecular template is imprinted into the polymeric structure during synthesis and thus increasing compound-specificity, are increasingly used, however they are, due to high selectivity, less applicable

to non-targeted analysis [55][56]. Recently, several advanced techniques have been developed and might in the future find application also in non-targeted screening. Such techniques are solid-supported extraction [57], dispersive liquid-liquid microextraction [58] and ultrasound-assisted emulsification microextraction [59], with the latter two being of high interest due to the possibility of miniaturization, which can prove advantageous particularly in biospecimens where volume is limited.

1.2.4 Instrumental analysis

Exposome analyses are based on high-throughput advanced mass spectrometry (MS) [60]. In metabolomics, nuclear magnetic resonance (NMR) spectroscopy has also proven itself as a very powerful technique, but due to its low sensitivity, it is currently not useful in the screening of xenobiotics [61], [62]. MS is based on detecting the mass to charge (m/z) ratio of ionized molecules and it has the advantage of high sensitivity, selectivity and the ability of chemical identification of unknown compounds. Prior to the MS analysis, compounds are ionized in an ion source. Hard ionization source is ionization by electron impact (EI) which is suitable for coupling with gas chromatography (GC), and produces a large amount of structural data because of extensive fragmentation of the molecule. Soft ionization modes provide less extensive molecular fragmentation and hence smaller number of ions and often intact molecular ions, which are important for molecular identification. Soft ionization sources include electrospray ionization (ESI), atmospheric pressure photoionization (APPI), atmospheric pressure chemical ionization (APCI) and fast atom bombardment (FAB). Recently instruments with dual ionization capabilities, such as ESI and APCI, have become available, and are useful for increased metabolite coverage.

Secondly, ions produced in an ion source are analyzed in mass analyzers, which differ in configuration and mass resolution. With the advance in technology, the trend has shifted from regular low-resolution mass spectrometry to high-resolution mass spectrometry (HRMS), which provides better qualitative performance, compound identification and data mining for biomarker discovery [63]–[67].

HRMS instruments such as Fourier-Transform ion cyclotron resonance (FT-ICR), Orbitrap and time of flight (TOF) are primarily used for full scan acquisition of MS. The combination of different mass analyzers yields hybrid systems, such as hybrid linear ion-trap/orbitrap (LTQ-Orbitrap), quadrupole-Orbitrap (Q-Orbitrap), quadrupole time-of-flight (Q-TOF), which provide MS/MS spectra with high mass accuracy and resolution. Currently, the most efficient combination in NT analysis is the combination of full scan acquisition to screen for chemical constituents and tandem MS which aids in compound identification, both performed under high-resolution conditions. Low (mass resolution from 10 000 to 20 000, full-width at half maximum (FWHM) and high (mass resolution above 60 000 FWHM) resolution mass analyzers and their properties are presented in Table 2.

Along with types of ionization and mass analyzers, data acquisition mode can also be varied. While for target analysis SRM and its extension MRM are popular, the NTS generally uses DDA or DIA. The first mode is data-dependent acquisition (DDA), where a preselected number of most intense ions are fragmented and their MS2 acquired [68]. The second, more recently explored mode, is data-independent acquisition (DIA), where the instrument within each scan cycle focuses on a narrow mass window of precursors, and fragments every precursor ion detected in that mass window [69]. The window is then cycled across all mass range, enabling measurement of MS2 of all precursors, which is beneficial for low abundance compounds, which are missed in DDA.

HRMS techniques are being further improved with the implementation of ion-mobility spectrometry (IMS), in which ions generated by MS ion source are separated in gaseous

phase prior to analysis with MS. Ions are separated based on their mobility in an electric field in the presence of a neutral gas. The mobility depends on ion's mass, charge and collision cross section (CCS), which corresponds to the rotationally averaged surface area of an ion [70].

Table 4: Mass analysers and their properties [71].

Analyser	**Resolution** ($m/z = 400$)	**Mass accuracy** [ppm]	**Detection limit on column**
QqQ	unit	50	fg-pg (SRM)
QIT	10 000	50	fg-pg (SRM)
LIT	10 000	50	pg (SRM)
IT-TOF/Q-TOF	20 000	3	pg (FS)
HR-TOF	60 000	2	pg (FS)
Q-Orbitrap/LTQ-Orbitrap	140 000	2	fg-pg (FS)
LTQ-FTICR/Q-FTICR	100 0000	≤1	pg (FS)

A great benefit of MS is its ability to be hyphenated to a separation method, such as high-performance liquid chromatography (HPLC), gas chromatography (GC) and capillary electrophoresis (CI), which provide compound separation prior to MS analysis. Using separation methods, the compounds are separated based on their physicochemical properties.

Using LC, compounds are separated according to distribution between liquid mobile phase and solid stationary phase. Stationary phase as well as mobile phases can be varied to achieve optimum separation. In the reversed phase LC, the stationary phase is usually silica modified by alkyl chains, usually eighteen carbon atoms in length. C18 columns enable the separation of non-polar and semi-polar compounds. Mobile phase is usually a mix containing high percentage of water and low percentage of organic solvents, such as acetonitrile, methanol, tetrahydrofuran etc., and the elution is either isocratic or gradient, where the latter provides faster analysis, narrower peaks and similar resolution power as the former. The problem of RPLC are highly polar compounds, which are eluted near or at the void volume. Convenient alternatives for the separation of polar compounds are aqueous normal phase chromatography and hydrophilic interaction chromatography (HILIC). For HILIC, the stationary phase is modified silica and mobile phase with high proportion of organic solvents and low proportion of aqueous solvents. It is useful for the separation of polar and highly polar compounds which cannot be retained on RP columns [72].

The basis of GC-MS is the mobility of compounds in the gaseous mobile phase, so volatility and thermal stability of the compounds is required. This is the main drawback of this technique, because a large number of metabolites are not sufficiently volatile. If the molecule contains appropriate reactive centers, this problem can be surpassed by derivatization, which increases its volatility. This step is usually time-consuming, limited to compounds containing reactive centers and increases difficulty of identification. The main advantage of GC-MS is however the reproducible EI ionization which enables the use of spectral libraries. Because of high reproducibility of GC-EI-MS spectra, the identification of metabolites with high confidence is enabled [52].

In CE, which is less used compared to LC and GC, the compounds are separated based on its migration through an electric field, which depends on compounds' charge [73][71][60].

1.2.5 Data processing

1.2.5.1 Raw data processing

Considering that a single human urine/blood sample contains several thousands of compounds [74] and the measurement results in thousands of features with various abundances, processing and analyzing of data is the most challenging part of non-targeted screening [75][76][77]. To be available for interpretation, complex data generated by HRMS requires extensive processing [78]. First off, raw data from the analytical instrument are converted into a format appropriate for processing software [79]. Although there is a number of mass spectral formats, mzML was created as a common open format that would be supported by any software, and is intended to enable processing across different software packages [80]. After conversion, the raw mzML data files are uploaded into the chosen data processing software, and various processing algorithms are applied. Noise filtering is used to remove instrumental interferences and chemical noise from the measurement signal, and to separate a component signal from the background. Chemical noise is produced by buffers and solvents and can be notably strong at the beginning and end of the elution. Peak or feature detection (picking) aims at identifying all signals caused by true ions and avoid detection of false positives. It is based on filtering MS signals above a set threshold, and separating analyte signals (features) from the noise. A variety of peak detection algorithms are available, such as vectorized peak detection, matched filter methods, derivative based methods, etc. The threshold for peak detection must be carefully selected, as a too low threshold will result in a large size of noisy data, whereas a too high threshold will result in the loss of low-abundance signals that may correspond to low level BoEs. Based on peak picking, extracted ion chromatograms are created for each feature. Peak overlapping is resolved by spectral and chromatographic deconvolution, which resolves overlapping peaks into individual peaks. A number of deconvolution algorithms are applicable, for example wavelet-based, which are relatively computationally demanding, due to wavelet fitting to each peak, and less computationally demanding (and with that achieving lower deconvolution efficiency), such as baseline cut-off or noise-amplitude-based algorithms. After deconvolution, alignment is applied to remove RT shifts of features across different samples. This is done to avoid doubling of features corresponding to the same compound within samples and is one of the most important steps in data processing. Like deconvolution, alignment is computationally demanding. A number of algorithms with different approaches are used, one example is MzMine's random sample consensus (RANSAC) alignment which models RT deviation from a master peak list, which is based on the first processed sample, and RT drifts corrected according to drift models. Other algorithms are, for example, based on time warping or Fourier transform recursive alignment. These steps are essential for generating a final, processable data matrix, however, a number of other steps can be employed in order to reduce bias and remove unwanted peaks. For example, sample normalization can be applied for removing unwanted systematic bias due to changes in signal intensities between measurements, gap filling is intended to correct deficient peak detection step, which causes missing peaks of actually detected compounds, while deisotoping removes redundant isotope features [81][82][83][84]. These processing algorithms are contained within publicly available or commercial software packages. The most commonly used are open-source MzMine2 [85], which is available with a graphical user interface (GUI), easy to use and with extensive data visualization, and XCMS, an R-based script [86], along with commercially available Thermo Fischer's Compound Discoverer™ (Table 5).

Table 5: Open-source data processing software.

Software	Features
MzMine2	Open source, compatible with LC-MS/MS and GC-MS data, based on Java and R, GUI available
XCMS	Open source, compatible with LC-MS/MS data, written as an R script and in cloud version
MS-Dial	Open source, compatible with GC-MS, GC-MS/MS, LC-MS/MS data, GUI available
Compound Discoverer™	Thermo-Fischer, compatible with GC-MS, LC-MS^n and IC*-MS^n data, GUI available

*IC – ion chromatography

1.2.5.2 Compound identification

The major bottleneck in NT metabolomics and exposomics is the annotation or identification of compounds. New approaches and software to facilitate compound identification and increase identification confidence are constantly being developed. The main issue is the confidence with which the compound is identified, which depends on the available data. Based on that, the following criteria for confidence of identification have been established [65][87]. One such criteria were defined by Schymanski et al. [65] (Table 3).

Table 6: Identification confidence levels.

Confidence Level	Description	Minimum data requirements
Level 1	Confirmed structure by reference standard	MS, MS2, RT, Reference standard
Level 2	Probable structure by either library spectrum match or by diagnostic evidence	MS, MS2, Library MS2, Experimental data
Level 3	Tentative candidate(s)	MS, MS2, Experimental data
Level 4	Unequivocal molecular formula	MS isotope/adduct
Level 5	Exact mass of interest	MS

To assign the identity to a feature, several options are available (Figure 4).

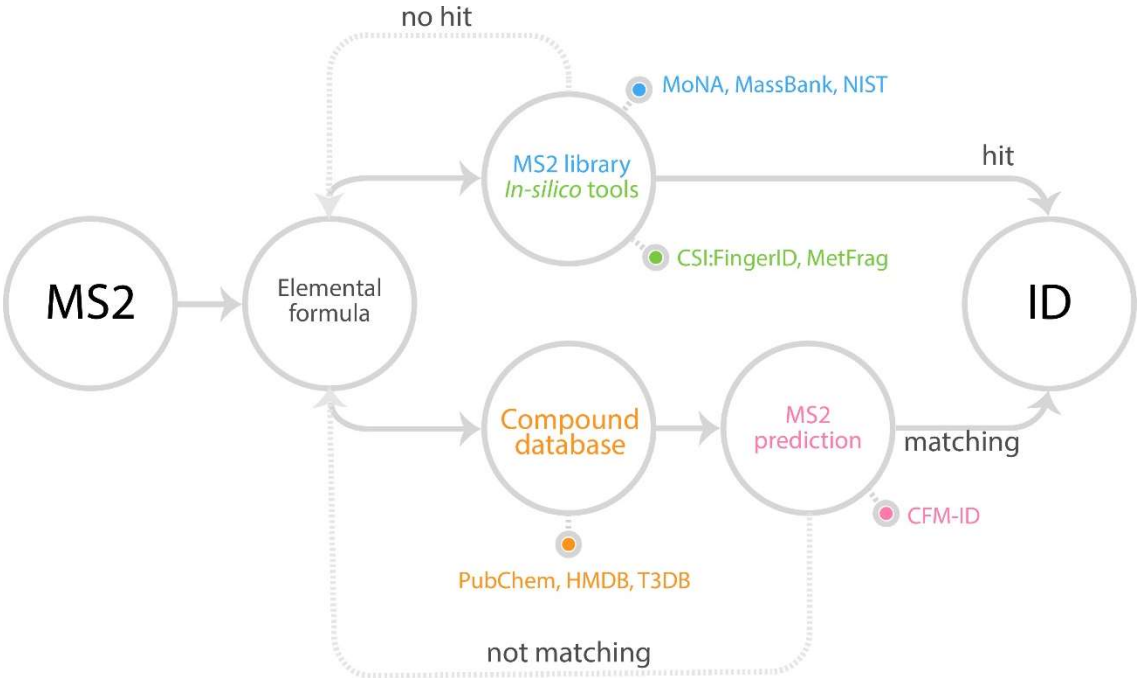

Figure 4: Identification workflow.

A common starting point of compound identification are small molecule databases (Table 7). Molecular formulas and accurate masses are contained within these databases, and candidates with matching molecular mass or formula can be retrieved. General databases, such as PubChem and CAS, contain a large number of compounds, for example PubChem contains more than three million entries. This is beneficial when searching novel compounds and BoEs to new chemicals, however with a large number of database entries, a large number of candidates are retrieved, out of which the majority are not relevant. Based on that, it is important to restrict the search space to more relevant compounds. For example, KEGG (Kyoto Encyclopedia of Genes and Genomes) contains data connected to metabolites, drugs and other chemical substances, along with genomic data and biological pathways [88], [89]. HMDB (Human Metabolome Database) contains data on to-date identified human metabolites [74]. In the light of exposome, these specific databases can be utilized when researching connection between exposure and subsequent biological effect, while it is less likely that relevant hits for BoEs will be found in this chemical space. For that, more specific databases have been established. The United States Environmental Protection Agency (EPA) established CompTox Chemistry Dashboard database, which contains data on potentially toxic chemicals, along with their physicochemical properties, environmental fate, exposure data, usage and toxicity [90]. In addition to that, PubChemLite was launched recently. It is a subset of PubChem contained chemicals that are relevant for exposomic research, and it is intended to be implemented into current workflows for HRMS-based exposomics [91]. Along with these databases, Exposome Explorer and T3DB have been created in order to facilitate exposomic research. Exposome Explorer is focused entirely on BoEs to environmental risk factors [2][92], while T3DB or the Toxic Exposome Database, as it will be referred to in the future, contains data on the common substances that are toxic to humans, with organ, DNA and proteins that are targeted by these compounds [93][94]. In 2021, CECScreen was launched and contains exposome-relevant CECs, metabolites and metadata [95]. As it is evident from Table 7, many databases target same or similar compounds and such redundancy inhibits streamlined harmonized workflow as one needs to consult several databases simultaneously.

Merging such databases in the future would be a great asset in reducing an already high complexity of compound identification.

Table 7: List of available compound databases and their focus or coverage.

Compound database	**Focus**
ChemSpider	General
PubChem	General
CAS	General
KEGG	Metabolites, gene data
HMDB	Human metabolites
Urine metabolome database	Urinary metabolites
CompTox Chemistry Dashboard	Potentially toxic compounds and their properties
PubChemLite	Subset of PubChem relevant for exposomics
T3DB	Chronically or acutely toxic compounds to which humans can be exposed
DrugBank	Drug data, drug targets and drug action information
Exposome Explorer	Biomarkers of exposure to dietary and environmental factors
CECscreen	CECs for exposome research
ChEBI	Chemicals of potential biological interest

Once potential candidates are found, their MS2 can be predicted and compared to experimentally acquired MS2 spectrum. For example, Competitive Fragmentation Modelling-ID (CFM-ID) uses in rule-based and combinatorial approaches to predict CDI-MS/MS and EI-MS spectra [96]. Experimental MS2 can be compared to the predicted one, and based on set matching thresholds, the identity is assigned. Furthermore, such *in-silico* predictors can be used to predict or construct *in-silico* MS2 of compounds of interest, which is particularly useful for suspect screening and identification of compounds for which experimental MS2 data is not available.

Experimental MS2 data are contained within mass spectral libraries (Table 8). Currently, there is a number of MS libraries, either commercial, such as NIST and WILEY, or freely available, publicly curated libraries, like MoNA (Mass Bank of North America) and MassBank. Certain libraries are dedicated to MS2 acquired by specific instruments, for example Metlin for QToF while mzCloud supports QOrbitrap data. This divide is founded on the fact that MS2 spectra are dependent on the type of analyser and the type of ionization and are less reproducible than EI acquired MS2.

Table 8: List of available MS libraries and the description of their focus.

MS libraries	**Description**
NIST	EI-MS, CID-MS/MS, vendor library
WILEY	EI-MS, CID-MS/MS, vendor library, largest collection of EI-MS data
MoNA	Large collection of CID-MS/MS spectra
MassBank	Community database
Metlin	Focused on QToF data

mzCloud	Focused on Orbitrap data
GNPS	Community database

An alternative to MS2 databases that effectively circumvent the problem of matching experimental and library MS2 are in-silico tools that use an array of different computational approaches for the identification of compounds (Table 9). [97]. As mentioned before, CFM-ID is an in-silico tool for the prediction of MS2 spectra, however it possesses another feature and that is matching of experimental MS2 query to predicted MS2 of compound database matches to molecular mass or molecular formula [98]. Similar to CFM-ID is MetFrag, which likewise predicts MS2 spectra, and scores it to molecular candidates from compound databases (PubChem, ChemSpider or KEGG). It differs however in prediction method, which is bond-dissociation-based [99]. Sirius-CSI:FingerID takes a different approach. First, elemental composition is calculated from the isotope pattern using deep neural network, followed by computation of fragmentation trees based on input experimental MS2 data. According to fragmentation trees, molecular fingerprint is predicted and matched to molecular fingerprints of candidates. Candidates are then sorted by matching score [100], indicating the most probable structure for the query compound. In contrast to previously mentioned software packages, where MS2 spectra are used as an input, xMSannotator and HaloSeeker use raw (U)HPLC-MS/MS data as an input, allowing automated streamlined workflow from processing to annotation within one software. xMSannotator is focused on metabolomics, using multistage clustering algorithm, where metabolic pathway associations are coupled to intensity profiles, retention time and MS data to cluster and annotate compounds [101]. HaloSeeker is focused on identifying halogen-containing compounds. After data processing, features are clustered together according to the number and type of halogen atoms. Specific plots are applied to visualize the results [102].

Table 9: *In-silico* tools for compound identification.

***In-silico* tool**	**Description**
CFM-ID	Machine learning-based fragmentation prediction and compound identification
CSI:FingerID	Kernel based fragmentation tree computation coupled with molecular fingerprint matching
MetFrag	Combinatorial fragmentation coupled with compound database search
MassFrontier	Experimental gas-phase reaction-based fragmentation prediction and compound identification
Feature-Based Molecular Networking	Clustering of features based on similarity of MS2 with MS-Cluster algorithm
xMSannotator	Works on raw data. Clustering of features according to their properties and common metabolic pathways, then annotating using compound databases
HaloSeeker	Annotation of halogenated compounds based on isotope pattern

1.2.6 Quality control and method validation

Due to intrinsic differences between established targeted and more recently developed non-targeted approaches, new procedures of quality control (QC) are necessary. The main difference between the two lies in the general aim, which with targeted analysis lies in the quantification of compounds, whereas identification is the main goal of non-targeted screening. As described in previous chapters, NT screening involves many stages, which need to be monitored to assure the quality of the results [103][104]. Firstly, during sample collection, samples are optimally aliquoted and frozen immediately after collection and stored at – 80 °C, with limited freeze-thaw cycles to reduce the loss of sample integrity due to chemical or biological degradation [13]. Afterwards, the use of QC samples is a common approach for quality assurance (QA) in targeted and semi-targeted analysis and likewise the QC samples can be applied to NT screening for QA and method validation. Typically, QC samples are prepared by pooling investigated matrix from different participants in order to cover interindividual variability and to be broadly representative of the whole sample set [105]. The samples can be additionally spiked by native and/or labelled standards of expected BoEs. Optimally, the standards cover maximum polarity and mass range and are spiked at the levels of expected BoEs.

By observing responses of spiked standards in QC samples, method parameters can be determined and method performance evaluated. Mass accuracy can be determined by calculating mass error of measured versus calculated m/z values of spiked standards. Repeatability of mass accuracy and retention times can be determined by following mass accuracies and retention times of standards in multiple runs of QC samples. Method reproducibility can be assessed by observing response of standards within QC samples injected on different time periods, as for example days and weeks [106]. NT screening methods are qualitative in nature and therefore LOD cannot be calculated, however it can be implied. This can be done with serial dilutions of QC samples by observing abundance of spiked standards. However due to simultaneous dilution of matrix, matrix effects which typically affect LOD will be reduced and estimated LODs will likely be lower than in actual samples. Similar to quantitative methods, carryover from previously injected samples can be monitored by injecting solvent blanks after every 5 – 10 samples [106]. Background levels for chemicals such as contaminants in mobile phases and solvents used in sample preparation or present in sample collection vessels and processing consumables can be addressed by using a procedural blank sample. To prepare the procedural blank, sample preparation procedure and analysis are performed using the same solvents, chemicals and consumables, however the biological sample is replaced by blank solvent [105].

For assuring the quality during the sample run, QC samples can be checked to assess precision by monitoring the standard deviation of peak areas from standards and/or known native metabolites [107]. Analytical system can be checked for non-random trends during batch run by following the area of spiked standards and native metabolites. Non-random effects due to chemical or instrumental events can be visualized for example by plotting batch samples and QC samples in principal component analysis. High quality batch runs would result in close clustering of QC samples in the center of the scores plot [105][108].

Furthermore, QC samples can be injected to the analytical system prior to measurement to condition or equilibrate the chromatographic system and with that improve the reproducibility of compound separation. During data processing and identification, QC samples can act as a base for the optimization of data processing parameters, which is particularly important when aiming at detection of low-level compounds, such as BoEs. QC and QA for the identification step focus on the reduction of false positive (Type I error) and false negative (Type I errors) rates [103], however general harmonized guidelines have

not been established yet [13]. Identification confidence is reported according to supporting evidence [65]. The requirements for reporting identification confidence are presented in Table 6, and present a guideline for consistent compound reporting. As the field advances, so will harmonized QC/QA guidelines; there are many efforts to produce such support and with that common ground for producing and reporting high quality NT results [9][19].

Chapter 2

Aims and Hypotheses

Literature reports the lack of methodology for non-targeted exposomics. Due to the challenges reported in Chapter 1.2, large scale non-targeted exposomic analysis, sample preparation, data processing and compound identification protocols are still underdeveloped. The aim of the dissertation is the development of non-targeted analytical workflow to be used in HBM. This includes the development of a sample preparation protocol, development of an HRMS-based instrumental method, development of data processing and identification workflow. By nature, a non-targeted workflow should be universal, and to demonstrate this attribute, its applicability to other fields of research is going to be tested and the following hypotheses addressed:

H1: The developed NTA workflow is suitable for application in HBM.
H2: The developed NTA workflow enables the identification of biomarkers of exposure in urine.
H3: NTA data processing and identification protocols can be extended to other fields.
H4: Biomarkers of exposure identified with NTA comply with the results of traditional, targeted HBM.

The specific goals of the thesis are:
- Development of a high-throughput sample preparation procedure for urine samples using solid-phase extraction suitable for NTA.
- Development, optimization and validation of the NTA instrumental method using UHPLC-HRMS.
- Optimization and validation of the targeted analytical method for endocrine disrupting chemicals, bisphenols, parabens and triclosan in order to connect targeted and non-targeted data of the same population.
- Development of data processing and compound identification protocols based on open-source software tools.
- Demonstration of the universality of data processing and compound identification protocols to other uses: an example of identification of environmental biotransformation products of cytostatic drug Imatinib.
- Application of the NTA workflow on urine samples of Slovenian children.

Chapter 3

Publications

The outcomes of the PhD work are presented in the following eight scientific papers, out of which three are published and five are currently under review at peer reviewed journals. According to the proposed hypotheses, the papers are divided into three sections:

1) Development of a NT workflow and its application

The papers in the first section address hypotheses **H1** and **H2**, and describe the development of the analytical method, the sample preparation procedure and compound identification for the search of BoEs in urine. The developed workflow was applied to a cohort of Slovenian children in order to describe their chemical exposome and identify urinary BoEs.

2) Application of the NT workflow to alternative scientific field

The papers in this section address **H3**, where the NT workflow, particularly data processing and compound identification, was adapted to environmental analysis. In this frame, the environmental fate of a cytostatic Imatinib was researched and its environmental transformation products were identified. The experimental data was submitted for publication in order to enable further data mining with tools developed and potential retrospective analysis.

3) Connecting the NTS workflow with the traditional targeted analysis

The papers in this section address the last hypothesis, **H4**, which proposed that the BoEs from NTS in children's urine match with the BoEs determined in targeted manner. Urine of children and adolescents was analyzed for bisphenols, parabens and triclosan within national HBM schemes. The results were compared to results of NTS to confirm findings and increase confidence of results obtained by novel methodology.

3.1 Development of NT Workflow and its Application

3.1.1 LC-HRMS-based method for suspect/non-targeted screening for biomarkers of exposure in human urine

Submitted: Tkalec, Ž., Codling, G., Klánová, J., Horvat, M., Kosjek, T., 2022

Due to the lack of analytical methodology for NT analysis of human samples in the search of BoEs, this study aimed to develop sample preparation and instrumental protocol to fill the gap. The main methodological platform for NT screening across more developed fields is currently high-resolution mass spectrometry (HRMS), which offers simultaneous detection of a large number of chemical entities within a single run [60]. Coupling to separation techniques, this offers efficient compound separation and detection, however, NT screening for BoEs faces several challenges, including 1) BoEs are present at very low concentrations, often several orders of magnitude lower than more abundant endogenous metabolites; 2) BoEs chromatographically overlap with high-abundance endogenous metabolites; 3) parent compounds undergo phase I and phase II metabolism, producing chemically distinct metabolites [31]. Some of these challenges can be addressed with optimized sample preparation procedures, which in NT context aim for a balance between removal of matrix and compound preservation [109]. There has been no universal sample preparation procedure established yet, with LLE and SPE commonly used for biological samples. The aim of this study was to develop a sample preparation procedure and an instrumental method suitable for NT screening in HBM. The development was founded upon urine matrix spiked with reference standards of chemicals of emerging concern. The sample preparation involved enzymatic hydrolysis followed by concentration and purification using SPE with instrumental analysis on UHPLC with HRMS detection. The method was validated by detection and identification of the spiked standards in the data matrix obtained from processing of HRMS data with MzMine2. The development was based on enzymatically hydrolysed urine spiked with 34 native standards with partition coefficients ranging from -2.29 – 4.80 and molecular masses from 122 to 612 Da. Prior to processing, urine was deconjugated using β-glucuronidase to generate glucuronide- and sulphate- free biomarkers of exposure. Sample preparation was performed by solid phase extraction using Oasis HLB 96-well plate with 10-fold sample concentration. Optimal elution was achieved with 10 % v/v methanol in acetonitrile and wash step with 5 v/v % of methanol in MQ. Chromatographic separation was achieved on Waters Acquity HSS-T3 column with acetonitrile and water as mobile phases. Suitability of the method was checked by analysing test samples by ESI(+)-Orbitrap-HRMS using full scan mode and identifying 11 out of 13 standards. Furthermore, limit of detection was estimated to be between 1-10 µg/L, with demonstrated low retention time drifts and consistent and high mass accuracy. The analytical workflow presented herein supports its application in a high-throughput non-targeted screening in human biomonitoring schemes.

This manuscript has been submitted to the Chemosphere journal.

LC-HRMS based method for suspect/non-targeted screening for biomarkers of chemical exposure in human urine

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ABSTRACT

Every day we are exposed to a cocktail of anthropogenic compounds many of which are biologically active and capable of inducing negative effects. The simplest way to monitor contaminants in a population is via human biomonitoring (HBM), however conventional targeted approaches require foreknowledge of chemicals of concern, often have compound specific extractions and provide information only for those compounds. This study developed an extraction process for human biomarkers of interest (BoE) in urine that is less compound specific. Combining this with an ultra-high resolution mass spectrometer capable of operating in full scan, and a suspect and non-targeted analysis (SS/NTA) approach, this method provides a more holistic characterization of human exposure. Sample preparation development was based on enzymatically hydrolysed urine spiked with 34 native standards and extracted by solid-phase extraction (SPE). HRMS data was processed by MzMine2 and 80 % of standards were identified in the final data matrix using typical NTA data processing procedures.

KEYWORDS: xenobiotic, exposome, biomonitoring, non-targeted, sample preparation, HBM4EU

1. Introduction

In the last century the advent of mass chemical production including pesticides, industrial compounds and flame retardants led to many compounds becoming ubiquitous in the environment. However, knowing compounds may be in the environment does not mean that they find their way into the human body. Due to the difficulties with obtaining human samples, the instrumental matrix effects, limitations on volume, metabolism of target chemicals and ethical considerations, human biomonitoring (HBM) has lagged in development of non-targeted monitoring methods.

However, understanding the body burden and trends in exposure are essential to formulate strategies to mitigate health impacts. HBM is a vital tool to provide the measure of individual and population exposure to chemicals from different source pathways and estimate potential risks associated with exposure (Ganzleben et al., 2017). Traditionally, HBM provided only quantifiable exposure data through targeted analysis of compounds of concern in various biological matrices such as blood and urine (Dennis et al., 2017). However, the number of emerging compounds of concern is increasing while legacy contaminants still require monitoring.

It is estimated that every year the number of new chemicals created increases by 4.4% (Llanos et al., 2019), with some 700 new compounds approved for use annually, while just a handful of existing compounds are removed from global production. Monitoring for these emerging contaminants and their metabolites becomes an impossible task if traditional targeted mass spectrometry approaches are used. The latest generation of ultra-high resolution mass spectrometers may provide a solution, as they are capable of acquiring accurate mass detection across a wide mass range, with sensitivity comparable to some targeted approaches. Non-targeted (NT) analysis is agnostic in nature and in ideal cases it provides information about 'every' compound in a sample that may be detected using the instrumental and extraction methods. NT potentially affords the opportunity to detect known-knowns (target), known-unknowns (suspect) and unknown-unknowns (unrecorded in databases or never determined).

In this regard NT screening is a starting, hypothesis-generating tool, and it has been widely applied in various fields such as in environmental and water quality monitoring and forensics (Caballero-Casero et al., 2021). However even in established fields of study the approaches for instrumental detection and identification of known-unknowns and unknown-unknowns is still evolving to remove false negative and positive reporting. In contrast, methods for NT screening for biomarkers of exposure (BoE) in human biological samples are even more limited (Pourchet et al., 2021).

To enable comprehensive screening for BoE, careful sample preparation and analysis procedures should be applied, however NT screening for BoEs faces several challenges, including that most synthetic chemicals are several orders of magnitude less abundant than some endogenous compounds. While the physical and chemical properties of many endogenous compounds overlap with BoEs making it difficult to exclude endogenous compounds without losing targets of interest. A further complication arises in that parent compounds may undergo phase I and phase II metabolism, producing chemically distinct metabolites. Phase

I metabolism involves reactions such as oxidation, hydroxylation and hydrolysis, while phase II reactions involve binding of very polar groups, such as glucuronide, sulphate or glutathione, rendering the compounds more polar and facilitating their excretion via urine (James, 2021).

Some of these challenges can be addressed with optimized sample preparation procedures, which in NT context aim for a balance between removal of matrix and compound preservation (Hajeb et al., 2022). To date, there has been no universally accepted sample preparation procedure. Both liquid-liquid extraction (LLE) and solid-phase extraction (SPE) are commonly used for biological samples. However, the choice of solvents, sorbents and clean-up is often selected to provide compounds that fit within the hypothesis of the research rather than a catch all approach.

Another key factor in HBM is which bio-material to sample with the most common being blood and urine. While blood reflects an equilibrium with the whole body, its collection is invasive, requiring qualified collection personnel, and often is volume limited. On the other hand, the collection of urine is non-invasive (Khamis et al., 2017), it is easily available and suitable for groups where blood samples are difficult to obtain, such as children. There are some limitations in that urine contains excreted compounds at low abundance which are often bio transformed. All in all, urine remains one of the few bio-fluids that may be used in large scale cohort studies.

The aim of this study was to develop workflow for NT screening in human urine, that includes sample preparation, an instrumental method, and data analysis. The development was founded upon urine matrix fortified with reference standards of chemicals of emerging concern. The sample preparation was based on enzymatic hydrolysis followed by concentration and purification using SPE with instrumental analysis on U(H)PLC with HRMS detection. The method was validated by detection and identification of the spiked standards in the data matrix obtained from processing of HRMS data with MzMine2. The study presents a high-throughput procedure for NT screening of urinary BoE applicable for small and large scale HBM studies.

2. Materials and methods

2.1 Chemicals and reagents

Analytical standards (n=34, minimum purity ≥ 99 %) were selected to cover a wide range of compound classes with a broad mass range (122 to 753 Dalton) and differing physical and chemical properties (see Table SI-1 for full details). The solvents used for sample preparation and chromatographic separation were LC-MS grade and were purchased from J.T. Baker (Deventer, the Netherlands). Oasis, SPE 96 well plates (60 mg) were purchased from Waters (Milford, USA).

2.2 Test sample preparation

A fortified human urine sample, was prepared by enzymatic digestion to validate the extraction method, clean-up and chromatographic separation. β-glucuronidase (Abalone, purified) was added to 1 mL of urine (to achieve 250 U/mL of enzyme in sample) and incubated by gently mixing for 18h at 37 °C. Then, the

deconjugated urine was spiked with the mixture of reference standards to the final concentration of 10 µg/L. Their full names, logP-values, elemental compositions reflecting their functionalization and monoisotopic masses are presented in Table 1. Ethical permissions were granted by Republic of Slovenia National Medical Ethics Committee (65/09/14 and 0120-118/2017/3).

Table 1: Full names of compounds for the test mix, with their names and abbreviations as used throughout the text and in the plots, with their corresponding logP-values, elemental formulas and monoisotopic masses.

Name/Abbreviation	Compound	logP*	Formula	Monoisotopic mass	RT (min)	Ionization mode**
AdipicAcid/AdipicA.	Adipic acid	0.08	C6H10O4	146.0579	0.96	ESI(-)
Erythritol/Eryth.	Erythritol	-2.29	C4H10O4	122.0579	1.32	ESI(-)
Acesulfame/Acesulf.	Acesulfame	-1.33	C4H5NO4S	162.9939	1.70	ESI(-)
Saccharin/Sacchar.	Saccharin	0.91	C7H5NO3S	182.9990	2.24	ESI(-)
Cyclamate/Cyclam.	Cyclamate	-1.61	C6H13NO3S	179.0616	2.54	ESI(-)
Omethoate/Ometh.	Omethoate	-0.74	C5H12NO4PS	213.0225	2.63	ESI(+)
Biphenol	Biphenol	2.8	C12H10O2	186.0681	3.31	ESI(+)
DemetonSS/DemSS	Demeton S Sulfone	0.07	C8H19O5PS2	290.0412	4.39	ESI(-)
Sucralose/Sucral.	Sucralose	-1	C12H19Cl3O8	396.0146	4.20	ESI(-)
Aspartame/Aspart.	Aspartame	-0.1	C14H18N2O5	294.1216	4.26	ESI(-)
Alitame	Alitame	-0.37	C14H25N3O4S	331.1566	5.30	ESI(+)
Mecoprop/Mecopr.	Mecoprop	3.13	C10H11ClO3	214.0397	6.53	ESI(-)
MeP	Methyl paraben	1.96	C8H8O3	152.0473	7.02	ESI(-)
BPS	Bisphenol S	1.65	C12H10O4S	250.0300	7.06	ESI(-)
Pravastatin/Pravast.	Pravastatin	0.59	C23H36O7	424.2461	7.72	ESI(-)
NDHH	Neohesperidin dihydrochalcone	0.2	C28H36O15	612.2054	8.13	ESI(-)
Acridone/Acrid.	Acridone	1.69	C13H9NO	195.0684	8.22	ESI(+)
EtP	Ethyl paraben	2.47	C9H10O3	166.0630	8.91	ESI(-)
Ketoprofen/Ketopr.	Ketoprofen	3.12	C16H14O3	254.0943	10.01	ESI(+)
Carbamazepine/Carbam.	Carbamazepine	2.77	C15H12N2O	236.0950	9.31	ESI(+)
Naproxen/Naprox.	Naproxen	3.18	C14H14O3	230.0943	10.57	ESI(-)
Aminoanthraquinone/AAQ	Aminoanthraquinone	3.74	C14H9NO2	223.0633	10.96	ESI(+)
BPA	Bisphenol A	3.32	C15H16O2	228.1150	11.32	ESI(-)
Diclofenac/Diclofen.	Diclofenac	4.51	C14H11Cl2NO2	295.0167	13.58	ESI(+)
DHBP	Dihydroxybenzophenone	2.96	C13H10O3	214.0630	11.73	ESI(-)
BuP	Butyl paraben	3.57	C11H14O3	194.0943	12.70	ESI(-)
BzP	Benzyl paraben	3.7	C14H12O3	228.0786	12.94	ESI(-)
BP8	Benzophenone 8, dioxybenzone	3.82	C14H12O4	244.0736	13.51	ESI(-)
TPP	Triphenylphosphate	4.59	C18H15O4P	326.0708	20.16	ESI(+)
Fenamiphos/Fenam.	Fenamiphos	3.32	C13H22NO3PS	303.1058	14.62	ESI(+)
NaDBS	Sodium dodecylbenzensulfonate	4.78	C18H30O3S	326.1916	17.93	ESI(-)

Chlorophene/Chlorop.	Chlorophene	3.6	C13H11ClO	218.0498	16.96	ESI(-)
Coumaphos/Coumap.	Coumaphos	4.13	C14H16ClO5PS	362.0145	20.12	ESI(+)
Phoxim/Phox.	Phoxim	4.39	C12H15N2O3PS	298.0541	34.78	ESI(+)

*Values obtained from PubChem (https://pubchem.ncbi.nlm.nih.gov/, accessed 21.10.2021)
**Many compounds are ionizable in both modes. The table shows the mode which produced better results.

2.3 Development of SPE for NT screening

Current trends in HBM involve large numbers of samples and low sample volumes making SPE with well plates an efficient option as opposed to larger SPE cartridges or LLE. HLB 60 well plates were selected as a broad-coverage sorbent for extraction, clean-up and concentration of urine samples. The sorbent was preconditioned with 1 mL of acetonitrile (ACN), methanol (MeOH) and water, respectively. Test sample (1 mL) was loaded to the sorbent, and allowed to pass through under gravity, the sorbent was then washed using 1.5 mL of a wash solvent. The sorbent was then dried under a gentle stream of nitrogen and each well was eluted with 1.5 mL of an elution solvent. Finally, the eluate was dried at 35 °C under a gentle stream of nitrogen and reconstituted in 100 µL 5% ACN in water creating a ten-fold concentration step. The selection of both wash solvent and eluent was optimised for this study.

To cover for the wide polarity of analytes, the elution solvent was selected based on a binary mixture of MeOH and ACN, from 10-100 v/v % of MeOH in ACN in increment steps of 10 %. For each mixture we evaluated two parameters, the elution efficiency and the matrix effects. Elution efficiency was monitored as absolute abundance of eluted analyte in test samples. Matrix effects were calculated for each mixture as a ratio of the absolute abundance of analyte in hydrolysed urine versus the absolute abundance in pure water. Optimal elution mixture was regarded as one with maximum elution efficiency and minimal matrix effects for the majority of analytes. In this study 10 v/v % MeOH in ACN was deemed as the optimum elution solvent.

For the wash solvent the aim was to enable the maximal detection of low-level BoEs, while removing very polar excess matrix constituents such as salts, sugars and small charged molecules. To remove very polar compounds, milli-Q water (MQ) is a natural choice for wash step, and to increase removal of less polar matrix a percentage of MeOH was added. We studied binary mixtures of 5, 10, 15, 20, 35 and 50 v/v % of MeOH in MQ. The wash step optimization was performed with 1.5 mL of 10 v/v % MeOH in ACN as the optimum elution solvent. More detailed information on the optimization of elution and wash solvents are provided in the results and discussion.

2.4 Instrumentation and chromatographic separation

2.4.1 NT method development on ESI(±)-UHPLC-QTrap-MS/MS

An ultra-high-performance liquid chromatography (UHPLC) separation was performed on Shimadzu Nexera X2. The standards were separated using the Waters Acquity HSS-T3 (2.1 × 100 mm, 1.8 µm) column with MQ (A) and ACN (B) as the mobile phases. Waters Acquity HSS-T3 column used for separation of polar and non-polar compounds. To maximize separation efficiency 100 mm column was used. MQ and ACN were selected as mobile phases and no modifiers were used in order to maximize detectability of standards in both, positive and negative ionization modes. Optimal separation was studied by varying elution program, solvents and column temperature. Using best parameters, standards in test samples were efficiently separated.Their RTs are presented in Table 1. The optimal elution gradient was: 5-15 % B (0.01-1 min), 15-25 % B (1-5 min), 25-40 % B (5-8 min), 40-60 % B (8-18 min), 60-75 % B (18-22 min), 75-85 % B (22-24 min), 85-100 % B (24-28 min), 100-5 % B (28-30 min), 5 % B, 30-35 min. Flow rate was 0.3 mL/min, while heating the column to 40 °C. The injection volume was 1 µL. The UHPLC was coupled to quadrupole-linear ion trap mass spectrometer (Sciex QTrap 4500). Electrospray ionization (ESI) was used as ionization source at the spray voltage of 4500 V and -3500 V and vaporizer temperature at 500 °C with curtain gas, gas 1 (GS1) and gas 2 (GS2) at 40,0 psi. Compounds were detected in multiple-reaction monitoring (MRM) mode. The MRM transitions and compound-specific MS parameters are presented in Table SI-1.

2.4.2 Method validation and proof-of-concept using ESI(+)-UHPLC-Q-IT-Orbitrap-MS/MS

The developed NT method was first applied to the sequence of 10 test samples in full-scan (FS) mode and then to 5 actual urine samples in order to test its suitability for NT screening in real samples. For the UHPLC separation the parameters were kept the same as those used for target analysis. The MS detection was performed on Orbitrap Fusion™ Tribrid™ Mass Spectrometer (Thermo Fisher Scientific Inc., Waltham, MA), in ESI(+) FS mode. Heated electrospray ionization (HESI) was used as the ionisation source, at the spray voltage of 4500 V, sheath gas flow 40 L/min, nebulizer auxiliary gas 15 L/min, and sweep gas 2 L/min. The ion transfer capillary was heated to 350 °C. The full-scan mass acquisition covered the mass range of 100-900 *m/z* and was performed at the resolution of 120 000 FWHM (full width at half maximum at *m/z* 200), while the MS2 data was acquired with the resolution of 60 000 FWHM. The automatic gain control (AGC) was 5×10^5 ions and maximum injection time 50 ms. Cycle time was 0.8 s.

2.5 Data processing and compound identification

HRMS FS data was processed using MzMine 2.53 (Pluskal et al., 2010) in the original Thermo .raw file format. Processing parameters were optimized to detect the features corresponding to spiked standards in test samples. Processing parameters are presented in SI-2. Spiked standards were identified as features with matching MH^+ at the maximum error of 5 ppm and the maximum retention time variation of 0.5 min.
For proof-of-concept, the compounds in real urine samples were identified by submitting all MS2 spectra within the feature list to Sirius-CSI:FingerID (Dührkop et al., 2019). Elemental formula match required to be 100 %, while candidate match score was required to be ≥ 60 % using PubChem, KEGG and CheBI compound databases. Candidates achieving the criteria were then filtered for

xenobiotics using T3DB (Wishart et al., 2015), Exposome Explorer (Neveu et al., 2017) and Chemistry Dashboard (Williams et al., 2017).

2.6 Validation and quality control procedures

Several quality control (QC) measures were taken. During extraction and instrument runs several blanks were generated. First, LCMS grade water (n=3) was processed as test samples served and as procedural blanks in order to identify contaminants. Deconjugated urine blanks (n=3) were used in order to monitor possible presence of the fortified compounds in urine. Instrumental solvent blanks, (injected every 5 samples) were used to monitor any possible carryover from injections on the HPLC system.

The limits of detection (LOD) of fortified standards in FS mode were determined by serial dilution of test samples with LC-MS grade water. Samples were prepared in concentrations 1000, 500, 250, 100, 50, 25, 10, and 0.1 µg/L. LOD was determined as lowest concentration at which features corresponding to standards were detected above 3 times the baseline. All features identified less than the LOD were excluded from the data set.

Retention time stability was assessed by monitoring retention time drifts for each identified standard across test samples. Stability of mass accuracy was monitored by following mass errors for MH+ of identified standards across test samples.

Non-specific activity of deconjugation enzyme was checked by spiking 1 mL of synthetic urine (CDC, 2009) with native standards and incubating them under the same conditions as those used for urine deconjugation. Spontaneous degradation was controlled by concurrently incubating the standards without the presence of the enzyme. Deconjugation stability was checked at the beginning of incubation and after 2, 8, 24, 30 and 48 h, while spontaneous degradation was monitored as a difference in abundance at time zero and at the end of the experiment. After incubation, the deconjugation samples were treated the same way as other samples.

3. Results and discussion

3.1 Test compounds and their chromatographic separation

Test samples used in the analytical method development were prepared by spiking enzymatically hydrolysed urine with native standards. The native standards served as model compounds and were selected in order to cover a wide range of polarities (described with partition coefficient, logP), molecular masses and functional groups (Table 1 and Figure 1). The native standards included pharmaceuticals, artificial sweeteners, flame retardants, bisphenols, parabens, benzophenones, surfactants and pesticides, and were variously functionalized, with moieties including oxygen, nitrogen, phosphorus, sulphur and chlorine. Compound masses ranged from 122.0579 to 612.2054, and logP from 0.96 to 4.51. Post-extraction labelled standards were included to adjust for any variations during the sample preparation and instrumental analysis. Chromatographic separation was studied by varying elution gradients and solvents. The optimal separation program, as described in section 2.4 produced chromatograms with no overlapping peaks, evenly distributed throughout chromatographic run (Figure 1). Even distribution is essential to avoid local chromatographic crowding of compounds, producing clearer

chromatograms and enhancing the chance of detecting low-level BoEs, which can overlap with high-abundance endogenous metabolites.

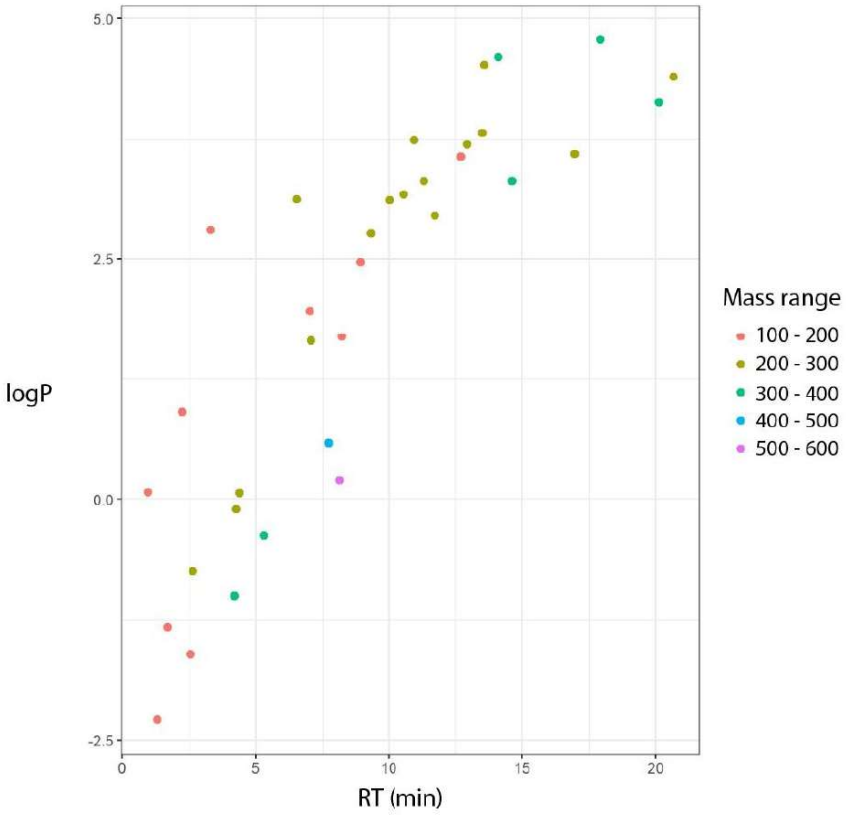

Figure 1: Separation of native and labelled standards used for NT method development according to their logP and molecular mass.

3.2 Deconjugation specificity

Xenobiotics can undergo phase I and II metabolism when introduced into the organism. Phase I reactions occur mostly in the liver and are driven by cytochrome P450 group of enzymes (James, 2021). They include oxidation, reduction and hydrolysis reactions to attach or transform into moieties suitable for the phase II metabolism. The latter involves formation of conjugates with, among others,

283 glucuronic acid, sulfuric acid and glutathione, which renders xenobiotics more polar
284 and hence easier to excrete via urine.

285 The deconjugation step, often part of a sample preparation procedure for urine, is
286 in principle the opposing reaction to the phase II metabolism. It cleaves the bond
287 between a xenobiotic and a polar conjugate molecule, releasing a parent
288 compound or phase I metabolite, depending on which was conjugated in the first
289 place (James, 2021). The deconjugation step can offer several advantages, which
290 we implemented in our NT analytical method. First, increased efficiency of sample
291 clean-up, as more non-polar compounds bind more strongly to reversed phase SPE
292 sorbent and with that a higher proportion of the more polar matrix can be removed.
293 Second, the efficiency of a reversed phase chromatographic separation can be
294 increased, since more polar compounds elute at shorter retention times, while the
295 more non-polar ones demonstrate stronger retention on the stationary phase,
296 which results in a more efficient separation. This further benefits in the higher
297 chance of detection of low-abundance compounds due to reduced coelution with
298 more polar high-abundance endogenous metabolites. Thirdly, the deconjugation
299 process reduces molecular complexity which is very beneficial for compound
300 identification. Also, from the MS perspective, phase II conjugated metabolites
301 would be more prone to electrospray negative ionisation, which generally yields a
302 weaker response and less informative mass spectra. On the contrary, the
303 electrospray positive ionisation is however expected to be a more viable alternative
304 for deconjugation products, i.e. the phase I metabolites and parent compounds,
305 also supporting the detection of low-abundance compounds.

306 Despite the several advantages offered by deconjugation, our concern was that
307 the enzymatic hydrolysis might change the chemical structure of the analytes due
308 to reported non-specific activity. For example, lipase activity has been reported,
309 which causes hydrolysis of ester bonds and was observed for phthalates (Blount
310 et al., 2000). To check the analyte integrity after deconjugation, we incubated the
311 standards with the deconjugation enzyme, β-glucuronidase at the activity levels
312 used for sample deconjugation (Figure 2, tabulated numeric results are reported
313 in SI-Deconjugation_stability.xlsx). The abundances were normalized to an
314 average for each standard in order to plot on the same scale. Locally estimated
315 scatterplot smoothing (LOESS) curves were plotted to visualize trends.

316

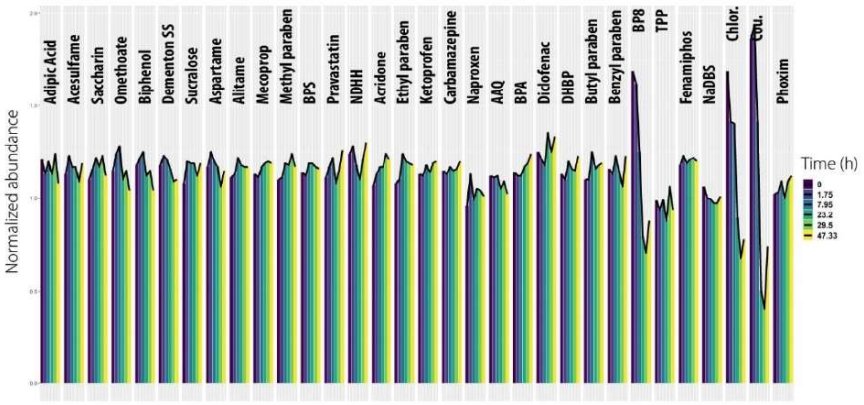

Figure 2: Abundance of standards when incubated with β-glucuronidase for different time periods.

Abundances of the majority of compounds have not significantly changed through the duration of the experiment, in fact final abundance did not differ more than 10 % for the majority of the tested compounds (31 out of 34). Exceptions were benzophenone 8, chorophene and coumaphos, for which the abundances reduced to 52 %, 46 % and 40 % of the starting abundance, respectively. However, we observed similar trends in the control experiments with no enzyme, where for the majority of samples abundances did not decrease for more than 20 %, while the abundances for benzophenone 8, chlorophene and coumaphos reduced to 42 %, 23 % and 66 %, respectively. This shows that degradation of these compounds is due to spontaneous breakdown processes rather than on account of the non-specific activity of β-glucuronidase.

The deconjugation stability experiment was conducted on a group of compounds with various functional groups, including esters, amides, ketones, ethers, phosphates, sulfones, polyols, phenols, carboxylic acids and so on. This increases the confidence that once the NT analytical method is applied to real urine samples, BoEs will not chemically change during urine deconjugation due to non-specific activity of β-glucuronidase.

3.3 Development of SPE for NT screening

We selected reversed-phase (RP) m-divinylbenzene N-vinylpyrrolidone copolymer sorbent Waters Oasis HLB, which absorbs both polar and non-polar compounds, and is not biased for acid, base or neutral compounds. This sorbent has been most widely used SPE sorbent across a variety of NT as well as targeted applications (Hajeb et al., 2022). Steps for SPE involve sorbent conditioning, sample loading, washing to remove high abundance endogenous matrix, drying and elution of analytes from the sorbent. As the first step we selected the elution solvent in order to recover the maximum of spiked standards with lowest eluted matrix, followed by selection of the wash solvent. Comparison of elution volumes showed that elution of 2.0 mL, 1.8 mL and 1.5 mL do not differ significantly in absolute

abundance of compounds in the eluted extract, therefore we opted for the lowest volume, 1.5 mL.

3.3.1 Selection of the elution solvent

As Oasis HLB retains polar and non-polar compounds, we opted for a binary mixture of a weak (MeOH) and stronger elution solvent (ACN). The elution efficiency was monitored through trends in abundance of eluted standards, while the amount of eluted matrix was monitored through the trends of matrix effects for each spiked standard (Figure 3, Numeric results are presented in table SI-Selection_Elution_Solvent.xlsx.).

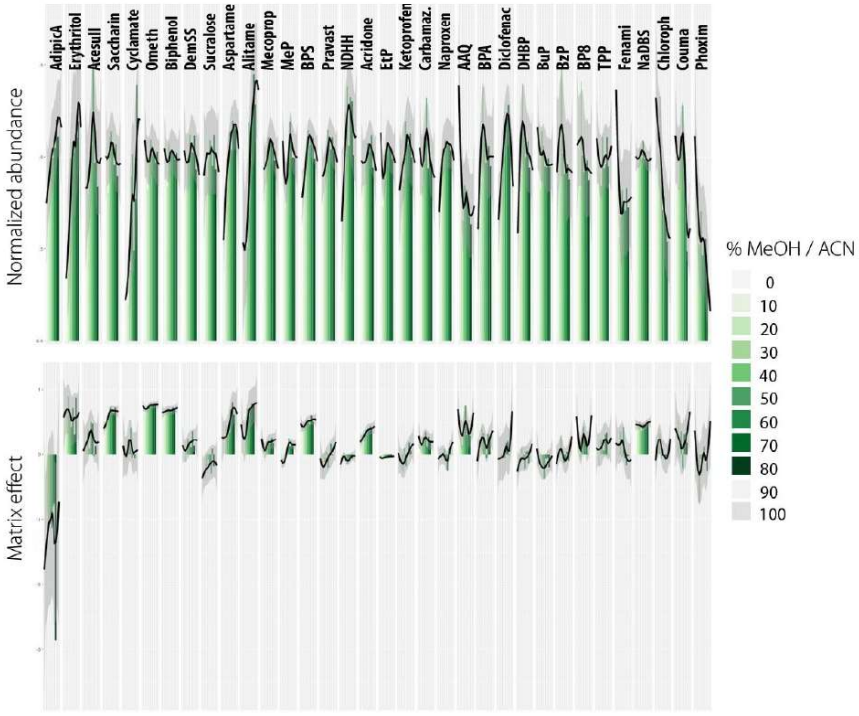

Figure 3: Elution efficiency (a) of native standards by mixture of MeOH and ACN. Standards are ordered according to their RT. Absolute abundances were scaled by normalization to average abundance for each compound. Matrix effects (b) of native standards when eluted by mixture of MeOH and ACN. LOESS curves have been added to visualize trends.

From the elution efficiency (Figure 3a), it is evident that with increasing MeOH, the abundance of early eluting polar compounds such as adipic acid, erythritol, acesulfame, saccharin, aspartame and alitame is increased. However, the late eluting/non-polar, compounds, such as aminoanthraquinone, fenamiphos, chlorophene, coumaphos and phoxim are retained on the sorbent, both events clearly in agreement with compounds' polarities. The matrix effects affect the instrumental sensitivity for the majority of compounds (Fig 3b), while adipic acid displays signal enhancement in the extracted urine as compared to water. The trend indicates that greater MeOH does not have a significant effect on matrix effects for any of the more polar, semi polar or non-polar compounds, and with that does not favour any group of compounds in terms of increased ionization and detectability. Therefore, according to elution efficiency, a lower percentage of MeOH in ACN is in favour of greater abundance of the more non-polar compounds, while not significantly influencing the matrix effects. Based on that, we identified 10 v/v % MeOH in ACN as the elution solvent of choice.

3.3.2 Selection of the wash solvent

After selecting the elution solvent, we studied the pertinence of wash solvents aiming to remove high abundance endogenous matrix constituents at the minimum loss of the test compounds. Accordingly, the wash step needed to be performed by a solvent mixture with low eluting power for more non-polar compounds, while eluting unwanted matrix constituents such as salts and highly charged species. Based on that, different mixtures of MeOH in MQ were tested. The level of matrix removal through matrix effects was monitored, and analyte loss through abundance of eluted analyte was measured for mixtures of 5, 10, 15, 20, 35 and 50 v/v % MeOH in MQ (Figure 4, numeric results are presented in table SI-Selection_wash_solvent.xlsx). The elution was done with the previously selected solvent, 10 v/v % of MeOH in ACN.

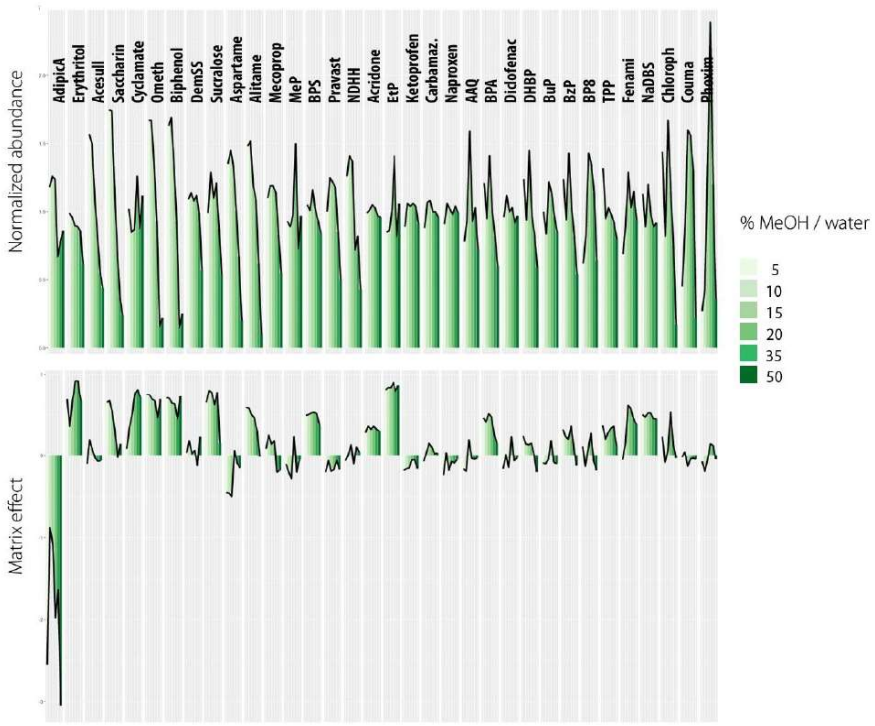

Figure 4: (a) Abundance of native standards when washed by mixture of MeOH and MQ. Standards are arranged from left to right according to their RT. Absolute abundances were scaled by normalization to average abundance for each compound. (b) Matrix effects of native standards when washed by mixture of MeOH and MQ. LOESS curves have been added to visualize trends.

Increasing MeOH percentage in MQ causes significant loss of more polar compounds such as adipic acid, erythritol, acesulfame, saccharine, omethoate and aspartame (Figure 4a). Abundances of more non-polar compounds, such as BPS, chlorophene, coumaphos and phoxim are first improved with increasing since the matrix is being washed out, but the compounds are then themselves eluted from the sorbent when MeOH content exceeds 15 v/v %. Certain compounds, such as saccharine, alitame, diclofenac, DHBP and BuP show reduction in matrix effects, with increasing MeOH leading to lower LODs. However, at the same time, this would cause systematic loss of abundance of more polar compounds, and at a certain point also non-polar compounds. As a compromise, 5 % v/v MeOH in MQ was selected as wash solvent.

3.4 Proof-of-concept and method performance

Detection and identification of fortified standards in the test mix acquired in FS mode and prepared according to the above described sample preparation

procedure served as a proof-of-concept and confirmation of the workflow's suitability. By application of the developed workflow, 12 out of 15 standards ionizable in the electrospray positive mode (Table 1) were identified using FS HRMS. Three compounds, BPA, demeton SS and biphenol were not detected. This is likely due to ion-suppression by matrix, subsequently lowering the signal abundance below the LOD. For the remainder of 12 standards, the corresponding features were successfully annotated. A feature was annotated as a molecular ion if the mass error compared to theoretical mass did not exceed 5 ppm and retention time did not differ for more than 0.5 min from that of a standard. Furthermore, for 11 of 12 successfully annotated standards, MS2 spectra were acquired and were used for further validation by comparing their MS2 spectrum to the MS2 spectrum of standards, (details of which are presented in table SI-MS2_matching.xlsx). The successful identification of 11 standards spiked into deconjugated urine at the expected level of xenobiotics, 10 µg/L served as a proof-of-concept and confirmed the method's suitability for NT screening of BoEs.

Estimation of method performance parameters (Table 2), based on analysing the features corresponding to spiked standards, showed the method's capability of achieving LODs in FS mode at as low as 1 µg/L, indicating that large number of BoEs present in urine could be detected when applied to samples used in HBM. However, it should be noted that LOD was determined by serial dilutions of the test sample with water, where along with the analytes matrix was diluted and so matrix effects were reduced and the effective LOD might differ. Retention times proved stable, with maximum deviation of ± 0.31 min, which was observed for pesticide phoxim, which was eluted from the column at the near end of chromatographic separation. This information is useful for setting processing parameters, especially for algorithms for retention time alignment during data processing. Large drifts in RT can cause doubling of features in the data matrix and increases the noise in the data. Furthermore, the instrumental method showed consistently low mass errors, with maximal mass error of 3.53 ppm for ketoprofen (Table 2, Retention time drifts and mass errors for each sample are tabulated SI-RT_drifts_mass_errors.xlsx and presented in SI-3).

Table 2: Estimated method performance parameters based on identified standards

Test compound	LOD (µg/L)	SD* of RT (± min)	Max. mass error (ppm)
Omethoate/Ometh.	10	0.10	0.47
Alitame	10	0.09	0.90
Acridone/Acrid.	10	0.05	1.02
Ketoprofen/Ketopr.	10	0.08	3.53
Carbamazepine/Carbam.	1	0.05	0.42
Aminoanthraquinone/AAQ	10	0.06	0.45
Diclofenac/Diclofen.	10	0.07	0.68
TPP	1	0.06	0.61
Fenamiphos/Fenam.	1	0.07	0.33
Coumaphos/Coumap.	10	0.06	0.83
Phoxim/Phox.	10	0.31	-0.33

*SD- Standard deviation

High mass accuracy is immensely important for finding the correct elemental composition and for reducing the number of candidates in the identification challenge. This is crucial when identifying BoEs to xenobiotics, as the chemical space (Milman and Zhurkovich, 2017) for xenobiotics is incredibly large and often the search for candidates is done through general databases such as PubChem, which contains data on more than 96 million unique chemical structures. This in turn produces a high number of potential hits.

Achieving good LODs and mass accuracies for spiked standards, the method was further applied to the preliminary NT study involving 5 real urine samples. With application of the developed workflow, two UV-filters, 4-hydroxybenzophenone and octabenzone, a surfactant residue octylphenol, two phthalate metabolites, monoethylhydroxyhexyl phthalate and monocarboxyisooctyl phthalate, and BoE of smoking, hydroxycotinine were tentatively identified at Schymanski confidence level 3 (Schymanski et al., 2014). The MS2 spectra and identification scores for the compounds are presented in SI-4. The number of identified compounds could be significantly enhanced by application of orthogonal identification methods and analysis of larger HBM cohorts, as exposure to certain chemicals is highly individual and therefore chance of detecting them lower. Nevertheless, this unambiguously demonstrates the method's effectiveness in identifying low level BoE and it's potential for application in large scale HBM studies. In spite of developing an optimized sample preparation and analysis procedure. compound annotation and subsequent identification are still the major bottlenecks in implementation of NTA to HBM, especially due to the high number of potentially relevant compounds. To circumvent this problem, the efforts to compile BoE, have been made through databases such as The Toxic Exposome Database or T3DB (Wishart et al., 2015), Exposome Explorer (Neveu et al., 2020), Chemistry Dashboard (https://comptox.epa.gov/dashboard/) and recently launched PubChemLite (Schymanski et al., 2021), a PubChem subset containing exposome-related entries. Creation of relevant databases and libraries along with *in-silico* identification methods, such as Sirius-CSI:FingerID (Dührkop et al., 2015, 2013), Metfrag (Ruttkies et al., 2016) and CFM-ID (Allen et al., 2014) aid in improvement of the confidence of BoE identification, and with that promote rapid real-time detection of contaminants and help reduce health risk associated with the exposure.

Conclusions

There is a clear need for the development of a less target-specific extraction and detection methods for BoEs in human urine. Thus, a UHPLC-MS/MS method and sample preparation procedure suitable for a wide range of compounds as presented here for non-targeted analysis of biomarkers of exposure in urine is essential to keep up with emerging compounds.
Deconjugation of phase II metabolites is a vital step in detection of parent or phase I molecules but concerns in stability and loss of molecules of potential interest have been considered for NT screening. In this study it is clear that some compounds may be affected during deconjugation, however this is due to spontaneous processes rather than non-specific enzymatic action. The elution for non-targeted screening is often a balance between compounds of interest and matrix interferences. The logical testing of 34 compounds as presented for differing elution strategies and washes as presented here provides a clear understanding of which potential compromises between recovery and ion-suppression may be

considered and an ideal method option was developed. The method was applied to a preliminary batch of samples and six biomarkers of exposure successfully identified, demonstrating the methods suitability for large scale HBM studies.

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3.1.2 Non-targeted and suspect screening-based human biomonitoring identifies 74 biomarkers of exposure in urine of children

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The NT workflow developed in the previous study (Section 3.1.1) was applied to a cohort of 200 children from Slovenia, of which 100 resided in the capital city of Slovenia and represented the urban part of the population. The remaining 100 participants resided in a region in the Eastern Slovenia, which is predominantly rural and characterised by intense agricultural activity. Due to the seasonal variations in agricultural practice, rural participants provided two samples, one in winter and the second at the beginning of summer, reflecting time periods with low and high intensity of nearby agricultural activity. Altogether, we obtained 300 samples of first morning urine. Samples were prepared according to the previous study and analysed using Orbitrap Fusion™ Tribrid™ Mass Spectrometer (Thermo Fisher Scientific Inc., Waltham, MA) in collaboration with RECETOX in Brno, Czech Republic. Data was processed using MzMine 2.53 [85]. BoEs were identified by submitting all acquired MS2 spectra to Sirius-CSI:Finger-ID [110], [111] and a selection of highest confidence hits. Furthermore, searching was conducted through MS2 library MoNA. Afterwards, all putatively identified BoE were submitted to Feature Based Molecular Networking (FBMN) [112], clustering features with similar fragmentation patterns together. In-silico library of suspects was generated for SS with CFM-ID [98], and experimental MS2 of suspects matched to predicted ones. Using this workflow, we tentatively identified 38 biomarkers of exposure using non-targeted approach and another 38 using suspect screening. Biomarkers of exposure show environmental burden of children to several classes of chemicals such as personal care products, plasticizers and plastic production chemicals, volatile organic compounds and polycyclic aromatic hydrocarbons, nicotine and caffeine, and pesticides, out of which three, atrazine, amitraz and diazinon, are restricted in the EU due to their high toxicity. Alongside the compounds with known use, we identified 7 chemicals with unknown use. Due to sampling in two time periods and in two locations, we elucidated the transitory and location-specific exposome. The results of the study identify and describe the exposome of children, which are exposed to many chemicals simultaneously. Many of these chemicals are concerning due to the potential of adverse effects. The work is to the author's knowledge among the first ones that demonstrate the practical approach of using NT analysis and SS in HBM.

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Suspect and non-targeted screening-based human biomonitoring identified 74 biomarkers of exposure in urine of Slovenian children

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ABSTRACT:
Human exposure to organic contaminants is widespread. Many of these contaminants show adverse health effects on human population and it is of paramount importance to analyse the human exposome. Human biomonitoring (HBM) follows the levels and the distribution of biomarkers of exposure (BoE), however, it is usually done in a targeted manner, meaning that it is oblivious to the presence and levels of any other exposures that are not targeted. Suspect and non-targeted screening (SS/NTS) approaches, on the other hand, tend to find BoE in a more agnostic way, without preselection of compounds, and with that they include finding evidence of exposure to predicted, unpredicted known and unknown chemicals.
This study describes the application of high-resolution mass spectrometry (HRMS)-based SS/NTS workflow for revealing organic contaminants in urine of a cohort of 200 children from Slovenia, aged 6-9 years. The children originated from two regions, urban and rural, and the latter were sampled in two time periods, summer and winter. We tentatively identified 74 BoE at confidence level 2 and 3 from several classes of pharmaceuticals, personal care products (PCP), plasticizers and plastic related products, volatile organic compounds (VOC), nicotine, caffeine and pesticides. Presence of three pesticides, atrazine, amitraz and diazinon is particularly concerning since they have been EU restricted. Among BoE we identified compounds not yet monitored in HBM schemes and of limited exposure data, such as bisphenol G and polyethylene glycols and polyethylene glycol ethers. Furthermore, 7 compounds with unknown use and sources of exposure were identified, either indicating new chemicals entering the market, or their metabolites and transformation products. Interestingly, several BoE showed location and time dependency.
This study presents high-throughput and cost-efficient approach to SS/NTS for HBM. The results shed a light on the exposome of Slovenian children, opening up questions on potential adverse health effects of such mixtures on this vulnerable population.

Keywords: exposure, non-targeted, high resolution mass spectrometry, exposome, HBM4EU, biomarker

1.Introduction

Humans are continuously exposed to a variety of external factors. Be they physical, psychological or chemical, all of these factors make up the exposome. Chemical exposome involves exposure to hundreds of different chemicals, which usually occur at low levels. They originate from variety of sources, as for the example the environment, diet and personal care products (PCP). With various biological effects and connected to genetic susceptibility, these chemicals are involved in etiology of many human diseases. As many as 80-85 % of diseases have been reported to be linked to the exposure to environmental chemicals (Uppal et al., 2016). Children, with their biological systems and organs at various stages of development and less advanced elimination of contaminants are particularly sensitive to adverse effects caused by exposure (Ferguson et al., 2017), so it is of great importance to comprehensively describe their chemical exposome and subsequent biological responses.

To study the exposome, several approaches have been applied, such as stationary sensing, wearable devices and personal sensors (Niedzwiecki et al., 2019), however measuring external levels does not directly reflect the internal dose which actually affects biological endpoints. Hence, more answers have been offered by human biomonitoring (HBM) that provides aggregated data of the exposures by targeting the chemicals of concern and their metabolites in biological matrices (Ganzleben et al., 2017). Conceptually, HBM can reflect temporal trends of exposure to environmental compounds as well as measure the efficiency of policy regulations (Pourchet et al., 2020). However, as a consequence of using traditional biomonitoring schemes and by following a limited number of only predetermined compounds, the chemicals that are not specifically targeted remain unidentified, with unknown levels, biological effects, and therefore uncharacterized risk. To define exposures holistically and to keep in time with rapidly emerging new chemicals in industrial and consumer products, non-targeted analysis (NTA) and suspect-screening (SS) approaches show promise to fill this gap. However, the methodology for SS/NTA human biological samples is severely vague (Pourchet et al., 2020).

Liquid chromatography (LC) hyphenated to high-resolution mass spectrometry (HRMS) is frequently used in non-targeted metabolomics (Schrimpe-Rutledge et al., 2016), however its employment in the exposomics involves several specificities. For example, a large number of endogenous metabolites are present in biological specimens in high quantities, while xenobiotics, being the focus of exposomics, are normally present at very low levels in the range of µg/L and lower. They often coelute and chromatographically overlap with endogenous metabolites, hindering their detectability. Furthermore, in many cases biomarkers of exposure (BoE), particularly in urine, are not parent compounds but their phase I and/or II metabolites, which may have not yet been identified and linked to their parent compounds. This increases the chemical diversity and complexity of compound identification, which is the bottleneck of NTA workflow. Given that BoE are present at very low levels, their tandem MS (MS2) spectra are difficult to obtain at sufficient quality. The MS2 spectra are used to identify the compounds by manual interpretation, by matching with spectral libraries, or by using *in-silico* fragmentation tools (Getzinger and Ferguson, 2020).

This study here presents an LC-MS based SS/NTA approach to characterize the chemical exposome of Slovenian children. We used enzymatic deconjugation to degrade phase II metabolites, which aided in compound separation using ultra-high performance liquid chromatography (UHPLC) as well as compound identification due to decreased molecular complexity. Deconjugated urine was extracted and concentrated using solid-phase extraction (SPE) and the BoE were separated and detected using UHPLC-QLIT-Orbitrap MS. Application of *in-silico* fragmentation tools and mass spectral library matching enabled the identification of 74 BoE. The presented study demonstrates a simple and high-throughput workflow for NTA and SS of HBM samples, and provides proof-of-concept analyses of the children's chemical exposome.

2. Materials and methods

2.1 Study design and population

The study involved children aged from 6 to 9 years from urban and rural regions of Slovenia. We recruited 200 participants, of which 100 participants resided in the capital city of Slovenia and represented the urban part of the population. The remaining 100 participants resided in a region in the Eastern Slovenia, which is predominantly rural and characterised by intense agricultural activity. Due to the seasonal variations in agricultural practice, rural participants provided two samples, one in winter and the second at the beginning of summer, reflecting time periods with low and high intensity of nearby agricultural activity. Altogether, we obtained 300 samples of first morning urine.

Urban participants were recruited within CROME-LIFE+ project ('Cross-Mediterranean Environment and Health Network', 2013-2017), while rural participants within the national project 'Exposure of children and adolescents to selected chemicals through their habitat environment (2016-2019)'. The eligibility criteria for the participants were to have resided in the selected region for at least 3 years and to not receive medication for any chronic liver or kidney disease. The population consisted of 50 % female and 50 % male participants, which was consistent also within location and sampling-time subgroups.

Parents or legal guardians provided the informed written consent and were able to withdraw from the study at any time. Ethical permissions were granted by Republic of Slovenia National Medical Ethics Committee (65/09/14 and 0120-118/2017/3).

2.2 Sample collection

Participants provided their first morning urine in the collection vessels, distributed by the organizer institution (Jožef Stefan Institute, Ljubljana, Slovenia). Samples were immediately aliquoted at the laboratory of the Clinical Chemistry and Biochemistry Institute of the University Medical Centre Ljubljana into 2 mL criovials, frozen on site with solid ice, and transported to Jožef Stefan Institute, where they were stored at -80 °C until the analysis.

2.3 Sample preparation

The sample preparation procedure was based on our in-house developed method (Tkalec et al., manuscript under review). Briefly, 1 mL of urine was spiked with the internal standard ($^{13}C_3$-caffeine) at 3 ng/mL and deconjugated with 250 U/mL of β-glucuronidase (Abalone, purified) for 18h at 37°C. The samples were then extracted on Oasis HLB 60 mg 96-well plates, which were preconditioned with 1 mL of acetonitrile (ACN), methanol (MeOH) and water, respectively. After loading, the sorbent was washed with 1 mL of 5 v/v % MeOH in water and eluted with 1.5 mL 10 v/v % MeOH in ACN. The eluates were dried under a gentle stream of nitrogen and kept frozen, before being reconstituted in 50 µL of MeOH prior to the analysis.

2.4 UHPLC-HRMS2

Compounds were separated based on previously published method (Tkalec et al., manuscript under review). In short, the separation was performed on the Shimadzu LC-30AD UHPLC using Waters Acquity HSS-T3 (2.1 × 100 mm, 1.8 µm) reversed phase column with water (A) and ACN (B) as mobile phases. Elution gradient was: 5-15 % B (0.01-1 min), 15-25 % B (1-5 min), 25-40 % B (5-8 min), 40-60 % B (8-18 min), 60-75 % B (18-22 min), 75-85 % B (22-24 min), 85-100 % B (24-28 min), 100-5 % B (28-30 min), 5 % B, 30-35 min. Flow rate was 0.3 mL/min, column was heated to 35 °C. The injection volume was 1 µL.
The UHPLC was coupled to Orbitrap Fusion™ Tribrid™ Mass Spectrometer (Thermo Fisher Scientific Inc., Waltham, MA). Heated electrospray ionization (HESI) was used as the ionisation source, at the spray voltage of 4500 V or -3500 V, sheath gas flow 40 L/min, nebulizer auxilliary gas 15 L/min, and sweep gas 2 L/min. The ion transfer capillary was heated to 350 °C and collision energy 20 eV. The full-scan mass acquisition covered the mass range of 100-900 *m/z* and was performed at the resolution of 120 000 FWHM (full width at half maximum at *m/z* 200) while MS2 data was acquired with the resolution of 60 000 FWHM. The automatic gain control (AGC) was 5 $\times 10^5$ ions and maximum injection time 50 ms. Cycle time was 0.8 s.

Inclusion list for suspect screening
To enable efficient suspect screening, a suspect inclusion list was used during data acquisition. Suspect list included 801 entries of calculated m/z values for molecular ions of xenobiotic compounds compiled from Exposome Explorer (Neveu et al., 2017), T3DB (Wishart et al., 2015) and HBM4EU priority substances and pesticides and their 1st phase metabolites considered in the Harmonized SPECIMEN study (https://www.hbm4eu.eu/). Each matching ion within the mass error of ± 3 ppm was fragmented and its MS^2 recorded in ion trap (IT) segment of Orbitrap Fusion™ Tribrid™ Mass Spectrometer. Hence, the MS2 of the matching ions from our suspect list were recorded at the unit resolution. Such procedure enabled simultaneous SS/NTA screening.

2.5 Quality control (QC) procedures

QC was maintained at several levels, i.e. by QC samples, procedural blanks, solvent blanks and by the predetermined acquisition order.

QC samples were prepared by pooling 200 µL of samples spiking with the internal standards to 3 ng/mL and with a mixture of non-labelled standards to the final concentration of 20 ng/mL. The list of standards used for QC is presented in Table 1. Caffeine-$^{13}C_3$ was used as an internal standard for following positive mode acquisition, while ethyl paraben-$^{13}C_6$ was used for negative ionization mode.

Table 1: List of native and labelled standards spiked to QC samples

Standard	Elemental composition	Monoisotopic mass	RT (min)
Omethoate	C5H12NO4PS	213.0225	2.63
Alitame	C14H25N3O4S	331.1566	5.30
Acridone	C13H9NO	195.0684	8.45
Ketoprofen	C16H14O3	254.0943	10.01
Carbamazepine	C15H12N2O	236.095	9.31
Aminoanthraquinone	C14H9NO2	223.0633	10.96
Fenamiphos	C13H22NO3PS	303.1058	14.62
Coumaphos	C14H16ClO5PS	362.0145	20.12
Phoxim	C12H15N2O3PS	298.0541	34.78
Caffeine-$^{13}C_3$	C5*13C3*H10N4O2	197.0904	3.89
Ethyl paraben-$^{13}C_6$	C3*13C6*H10O3	172.0831	8.91

After spiking, the QC samples were processed following the same way as the investigated samples.
The standards were monitored in real-time during data acquisition to ensure the control over the performance of the analytical system. After data acquisition, mass errors, RT drifts and trends in areas of native standards were checked to assess the quality of data acquisition. Batch acquisition has been deemed satisfactory within deviation in RT of ± 0.4 min, and mass accuracy of ± 5 ppm for each standard. Areas of standards were plotted and screened for any increasing or decreasing trends. Figure SI-1 presents areas of native standards across each QC sample while SI-QC_parameters.xlsx tabulates areas, mass accuracies and retention time drifts for each standard in QC samples. Internal standards were checked after acquisition in the final aligned feature list and each sample without corresponding feature with RT drifts and mass error within specified limits of 0.4 min and 5 ppm, respectively, was rejected. Areas of internal standards for each sample are plotted in Figure SI-2. Accompanying plotting of standards in QCs, IS area plot served as an additional measure of quality for data acquisition.

Procedural blanks were prepared by freezing LC-MS water three months before sample preparation in same criovials used for storing actual samples. They were subsequently spiked with isotopically labelled internal standards at the same concentration as the actual urine samples, and processed using the same sample preparation procedure as the investigated samples to check for contamination during sample preparation. The procedural blanks were prepared in triplicate.
Solvent blanks were used to control sample carryover and were injected after every 10th sample.

Acquisition order

At the beginning of the sequence, four solvent blanks and five QC sample replicates were injected to check the starting system's performance and the integrity of the QC samples. Afterwards, procedural blanks were injected. Randomly ordered samples were analysed in batches. At the beginning of each batch, a solvent blank and a QC sample were injected. QC samples and solvent blanks were injected per every 15 investigated samples. Each QC sample replicate was injected no more than ten times and the order of the injected QC replicates was alternating to avoid non-random trends.

2.6 Data processing

Raw data was first transformed from raw format to mzXML using MSConvert and analysed using MzMine 2.53 (Pluskal et al., 2010). The processing parameters were optimized to allow for the detection of the spiked non-labelled standards and are presented in SI-1. Processing thresholds to detect BoE were rather low, resulting in computationally demanding amounts of data. Samples with missing peaks for internal standard caffeine-$^{13}C_3$ were excluded from further processing. Any features present in the procedural blanks were removed from dataset.
Only features with acquired MS2 data were filtered and further processed.

2.7 Compound identification

2.7.1 Non-targeted

Identification procedure is presented in Figure 1. After processing we submitted all acquired MS2 spectra in one joined .mgf file to Sirius-CSI:Finger-ID (Dührkop et al., 2015, 2013). Once candidates were generated, we selected only the hits with 100 % elemental formula matching score within 5 ppm of mass error and highest library match (KEGG, ChEBI or PubChem) with the minimum matching score of 60 % as candidates.

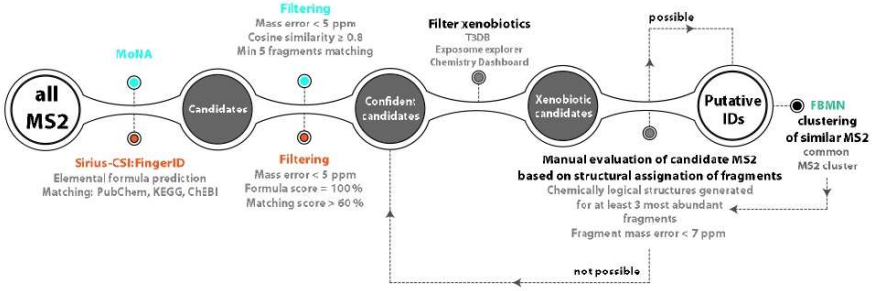

Figure 1: Identification workflow for NTS.

The prioritized list of candidates, which passed the criteria was exported as .csv file and subsequently manually filtered to retain only features matching a potential BoE. This was done based on reviewing origin and use of candidates by compound

databases T3DB (Wishart et al., 2015), Exposome Explorer (Neveu et al., 2020) and Chemistry Dashboard (Williams et al., 2017). MS2 of each filtered match was then scrutinized by manually explaining MS2. For at least 3 most abundant fragments chemically logical structures were required to be generated. Maximum mass error for successful match between proposed structure and the fragment was permitted to 7 ppm. If MS2 was successfully explained, the candidate identity was tentatively assigned. The NT identification data along with molecular formulas of proposed fragments is presented in the document SI-NT_spectral_data.xlsx. Structurally assigned MS2 spectra are presented in SI-4.

The same process was repeated with MoNA (Mass Bank of North America), mass repository with experimental MS2 data (https://mona.fiehnlab.ucdavis.edu/). The criteria for successful identification were the mass error of ≤ 5 ppm and cosine similarity score ≥ 0.8, with at least 5 fragments matching. If any of MS2 spectra consisted of less than 5 fragments, then matching of all fragments was required. Matching of spectra with less than 3 fragments in MS2 was regarded as insignificant. Like above, MS2 of all matches was then structurally assigned.

All putatively identified BoE were then submitted to Feature Based Molecular Networking (FBMN) (Wang et al., 2017), clustering features with similar fragmentation patterns together. Clustering features were then identified by manual interpretation of their MS2 spectra, i.e. by comparing MS2 spectra of an unknown feature with structurally assigned MS2 spectrum of previously identified compound and then proposing structure of the candidate.

2.7.2. Suspect screening

When available, experimental MS2 data were collected from MoNa, however, due to scarcity of experimental mass spectral data for several compounds included in the list, the *in-silico* MS2 library was generated using CFM-ID (Allen et al., 2014). MH+ were measured with orbitrap analyser at high-resolution, while the MS2 of candidates from the suspect list were acquired in linear ion trap and were therefore of unit resolution. In this view, the identification parameters were adjusted for SS: mass error of molecular error was required to be ≤ 5 ppm, cosine similarity score was required to be ≥ 0.8, with at least 5 fragments matching. If any of MS2 spectra consisted of less than 5 fragments, then matching of all fragments was required. Matching of spectra with less than 3 fragments in MS2 was regarded as insignificant.

2.8 Regional and temporal differences

OPLS-DA model was constructed based on putatively identified BoE to check for differences between 1) exposure of children in rural and urban regions and 2) difference between winter and summer sampling of rural population. For statistical analyses urine dilution was corrected using specific gravity adjustment (Suwazono et al., 2005), while compound loss during sample preparation and data acquisition was corrected by normalizing the data to area of labelled internal standard caffeine-$^{13}C_3$. Models were constructed using Simca 15.0.2 (Sartorius Stedim Data Analytics) on natural logarithm transformed and Pareto scaled data. Missing values were replaced by lowest non-zero value divided by two for each BoE. Number of

components was determined using 7-fold cross-validation. Overfitting was checked with permutation test (N=100). Discriminatory features were determined using S-plot and Variable Importance for the Projection (VIP) score. Discriminatory features were checked using univariate non-parametric Mann-Whitney U-test.

3. Results and discussion

3.1 Quality control

According to the described procedure, whose development and optimization are in detail discussed elsewhere (Tkalec et al., manuscript under review) we aimed to characterize the chemical exposome using UHPLC-HRMS in NT and SS mode. LC-MS data was acquired in both positive and negative mode. However, in the negative mode we did not observe labelled standard ethyl paraben-$^{13}C_6$, intended for monitoring quality of data acquisition in negative mode. This was attributed to inappropriately set data acquisition threshold. As the result, we rejected all the data acquired in the negative mode and hence continued only with the data acquired in the positive mode.

To ensure data quality, QC measures were held on several levels. Areas, mass accuracies and retention time drifts of nine native standards were checked after data acquisition (see Figure SI-1 and SI-QC_parameters.xlsx). No trends were observable in the areas of the native standards, except fluctuating trend which is the consequence of alternating order of injection of different QC samples. Due to missing peaks for phoxim, this standard was excluded from monitoring. Mass accuracies did not exceed 5 ppm, in fact for the majority of standards, the average mass error did not exceed 3 ppm, showing high mass accuracy of the acquired data. Retention times remained relatively constant throughout the run, with maximum retention time drift of 5 % for the standard omethoate. Non-random trends in acquisition were monitored by observing areas of internal standard in each sample as presented in Figure SI-2, which shows no non-random trends.

To avoid erroneous conclusions about the exposure, all features present in solvent and procedural blanks were excluded from further consideration. Even though certain compounds that leach from laboratory equipment and are present in solvents, as for example phthalates, could be present in the investigated urine samples, we avoided identifying the parent compounds, that would have inconclusive source. The rationale was that it is more probable their metabolites will be identified, which originate almost exclusively from the exposure.

No sample to sample carryover was observed from analysing solvent blanks which were injected following each QC within the batch.

3.2 Detection and identification of BoE in urine of children

Resulting data was processed using MzMine2.53 (Pluskal et al., 2010). Processing parameters were set to allow for detection of low abundance features, therefore very low thresholds for mass detection and subsequent steps were required. The criterion for the retention time (RT) drift was set according to the RT of standards spiked into QC samples (Tkalec et al., manuscript under review). The maximum RT drift was ± 0.31 min, so RT limit was set at ± 0.4 min. A too narrow RT limit

can cause doubling of features, while a too high one can cause merging of non-identical features. Both events can negatively impact the identification ability, so this parameter should be carefully set. The detection frequencies of the identified BoE offer only a mere approximation, as a compound might appear in final data matrix as two or more distinct features.

Along with the stability of RT, the stability of instrument's mass accuracy was precisely followed (Tkalec et al., manuscript under review), through monitoring of the drifts of standards' mass accuracies in the QC samples. The maximum mass error was determined for the protonated molecule of the standard ketoprofen at +3.53 ppm, demonstrating that errors were generally low. Nevertheless, to be on the safe side, we set the permitted mass error for the MS1 level at 5 ppm.

As we filtered out only the features with corresponding MS2 data, the final data matrix contained 34015 features. As mentioned before, data acquisition and processing thresholds were set low to maximize the probability of acquiring data on low levels BoE, therefore the number of the features with acquired MS2 data in the resulting aligned list is reasonably high. From this data matrix we tentatively identified 74 BoE in total, out of which 36 were found using NT analysis (Table 2) and 38 using SS (Table 3). As a result of identification workflow through mass spectral library matching or *in-silico* identification connected to the structural assignation of their MS2 spectra, BoE identified with NTA were at the Schymanski confidence level of 2 (Schymanski et al., 2014). Of NT identified BoE, 18 were identified using Sirius-CSI:FingerID, 10 through mass spectral database MoNA and 10 using feature-based molecular networking (FBMN) (Wang et al., 2017). The latter revealed the identification of two compound clusters, the penicillins and polyethylene glycol cluster. Phenoxymethyl penicillin (MH+ 351.1004) and decaethylene glycol (MH+ 459.2800) were identified using Sirius-CSI:FingerID, and served as the basis for identification of other congeners within molecular networks. Assigned MS2 spectra with identification data and corresponding identification scores are presented in SI-NT_spectral_data.xslx.

NT identification protocol considered only the highest scoring matches. If the best match could not be confirmed by subsequent procedures, the candidate was rejected and the next highest-scoring candidates not considered. This enabled more efficient and less time-consuming screening, however it also increased loss of potential matches and higher number of false negatives.

Table 2: Compounds identified using NT analysis. Presented are their elemental formulas, mass errors, RT, detection frequencies and classification.

Identity	Metabolite of	Biomarker group	Elemental composition	Theoretical mass (MH+)	Experimental mass (MH+)	Mass error (ppm)	RT (min)	Detection frequency
3-Hydroxycotinine	Nicotine (Raja, 2016)	Biomarker of smoking	C10H13N2O2+	193.0977	193.0972	2.589	2.36	0.24
Nonaethylene glycol	/	PCP	C18H39O10+	415.2538	415.2529	2.167	4.7	0.13
Undecaethylene glycol	/	PCP	C22H47O12+	503.3062	503.3091	-5.762	4.71	0.86
Decaethylene glycol	/	PCP	C20H43O11+	459.28	459.2821	-4.572	5.21	0.2
Dodecaethylene glycol	/	PCP	C24H51O13+	547.3324	547.3307	3.106	5.56	0.68
Tetradecaethylene glycol	/	PCP	C28H58O15+NH4+	652.4152	652.4136	2.452	5.69	0.53
Pentaethylene glycol decyl ether	/	PCP	C20H43O6+	379.3054	379.3062	-2.109	20.15	0.24
Tetraethylene glycol decyl ether	/	PCP	C18H39O5+	335.2792	335.2794	-0.597	20.44	0.1
DEET	/	PCP, repellent	C12H18NO+	192.1383	192.1383	0.000	11.11	0.01
Icaridin	/	PCP, repellent	C12H24NO3+	230.1751	230.1756	-2.172	13.06	0.02
4-Hydroxybenzophenone	/	PCP, UV-filter	C13H11O2+	199.076	199.0758	1.005	10.61	0.33
Octabenzone	/	PCP, UV-filter	C21H27O3+	327.1955	327.1952	0.917	27.91	0.01
N-(2,6-dimethylphenyl)-2-hydroxyacetamide	Metalaxyl M (EPA, 1988)	Pesticide, Fungicide	C10H14NO2+	180.1019	180.1024	-2.776	2.94	0.31
N-(2,4-dimethylphenyl)formamide	Amitraz (Lazarus et al., 2021)	Pesticide, Fungicide	C9H12NO+	150.0913	150.0918	-3.331	5.68	0.55
Naphthoxyacetic acid	/	PGR	C12H11O3+	203.0703	203.0707	-1.97	5.81	0.14
Trinexapac	/	PGR	C11H13O5+	225.0757	225.0763	-2.666	6.32	0.16
Phenoxymethyl penicillin / Penicillin V	/	Pharmaceutical	C16H19N2O5S+	351.1009	351.1004	1.424	6.18	0.01
Phenoxymethyl penicilloyl	Penicillin V (CHEBI:53703)*	Pharmaceutical	C16H21N2O6S+	369.1115	369.1116	-0.271	4.62	0.1
N-methylphenoxymethyl penicilloyl	Penicillin V**	Pharmaceutical	C17H23N2O6S+	383.1271	383.1282	-2.871	5.14	0.02
Amoxycilloyl	Amoxycillin (CHEBI:53705)*	Pharmaceutical	C17H24N3O5S+	382.1431	382.1434	-0.785	5.83	0.11
Levetiracetam	/	Pharmaceutical	C8H15N2O+	171.1128	171.1133	-2.922	2.79	0.07
N-acetylphenoxymethyl penicilloyl	Penicillin V**	Pharmaceutical	C18H23N2O7S+	411.122	411.1234	-3.405	4.49	0.11
Carbamazepine epoxide	Carbamazepine (Potter and Donnelly, 1998)	Pharmaceutical	C15H13N2O2+	253.0972	253.0974	-0.790	5.13	0.1
Aminophenol	Paracetamol (Athersuch et al., 2018)	Pharmaceutical	C6H8NO+	110.06	110.0604	-3.634	5.95	0.29
Lauramide DEA	/	Surfactant	C16H34NO3+	288.2533	288.2536	-1.041	18.82	0.11
Octylphenol	/	Surfactant byproduct	C14H23O+	207.1743	207.1744	-0.483	19.7	0.02
Propenylaniline	/	Unknown use	C9H12N+	134.0964	134.0968	-2.983	5.5	0.83
6-Phenylpicoline	/	Unknown use	C12H12N+	170.0964	170.0968	-2.352	5.7	0.06
Cycloheptylamine	/	Unknown use	C7H16N+	114.1277	114.1281	-3.505	8.28	0.05
Chloroisoquinoline	/	Unknown use	C9H7NCl+	164.0262	164.0266	-2.439	8.56	0.02
Dicyclohexyl urea	/	Unknown use	C13H25N2O+	225.1961	225.1965	-1.776	12.26	0.01
Isoquinoline	/	Unknown use	C9H8N+	130.0651	130.0654	-2.307	4.74	0.9
Methylacridine	/	Unknown use	C14H12N+	194.0964	194.0968	-2.061	9.81	0.01
Naphthylamine	/	VOC	C10H10N+	144.0808	144.0812	-2.776	5.93	0.15
Cresol	/	VOC	C7H9O+	109.0648	109.0652	-3.668	4.31	0.41
Benzaldehyde	/	VOC	C7H7O+	107.0498	107.0496	1.868	4.43	0.65

*CHEBI database entry (https://www.ebi.ac.uk/chebi/init.do, accessed June 2021), numerical value specifies CHEBI entry
**Proposed identity based on mass spectrum, not available in literature

By using SS approach 11 BoE were identified through *in-silico* generated library employing CFM-ID (Allen et al., 2014) and one compound by comparison with the library spectrum from MoNA. The remaining 26 compounds were identified by manual assignation of the MS2 spectra of molecular formula candidates matching entries in the suspect list. IUPAC names with SMILES, InCHI, InCHI-Keys, matching scores where applicable and characteristic ions are presented in SI-SS_data.xlsx. Due to identification through experimental and *in-silico* mass spectral libraries the BoEs identified using SS approach were identified at Schymanski confidence level 3 (Schymanski et al., 2014). To be as comprehensive as possible, 801 entries were included into SS list, which is relatively exhaustive and generated a large number of hits. The hits required to be subsequently individually checked, which demanded extensive identification time. Other SS studies employed even more extensive SS lists, for example with 1158 (Caballero-Casero et al., 2021) and 1450 (Chen et al., 2021) entries. To reduce the identification time and workload required for SS, a compromise between number of entries and subsequently large number of hits and time-efficacy could be made by prioritizing suspect list according for population specificities. For example, to include larger number of pesticide-related entries in study of rural population while reducing the number of traffic-related ones, and the opposite for an urban population. This would reduce the number of hits, however it might lead to a significant bias due to, for example, multiple sources of certain compounds, transit and relocation of people and dietary differences.

Using a tribrid instrument enabled us to perform SS and NT screening simultaneously. During data acquisition, inclusion list of suspects was applied, as described in section 2.4. For each scan, the detected *m/z* matching that of any in the suspect list (within mass error) were automatically fragmented in linear ion trap, while orbitrap part was operating at the same time in DDA mode, fragmenting 10 most abundant ions. Therefore, MS2 spectra of compounds matching m/z values of those in the inclusion list are recorded in low resolution, whilst MS2 of other features are recorded in HR.

This process ensured acquisition of MS2 of very low abundance BoEs in the suspect list, that would otherwise not be fragmented in typical DDA mode. The main drawback is, that the MS2 of suspect hits is acquired of low-resolution. Due to the same reason, hits from SS do not appear in the list of the hits from NTS, as identification protocols used different thresholds and approaches.

Table 3: Compounds identified using SS approach. Presented are their elemental formulas, mass errors, RT, detection frequencies and mode of identification

Identity	Metabolite of	Group	Elemental composition	Theoretical mass (MH+)	Experimental mass (MH+)	Mass error (ppm)	Compound_RT	Detection frequency
Cotinine	Nicotine (Raja, 2016)	Biomarker of smoking	C10H13N2O+	177.1028	177.1028	-0.068	2.83	0.22
Caffeine	/	Biomarker of soda, tea	C8H11N4O2+	195.0882	195.0882	0.005	2.36	0.98
Celestolide	/	PCP, fragrance	C17H25O+	245.1905	245.1902	1.387	8.55	0.06
1-Methyl-alpha-ionone	/	PCP, fragrance	C14H23O+	207.1749	207.1744	2.365	19.7	0.02
Ethylparaben	/	PCP, preservative	C9H11O3+	167.0708	167.0706	1.311	6.05	0.17
Butylparaben	/	PCP, preservative	C11H15O3+	195.1021	195.102	0.61	6.08	0.06
Dioxybenzone	/	PCP, UV-filter	C14H13O4+	245.0814	245.0811	1.159	7.53	0.04
Terbuthylazine	/	Pesticide, Algicide	C9H17ClN5+	230.1172	230.1173	-0.226	8.2	0.03
Pyrimethanil	/	Pesticide, Fungicide	C12H14N3+	200.1188	200.1186	0.859	6.37	0.04
Tebuconazole	/	Pesticide, Fungicide	C16H23ClN3O+	308.153	308.1535	-1.736	6.37	0.14
4-[(4,6-dimethylpyrimidin-2-yl)amino]phenol	Pyrimethanil (Faniband et al., 2019)	Pesticide, Fungicide	C12H14N3O+	216.1137	216.114	-1.448	8.27	0.11
4-Hydroxychlorpropham	Chlorpropham (Carrera et al., 1998)	Pesticide, Herbicide	C10H13ClNO3+	230.0584	230.0571	5.633	2.34	0.18
Metholachlor	/	Pesticide, Herbicide	C15H23ClNO2+	284.1417	284.1401	5.744	4.93	0.1
Desisopropyl atrazine	Atrazine (Joo et al., 2010)	Pesticide, Herbicide	C5H9ClN5+	174.0546	174.0554	-4.32	4.29	0.55
Desethyl atrazine	Atrazine (Joo et al., 2010)	Pesticide, Herbicide	C6H11ClN5+	188.0703	188.0709	-3.201	8.98	0.02
2-Isopropyl-6-methyl-pyrimidin-4-ol	Diazinon (Shemer and Linden, 2006)	Pesticide, Insecticide	C8H13N2O+	153.1028	153.1025	2.123	6.22	0.08
Prohexadione	/	PGR	C10H13O5+	213.0763	213.0765	-0.948	2.46	0.33
Bisphenol G	/	Plasticizer, bisphenol	C11H13O5+	313.2168	313.2169	-0.463	5.91	0.09
Bisphenol F	/	Plasticizer, bisphenol	C21H29O2+	201.0916	201.0911	2.263	7.77	0.06
Bisphenol A	/	Plasticizer, bisphenol	C13H13O2+	229.1229	229.1231	-1.069	8.23	0.02
Monobenzyl phthalate	BBP (Huang et al., 2021)	Plasticizer, phthalate	C15H17O2+	257.0814	257.0812	0.716	8.34	0.48
Monobutyl phthalate	DBP (Huang et al., 2021)	Plasticizer, phthalate	C15H13O4+	223.0965	223.0949	7.172	5.06	0.04
Monohydroxybutyl phthalate	DBP, BBP (Huang et al., 2021)	Plasticizer, phthalate	C12H15O4+	239.0919	239.092	-0.314	6.63	0.11
Monocyclohexyl phthalate	DCHP (Huang et al., 2021)	Plasticizer, phthalate	C12H15O5+	249.1127	249.1126	0.337	6.72	0.15
Mono-2-ethyl-5-hydroxyhexyl phthalate	DEHP (Huang et al., 2021)	Plasticizer, phthalate	C14H17O4+	295.1545	295.1551	-1.867	7.97	0.35
Mono-2-ethyl-5-oxohexyl phthalate	DEHP (Huang et al., 2021)	Plasticizer, phthalate	C16H23O5+	293.1389	293.1393	-1.368	8.44	0.24
Monoethylhexyl phthalate	DEHP (Huang et al., 2021)	Plasticizer, phthalate	C16H21O5+	279.1591	279.1581	3.582	9.67	0.14
Monoethyl phthalate	DEP (Huang et al., 2021)	Plasticizer, phthalate	C16H23O4+	195.0657	195.0658	-0.338	7.76	0.05
Monooxoisodecyl phthalate	DIDP (Huang et al., 2021)	Plasticizer, phthalate	C10H11O4+	321.1702	321.1701	0.308	6.34	0.06
Monocarboxyisodecyl phthalate	DIDP (Huang et al., 2021)	Plasticizer, phthalate	C18H25O5+	337.1651	337.1638	3.896	6.5	0.13
Monohydroxyisodecyl phthalate	DIDP (Huang et al., 2021)	Plasticizer, phthalate	C18H26O6+	323.1858	323.1858	0.151	8.12	0.06
Monoisononyl phthalate	DINP (Huang et al., 2021)	Plasticizer, phthalate	C18H27O5+	293.1753	293.1756	-1.078	7.17	0.06
Monohydroxyisononyl phthalate	DINP (Huang et al., 2021)	Plasticizer, phthalate	C17H25O4+	309.1702	309.1699	0.967	7.98	0.07
Monooxoisononyl phthalate	DINP (Huang et al., 2021)	Plasticizer, phthalate	C17H25O5+	307.1545	307.1541	1.461	8.06	0.08
Monoisononyl phthalate	DINP (Huang et al., 2021)	Plasticizer, phthalate	C17H23O5+	293.1747	293.173	5.799	9.39	0.93
Monocarboxyisooctyl phthalate	DIOP, DINP (Huang et al., 2021)	Plasticizer, phthalate	C17H23O6+	323.1494	323.1501	-2.089	7.21	0.11
Monooctyl phthalate	DOP (Huang et al., 2021)	Plasticizer, phthalate	C16H23O4+	279.1591	279.1578	4.657	16.19	0.01
Monopentyl phthalate	DPP (Huang et al., 2021)	Plasticizer, phthalate	C13H17O4+	237.1127	237.1127	-0.067	6.39	0.29

Low-abundance compounds are hard to detect in complex matrices such as urine and even harder to identify due to sometimes insufficient quality of acquired MS2 spectra. Enzymatic hydrolysis enabled the cleavage of conjugate bonds, thus forming parent BoE or their 1st stage metabolites, which greatly benefitted their identification. First of all, deconjugation increases the concentration of BoE or its 1st stage metabolite, since different glucuronide or sulphate metabolites are transformed back into one species, and with that the chance of acquiring high quality MS2 is increased. Furthermore, MS data on conjugated species are very scarcely included in databases and MS2 libraries used for searching for potential molecular candidates, which results in the failure of identification. Third, conjugated species are, due to the nature of the conjugate moiety, more prone to ESI negative ionisation. On the contrary, parent BoE or their 1st stage metabolites, when ionised under electrospray positive conditions, yield more abundant and richer MS2 spectra, providing more structural information to enable successful identification. Furthermore, attaching aforementioned moieties to parent molecules increases molecular complexity and with that their acquired MS2, which in turn detrimentally affects identification through either *in-silico* approaches or if necessary, manual assignment of MS2 spectra. On the other hand, even though deconjugation step increases chances of detecting and identifying at least one metabolite, this results in missing of information on conjugated metabolites and for example, free vs conjugated ratio, which is typically very relevant in toxicological research. To assess this and more comprehensively address the issue, deconjugated and non-deconjugated samples could be analysed in parallel, however this would result in significant prolongation of analysis time.

Furthermore, enzymatic deconjugation by glucuronidase/arylsulfatase can in some cases chemically affect BoE by non-specific activity, and produce new chemical species (Blount et al., 2000).

Sample preparation protocol involved solid-phase extraction and although broad polarity-range sorbent was used it is highly likely that very polar compounds have been eliminated. This contributed to less chromatographic overlapping at low retention times, a chromatographic region which is inherently rich due to polar nature of urine, however this also resulted in loss of some information on very polar BoEs and enhanced a focus on medium to low polarity compounds. Because of a lack of universal sample preparation and analytical techniques, laboratory procedures require a certain compromise. To broaden the coverage of compounds even more, the sample preparation procedures could be multiplexed to several fractions and analysed using multiple separation techniques. This would provide a larger overview of potential BoEs, however at the same time multiply the analytical time and cost.

In the applied identification approach, each feature was submitted for identification and only hits at the high degree of matching were retained. The high probability hits were screened for potential BoE. Due to that, this approach missed a large number of BoE to potentially concerning chemicals, which were detected with low quality MS2. As mentioned before, for example, using suspect list with 801 suspect compounds, along with 38 tentatively identified, we observed 980 total hits with matching at least one theoretical molecular mass on suspect list, matching in total unique 233 xenobiotics and their BoEs, showing that acquired data still holds a

large amount of un-retrieved information and potentially much higher number of BoE. Furthermore, even though our focus was on identifying the BoE, the identification procedure produced large amount of other tentatively identified compounds, as for example regarding dietary chemicals and their metabolites which were out of scope of this paper and not studied further.
NTS/SS in this study were used as an initial screening operable within HBM to derive first overview of the populations' exposure. The compounds were therefore identified at confidence levels 2 and 3, and to lead to definitive conclusions should be in future confirmed by standards and be followed in detail by a subsequent targeted full-scale HBM study.

3.2 Implications for the exposure of children

Compounds found by NT and SS are identified tentatively, acting as a basis for subsequent targeted studies to confirm the exposure and accurately quantify the exposure and explore exposure pattern in detail. However, within the scope of certainty of NT and SS, certain conclusions on the exposure can be implied.
Tentatively identified BoE indicate that children are exposed to a wide set of organic contaminants, falling into several compound classes.
BoE to 12 pesticides were identified. We found the evidence of exposure to fungicides pyrimethanil, metalaxyl and tebuconazole, algicide terbuthylazine, herbicides metholachlor and chlorpropham. The latter was previously detected by similar SS approach in urine of pregnant women (Bonvallot et al., 2021). Further, we found BoE to several plant growth regulators (PGR), prohexadione, naphthyloxyacetic acid and trinexapac-ethyl. All of the above are EU approved products, however we identified BoE to three restricted pesticides. Atrazine, amitraz and diazinon were restricted from use in EU, atrazine and amitraz in 2004 (documents 2004/248/EC and 2004/141/EC), and diazinon in 2007 (document 2007/393), however the data here indicates continued exposure.
Personal care products (PCPs) are used daily and contain many groups of different compounds, many of which are continuously monitored in established HBM schemes. BoE to 16 PCPs were identified in urine of children, namely parabens, UV-filters, fragrances, repellents, surfactants and polyethylene glycols and polyethylene glycol ethers. Out of parabens, we identified two, ethyl and butyl paraben. This is in agreement with the results of previously published targeted analysis of the same population, where we quantified the levels of several parabens, among others also ethyl and butyl paraben (Tkalec et al., 2021). Parabens have also been previously identified by similar NT/SS approaches in urine (Caballero-Casero et al., 2021) and serum (Chen et al., 2021) and by a large number of targeted studies (Wei et al., 2021). Out of remaining compounds, the most commonly detected compound was undecaethylene glycol (86 %), a congener of polyethylene glycol (PEG) and polyethylene glycol ethers (PEGE). This class of compounds is used for various applications, particularly in cosmetics as emulsifiers, thickeners, humectants, cleansing agents and non-ionic surfactants, as excipients in pharmaceutical formulations and others (Fruijtier-Pölloth, 2005). Even though they are widely present in everyday life, there is scarcely any study considering human exposure to PEGs and PEGEs. Same is true for another tentatively identified compound, lauramide DEA, a surfactant widely used in

cosmetics and other PCPs like shampoos and soaps (Mathews et al., 1996). Although considered safe, both classes of compounds could be in future considered as contaminants of emerging concern (CECs).

Similar to PCPs, a substantial group of compounds, which are likewise already widely monitored using HBM are plasticizers and plastic-related compounds. Bisphenols are used in production of polycarbonate plastic and epoxy resins. In the samples of Slovenian children, we found BoE to BPA and its alternative, BPF along with much less reported and monitored alternative, BPG. As for parabens, this is with the agreement with results of targeted analysis of same samples, where BPA and BPF have been quantified in the majority of samples (Tkalec et al., 2021). BPG however, was not analysed in that population, and the exposure to this compound has not been yet widely studied in literature. Identifying it using SS/NT approach in urine of children suggests its inclusion in the existing HBM programmes to determine its distribution and levels in a wider population.

By far the most detected class of compounds were BoEs to phthalates, out of which, monoisononyl phthalate was most commonly detected (93 %). As for parabens, phthalates have likewise been largely monitored and quantified in many targeted studies (Eales et al., 2022) and moreover identified by similar SS studies in urine, where, hydroxylated monoisononylphthalate was detected in even higher frequency (100 %) (Caballero-Casero et al., 2021) and in serum, where the most commonly detected BoE was monodecyl phthalate (Chen et al., 2021), both of them similarly members of high-molecular weight phthalates.

Furthermore, we identified several volatile organic contaminants (VOCs), such as cresol, benzaldehyde and naphthylamine and BoEs to tobacco smoke, cotinine and 3-hydroxycotinine. All of these compounds can be linked to exposure to cigarette smoke, as cresol is released by automobile exhaust, found in air of areas of high traffic and vicinity of gas stations (Risne and Cash, 1990), while naphthylamine is used in various industrial applications, however it has been detected as a product of incomplete combustion in the cigarette smoke (Niu et al., 2018; Yu et al., 2014).

Using NTS we identified BoEs to several pharmaceuticals. Pharmaceuticals are being used intentionally to treat or prevent chronic or acute diseases, or to weaken their symptoms. While an unintentional exposure to pharmaceuticals is possible through contaminated food or water, their doses are significantly lower and probably not detected by SS/NTS. Hence, pharmaceuticals play a rather special role in the exposome analysis, however we still considered them for several reasons. First of all, they served for the confirmation of the applied workflow. During sampling, participants answered an extensive questionnaire involving medication during time of sampling. This data was used *post-hoc* to connect self-reported data to the identified biomarkers and with that confirm the analytical workflow of the study. Second, due to high level of biological activity, pharmaceuticals influence a large array of metabolic pathways, which might be of great significance when considering health implications of exposures. We identified phenoxymethyl penicillin (Penicillin V) and its metabolites, phenoxymethyl penniciloyl, *N*-methyl and *N*-acetyl phenoxymethyl penniciloyl. They were identified in the participants' samples, who reported to take phenoxymethyl penicillin to treat acute tonsillitis. Similarly, amoxycilloyl being a metabolite of amoxycillin, was identified in urine of the participants, who reported using amoxycillin for the treatment of acute ear infection. Further, carbamazepine

epoxide is a pharmacologically active metabolite of an anticonvulsant carbamazepine. Both, carbamazepine epoxide and levetiracetam, another antiepileptic drug, were identified in samples of the participants reporting being treated for epilepsy. Finally, we found aminophenol, a metabolite of paracetamol in the corresponding urine samples of those participants, who reported the use of this pharmaceutical.

Along with compounds with well-defined source, we tentatively identified propenyl aniline, phenyl picoline, cycloheptyl amine, methyl acridine, isoquinoline, chloroisoquinoline and dicyclohexyl urea, for which we were unable to retrieve conclusive information about their use or sources of exposure. Dicyclohexyl urea was, however, also previously detected and identified in human serum using similar non-targeted approach (Hall et al., 2012). These compounds are, to the authors' knowledge, reported being present in human urine for the first time. This urges for monitoring of these chemicals in a larger population, to describe the extent of exposure, sources, toxicological parameters, and to assess health risks connected to the exposure.

3.3 Statistical differences between populations

Specific differences between the presence of BoE at two locations and at two sampling times were investigated using OPLS-DA, where the models demonstrated that the exposure of children differed according to the location and time (Table 4). Even though detection frequencies were low for certain BoEs, model parameters show sufficient explanation and predictability, while avoiding overfitting, confirmed by permutation test. Nevertheless, the results should be still considered with caution.

Discriminatory BoE with VIP-score higher than 0.5 were further validated for significance using non-parametric univariate Mann-Whitney test. The results are presented in Table SI-1 and Table SI-2 and visualized in Figure 1 and Figure 2.

Table 4: OPLS model parameters, goodness of fit (R2Y), predictability (Q2) and number of components used to build the model.

	R2Y (cum)		Q2 (cum)		N	
Model	Winter vs Summer	Urban vs Rural	Winter vs Summer	Urban vs Rural	Winter vs Summer	Urban vs Rural
Experimental	0.562	0.525	0.285	0.298	2	3
Permuted	0.292	0.238	-0.341	-0.51		

3.3.1 Rural vs Urban

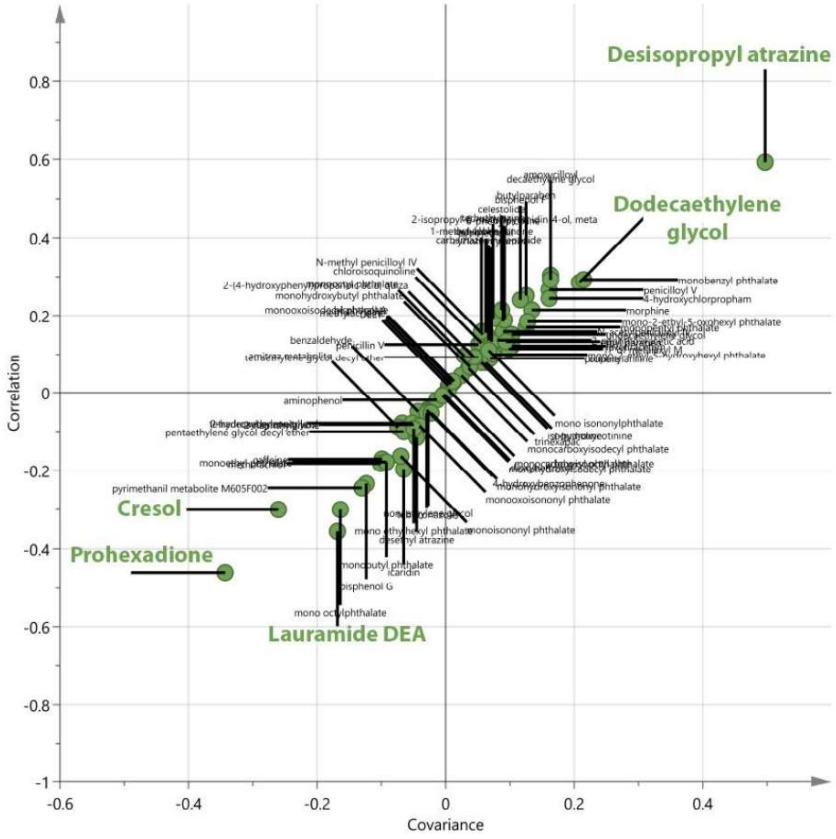

Figure 2: S-plot (correlation vs covariance) visualizing discriminatory BoE between urban and rural samples. Significant features have higher non-zero covariance and correlation (top right and bottom left)

Results of statistical analysis, presented in Table SI-1 show the difference between relative abundances of contaminants among individuals from urban and rural regions. Differential BoE are presented in S-plot of OPLS-DA (Figure 2). A handful of BoE were differently represented in rural and urban populations. BoE to pesticides atrazine and diazinon were elevated in rural region, which could indicate higher intensity of local agricultural activity, typical for the region. PGR prohexadione was, however, higher in urban population, which might indicate dietary exposure of imported food items. The levels of cresol, normally found in the contaminated air of high traffic areas, were expectedly higher in samples of the individuals residing in urban region. Insect repellents are used only when required to avoid tick and mosquito bites, so a higher level of icaridin in urban population could speculatively indicate a group situation, such as a school excursion.

Several other BoE followed location-specific pattern, however the differences were difficult to interpret due reasons such as a large array of applications, for example of surfactant lauramide DEA and cosmetic ingredient decaethylene glycol, or inconclusive sources such as phenyl picoline, bisphenol G and bisphenol F.
Urinary BoE to organic contaminants are often short-lived, reflecting exposure happening at most few days before sampling. Longitudinal samples would more accurately describe the differences, however conclusions from spot samples might still reflect some differences in exposure of urban and rural individuals.

3.3.2 Winter vs Summer

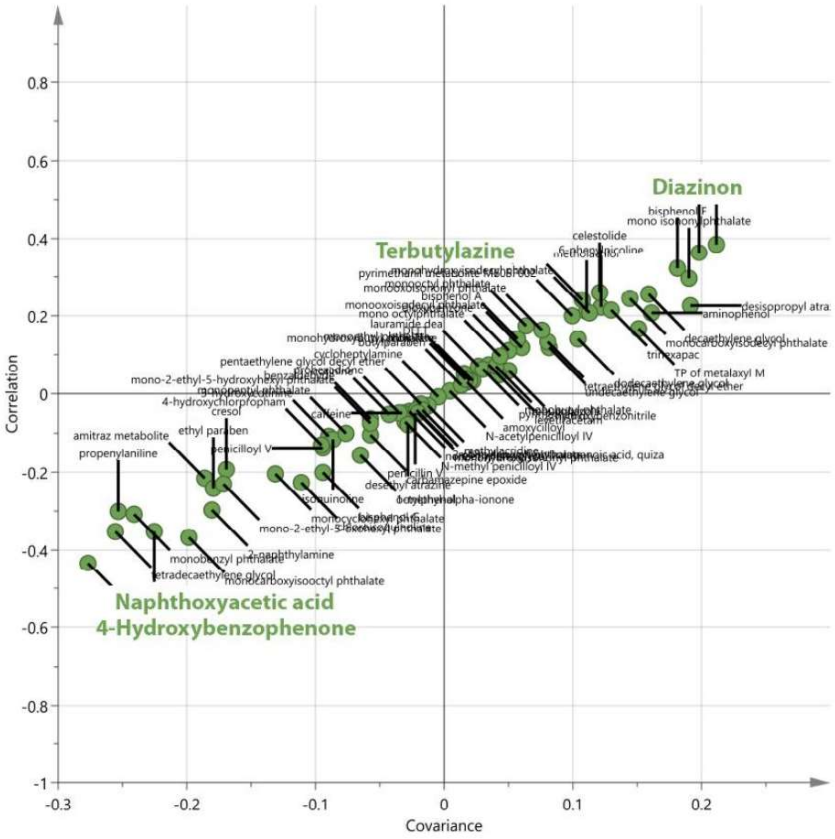

Figure 3: S-plot (correlation vs covariance) visualizing discriminatory BoE between winter and summer sampling. Significant features have higher non-zero covariance and correlation (top right and bottom left)

Exposure is highly individual, some stressors can be present permanently, while some vary with season, changing of location and consumption of seasonal food. Due to varying intensity of nearby agricultural activity, individuals from rural regions were sampled twice, once in winter/early spring 2019 and once during the summer of the same year. Discriminatory BoE for winter versus summer sampling are visualized in S-plot of OPLS-DA (Figure 3), which shows that BoE to pesticides diazinon, terbuthylazine and pyrimethanil with PGR naphthoxyacetic acid were higher in the summertime samples (Table SI-2). This coincides with higher agricultural activity in the region during summer season which is consistent with literature as proximity to fields, field area and spraying season have been identified as determinants of exposure to pesticides before (Teysseire et al., 2021). Levels of BoE to UV-filter 4-hydroxybenzophenone were, contrary to the expected, higher in winter. Seasonal differences of other compounds are, similarly to the regional differences, more difficult to discuss. It should be noted however that by using spot samples such differences are sometimes difficult to explain due to short life time of many chemicals of which BoE are found in urine.

Conclusions

Within the scope of this study the chemical exposome of 300 children aged 6-9 years from Slovenia was characterized using non-targeted analysis and suspect screening (NTA/SS), which allowed identification of a large number of xenobiotics and their biomarkers of exposure within one run. In contrast to metabolomics workflow, the thresholds for data processing for low-level biomarkers of exposure require to be low, generating a large amount of noisy data, increasing the difficulty of confident compound identification, which is still the main bottleneck in applying NTA/SS in human biomonitoring.

In this study, 36 biomarkers of exposure were tentatively identified using non-targeted approach and another 38 using suspect screening. Biomarkers of exposure indicate environmental burden of children to several classes of chemicals such as personal care products, plasticizers and plastic production chemicals, volatile organic compounds, nicotine and caffeine, and pesticides, out of which three, atrazine, amitraz and diazinon are restricted in EU due to their high toxicity. In addition, compounds not yet monitored in HBM schemes, such as bisphenol G and polyethylene glycols and polyethylene glycol ethers were identified. Alongside the compounds with known use, we identified 7 chemicals with unknown use, which might become in the future the chemicals of emerging concern and should be included in targeted HBM schemes in order to monitor their occurrence in a wider population. Due to sampling in two time periods and in two locations, we were able to demonstrate the transiency of the exposome and its location dependence. The results of the study show the complexity of the children's exposome, with them being exposed to many chemicals simultaneously. The work demonstrates the practical approach and emphasizes the potential of using non-targeted analysis and suspect screening in human biomonitoring,

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3.1.3 UHPLC-HRMS data from non-targeted and suspect screening for biomarkers of exposure in urine of children

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To facilitate further mining of the data and the transparency of the applied protocol, the NT data was published within this data article. The paper was submitted to the Data in Brief journal.

Article information

Article title
UHPLC-HRMS data from non-targeted screening for biomarkers of exposure in urine of children

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Abstract
Exposomic analysis is intended to find environmental stressors that can influence human health. Here, enzymatically hydrolysed urine samples of 200 children were analysed by UHPLC-ESI(+)-LIT-Orbitrap-HRMS/MS in full-scan mode. The participants originated from urban (n = 100) and rural (n = 100) residential areas. The latter were sampled twice, once winter and once in summer to reflect seasonal variations of nearby agricultural activity. This data is intended to be available for further processing to find new biomarkers of exposure and endogenous biomarkers of effect. Importantly, the data is available for retrospective analysis for newly emerging contaminants, which are currently unknown but may become of concern in the future.

Specifications table

Subject	*Analytical chemistry*
Specific subject area	*Non-targeted analysis of urine samples*
Type of data	Raw
How the data were acquired	*Data was acquired using Shimadzu LC-30AD UPLC system coupled to a Thermo Scientific Orbitrap Fusion™ Tribrid™ Mass Spectrometer (San Jose, CA, USA) operating in positive mode ESI(+). Chromatographic separation was achieved on a Waters Acquity HSS-T3 (100 × 2.1 mm, 1.8 µm).*

Data format	*UHPLC-HRMS data are in .raw format* Inclusion list for suspect screening is in .csv format
Description of data collection	*Data was collected in the full scan range of 100-900 m/z with mass resolution of 120,000 full width at half maximum (FWHM) at 200 m/z. MS/MS data was collected using data dependant acquisition fragmenting the 10 most abundant ions with resolution of 60,000 FWHM at 200 m/z. Data acquisition used inclusion list of 801 suspected chemicals.*
Data source location	• *Institution: Jožef Stefan Institute, Department of Environmental Sciences* • *City/Town/Region: Ljubljana* • *Country: Slovenia*
Data accessibility	***All data referred to in your data article must be made publicly available prior to publication.***

Value of the data

- *This data was obtained with non-targeted analysis of urine of children, and is relevant to any researchers that may have hypothesis confirmable by reprocessing non-targeted data*
- *This data is of value for people researching exposomics, metabolomics, nutrition and developers of new processing and identification tools*
- *The data can be further processed by new identification and data mining tools and deliver even more valuable insights on exposure and subsequent metabolomic responses*
- *The data can be retrospectively analysed for biomarkers of exposure and effect of any chemical, that might become of health concern in the future*

Data description

Raw data published here was generated by analysis of enzymatically hydrolysed first morning urine samples by UHPLC-ESI(+)-LIT-Orbitrap-HRMS operating in full-scan mode with inclusion list of 801 suspected compounds. File name reflects participants sex, F – female, M – male and residential area, U – urban, R – rural. Inclusion list is a .csv file and contains compound name, IUPAC name, monoisotopic mass, InChiKey and Canonical SMILES.

Experimental design, materials and methods

Experimental design

Participants were aged between 6-9 years and resided in urban (n = 100) and rural (n = 100) regions in Slovenia. Urban children resided in the capital Ljubljana, while rural children resided in an eastern part of the country, characterized by intense agricultural activity. This subpopulation was sample twice, one in winter and once in summer to reflect potential variations in agricultural activities. All in all, 300 samples were analysed.

Sample preparation

Samples were prepared by enzymatic hydrolysis by incubating 1 mL of urine with 250 U/mL β-glucuronidase (Abalone, purified) for 18 h at 37 °C. Samples were then extracted and concentrated using Oasis HLB (60 mg), which were preconditioned with 1 mL acetonitrile, methanol and water. Sorbent was then washed with 1 mL of 5 v/v % methanol in water and eluted with 1.5 mL of 10 v/v % methanol in acetonitrile. Dried under nitrogen stream, the samples were reconstituted in 50 µL of methanol.

Chemical analysis

Chromatographic separation was conducted on the Shimadzu LC-30AD UHPLC using Waters Acquity HSS-T3 (2.1 × 100 mm, 1.8 µm) column with water (A) and ACN (B) as mobile phases. Elution gradient was: 5-15 % B (0.01-1 min), 15-25 % B (1-5 min), 25-40 % B (5-8 min), 40-60 % B (8-18 min), 60-75 % B (18-22 min), 75-85 % B (22-24 min), 85-100 % B (24-28 min), 100-5 % B (28-30 min), 5 % B, 30-35 min. Flow rate was 0.3 mL/min, column was heated to 35 °C. The injection volume was 1 µL.
The UHPLC was coupled to Orbitrap Fusion™ Tribrid™ Mass Spectrometer (Thermo Fisher Scientific Inc., Waltham, MA). Heated electrospray ionization (HESI) was used as the ionisation source, at the spray voltage of 4500 V or -3500 V, sheath gas flow 40 L/min, nebulizer auxilliary gas 15 L/min, and sweep gas 2 L/min. The ion transfer capillary was heated to 350 °C. The full-scan mass acquisition covered the mass range of 100-900 *m/z* and was performed at the resolution of 120 000 FWHM (full width at half maximum at *m/z* 200) while MS2 data was acquired with the resolution of 60 000 FWHM. The automatic gain control (AGC) was 5 $\times 10^5$ ions and maximum injection time 50 ms. Cycle time was 0.8 s.

CRediT author statement

Žiga Tkalec: *Investigation, Formal analysis, Visualization, Writing-original draft, Writing – review & editing.* ***Garry Codling:*** *Formal analysis, Writing – review & editing.* **Jana Klánová***: Writing – review & editing.* ***Milena Horvat:*** *Writing – review & editing.* ***Tina Kosjek:*** *Conceptualization, Investigation, Validation, Writing – review & editing, Supervision.*

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Ethics Statement
Ethical permissions for the study and data acquisition were granted by Republic of Slovenia National Medical Ethics Committee (65/09/14 and 0120-118/2017/3).

Declaration of interests

☒ 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.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

3.2 Application of the NT Workflow to an Alternative Scientific Field

3.2.1 A novel workflow utilizing open-source software tools in the environmental fate studies: The example of imatinib biotransformation

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NT workflow that was developed in the study described in Section 3.1.1 was applied to an alternative field of research in order to test its universality [113]. Imatinib – IMA is a clinically approved targeted anticancer drug, which acts as a tyrosine kinase inhibitor and is used to treat chronic myeloid leukemia and gastrointestinal stromal tumors [114]. After administration it is excreted mainly through urine and ends up in wastewater headed to municipal wastewater treatment plants. Several studies have suggested that the presence of IMA in the environment poses a great risk to aquatic life and human health (REF). The fate of IMA during wastewater treatment and in the environment is not yet known. Therefore, in the present study we aimed to describe environmental fate and identify IMA's TPs using identification protocol that we had developed for the study described in Section 3.1.1. For that, we studied the biodegradation of IMA by using a modified Zahn-Wellens test setup, under specific conditions including carbon-rich and carbon-deficient media, at two concentrations of activated sludge obtained from a municipal wastewater treatment plant. For chemical analysis an UPLC hyphenated to quadrupole-Orbitrap mass spectrometer was used. Nontargeted UHPLC-HRMS data was processed using freely available MzMine2 software, while TPs were determined using multivariate statistical analysis (MVSA), namely principal component analysis (PCA) and orthogonal projections to latent structures discriminant analysis (OPLS-DA) using freely available tool Metaboanalyst. Furthermore, IMA and two of its major TPs were screened for in real samples of WWTP influents and effluents and toxicity of TPs was estimated using QSAR models. The results showed that IMA is readily biodegraded. We identified eight TPs; one was previously identified and another one reported as a metabolite, while six were new to the science. Two major TPs were formed by hydrolysis of the IMA amide bond, one of which undergoes further biological transformations, including conjugation and deamination. TPs formed by oxidation and demethylation were also determined, with all transformations implying that IMA undergoes complex breakdown processes in the environment. We validated the results by searching for TPs in actual wastewater, and we found one of the major ones in real wastewater. This is concerning due to the fact that anticancer agents do not have a threshold level to reveal their mutagenic, carcinogenic or cytotoxic activity.

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A novel workflow utilizing open-source software tools in the environmental fate studies: The example of imatinib biotransformation

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HIGHLIGHTS

- A rapid and efficient workflow for identification of TPs is proposed.
- Open-source software tools are utilized in the workflow.
- Imatinib is readily degraded by activated sludge.
- Six novel transformation products are identified.
- A transformation product is found in wastewater treatment plant effluents.

GRAPHICAL ABSTRACT

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ABSTRACT

The aim of this study is to utilize novel and powerful workflows with publicly available tools to efficiently process data and facilitate rapid acquisition of knowledge on environmental fate studies. Taking imatinib (IMA) as an example, we developed an efficient workflow to describe IMA biodegradation with activated sludge (AS) from wastewater treatment plants (WWTP). IMA is a cytostatic pharmaceutical; a selective tyrosine kinase inhibitor used to treat chronic myeloid leukemia. Its reported ecotoxic, endocrine and genotoxic effects imply high risk for aquatic wildlife and human health, however its fate in the environment is not yet well known. The study was conducted in a batch biotransformation setup, at two AS concentration levels and in presence and absence of carbon source. Degradation profiles and formation of IMA transformation products (TPs) were investigated using UHPLC-QqOrbitrap-MS/MS which showed that IMA is readily biodegradable. TPs were determined using multivariate statistical analysis. Eight TPs were determined and tentatively identified, six of them for first time. Hydrolysis of amide bond, oxidation, demethylation, deamination, acetylation and succinylation are proposed as major biodegradation pathways. TP235, the product of amide bond hydrolysis, was detected and quantified in actual wastewaters, at levels around 1 ng/L. This calls for more studies on the environmental fate of IMA in order to properly asses the environmental risk and hazard associated to IMA and its TPs.

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1. Introduction

With increasing incidence of cancer, the use of anticancer drugs is growing and so is their presence in the environment. Many anticancer agents exhibit mutagenic, carcinogenic and teratogenic effects and are classified as highly hazardous compounds. Therefore, their presence in

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the environment poses a great risk to wildlife and human health (Heath et al., 2016). Once administered to a patient, the majority of a drug is metabolized in the body. However, a percentage of unaltered compound can be excreted via urine and feces. Therefore, cytostatic drugs along with their metabolites can enter the water cycle via oncological clinic, hospital, and household (after outpatient treatment) wastewater effluents and reach municipal wastewater treatment plants (WWTPs) and ultimately the aquatic environment (Kosjek and Heath, 2011).

Imatinib mesylate – IMA (Gleevec, Glivec) is the first clinically approved targeted anticancer drug, which acts as a tyrosine kinase inhibitor and is used to treat chronic myeloid leukemia and gastrointestinal stromal tumors (Busse et al., 2001). Demethylation, *N*-oxidation and hydroxylation are the main routes of IMA metabolism in the human body (Friedecký et al., 2015; Marull and Rochat, 2006; Rochat, 2016). Once excreted, the cytostatic drugs and their metabolites reach the environment via WWTPs, where they can undergo biotic and abiotic transformations, forming transformation products (TPs). These may exhibit even greater toxicity than their parent compounds, particularly in a mixture (Donner et al., 2013; Heath et al., 2016; Kümmerer, 2009).

Several studies have suggested that the presence of IMA in the environment poses great risk to aquatic life and human health. Environmental risk assessment showed hazard quotients (HQs) for IMA in hospital effluents well above 10 (Olalla et al., 2018) and risk quotients (RQs) of 6.07 and 54.6 for acute toxicity and genotoxicity (Mišík et al., 2019), respectively, representing a high risk for aquatic life. A study of chronic exposure effects on crustaceans showed that IMA induced 50% reproduction inhibition at levels of several hundred μg/L (Li et al., 2021; Parrella et al., 2014a), while a chronic exposure study on zebra fish showed minor genotoxic effects and whole-transcriptome changes (Novak et al., 2021). It is also toxic to algae, with half maximal effective concentration (EC50) at the level of few mg/L (Bialk-Bielinska et al., 2017; Brezovšek et al., 2014; Hena et al., 2021), while the results regarding its mutagenicity are contradictory (Novak et al., 2017; Parrella et al., 2015). Furthermore, high estrogenic activity of IMA was detected in-vitro on human breast cancer ER^+ MCF-7 cells at a level of 61 pg/L (Parrella et al., 2014b), indicating a potential risk for the environment and humans at concentrations lower than expected in the environment. Data on IMA levels in wastewaters (WWs) is scarce, with Olalla (Olalla et al., 2018) reporting average concentrations at 164 ng/L in Spanish hospital wastewaters, Gouveia (Gouveia et al., 2020) reporting levels up to 149 ng/L in WWTP influents from Portugal, whereas Isidori found levels below the corresponding method limit of quantification (LOQ) (<120 ng/L) in WWs from Spain and Slovenia (Isidori et al., 2016). While we found no studies on the occurrence of IMA in surface waters, several studies have reported predicted environmental concentrations (PEC) of 5 ng/L (Besse et al., 2011), 0.5 ng/L (Booker et al., 2014) and 8 ng/L (Santos et al., 2018) in France, UK, and Portugal, respectively.

The fate of IMA during WW treatment and in the environment is not yet well known. It has been shown that IMA is fairly stable in wastewater at neutral pH (Negreira et al., 2014) and in deionised water under hydrolytic, oxidative and photolytic conditions (Szczepek et al., 2007). Furthermore, photocatalytic degradation pathways have been described, proposing several photodegradation TPs (Secrétan et al., 2019). However, biodegradation data is not yet available, nor the data describing TPs deriving from it. To fill in this gap, the present study aims to present an efficient and rapid workflow utilizing freely available tools to identify IMA TPs formed during WW treatment. Such workflow is applicable in other studies dealing with the identification of TPs. We studied the biodegradation of IMA by using a modified Zahn-Wellens test setup, under specific conditions including carbon rich and carbon deficient media, at two concentrations of activated sludge (AS) obtained from a municipal WWTP. For chemical analysis ultra-high performance liquid chromatography (UHPLC) hyphenated to high resolution quadrupole-Orbitrap mass spectrometry (HRMS) was used. Nontargeted UHPLC-HRMS data was processed using freely available MzMine2 software, while TPs were determined using multivariate statistical analysis (MVSA), namely principal component analysis (PCA) and orthogonal projections to latent structures discriminant analysis (OPLS-DA) using freely available tool Metaboanalyst. Furthermore, IMA and two of its major TPs were screened for in real samples of WWTP influents and effluents and toxicity of TPs was estimated using QSAR models.

2. Experimental

Chemicals used in experiments are presented in SI-1.

2.1. Setup of the biotransformation experiment

Biotransformation experiments were conducted in 0.5 L glass bottles with a total wetted volume of 0.4 L. Test bottles were aerated with an aquarium pump. The experiments were conducted at room temperature, protected from light. Into each bottle, 50 mL or 10 mL of settled AS was added. AS was obtained from a municipal wastewater treatment plant (Ljubljana, Slovenia, 360,000 population units) (Kosjek et al., 2015). The concentration of AS was determined after the conclusion of the experiment: 15 mL of homogenized sample was filtered through previously dried glass microfiber filters (MN GF-3, Macherey Nagel, Düren, Germany). After that, the filtrate was dried to constant weight at 150 °C, and the mass of AS was determined as the mass difference. The media for the experiments were nutrient-mineral medium (NMM) and mineral medium (MM). NMM mimics the composition of municipal wastewater. NMM was prepared in tap water and contained organic constituents, such as meat extract (130 mg/L), casein peptone (130 mg/L), yeast extract (13 mg/L) and ammonium acetate (317 mg/L) and salts calcium carbonate (100 mg/L), magnesium carbonate (100 mg/L), sodium chloride (40 mg/L), ammonium chloride (40 mg/L), potassium hydrogenphosphate (24 mg/L), potassium dihydrogenphosphate (8 mg/L) and iron (II) sulfate heptahydrate (5 mg/L) (Kosjek et al., 2007). NMM was applied to experiments A, B, 2A, 2B (Table SI-1). MM was prepared in the same manner, excluding carbon sources, i.e., meat extract, casein peptone, yeast extract and ammonium acetate. MM was applied to bottles C, D, 2C, 2D. To account for abiotic degradation and sorption of IMA to AS, formaldehyde (FDH) was added to bottles B, D, E, 2B, 2D, 2E (Table SI-1). The solution was mixed for 4 h to achieve complete biomass inhibition. In the experiments without biomass inhibiting agent FDH, the same volume of deionised water (DIW) was added. All the experiments were performed protected from UV and visible light. Detailed justification of the applied experimental conditions is given in our previous paper (Kosjek et al., 2018).

Sampling was performed in 24-h intervals by withdrawing 4-mL of test mixture on eight consecutive days and storing the samples in 10-mL PTFE vials at −20 °C until analysis.

2.2. Sample preparation

300 μL of filtered samples (0.2 μm regenerated cellulose filters, Phenomenex) were transferred into LC vials and internal standard *N*-desmethyl imatinib-*d*8 was added at a final concentration of 0.2 μg/mL prior to injection into the UHPLC-MS/MS system.

2.3. Nontargeted UHPLC-ESI(+)-QqOrbitrap-MS/MS analysis

The analysis was performed on a Waters Acquity (Waters, Milford, MA, USA) UHPLC system hyphenated to a Thermo Scientific Q-Exactive hybrid quadrupole - Orbitrap (San Jose, CA, USA) mass spectrometer.

Chromatographic separation was achieved on a reversed-phase UHPLC column Hibar HR (50 × 2.1 mm, 2 μm particle size) from Merck (Darmstadt, Germany). The separation of TPs was achieved at 25 °C with water (A) and methanol (B) as mobile phases and the following gradient: 0–1 min, 5% B; 3 min, 20% B; 6 min, 80% B; 7 min 100% B; 10–12 min, 5% B. The flow rate was 0.3 mL/min and 10 μL of sample was injected into the system.

The electrospray (ESI) interface was operated in positive mode under the following conditions: spray voltage, 3000 V; sheath gas flow rate, 40 PSI; auxiliary gas, 10 PSI; capillary temperature, 350 °C and vaporizer temperature, 400 °C. Nitrogen (>99.98%) was used as sheath, auxiliary and sweep gas.

The mass spectrometer performed data-dependent acquisition (DDA), with full scan range of 70–1000 m/z achieving mass resolution of 70,000 full width at half maximum (FWHM) at m/z 200, and DDA achieving resolution of 17,500 FWHM at m/z 200. The five most intense ions detected were selected for fragmentation and their MS^2 recorded. The precursor ions were fragmented by higher-energy collisional dissociation (HCD) with normalized collision energy of 40% and an activation time of 120 ms.

The software Xcalibur™ 2.2 (Thermo Fischer Scientific) was used for data acquisition. External calibration for mass accuracy was carried out following the manufacturer's guidelines one day before analysis.

2.4. Data processing

Data matrix was generated by MZmine 2.36 (Pluskal et al., 2010), a software for automatic processing of chromatographic data. Processing parameters are described in SI-3. Based on the degradation curve of IMA, the features detected in less than 2 of 8 time points (i.e. samples in a series) were removed.

2.5. Data analysis

The data matrix was analysed using the R-package Metaboanalyst (Chong and Xia, 2018). The analysis was based on multivariate statistical analyses, namely PCA and OPLS-DA. Prior to analysis the feature intensities obtained by MzMine 2.36 were log transformed and Pareto scaled.

Based on scores and loadings plots, similar groups identified by PCA were joined together and used to build the OPLS-DA model. The OPLS-DA model was evaluated using R2Y, the goodness of fit of the model, and Q2, the predictability of the model. The model was validated using the permutation test (N = 1000). Differential features were discriminated by S-plot.

The differential features determined with PCA and OPLS-DA were regarded as candidates for TPs. Time-dependant curves were generated for these features. Features exhibiting time-dependant change in concentration were considered significant and their structures were elucidated. Compounds at the constant level throughout the biodegradation experiment were considered insignificant and probably sourcing from the biomass, media or FDH rather than degradation of IMA. After structural elucidation, correlation analysis was conducted to check for any correlated features that would indicate formation of additional TPs (Fig. 1).

2.6. Compound identification

The structures of significant features were proposed by analysis of their MS^2 spectra. Manual analysis of MS^2 spectra of TPs candidates was conducted using Sirius-CSI:FingerID tool. Sirius (Rasche et al., 2011) (Dührkop et al., 2013) is a java-based framework aimed at metabolite identification. CSI:FingerID (Dührkop et al., 2015) utilizes fragmentation trees and fragmentation patterns to predict a molecular fingerprint, which is used in searching a molecular structure in databases such as PubChem and KEGG. This approach was used to identify already known compounds.

If search provided no hits of known compounds, then a molecular structure of a candidate was proposed based on predicted fragment elemental compositions and fragmentation pattern. Finally, the proposed molecular structure was *in-silico* fragmented using CFM-ID (Allen et al., 2014) and the experimental and predicted spectra were compared for further confidence in the proposed molecular structures of TPs.

2.7. Quantitative analysis of real WW samples

To prove the validity of the TPs found in the batch biodegradation experiment, IMA and its most abundant TPs were screened for in municipal WW samples.

2.7.1. Sample collection

Wastewater samples were obtained from two Slovenian wastewater treatment facilities, namely, WWTP Ljubljana (46°04′06.5″N, 14°37′24.6″E) and WWTP Domžale-Kamnik (46°07′01.7″N, 14°36′35.5″E), with capacities of 149,000 and 36,000 population equivalents, respectively (Gornik et al., 2020). Treatment on WWTPs is mechanical and biological, with WWTPs receiving hospital, industrial and municipal wastewaters. Sampling of WW effluent and influent was conducted

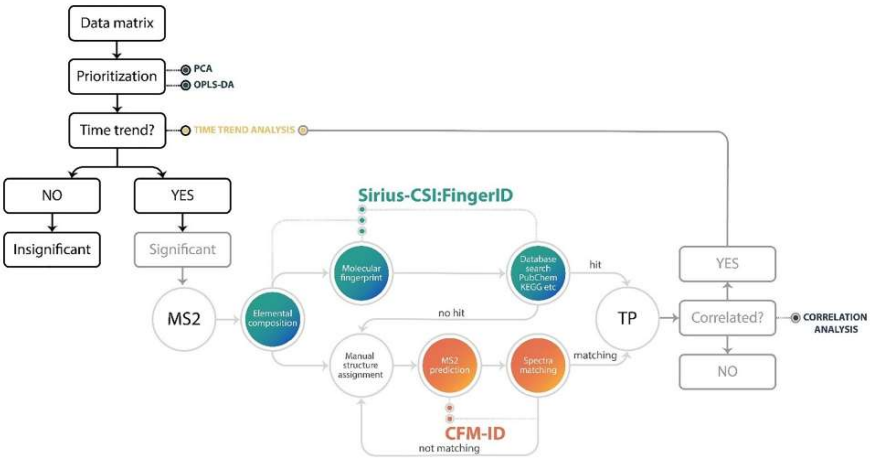

Fig. 1. Workflow for determination of IMA TPs based on MVSA and time-trends of their formation.

every day for 7 consecutive days in August 2020. All collected samples were 24-h composite samples.

2.7.2. Sample preparation

To 150 mL of homogenized WW sample *N*-desmethyl IMA-*d8* internal standard was added at the final concentration of 30 ng/L. Samples were filtered through 0.45 μm cellulose nitrate filter to remove any particulate matter and pH was adjusted to 2 with concentrated HCl. Oasis MXC (150 mg, 6 mL) were sequentially preconditioned with 5 mL of ACN, MeOH, and water; then samples were loaded and the cartridges were washed with 5 mL of water. After drying of the sorbent for 60 min, extracts were eluted with 5 mL of 5% NH_3 in ACN/MeOH (1:1), dried under nitrogen stream and reconstituted in 500 μL 5% ACN in water. Prior to injection, samples were filtered through 0.2 μm regenerated cellulose syringe filters (Phenomenex).

2.7.3. UHPLC-ESI(+)-Qtrap-MS

Quantification of IMA and two of its most abundant TPs (TP235 and TP278) was performed on a Shimadzu Nexera UHPLC system coupled to a Sciex 4500 Qtrap mass spectrometer. Chromatographic separation was achieved on a Waters Acquity HSS-T3 column (100 × 2.1 mm, 1,8 μm) with 0,1% formic acid in water (A) and ACN (B) as mobile phases. Injection volume was 4 μL. Flow was 0.4 mL/min with gradient elution: 95–50% A, 0–5 min, 50–0% A, 5–5.10 min, 0% A, 5.10–7 min, 95% A, 7.01–10 min. The column was heated at 40 °C. Ions were detected using ESI(+) ionisation in multiple reaction monitoring (MRM) mode. Transitions and compound-specific parameters are presented in Table SI-2, where transitions used for quantification are bolded. Three transitions per analyte were used. Curtain gas (CUR) was set to 40 PSI, collision gas was set to medium, ion-spray voltage was 5500 V, source temperature was 550 °C, ion source gas 1 (GS1) and 2 (GS2) were both set to 40 PSI. Dwell time for each analyte was 50 ms. Total cycle time was 0.6 s, which allowed for approximately 12 points per peak. Data acquisition and sample quantification were performed using Analyst software (ABSciex, Foster City, CA, USA). Method validation is presented in SI-4.

2.8. Toxicity assessment

The toxicity assessment of IMA and its TPs was based on QSAR models, which predict toxicity from structure and physico-chemical properties of compounds. Calculation was carried out by Toxicity Estimation Software Tool T.E.S.T 5.1 (U.S. EPA, 2020), where the consensus method, consisting of five QSAR models was applied.

3. Results and discussion

3.1. Degradation of IMA

Degradation profiles of IMA are presented in Fig. 2. IMA shows stability in distilled water (2E) at room temperature and does not undergo spontaneous degradation for the duration of the experiment. IMA degradation is evident only in experiments 2A, 2C, A and C, which contain living biomass. In the experiments 2D and 2B, which contained inhibited biomass, the degradation did not occur, showing that degradation of IMA depends on the presence of living biomass.

Different concentrations of AS influenced the rate of the degradation of IMA. As evident in Fig. 2, at the higher concentration of AS (experiments A, C) the degradation occurred faster than in the experiments with lower concentration (2A, 2C). 70% and 100% of IMA were removed at the higher concentration of AS (experiments A and C), while 22% and 70% of IMA were removed at lower volume of AS inoculum (2A and 2C) after the first day of biodegradation. Likewise, the medium for AS influenced the rate of the degradation. The degradation occurred faster in the MM (C, 2C) than in NMM (A, 2A), which suggests that IMA can be utilized as a sole carbon source.

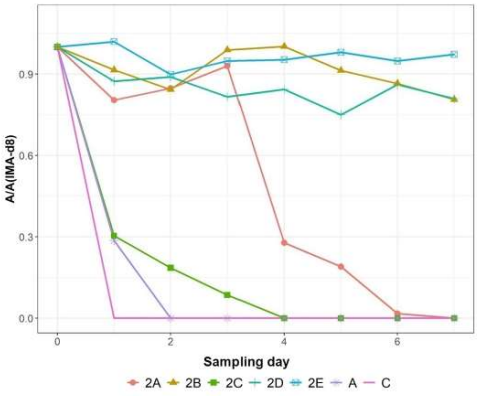

Fig. 2. Degradation of IMA is dependent on the presence of living biomass. IMA levels are decreasing in experiments with living biomass (2A, 2C, A, C), while remaining constant in experiments with deactivated biomass (2B, 2D) and in control (2E).

The samples obtained from the experiments B and D, which contained higher concentration of AS inhibited by FDH were resistant towards cellulose nitrate filtering due to interaction of FDH with AS, and were therefore not analysed.

3.2. Data processing and data analysis

The processing of UHPLC-MS/MS data resulted in a data matrix with 5231 features after duplicate filtering and removal of features present in less than 25% of all samples. A feature was required to be present in at least two of eight samples within a group. This threshold clarifies the data of noise to some extent, but considers the possibility of further biotransformation of the potential TP.

As evident from Fig. 3, PCA scores plot of the data matrix shows clear separation of the different experimental groups, explaining 45.0% variance on the first two PCs. Groups A, C, 2A, 2C cluster together, indicating the same response to the experimental factors. Experimental groups containing FDH, namely 2B, 2D and E cluster separately. 2E group is clustered with A, C, 2A, 2C group due to the presence of non-degraded IMA, which is also present in first time points of groups A, C, 2A, 2C.

PCA (Fig. 3 top) demonstrates a difference between the degradation of IMA in the presence of living biomass (2A, 2C, A, C) and the presence of inhibited biomass (2B, 2D), which is in line with the trends exhibited by degradation curves (Fig. 2). It is also evident, that the amount of AS did not cause discrimination of the groups (A vs 2A, C vs 2C), which indicates that the amount of AS influences the rate of the degradation, but not the chemical mechanism. Similarly, the close grouping of A and C (and similarly 2A and 2C) shows that the different mediums, namely NMM and MM, do not affect the chemistry of the degradation.

Loadings plot (Fig. 3, top right) of PCA indicates features causing group discrimination. Several differential features (including features representing IMA and desIMA-*d8*) can be distinguished from loadings plot. Feature with *m/z* of 494.2645 corresponds to the non-degraded IMA present in first few samples in groups A, C, 2A, 2C and blank group 2E. Features with *m/z* 235.1433, 278.1390, 320.1493 and 378.1544 are present in groups A, C, 2A and 2C, discriminating groups with active or inhibited biomass. These same features also exhibited time-dependent rise in concentrations and were considered good candidates for TPs and were identified in the next step.

Further search for TPs was based on building OPLS-DA model (Fig. 3 bottom). The OPLS-DA model discriminates between two groups and

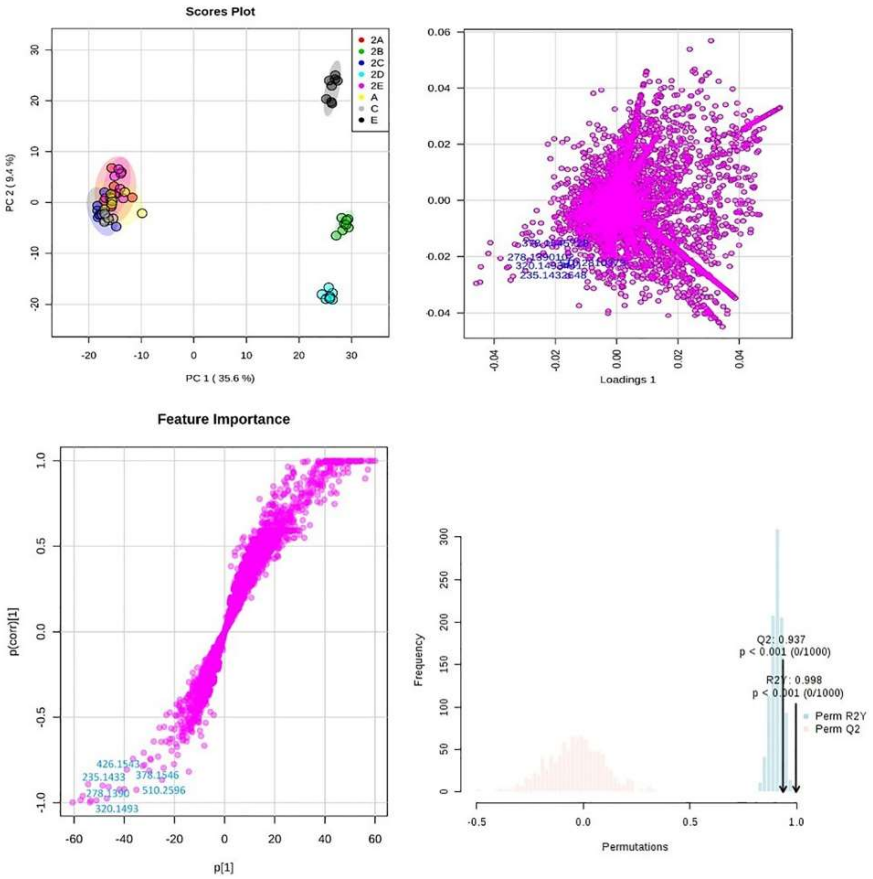

Fig. 3. Scores (top left) and loadings (top right) plot of PCA. Scores plot shows similarity of groups A, C, 2A and 2C with living biomass opposing separated groups 2B, 2D with inhibited biomass and FDH blank E. Loadings plot indicate several possible TPs. S-plot (bottom left) indicating same differential features as PCA with addition of several other potential TPs. Validation plot of OPLS-DA model (bottom right), based on 1000 permutations shows permuted Q2 and R2Y values being lower than the model Q2 and R2Y values, supporting the validity of the model.

the clustering from PCA acted as basis for clustering similar groups together.

The model was built upon joined groups 2A and 2C opposing joined groups 2B and 2D. The model was validated using the permutation test, using 1000 permutations. In our study model, the predictability, Q2, was 0.937 ($p < 0.001$). The goodness of fit value of the models was 0.998 ($p < 0.001$). Permuted Q2 values were close to zero and permuted R2Y lower than the study model value, supporting the validity of the model. The validation plot is presented in Fig. 3 (bottom right). Visualization of the differential features was achieved using S-plot (Fig. 3, bottom left), which emphasizes discriminative features using covariance and correlation. Same differential features with appropriate time-dependant behaviour were detected in OPLS-DA models as in PCA, namely features with *m/z* 235.1433, 278.1390, 320.1493 and 378.1544, indicating several TPs. Furthermore, features with *m/z* 510.2596 and 426.1543 were observed in S-plot indicating additional TPs. The same model was built for joined group A-C and 2B-2D, which emphasized same differential features.

Differential features observed from PCA and OPLS-DA were further screened for any features with similar pattern with correlation analysis. Clustering of correlated features used Pearson's correlation coefficient, which clustered together previously discovered TP candidates with IMA and des-IMA-*d*8 along with several other features. Out of these, feature with *m/z* 221.1276 was newly determined as a potential TP. Heat map of correlation analysis is presented in Fig. SI-1.

As evident from Fig. 4, TP235 and TP278 are the most abundant TPs of IMA pinpointing its main TPs. After the formation, the level of TP235 remains constant, whereas the abundance of TP278 decreases over time, indicating that it further transforms into a secondary TP. Fig. 4 right shows the TPs which have lower abundances during the observation period, of which TP221, TP263, TP378, and TP426 emerge later in the experiment, so these might be transformed from primary TPs.

3.3. Identification of TPs

Table 1 shows retention times, assigned molecular formulas, measured and observed molecular masses with mass errors of molecular ions and fragments.

For identification, the MS^2 spectra of TP candidates were first run through Sirius-CSI:FingerID in order to check for known compounds reported in databases. Using PubChem library, TP235 was thus automatically identified as 4-((4-methylpiperazin-1-yl)methyl)benzoic acid (formula matching score: 100%, database matching score: 56.93%). The remaining TPs were identified manually by annotation of their MS^2. Based on elemental formula and annotated MS^2, a molecular structure candidate was proposed for each TP. The proposed chemical structures of the TPs were further evaluated by comparing the experimental MS^2 fragments and *in-silico* generated MS^2 using CFM-ID. Candidates were confirmed when a minimum of 5 fragment peaks matched *in-silico* generated MS^2. Fragmentation pattern of IMA is presented in Fig. SI-2, proposed TPs and their fragment ions are presented in Table 1, while Fig. SI-3 shows their MS^2 spectra.

Fragmentation of IMA was studied and further used to aid in assignment of TPs. Peak *m/z* 494.2654 corresponds to the protonated IMA (Fig. SI-2). Peak *m/z* 394.1649 corresponds to a fragment formed by cleavage of methyl piperazine from the parent molecule, while the peak *m/z* 99.0919 corresponds to the methyl piperazine fragment itself. Fragment *m/z* 380.1501 corresponds to loss of dimethylpiperazine moiety from parent molecule. Fragment *m/z* 222.0906 corresponds to methyl tolylbenzamide ion.

The main transformation products of IMA are TP235 and TP278, which are formed by hydrolysis of the amide bond. TP235 was identified automatically using Sirius-CSI:FingerID. Peak *m/z* 235.1432, corresponds to the protonated molecule of TP235 (Fig. SI-3A). Fragment ion *m/z* 135.0437 corresponds to the ion formed by loss of methylpiperazine at benzyl C-atom. Fragment *m/z* 100.0998 corresponds to methylpiperazine fragments, supporting the identity of TP235 as 4-((4-methylpiperazin-1-yl)methyl)benzoic acid.

MS^2 of TP221 shows a protonated molecule at 221.12976 (Fig. SI-3B). The difference between molecular ions of TP235 and TP221 indicates the loss of a methyl group. Fragment *m/z* 135.0437 in the mass spectrum of TP221 corresponds to the one in the MS^2 spectrum of TP235, while presence of fragment *m/z* 85.0764, that corresponds to piperazine fragment, suggests that the methyl group is lost at the piperazine ring. Accordingly, TP221 is tentatively identified as 4-(piperazin-1yl)benzoic acid.

The protonated molecule of TP278 suggests elemental composition of $C_{16}H_{15}N_5+H^+$ (Fig. SI-3C). The difference of 17.0264 between *m/z* 278.1390 and *m/z* 261.1126 corresponds to the loss of NH_3, suggesting the presence of a primary amine moiety in the TP278, while difference between *m/z* 278.1390 and *m/z* 173.0814 indicates the loss of pyridine. Fragment *m/z* 106.0652 corresponds to the protonated 4-methylaminobenzene, suggesting the identity of TP278 as 6-methyl-N^1-(4-(pyridin-3-yl)pyrimidin-2-yl)benzene-1,3-diamine.

Similar MS^2 fragmentation pattern is shown by TP263 (Fig. SI-3D), which gives the protonated molecule at $[M+H]^+$ 263.1279, and corresponds to $C_{16}H_{15}N_4$. The mass difference between the protonated molecules of TP278 and that of TP263 indicates the cleavage of the amino group from the TP278. By the loss of the methyl group from TP263 the fragment ion at *m/z* 247.0966 is formed, while fragment *m/z* 184.0860 indicates the loss of pyridine from the protonated molecule. This suggests the identity of TP263 as 4-(pyridin-3-yl)-*N*-(2-methylphenyl) pyrimidin-2-amine.

The mass spectrum of TP320 (Fig. SI-3E) shows the protonated molecule at $[M+H]^+$ 320.1495, which corresponds to the elemental composition of $C_{18}H_{18}N_5O$. Its mass spectrum shares several fragment ions with that of TP278, including the *m/z* 278.1392, *m/z* 261.1122, *m/z* 173.0821 and *m/z* 106.0652. The difference between the elemental compositions of TP320 and TP278 corresponds to C_2H_2O, which implies the presence of an acetyl group. The loss of water is observed from *m/z* 320.1495 to *m/z* 302.1391, leaving an acetylene group attached to the nitrogen carbon, supporting the identification of TP320 as acetylated TP278, *N*-(4-methyl-3-((4-(pyridine-3-yl)pyrimidin-2-yl)amino)phenyl)acetamide.

Similarly, the core of the TP378 mass spectrum is TP278, based upon the presence of several identical fragments (Fig. SI-3F). The mass difference between their protonated molecules is 100.0180, which corresponds to $C_4H_4O_3$, and indicates the presence of a succinyl moiety in TP378. This is supported by sequential cleavage of the succinyl group as presented in Fig. SI-3F. This cleavage resembles the loss of water (from $[M+H]^+$ 378.1566 to *m/z* 360.1439) and subsequent loss of carbon monoxide (from *m/z* 360.1439 to *m/z* 332.1489), together indicating the presence of a carboxylic acid. Further loss of a water molecule from *m/z* 332.1489 to *m/z* 314.1387 implies the presence of another oxygen in the molecule, thus identifying this TP tentatively as 4-((4-methyl-3-((4-(pyridine-3-yl)pyrimidin-2-yl)amino)phenyl) amino)-4-oxobutanoic acid. From the mass spectra of TP320 and TP378 it is not evident which amine group is acetylated and succinylated. However, we propose that the acetylation and succinylation reactions

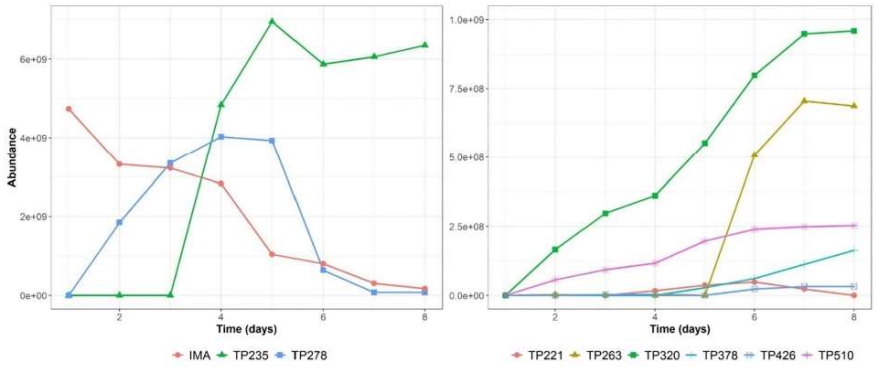

Fig. 4. Left: Time trends of the formation of the IMA TPs. TP235 and TP278 are the most abundant. Right: Lower abundance TPs.

Table 1
Accurate mass measurements of protonated IMA, TPs and their fragment ions.

	Retention time	Theoretical molecular mass	Elemental composition of parent compound	Elemental composition of fragment	Theoretical *m/z*	Observed *m/z*	Mass error (ppm)
IMA	6.63	493.2590	$C_{29}H_{31}N_7O$	$C_{29}H_{32}N_7O$	494.2663	494.2654	1.82
				$C_{29}H_{30}N_7$	476.2557	476.2534	4.82
				$C_{24}H_{20}N_5O$	394.1662	394.1649	−3.30
				$C_{23}H_{17}N_5O$	380.1506	380.1501	1.3
				$C_{15}H_{12}NO$	222.0913	222.0906	3.15
				$C_{12}H_{17}N_2$	189.1386	189.1382	−2.11
				$C_5H_{11}N_2$	99.0917	99.0919	−2.02
TP221	1.15	220.1212	$C_{12}H_{16}N_2O_2$	$C_{12}H_{17}N_2O_2$	221.1290	221.1276	−6.33
				$C_8H_7O_2$	135.0441	135.0437	−2.96
				C_8H_7O	119.0491	119.0490	−0.84
				$C_4H_9N_2$	85.0766	85.0764	−2.35
TP235	1.55	234.1368	$C_{13}H_{18}N_2O_2$	$C_{13}H_{19}N_2O_2$	235.1441	235.1432	−3.83
				$C_8H_7O_2$	135.0441	135.0437	−2.96
				$C_6H_{12}N_2$	100.1000	100.0998	−2.00
				$C_5H_{11}N_2$	99.0917	99.0919	−2.02
TP263	6.60	262.1218	$C_{16}H_{14}N_4$	$C_{16}H_{15}N_4$	263.1297	263.1279	−6.84
				$C_{15}H_{11}N_4$	247.0978	247.0966	−4.86
				$C_{15}H_{14}N_3$	236.1188	236.1169	−8.05
				$C_{11}H_{10}N_3$	184.0869	184.0860	−4.89
TP278	5.75	277.1327	$C_{16}H_{15}N_5$	$C_{16}H_{16}N_5$	278.1400	278.1390	−3.60
				$C_{16}H_{13}N_4$	261.1135	261.1126	−3.45
				$C_9H_9N_4$	173.0827	173.0814	−7.51
				$C_8H_{10}N_3$	148.0869	148.0864	−3.38
				C_7H_8N	106.0651	106.0652	0.94
TP320	5.81	319.1433	$C_{18}H_{17}N_5O$	$C_{18}H_{18}N_5O$	320.1511	320.1495	−5.00
				$C_{18}H_{16}N_5$	302.1406	302.1391	−4.96
				$C_{16}H_{16}N_5$	278.1406	278.1392	−5.03
				$C_{16}H_{13}N_4$	261.1135	261.1122	−4.98
				$C_9H_9N_4$	173.0827	173.0821	−3.47
				C_7H_8N	106.0651	106.0652	0.94
TP378	4.77	377.1488	$C_{20}H_{19}N_5O_3$	$C_{20}H_{20}N_5O_3$	378.1566	na	na
				$C_{20}H_{18}N_5O_2$	360.1455	360.1439	−4.44
				$C_{19}H_{18}N_5O$	332.1506	332.1489	−5.12
				$C_{19}H_{16}N_5$	314.1400	314.1387	−4.14
				$C_{16}H_{16}N_5$	278.1406	278.1389	−6.11
				$C_{16}H_{13}N_4$	261.1135	261.1125	−3.83
				$C_9H_9N_4$	173.0827	173.0821	−3.47
				C_7H_8N	106.0651	106.0653	1.89
TP426	5.04	425.1488	$C_{24}H_{19}N_5O_3$	$C_{24}H_{20}N_5O_3$	426.1561	426.1546	−3.52
				$C_{24}H_{18}N_5O_2$	408.1455	408.1443	−2.94
				$C_{23}H_{18}N_5O$	380.1506	380.1496	−2.63
				$C_8H_5O_3$	149.0233	149.0231	−1.34
TP510	5.87	509.2539	$C_{29}H_{31}N_7O_2$	$C_{29}H_{32}N_7O_2$	510.2617	na	na
				$C_{24}H_{20}N_5O$	394.1662	394.1647	−3.81
				$C_{23}H_{18}N_5O$	380.1506	380.1490	−4.21
				$C_{16}H_{15}N_5$	277.1327	277.1311	−5.77
				$C_{15}H_{12}N_5$	262.1087	262.1078	−3.43
				$C_{15}H_{12}NO$	222.0913	222.0905	−3.60
				$C_5H_{11}N_2O$	115.0871	115.0866	−4.34
				$C_5H_{11}N_2$	99.0917	99.0919	−2.02

took place on the primary amine atom, due to the lesser steric hindrance, as compared to the secondary amine group connecting the 4-methylaminobenzene part with 3-pyridinylpyrimidine. In support to our assumption, the secondary amine nitrogen atom is deemed less nucleophilic than the primary amine nitrogen atom due to its proximity to two electronegative nitrogen atoms in the pyridine ring. However, because transformations were conducted under the influence of living biomass, it is highly probable that the acetylation and succinylation are enzymatic, and that includes the possibility of different selectivity.

The elemental composition derived from the protonated molecule of TP426 is $C_{24}H_{19}N_5O_3+H^+$, indicating that the core of the IMA structure is retained in this TP (Fig. SI-3G). However, the loss of two nitrogen atoms suggests cleavage of methylpiperazine while the two additional oxygen atoms indicate oxidation. The mass difference between $[M+H]^+$ 426.1546 and *m/z* 408.1443 corresponds to the loss of a water molecule, and further loss of CO at *m/z* 380.1496, while both combined indicate the presence of a carboxylic acid. The presence of fragment ion at *m/z* 149.0231 corresponds to 4-carbonylbenzoic acid fragment, identifying TP426 as 4-((4-methyl-3-((4-(pyridine-3-yl) pyrimidin-2-yl)amino)phenyl)carbamoyl)benzoic acid.

The protonated molecule of TP510 corresponds to the molecular composition of $C_{29}H_{31}N_7O_2+H^+$, which suggests oxidation of IMA (Fig. SI-3H). Their MS2 spectra show several common fragment ions (*m/z* 394.1647, 380.1490, 222.0905, 90.0917), with exception of at *m/z* 115.0871, which corresponds to the oxidized methylpiperazine ion. The fragment ion *m/z* 99.09192 corresponding to non-oxidized methylpiperazine is also evident, which implies weak oxygen bonding and thus TP510 was identified as imatinib piperazine-*N*-oxide, which has been previously reported as IMA metabolite (Gschwind et al., 2005). In summary, we identified 8 TPs of IMA, of which one, TP510 was reported before (Secrétan et al., 2019), while TP426 was reported as a human metabolite (Gschwind et al., 2005). TP235, TP221, TP278, TP263, TP320 and TP378 are herein identified for the first time.

The proposed biodegradation pathway is presented in Fig. 5. The most abundant TPs of IMA are TP235 and TP278, which are formed by hydrolysis of the amide bond. TP235 is present in samples at constant

level (Fig. 4), since the demethylation of its piperazine ring results in a minor TP221. On the contrary, the levels of TP278 start to decrease after day 5, indicating its further transformation, as evident in Fig. 4. Accordingly, TP263 forms by deamination of TP278. TP278 also undergoes conjugation reactions, i.e. acetylation and succinylation, forming TP320 and TP378, respectively. *N*-acetylation and *N*-succinylation are conjugations frequent for molecules with primary amine groups and were previously described on similar compounds (Gulde et al., 2016). IMA is also subjected to oxidation, where the methylated piperazine nitrogen atom forms *N*-oxide, TP510. Alternatively, oxidation of the reactive benzylic carbon followed by cleavage of the piperazine ring results in formation of TP426. Both oxidation products, TP510 and TP426, are also known IMA human metabolites (Gschwind et al., 2005).

Many other features were present in the degradation data matrix; however, the concentration was too low for their MS^2 acquisition, making their identification impossible. This implies that many more TPs of IMA may be formed in the environment, which might impose serious risks for the environment.

3.4. Determination of IMA, TP235 and TP278 in real WW samples

We searched for IMA and its two most abundant TPs, TP235 and TP278 in real WW samples. We analysed 24 h composite samples of influent and effluent from two different WWTPs sampled on 7-consecutive days. Results of the analysis are presented in Table SI-3 and Table SI-4.

The levels of IMA and TP278 were <LOQ in all samples, however TP235 was quantified in effluents of the larger WWTP in Ljubljana, which receives also wastewaters from an oncological ward, and in one effluent from the smaller WWTP Domžale-Kamnik. This confirms the relevance of this research, which demonstrates the formation of TPs during water treatment with AS in batch experiments and respectively, their occurrence in real treated waters.

IMA was, however, not quantified in any of the real wastewater samples which is probably due to rapid biodegradation. It could also be due to the comparatively lower LOQ of the TPs (1 ng/L) as compared to that of IMA (5 ng/L). Similarly, TP278 was not quantified, which could be reasoned by its further transformation, which was observed in the batch experiment. Certainly, the results of the analysis rise concern and call for further studies in environmental fate of the cytostatic IMA and subsequent environmental risk assessment, as anticancer agents do not have a threshold level to reveal their mutagenic, carcinogenic or cytotoxic activity. In this view, finding the residues of such drug in the outflow of WWTPs is much more of an emerging issue as compared to other pharmaceuticals.

3.5. Assessment of aquatic toxicity of identified TPs

We estimated the potential aquatic toxicity of IMA residues using *in-silico* QSAR models, as presented in Table SI-5. The QSAR models use relationship between molecular structure and toxicity of known compounds to predict toxicological properties of new compounds of interest.

To predict the aquatic toxicology of IMA and its TPs, which enter the aquatic environment through WW effluents, we employed relevant and commonly used organisms, *Daphnia magna* and *Pimephales promelas* as toxicological targets. 48-h and 96-h 50% lethal concentration (LC50) were selected as prediction endpoints for *Daphnia magna* and *Pimephales promelas*, respectively. For *Pimephales promelas* predicted 96-h LC50 for IMA was 0.08 mg/L, while the TPs showed LC50 in a range from 0.26 to 154.39 mg/L, with relative toxicities between 0.303 and 0.001. This indicates lower toxicities of TPs than of IMA. However, for IMA, TP378 and TP426 the predicted LC50 values were below 1 mg/L, which classifies them as hazardous to the aquatic environment, according to the European Commission in Regulation EC No 1272/2008 on classification, labelling and packaging of substances and mixtures.

In *Daphnia magna* the predicted 48-h LC50 for IMA was 7.55 mg/L and for TPs in a range 5.56–441.01 mg/L. The relative toxicities for this test show again that the majority of TPs are likely to be less toxic than IMA, however for crustaceans TP263 and TP320 present higher toxic potential as compared to the parent IMA. It should be noted that QSAR models are predictive and not experimental and should be as such considered with caution. Regardless, the models offer quick initial screening of TP toxicity and show some TPs with potentially higher toxicities than the parent compound IMA, while urging that further studies are required to fully assess the environmental risk.

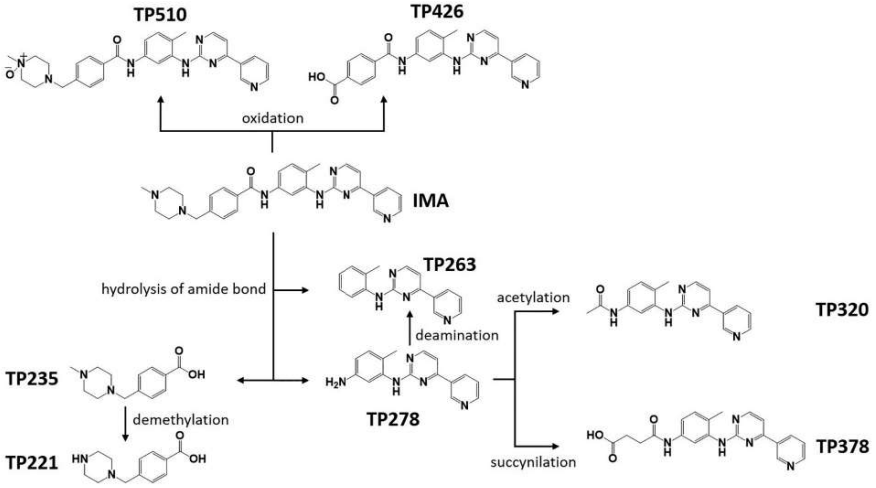

Fig. 5. Proposed microbial degradation pathway of IMA.

3.6. Study limitations

Slovenia is a country with high water flow, high precipitation and environmentally less burdened waters. Further, its population density is low, so the levels of IMA residues can be explained by its relatively low consumption. The consumption of the active substance was on the average 24 kg/year and remained stable through the period between 2010 and 2019. In this view, an occurrence study on WWTPs with lower capacities and higher IMA prescription would predictably show more concerning results, so further studies including countries with lower water flow and higher population density are needed to obtain additional data on its occurrence and transformation.

The second limitation is in the prediction of toxic effects of IMA and its TPs, which was conducted using short term ecotoxicological endpoints on two aquatic organisms. It should be emphasized that even though this data presents an insight into potential short term ecotoxicological effects, it does not reflect cancerogenic, mutagenic and genotoxic effects, which have no threshold levels, so the occurrence at any level may provoke the corresponding effect.

4. Conclusions

The present study aimed to provide a novel, efficient, and rapid workflow, making use of publicly available tools, for the study of the formation and identification of TPs of contaminants expected to be present in waters, using as case study the cytostatic drug IMA. Simulation of a wastewater treatment was conducted in order to monitor degradation of IMA under controlled conditions and to determine the TPs produced by its biodegradation. The findings of this study suggest that IMA is readily biodegraded, but instead of being completely mineralized, it forms TPs. We identified eight TPs using the principles and workflows employed in nontargeted analysis. UHPLC-HRMS^2 data was processed using the free access software MZMine2 to prepare the data matrix, which was then analysed using MVSA. PCA and OPLS-DA models were built in Metaboanalyst and used to visualize experimental group differences and to determine their differential compounds. Identification was achieved by MS^2 analysis, aided by Sirius-CSI:FingerID and CFM-ID. Among the identified TPs, one was previously identified and another one reported as a metabolite, while six are new to the science. Two major TPs are formed by hydrolysis of the IMA amide bond, one of which undergoes further biological transformations, including conjugation and deamination. TPs formed by oxidation and demethylation were also determined, with all transformations implying that IMA undergoes complex breakdown processes in the environment. Many other features were present in the degradation data matrix; however, the concentration was too low for their MS^2 acquisition, making their identification impossible. This implies that many more TPs of IMA may be formed in the environment, which might impose serious risks for the environment. Furthermore, TP235 was also found in real WW samples from WWTPs, which is even more concerning due to the fact that anticancer agents do not have a threshold level to reveal their mutagenic, carcinogenic or cytotoxic activity. In this view, finding the residues of such drug in the outflow of WWTPs is much more of an emerging issue as compared to other pharmaceuticals, even though *in-silico* QSAR models predict lower toxicity than that of the parent compound. The outcomes of this study suggest that further investigations into the occurrence and toxicity of IMA residues are necessary to properly assess the risk and hazard associated with the release of IMA in the environment.

CRediT authorship contribution statement

Žiga Tkalec: Investigation, Formal analysis, Visualization, Writing – original draft, Writing – review & editing. **Noelia Negreira:** Investigation, Formal analysis, Visualization, Writing – review & editing. **Miren López de Alda:** Project administration, Conceptualization, Validation, Writing – review & editing, Supervision, Funding acquisition. **Damià Barceló:** Writing – review & editing. **Tina Kosjek:** Conceptualization, Investigation, Validation, Writing – review & editing, Supervision.

Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2021.149063.

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3.2.2 UHPLC-HRMS data from non-targeted screening for biotransformation products of cytostatic drug imatinib

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To increase transparency and support further data mining, the raw and processed data acquired in the previous study (Section 3.2.1) was published [115]. This enables new data mining approaches, which are rapidly developing to be applied to already existing datasets and with that discover pieces of information unavailable before. The revised version is currently under second revision at the Data in Brief journal and the data is available under the doi-number: 10.17632/bby22v4bdr.1.

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Data Article

UHPLC-HRMS data from non-targeted screening for biotransformation products of cytostatic drug imatinib

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ABSTRACT

Imatinib is a selective tyrosine kinase inhibitor used to treat chronic myeloid leukemia. It enters the environment by excretion from the body through urine and feces and is transferred with wastewater to a wastewater treatment plant. There, it can be degraded by activated sludge, forming a number of biotransformation products. Presence of imatinib and its potential transformation products in the environment can impose a high risk to aquatic organisms and human health, therefore it is important to obtain knowledge of its environmental fate. The data presented here is a result of a simulated biodegradation of imatinib at two levels of activated sludge using a batch biotransformation setup, with and without carbon source. The data was acquired with UHPLC-HRMS/MS and processed by MzMine2.36 [1]. The dataset presents a table of $[M+H]^+$ features with retention times and corresponding MS/MS data. With development of new data mining tools this data can be used to identify new transformation products of imatinib and with it fully understand its environmental fate and the risk associated with its presence in the environment.

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Specifications Table

Subject	*Analytical chemistry*
Specific subject area	*Non-targeted analysis of simulated environmental samples*
Type of data	Raw and table
How the data were acquired	*Data was acquired using HPLC coupled to a Thermo Scientific Q-Exactive triple quadrupole-Orbitrap high-resolution mass spectrometer (San Jose, CA, USA) operating in positive mode ESI(+). Chromatographic separation was achieved on a Hibar HR column (50 × 2.1 mm, 2 µm, Merck).*
Data format	*Abundance table in .csv, mass spectrometry data in .mgf and .raw files of the acquired data*
Description of data collection	*Mass spectrometry data was collected in the full scan range of 70-1000 m/z with mass resolution of 70,000 full width at half maximum (FWHM) at 200 m/z. MS/MS data was collected using data dependant acquisition fragmenting the 10 most abundant ions with resolution of 17,500 FWHM at 200 m/z. This data was pre-processed using MzMine 2.36.*
Data source location	• *Institution: Jožef Stefan Institute, Department of Environmental Sciences* • *City/Town/Region: Ljubljana* • *Country: Slovenia*
Data accessibility	Mendeley data: doi:10.17632/bby22v4bdr.1
Related research article	***For a published article:*** [1] Ž. Tkalec, N. Negreira, M. López de Alda, D. Barceló, T. Kosjek, A novel workflow utilizing open-source software tools in the environmental fate studies: The example of imatinib biotransformation, Sci. Total Environ. 797 (2021) 149063. https://doi.org/10.1016/j.scitotenv.2021.149063.

Value of the Data

- The data describes the biotransformation of the cytostatic drug imatinib. It consists of degradation kinetics at two levels of activated sludge in carbon rich and carbon poor media. Non-targeted screening data contains structural information about the biotransformation products formed from imatinib during microbial degradation.
- This data is of value for people researching the environmental fate of organic contaminants as well as data scientists interested in data mining and developing new identification tools.
- The data can be further mined by ever-developing identification and data mining tools and produce even more valuable insights on biotransformation and lead to broader understanding of processes during wastewater treatment. Further identification of other biotransformation products can help enhance the current knowledge on the risk associated with the introduction of the cytostatic imatinib into the environment.

1. Data Description

The dataset contains MzMine 2.32 processed non-targeted data and consists of:

- raw LC-MS data files obtained with Q-Orbitrap mass spectrometer
- quantitative data file "Imatinib_biotransformation_quant.csv" contains abundance and retention time data for each detected ion for which MS/MS data was acquired. The data table includes row ID, feature m/z and retention time and abundances in each sample.
- The file Imatinib_biotransformation_MSMS.mgf contains merged MS/MS data for each ion presented in the first table. The dataset is not filtered to allow processing for future use.

2. Experimental Design, Materials and Methods

2.1. Experimental design

Imatinib was spiked into an artificial wastewater matrix at the level of 1 mg/L. Its biodegradation was studied in two media, i.e. the nutrient-mineral medium (IMA-A, IMA-B, IMA-2A, IMA-2B) which simulated the composition of actual wastewater [2], and in the mineral medium (IMA-C, IMA-D, IMA-2C, IMA-2D), which was devoid of other carbon sources except for the spiked compound imatinib. To account for possible abiotic degradation and sorption the biomass was inhibited *via* the addition of formaldehyde (IMA-2B, IMA-2D). To follow possible differences in biotransformation kinetics each biodegradation setup was studied at two levels of activated sludge, e.g. high – 50 mL (IMA-A, IMA-B) and low – 10 mL (IMA-2A, IMA-2B, IMA-2C, IMA-2D) per 400 mL of total batch volume. Spontaneous degradation of the parent compound was controlled by adding imatinib into the distilled water (batch IMA-2E), while concurrent matrix biodegradation was followed in a non-spiked batch recorded as IMA-E. Sampling was performed in 24-h intervals on eight consecutive days, which is reflected by the last number of the datafile name.

2.2. Chemical analysis

Chromatographic separation was performed on a Waters Acquity (Waters, Milford, MA, USA) UHPLC system using a reversed phase Hibar HR (50 × 2.1 mm, 2 µm, Merck) column. The separation was achieved using water (A) and methanol (B) as mobile phases at a flow of 0.3 mL/min and at 25°C with an injection volume of 10 µL. The elution gradient was 0-1 min, 5% B; 3 min, 20% B; 6 min, 80% B; 7 min 100% B; 10-12 min, 5% B. Mass spectrometry data was acquired on a Thermo Scientific Q-Exactive quadrupole-Orbitrap (San Jose, CA, USA) mass spectrometer operating in positive electrospray ionisation mode (ESI(+)). Data was acquired using data dependant acquisition with the 5 most abundant ions being fragmented by higher-energy collisional dissociation (HCD) with normalized collision energy at 40 % and activation time of 120 ms. With that, full scan data was acquired with resolution of 70,000 FWHM at *m/z* 200 and data dependant acquisition of 17,500 FWHM at *m/z* 200. Spray voltage was 3000 V, sheath gas flow was set at 40 PSI, auxiliary gas at 10 PSI with capillary and vaporizer temperatures at 350°C and 400°C, respectively. Acquisition was controlled by Xcalibur 2.2 (Thermo Fischer Scientific).

2.3. Data pre-processing

Raw data was imported to MzMine 2.36 [3], a software widely used for processing of mass spectrometry non-targeted data (Sailwal et al., 2020; Sarpe and Schriemer, 2017). Mass detection was done with Exact mass module using 1.0E06 as noise threshold. Chromatograms were built with minimum time span of 0.02 min and minimum height 1.0E06. Mass error tolerance was 0.005 *m/z*. Chromatograms were deconvoluted using Wavelets (ADAP) module with signal-to-noise threshold of 10 and intensity window SN as signal-to-noise estimator. Minimum feature height was set to 1.0E06. Area threshold was set at 100 with peak duration range and retention time wavelet range of 0.02-0.90 min. Isotopic peaks were grouped with 0.005 *m/z* of mass tolerance and 0.1 min of retention time tolerance. Maximum allowed charge was 1. Ions were aligned using RANSAC alignment module with 0.005 *m/z* of mass tolerance and 0.2 min of retention time tolerance. Tolerance of 0.1 min was allowed after correction. Number of points was set to 20 % with threshold value of 3. After alignment, ions were filtered for duplicates with New average filter mode and 0.005 *m/z* of mass tolerance and 0.2 min of retention time tolerance.

CRediT Author Statement

Žiga Tkalec: Investigation, Formal analysis, Visualization, Writing – original draft, Writing – review & editing; **Noelia Negreira:** Investigation, Formal analysis, Visualization, Writing – review & editing; **Miren López de Alda:** Project administration, Conceptualization, Validation, Writing – review& editing, Supervision, Funding acquisition; **Damiá Barceló:** writing – review & editing; **Tina Kosjek:** Conceptualization, Investigation, Validation, Writing – review & editing, Supervision.

Ethics Statement

The authors declare that the manuscript meets all the rules and conditions described in the "Ethics in publishing" section standards (https://www.elsevier.com/journals/data-in-brief/2352-3409/guide-for-authors). This work did not include any investigations involving animal experimentation or human participants.

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.

Data Availability

Imatinib biotransformation (Original data) (Mendeley Data)

Acknowledgments

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3.3 Connecting the NT Workflow with the Traditional Targeted Analysis

3.3.1 Exposure of Slovenian children and adolescents to bisphenols, parabens and triclosan: Urinary levels, exposure patterns, determinants of exposure and susceptibility

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The study presented herein [116] aimed to quantify endocrine disrupting chemicals (EDCs) bisphenols, parabens and triclosan using targeted analysis. EDCs are exogenous compounds which are involved in endocrine functions such as synthesis, secretion, activity and clearing of native hormones, causing adverse health effects [117]. Although their acute toxicity is thought to be low, long-term chronic exposure and combined effects of exposure are not yet well understood. Bisphenol A (BPA) and its alternatives bisphenol F (BPF) and bisphenol S (BPS) are used in the synthesis of polycarbonate plastic and epoxy resins, with human population exposed due to their widespread occurrence in food and environment. Parabens are alkyl esters of para-hydroxy benzoic acid and are used as antimicrobials and preservatives in cosmetics, personal care products, pharmaceuticals, food and beverages [118]. Similarly, triclosan (TCS) is used as an antimicrobial agent in personal-care products, such as cosmetics, toothpastes, shower gels, deodorants as well as in household cleaning products, plastics and toys [119]. In this study, we investigated the levels of EDCs in urine from Slovenian children and adolescents living in a rural region in the north-eastern part of Slovenia. We searched for the exposure patterns and connected the EDC levels with potential exposure sources by analysing questionnaire data related to their dietary habits, food storage and the use of personal care products. Participants were genotyped for the presence of SNP in UGT2B15 gene (rs1902023) in order to investigate the associations between the SNP and EDCs levels in urine. In the study we found that urinary levels of selected EDCs found in Slovenian population were comparable to the levels found across the world, with differences in levels of certain EDCs, such as generally lower levels of propyl paraben (PrP) and TCS and higher levels of ethyl paraben (EtP). Exposure turned out to be location dependent, as samples from individuals in one specific subregion contained higher levels of BPF and EtP than in other locations. Furthermore, we elucidated several determinants of exposure, BPA was significantly associated with high fat food items, while MeP with personal care products. UGT2B15 has been found to play a role in MeP and EtP metabolism, while contrary to expectation, not with BPA. Participants were exposed to a variety of EDCs, and even though the urinary levels were relatively low and below the current adverse effect levels for individual EDCs, mixture effects should be considered in the future.

Importantly, this study validated the results of NTS described in Section 3.1.2, as it determined in a targeted manner the same EDCs (BPF, EtP and butyl paraben) within the samples from the same population. BPA and MeP were however not determined using NTS, despite being quantified in 99 % and 80 % of samples with the targeted method. This shows that the results of NTS are in line with the results of traditional targeted screening and confirms hypothesis H4. The results demonstrate that there is still an immense potential for post-acquisition data processing tools to enable the detection of numerous other chemicals including BPA and MeP, for which it was demonstrated via targeted analysis that they were present in the investigated samples.

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Exposure of Slovenian children and adolescents to bisphenols, parabens and triclosan: Urinary levels, exposure patterns, determinants of exposure and susceptibility

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ABSTRACT

Chemicals such as bisphenols, parabens and triclosan are endocrine disrupting chemicals. They are used in a wide variety of consumer products, making human exposure to those chemicals widespread.

In the present study, levels of three bisphenols (bisphenol A, F and S), 7 parabens (methyl-, ethyl-, isopropyl-, propyl-, isobutyl-, butyl-, benzyl paraben) and triclosan were measured in first morning void from 246 Slovenian children and adolescents, aged 6–9 and 11–15 years and living in a rural region of Slovenia. Median levels of specific-gravity corrected levels for bisphenol A, bisphenol F, methyl paraben and ethyl paraben were 1.9, 0.085, 5.4 and 2.5 μg/L for children and 1.6, 0.11, 7.2 and 6.0 μg/L for adolescents, respectively. Median levels for all other endocrine disrupting chemicals were < LOQ. The levels are comparable with the levels reported in studies across the world. Exposure was age, sex, and location specific. Higher levels of bisphenol F and ethyl paraben were found in the samples of adolescents, while higher levels of methyl paraben were found in samples from girls. Furthermore, individuals living in one of the sampling locations, Goričko, were exposed to higher levels of bisphenol F and ethyl paraben than those in the remaining two sampling locations. Information about participants' dietary habits, use of food packaging and personal care products was obtained through questionnaires, and used to investigate associations between urinary levels of the biomarkers and potential exposure sources. High fat foods were associated with bisphenol A exposure, and cosmetics items such as lipstick and perfume with methyl paraben exposure. Significant correlation between methyl- and propyl paraben was observed in children's samples, suggesting similar exposure sources, while other compounds were not largely correlated, indicating independent sources. Furthermore, association between a single nucleotide polymorphism (SNP) in *UGT2B15* gene and urinary levels of methyl and ethyl paraben was observed, showing the role of UGT2B15 isoform in methyl and ethyl paraben metabolism as well as indicating the SNP rs1902023 as a potential biomarker of susceptibility to adverse effects caused by the exposure.

The present study reports exposure of children and adolescents in Slovenia to a wide range of different endocrine disrupting chemicals for the first time, connecting it to exposure patterns and exposure sources. The study is to the authors' knowledge the first that investigates direct connection between levels of urinary endocrine disrupting chemical biomarkers and genetic polymorphism in *UGT2B15*.

1. Introduction

Humans are exposed to an increasing load of environmental contaminants, many of which play a role in development of chronic diseases. (Woodruff, 2015) Bisphenols, parabens and triclosan are found in wide variety of consumer products and are endocrine disrupting chemicals (EDCs). EDCs are exogenous compounds which are involved in endocrine functions such as synthesis, secretion, activity and clearing of native hormones, causing adverse health effects. (Darbre, 2019) Although their acute toxicity is thought to be low, long-term chronic

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exposure and combined effects of exposure are not yet well understood.

Bisphenol A (BPA) is an industrial chemical which is used as a monomer in production of polycarbonate plastic and in the production of bisphenol-based epoxy resins. Due to properties such as shatter-resistance, being optically clear and lightweight, polycarbonate plastic is widely used for reusable water bottles and food storage containers, while epoxy resins are used for coating of metal cans coming into direct contact with food. BPA is likewise used for tooth fillings, cash receipts and plastic toys for children. (Geens et al., 2012; Russo et al., 2017) In the body BPA is rapidly metabolised. The majority of BPA is metabolized to glucuronide form and excreted from the body almost completely within 24 h. (Thayer et al., 2015) Several studies have observed associations between exposure to bisphenols and several non-communicable diseases, for example type II diabetes (Duan et al., 2018), cardiovascular diseases and obesity. (Andra et al., 2015; Jiang et al., 2020) BPA was shown to affect reproductive system, possess developmental toxicity and negatively impact respiratory system, liver, kidneys, immune system, mental health and also to play a role in cancer development. (Ma et al., 2019)

Due to concerns about toxicity and continuous use of BPA, manufacturers have started to remove BPA from products and implement its structurally related alternatives. Among these, bisphenol F (BPF) and S (BPS) are the most common. Similarly to BPA, BPS is used in the production of polycarbonate plastic, epoxy resins and thermal paper (Skledar et al., 2016), while BPF is used for food packaging, pipe linings, epoxy resins, dental materials, coatings and adhesives. (Usman et al., 2019) BPF and BPS were found to have comparable estrogenic, androgenic, antiandrogenic, antiestrogenic potency as BPA, (Pelch et al., 2019; Rochester and Bolden, 2015) raising similar concerns when used as BPA alternatives. In the body, BPF is mainly metabolised to sulphate conjugate (Cabaton et al., 2006), while glucuronide is the predominant urinary metabolite of BPS (Gys et al., 2018; Song et al., 2018). Both compounds are almost completely cleared from the body within 48 h. (Oh et al., 2018)

Parabens are alkyl esters of para-hydroxy benzoic acid (PHBA) and are used as antimicrobials and preservatives in cosmetics, personal care products, pharmaceuticals, food and beverages. (Liao et al., 2013) Parabens differ by the length of the chain, the most common parabens are methyl paraben (MeP), ethyl paraben (EtP), propyl and iso-propyl paraben (PrP, iPrP), butyl and iso-butyl paraben (BuP, iBuP) and benzyl paraben (BzP). Parabens have been found exhibiting uterotropic, estrogenic and aromatase-inhibitory effects, while all except BzP have been found anti-androgenic in several *in-vitro* studies, raising significant concern for endocrine disruption. The two long chained parabens, PrP and BuP, have been raising highest concern (Boberg et al., 2010), as toxic effects of parabens on several bodily endpoints increase with the length of alkyl chain. Parabens are cleared from the body mainly by hydrolysis of the ester bond, forming PHBA which is subsequently conjugated with glucuronic acid, sulphuric acid or glycine, forming para-hydroxy hippuric acid (PHHA). Although PHHA is the major metabolite, it is widely non-specific. Glucuronidated parent compounds are therefore followed as biomarkers of exposure to parabens. (Abbas et al., 2010) Parabens are rapidly metabolised, on the average 80% of the compound is cleared within 24 h. However, different parabens have slightly different rate of clearing, where the long-chained parabens are more rapidly hydrolysed to PHBA and corresponding alcohol. (Moos et al., 2016).

Triclosan (TCS) is used as an antimicrobial agent in personal-care products, such as cosmetics, toothpastes, shower gels, deodorants as well as in household cleaning products, plastics and toys. (Bedoux et al., 2012) Once introduced in the body, it is rapidly metabolised to its glucuronide and sulphate conjugates and excreted in urine, with the half-life of 11 h. (Yueh and Tukey, 2016) Disruption of hormone metabolism, steroidogenic enzyme activity and displacement of hormones from hormone receptors have been implied as the toxicological mechanisms of TCS. (Wang and Tian, 2015) TCS has also been reported to have the effect on reproductive hormones mediated by steroidogenic enzymes in prenatally exposed infants. (Wang et al., 2018)

Most of EDCs included in this study are excreted mainly as glucuronide conjugates. UDP-glucuronosyltransferases (UGTs) are a superfamily of enzymes that catalyse conjugation of endogenous compounds and xenobiotics with glucuronic acid via uridine diphosphate (UDP) glucuronic acid. Glucuronides are more hydrophilic, which facilitates their excretion with urine. UGT superfamily consists of 19 isoforms with different substrate specificities. Among them, UGT2B15 has been reported to have a high affinity for several EDCs included in this study. (Abbas et al., 2010; Hanioka et al., 2008) A SNP in UGT2B15 (rs1902023), which involves 253A > C substitution in coding region of *UGT2B15* gene and causes amino acid change from tyrosine (Tyr, A allele) to aspartic acid (Asp, C allele) at codon 85, has been linked with decreased enzymatic rate of 85Tyr UGT2B15 variant and thus increased levels and toxicity of its substrates in individuals possessing A allele. (Hanioka et al., 2011) As susceptibility to adverse effects of the chemicals is related to individual's genetic predisposition, it is important to find connection between genes involved in metabolism of EDCs and biomarker levels.

Within the scope of human biomonitoring (HBM) actions, which have been implemented in various countries, several studies investigated exposure of different vulnerable populations, such as children and adolescents to EDCs. (Casas et al., 2011; Frederiksen et al., 2013; Larsson et al., 2017; Liu et al., 2019; Myridakis et al., 2015; Rocha et al., 2018) Furthermore, many studies indicated potential determinants and sources of exposure to EDCs, such as, among others, consumption of canned food and social class for BPA (Covaci et al., 2015) and use of personal care products, such as makeup and skincare products for parabens. (Sanchis et al., 2019) Even though certain determinants have been previously described, further research is needed to provide more comprehensive data on specific exposure sources, especially for vulnerable populations. Despite the number of HBM studies in other countries, there is a lack of data on exposure of general population and especially children and adolescents to EDCs in Slovenia. In 2011, a study on BPA levels in urine of Slovenian children was conducted (Snoj Tratnik et al., 2019), followed by risk characterization study in 2019. (Sarigiannis et al., 2019) However, the present study advances the work with an investigation on a larger number of EDCs, involving children as well as adolescents. We investigated the levels of 3 bisphenols, 7 parabens and triclosan in urine from Slovenian children and adolescents living in a rural region in the northeastern part of Slovenia. We searched for the exposure patterns and connected the EDC levels with potential exposure sources by analysing questionnaire data related to their dietary habits, food storage and the use of personal care products. Participants were genotyped for the presence of SNP in *UGT2B15* gene (rs1902023) in order to investigate the associations between the SNP and EDCs levels in urine.

2. Material and methods

2.1. Recruitment and sampling

The study was focused on addressing exposure of children from Prekmurje region in Slovenia and was conducted within the national project 'Exposure of children and adolescents to selected chemicals through their habitat environment (2016–2019)'. Prekmurje region is characterized as predominantly rural with high intensity of agricultural activity. For this region the exposure of inhabitants and especially children and adolescents to EDCs is unknown. Children and adolescents aged 6–10 years and 12–15 years, were sampled in first half of the year 2018. Participants provided first morning urine, blood and saliva. They were recruited from 7 different schools, belonging to three distinct subregions, namely Goričko (further on referred to as location 1, L1), Ravninski del (further on referred to as location 2, L2) and Lendavske Gorice (further on referred to as location 3, L3). Participants were

recruited based on the following inclusion criteria: 1) participant resided in the region, 2) participant resided in the same region for at least 3 years and 3) participant was not medicated for any chronic liver or kidney disease. The participants provided first morning urine, and when this was not possible, at least 5 h had to pass since last urination. The participants collected their urine into collection vessels provided by the organizer. Samples were then aliquoted at the laboratory of the Clinical Chemistry and Biochemistry Institute of the University Medical Centre Ljubljana into 2-mL criovials, transported to Jožef Stefan Institute and stored at −80 °C until analysis.

Ethical permission for the study was granted by Republic of Slovenia National Medical Ethics Committee, with number of accordance 0120–118/2017/3. Parents or legal guardians of all the participants signed the informed written consent and were able to withdraw from the study at any time.

2.2. Questionnaires

Questionnaires were targeting living environment, food consumption and nutritional habits, lifestyle, socioeconomic status, and use of personal care products. EDC-specific questions included data on type of personal care products that the participants were using (cosmetic cream, lipstick, perfume, deodorant etc.), food-storage containers (plastic, glass, metal), food reheating in the containers, and frequency of consumption of canned food. Questionnaires were answered by the participants (and their families) during interviews held in schools, with the help of trained field workers.

2.3. Chemical analysis

2.3.1. Sample preparation

Sample preparation procedure was based on previously reported method. (Snoj Tratnik et al., 2019) Briefly, glucuronide and sulfate metabolites were deconjugated using *β*-glucuronidase/arylsulfatase (Helix Pomatia, type H-2) at the concentration of 200 U/mL. 1-mL samples were incubated at 37 °C for 18 h. Afterwards, samples were extracted on Oasis HLB 96-well plates (60 mg, Waters). Dried extracts were reconstituted in 100 μL of ACN and derivatized with 20 μL *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA, 1 h, 50 °C). Derivatized samples were cleaned on two Strata Si (100 mg/1 mL) 96-well plates. Each plate was conditioned with 1 mL of DCM and 2 mL of EtAc. The first Strata Si plate was mounted on the second one to achieve sample cleaning through two Si columns. Derivatized samples were loaded on the first plate and eluted with 1.5 mL of DCM into 2-mL collection plate. Eluates were concentrated under nitrogen stream to the final volume of approximately 100 μL and transferred to glass vials containing glass inserts. Chemicals used in the analysis are listed in SI-1.

Each sample was injected twice. In the first injection, BPA and BPF were analyzed. Afterwards, additional 20 μL of MSTFA were added to slow down the degradation of derivatives and the sample was measured again. This time, BPS, parabens and TCS were analyzed.

Each compound was normalized against its corresponding ^{13}C- or *d*-labelled internal standard. The internal standards covered for the entire sample preparation procedure; they were added after deconjugation step at the final concentration of 4 μg/L. $^{13}C_6$-propyl paraben was used to normalize propyl and isopropyl paraben, while $^{13}C_6$-butyl paraben was used for butyl and isobutyl parabens, and all the remaining internal standards were isotopically labelled target analytes.

2.3.2. GC-MS/MS analysis

Agilent 7890B gas chromatograph coupled to the triple quadrupole mass analyser Agilent 7000 was used for quantification. Injector temperature was set to 270 °C. Injections were performed in splitless mode with the injection volume of 2 μL. Chromatographic separation was achieved by using Agilent DB5-MS UI column (30 m, 0.25 mm, 0.25 μm). Helium was used as carrier gas at 1 mL/min. The temperature program was 120 °C, held for 2 min, ramped at 16 °C/min until 300 °C, and finally held for 12 min. The temperature of transfer line was 280 °C. Molecules were ionized by electron impact ionization at 70 eV. Nitrogen was used as collision gas at 1.5 mL/min.

The analyses were performed in the multiple reaction monitoring mode using two transitions per molecule. The more intensive transition was used for quantitation and the second transition to confirm the analyte identity. Collision energies were optimized for each transition. Transitions with collision energies and retention times are reported in table SI-1. Dwell time of 50 ms per transition allowed for approximately 10 points per peak.

2.3.3. Method validation

Method linearity, range, accuracy error, repeatability, reproducibility, limit of quantitation and measurement uncertainty were evaluated. Linearity was evaluated on the basis of R^2 values of calibration curve within the range of each compound. Calibration curve was prepared by spiking synthetic urine in triplicate for each level. Synthetic urine was prepared according to the published procedure. (CDC, 2009). The calibration curve was prepared in the relevant concentration range, from the limit of quantification (LOQ) to 100 μg/L for bisphenols, and from LOQ to 500 μg/L for parabens and triclosan. Validation parameters are presented in table SI-2. Accuracy error was evaluated by using spiked synthetic urine at three concentration levels, namely LOQ, 1 and 10 μg/L. Instrumental repeatability was evaluated as the relative standard deviation (RSD) of three injections of the accuracy error samples. Method repeatability was determined as RSD of three methodological replicates at three concentration levels, LOQ, 1 and 10 μg/L. Reproducibility was calculated as RSD in response ratio of QC samples recorded each week. Measurement uncertainty (MU) was estimated based on QC samples. We took into account accuracy error and reproducibility of QCs, which covered uncertainty contributed from different analysts, different days of analysis, and uncertainty in concentration contributed by preparation of standards and calibration samples. Extended description of the calculation is reported in SI-4.

Since BPA, BPS, MeP, EtP and TCS were present in procedural blanks, limit of detection (LOD) could not be determined for the investigated compounds. The concentrations in calibration curve and samples were corrected for their levels in the procedural blanks. The blank samples were prepared in triplicates and each sample was injected three times, so the average of 9 injections was used for the correction. Procedural blank for calibration curve was synthetic urine spiked with isotopically labelled internal standards, while LC-MS purity grade water spiked with labelled internal standards was used to correct real samples. Blank subtraction introduces more uncertainty, especially in quantification accuracy at levels close to background values, therefore the lowest point of the calibration curve with the accuracy error of ≤20% was used as LOQ (Table SI-2).

2.3.4. Quality control

Contribution of background contamination was controlled by using blank samples as mentioned in section 2.3.3. Carryover was monitored by using solvent blanks, which were injected after each five real samples.

QC samples were used to monitor the analytical system performance and were injected after each solvent blank. QC samples were prepared by spiking pooled deconjugated urine with investigated compounds and internal standards at 5 μg/L and 4 μg/L, respectively. System performance was checked using control charts in which response ratio was plotted against the number of injections. Batches exhibiting large deviations or trends in responses were rejected and their analysis repeated, after the cause was determined and eliminated.

The analytes were tested for degradation by potential nonspecific lipase activity of β-glucuronidase/arylsulfatase used for urine deconjugation. Briefly, 1 mL of synthetic urine was spiked with the internal standards and EDC standards to 5 μg/L. 20 μL of enzymatic solution with

10 kU/mL of β-glucuronidase/arylsulfatase was added and the sample was deconjugated according to the above described procedure. The process was controlled by replacing the enzyme with the equal volume of LC-MS water. Test and control samples were prepared in triplicates. After deconjugation, samples were extracted, cleaned and analyzed according to the above described procedure. Deconjugation specificity was calculated as the percentage difference in responses of the test and control samples.

Integrity of samples was investigated by analysing the stability of EDC derivatives in urine extracts at three levels, at LOQ, 1 and 10 μg/L, each level was prepared in duplicate. Samples were spiked with native and isotopically labelled standards to the desired level and prepared according to the sample preparation procedure described above. Finally, we added 20 μL of ethyl acetate to the first set of the duplicates and 20 μL of MSTFA to the second set, in order to quench the degradation of derivatives. The difference between responses of analytes in each sample was monitored. Samples were injected 20 times in the alternating order.

2.4. Specific gravity adjustment

To account for sample dilution the concentrations of EDCs were adjusted using specific gravity adjustment. SG-corrected concentrations were calculated by the formula

$$C_{adjujsted}(\mu g/L) \;=\; C_{measured} \times \; (SG_s - 1)/(SG_i - 1)$$

where $C_{adjusted}$ is the adjusted concentration, $C_{measured}$ is measured concentration, SG_s is standard specific gravity, which was calculated as average specific gravity for boys ($SG_s = 1.024$) and girls ($SG_s = 1.023$) separately, SG_i is measured specific gravity of an individual sample.

2.5. DNA extraction and SNP genotyping

FlexiGene® DNA Kit (Qiagen, Hilden, Germany) and PrepIT-L2P (DNA, Genotec Inc.) were used for DNA extraction for blood and saliva, respectively. Extraction was conducted according to the protocol included in the kit. Saliva was used when the blood sample of a participant was not available. Quality of the extracted DNA was checked using a NanoDrop 2000c UV–VIS spectrometer (ThermoFisher Scinetific, USA). SNP in UGT2B15 (rs1902023) was genotyped using TaqMan® SNP Genotyping Assays (Applied Biosystems, Foster City, CA, USA).

Reaction volume was 5 μL and consisted of 2.5 μL TaqMan® Universal master Mix II with UNG (Applied Biosystems, Foster City, CA, USA), 0.125 μL of 44x TaqMan probe/primer mix, 1.875 μL ultrapure nuclease-free water (Life Technologies, CA, USA) and 0.5 μL of genomic DNA.

LightCycler® 480 Instrument II and LightCycler® 480 1.5.1 Software (Roche) were used for amplifications and fluorescence detection. PCR cycling consisted of pre-PCR (1 cycle: 50 °C, 2 min), activation (1 cycle: 95 °C, 10 min), annealing and amplification (50 cycles, 95 °C, 15 s, 61 °C, 1 min) and post-PCR (1 cycle: 40 °C, 30 s). 10% of samples were analysed in two replicates for method control. The χ^2 test was performed to check if the variant was in Hardy-Weinberg equilibrium for the studied population and confirm validity of genotyping.

2.6. Statistical analyses

2.6.1. Group differences

Compounds with more than 50% of values below LOQ were excluded from the statistical analysis. For the remaining compounds, concentrations below LOQ were replaced by half of the LOQ. Data normality was checked using Shapiro-Wilk test. Differences between groups (location, gender, age) were analysed with ANOVA and Student's *t*-test at the significance level of 0.05, on natural logarithm transformed data to approach normality.

2.6.2. Correlations among EDCs

Correlations among different EDCs were analyzed using Pearson correlation coefficient on logarithm transformed data. Correlation coefficients with significance level lower than 0.1 were retained.

2.6.3. Questionnaire analysis

Questionnaire analysis was performed using multiple linear regression (LR) and partial-least squares regression (PLSR). Stepwise linear regression was conducted in R (package: olsrr (Aravind Hebbali (2018). Olsrr: Tools for building OLS regression models. Rpackage version 0.5.2, https://CRAN.R-project.org/package=olsrr).

Software for construction of PLSR model was SIMCA 15.0.2 (Sartorius Stedim Data Analytics AB). Number of principal components was determined by 7-fold cross-validation. PLSR models were optimized by exclusion of insignificant variables based on the Variable Importance for the Projection (VIP) plot and PLS regression coefficients. After model optimization, overfitting was checked by permutation test (N = 100).

The studied variables were gender, geographical region, use of personal care products (lipstick, cream, hair balsam, hair styling products, perfume, deodorant, body oil and nailpolish), food container materials (frequencies of storage in plastic, ceramic, metal, glass, PVC, paper, tetrapak), heating in PVC, frequency of consumption of canned food in last month, frequency of use and age of sports bottles (PVC, metal), frequency of using water dispenser. Additionally, frequency and amount of food consumed were studied, i.e. 64 distinct foods from 8 different categories (dairy products, fats, fruit and vegetables, meat and eggs, carbohydrate rich foods, condiments, snacks, and beverages). Along with dietary sources, nearby living environment was studied such as proximity of industry, waste disposal, waste incineration, gas plant, agriculture, orchard, vineyard and gardens.

Variables determined as significant in both LR and PLSR were regarded as candidates for determinants of exposure. Each candidate was validated by searching for the differences in EDC urinary levels between groups defined by questionnaire data for the determinant candidate with Student's *t*-test.

2.6.4. Associations between EDC levels and UGT2B15 genotype

Associations between EDC urinary levels and UGT2B15 genotype were studied with ANOVA. Individuals with genotypes showing similar trend in urinary EDC levels, i.e. children and adolescents with adenine-adenine (AA) and adenine-cytosine (AC) genotype were grouped together, while individuals possessing cytosine-cytosine (CC) genotype were placed in the other group. Student's *t*-test was used to check for the differences in concentration of EDCs between the two groups.

3. Results and discussion

3.1. Analytical method performance

To achieve reliable and robust results, which accurately reflect exposure of children and adolescents to EDCs, the performance of analytical method was carefully evaluated. EDCs exhibited linear response in the whole analytical range, R^2 was 0.998 or higher for each compound. LOQs, determined as the lowest calibration point with accuracy error ≤20% are presented in Table SI-2. LOQs varied from the lowest, 0.03 μg/L for BPF, to the highest, which was 1.0 μg/L for EtP.

Method proved to be accurate, repeatable and reproducible. Accuracy errors were within 10% of the nominal concentration for each EDC. Likewise, concentrations of EDC did not vary more than 10% when performed by a different analyst at a different time. Furthermore, we estimated the method's extended measurement uncertainty by including all relevant validation parameters (see SI-4), which showed that our results are within ±20% of the reported value for each EDC at 95% confidence interval.

Nonspecific lipase activity of β-glucuronidase/arylsulfatase from Helix Pomatia has been reported on certain EDCs, such as phthalates.

(Blount et al., 2000) Analogously to phthalates, parabens are esters of a benzoic acid congener, and are therefore at risk of being hydrolysed to PHBA and corresponding alcohols during deconjugation, biasing the results towards lower values. Hence, we tested the enzyme's specificity (see SI-5) by incubating EDCs with (1) enzyme and (2) water as a control. The results of the test show that the difference between concentration of enzyme and water incubated EDCs is lower than the RSD of the methodological replicates for each EDC (Table SI-3). This confirms that β-glucuronidase/arylsulfatase from Helix Pomatia (type H-2), at the concentration of 20 U/mL exhibits no nonspecific enzymatic activity towards the tested EDCs at 37 °C in 18 h, and therefore the reported results contain no bias contributed by the deconjugation step.

For GC analysis, EDCs are transformed to trimethylsilyl derivatives during sample preparation, thus the integrity of the samples is limited by the stability of the derivatives in urine. By checking the stability of the derivatives in urine extract we showed that all compounds, except BPS $(TMS)_2$, were stable in processed urine sample, as presented in Figure SI-1. BPS$(TMS)_2$ degraded in urine more rapidly than derivatives of any other EDC, however degradation was halted by adding excess MSTFA into the sample prior to the second injection into the instrument. Degradation curve of BPS$(TMS)_2$ is presented in Figure SI-2.

Our laboratory has successfully participated in several interlaboratory comparison investigations and external quality assurance schemes for all three bisphenols within HBM4EU (https://www.hbm4eu.eu/) project. Positive results reinforce reliability of the method not only for bisphenols but also for parabens and TCS since all compounds are analysed following the same procedure simultaneously.

3.2. Characteristics of the population

In total, 246 children and adolescents were recruited at three locations in Prekmurje region (Table 1). The participant response rate was 32%. Majority of participants (53%) were from L1, followed by L2 (26%) and L3 (21%). Both sexes were represented almost equally, 49% were male and 51% female. Participation was greater by children, which were aged 6–10 years and comprised 61% of the population, while adolescents, aged 11–15 years amounted to 39%.

Based on CDC online calculator (https://www.cdc.gov/healthyweight/bmi/calculator.html) 4% of participants were underweight, 73% of healthy weight, 13% overweight and 10% obese. 22% had medically confirmed allergies or food intolerances. 21% are currently medicated or using food supplements. Majority of participants resided in single-apartment house (87%) on the countryside or in a village (72%).

3.3. EDC levels in urine

The high detection rates of BPA, BPF, MeP and EtP along with moderate detection rates of BPS, other parabens and TCS show widespread exposure of Slovenian children and adolescents to numerous EDCs, as presented in Table 2. Levels are plotted in Fig. SI-3 and Fig. SI-4.

Bisphenols were commonly present in children's and adolescents' urine. BPA was determined in 99 and 100% of samples, while BPF and BPS were found in 85 and 87% and in 27 and 35% of samples, for children and adolescents, respectively. The highest concentration of BPA was 40 μg/L, while the maximum determined concentrations of BPF and BPS were 11 μg/L and 23 μg/L. Although there is an effort to replace BPA with its alternatives, of bisphenols, BPA was still found in the highest concentrations throughout the studied population (QR = 99 and 100 %, GM = 2.1 and 1.9 μg/L), followed by BPS (QR = 27 and 35 %, GM = 0.30 and 0.36 μg/L) and BPF (QR = 85 and 87 %, GM = 0.11 and 0.17 μg/L). BPA urinary levels are widely reported in literature, however data on BPF and BPS levels in children's and adolescents' urine is currently scarce (Table 3). Median urinary levels of BPA in Slovenian children and adolescents are comparable to the levels across the world. Compared to American population (Centers for Disease Control and Prevention, 2015), Slovenian children and adolescents seem to be exposed to higher levels of BPA, but to lower levels of BPF and BPS. This indicates lower use of BPA alternatives in Slovenia compared to USA. However, due to varying LOD and LOQ values of different reported methods, levels of BPF and BPS are difficult to compare.

Similarly to bisphenols, parabens were likewise frequently present in urine of Slovenian children and adolescents. Among parabens, the most common and abundant was MeP (QR = 83 and 80%, GM = 5.0 and 5.0 μg/L), followed by EtP (QR = 60 and 72%, GM = 2.7 and 4.4 μg/L). Other parabens were quantified in the whole population in less than 50% of samples, namely in 22%, 20%, 8%, 1% and 0.4% of samples for BzP, PrP, BuP, iPrP and iBuP, respectively.

The detected concentrations varied greatly within the studied population, MeP ranging from < LOQ to 1020 μg/L, while EtP was found at the maximum concentration of 200 μg/L. Although PrP was found only in 20% of all the samples, its highest determined concentration was relatively high, 320 μg/L. The antimicrobial TCS was found in 24 and 35% of samples, reaching up to 25 μg/L.

Compared to other studies (Table 4), our study reveals generally lower median urinary levels of MeP and PrP, but contrastingly, higher levels of EtP. As shown in Table 4, the urinary median EtP levels found in this study are only lower than in Spanish (Casas et al., 2011) and Korean (Kim et al., 2018) population.

This indicates the usage of different paraben combinations in consumer products in different countries. Presence of less used parabens such as iPrP and iBuP in the urine of children and adolescents is very low and in accordance with other studies mentioned in Table 4. Interestingly, median levels of TCS found in our study are <LOQ (which is 0.20 μg/L), indicating TCS levels that are lower than in most other populations.

As the results show, Slovenian children and adolescents are exposed to numerous EDCs, even though the levels are low. Apel et al. (2017) distinguishes between HBM I and HBM II levels of emerging substances. The HBM I value is attributed to the concentration of the compound found at or below which there is no risk of adverse effect, while HBM II values represent concentration at or above which adverse health effects are possible. Currently, of EDCs included here, HBM values are only

Table 1
Regional distribution and characteristics of the sampled population.

Location	L1		L2		L3		Total
Sex	Male	Female	Male	Female	Male	Female	121 (49% male) / 125 (51% female)
Age 6–10 y	28	36	23	26	16	20	149 (61%)
Age 12–15 y	34	33	12	2	8	8	97 (39%)
Total	131 (53%)		63 (26%)		52 (21%)		246
Characteristics	**Age (years)**		**Weight (kg)**		**Height (cm)**		
Sex	Male	Female	Male	Female	Male	Female	
Age 6–10 y	9	9	33	33	137	137	
Age 12–15 y	14	14	60	59	169	165	

Table 2
Urinary concentrations in μg/L of bisphenols, parabens and TCS in children and adolescents from Slovenia.

	Compound	N	QR	AM	GM	SD	Median	Min	Max	P5	P10	P90	P95	P99
Children	BPA	149	99	3.3	2.1	5.2	1.9	<LOQ	40	0.75	0.91	5.6	9.5	30
	BPF	149	85	0.37	0.11	1	0.085	<LOQ	7.2	<LOQ	<LOQ	0.78	1.5	5.9
	MeP	149	83	23	5	90	5.4	<LOQ	1020	<LOQ	<LOQ	49	73	260
	EtP	149	60	9.1	2.7	18	2.5	<LOQ	160	<LOQ	<LOQ	20	34	88
	iPrP	149	0.67	<LOQ	0.13	0.99	<LOQ	<LOQ	1.3	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
	PrP	149	18	5.6	0.21	33	<LOQ	<LOQ	320	<LOQ	<LOQ	0.76	3.6	140
	iBuP	149	0.67	<LOQ	<LOQ	0	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
	BuP	149	8	<LOQ	0.15	0.57	<LOQ	<LOQ	3.7	<LOQ	<LOQ	<LOQ	1.2	3.1
	TCS	149	24	0.53	0.19	1.6	<LOQ	<LOQ	14.4	<LOQ	<LOQ	0.64	2.7	8
	BzP	149	21	<LOQ	0.064	0.07	<LOQ	<LOQ	0.54	<LOQ	<LOQ	0.13	0.22	0.37
	BPS	149	27	0.33	0.3	0.2	<LOQ	<LOQ	1.7	<LOQ	<LOQ	0.59	0.7	1.1
Adolescents	BPA	97	100	2.7	1.9	2.8	1.6	0.49	18	0.74	0.87	6	7.3	15
	BPF	97	87	0.85	0.17	2	0.11	<LOQ	11	<LOQ	<LOQ	2.4	4.5	9.5
	MeP	97	80	20	5	41	7.2	<LOQ	310	<LOQ	<LOQ	40	105	140
	EtP	97	72	12	4.4	19	6	<LOQ	97	<LOQ	<LOQ	27	63	84
	iPrP	97	1	<LOQ	0.13	0.34	<LOQ	<LOQ	3.1	<LOQ	<LOQ	<LOQ	<LOQ	1.7
	PrP	97	22	1.4	0.22	6.7	<LOQ	<LOQ	54	<LOQ	<LOQ	1.4	3.1	39
	iBuP	97	0	/	/	/	/	/	/	/	/	/	/	/
	BuP	97	6.2	<LOQ	0.15	0.59	<LOQ	<LOQ	3.6	<LOQ	<LOQ	<LOQ	1.7	3.1
	TCS	97	35	0.99	0.26	3.1	<LOQ	<LOQ	25	<LOQ	<LOQ	2.2	3.6	14
	BzP	97	22	<LOQ	0.066	0.074	<LOQ	<LOQ	0.53	<LOQ	<LOQ	0.18	0.2	0.32
	BPS	97	35	0.81	0.36	2.7	<LOQ	<LOQ	23	<LOQ	<LOQ	1	1.8	13

*QR – Quantification rate, AM – arithmetic mean, GM – geometric mean (imputed data), SD – standard deviation in AM, P – percentile

Table 3
Comparison of median urinary concentrations of bisphenols in children and adolescents from other studies.

N	Sampling year	Country	Age of participants (yrs)	Adjustment*	BPA (μg/L)	BPF (μg/L)	BPS (μg/L)	Reference
134	2012–2013	Brazil	6–10	none	1.76	<LOD	<LOD	(Rocha et al., 2018)
653	2011–2012	EU**	6–11	none	1.96	–	–	(Covaci et al., 2015)
113	2011–2012	Sweden	3–5	sg	1.40	–	0.16	(Larsson et al., 2017)
80	2016	China	3–5	none	0.369	<LOD	0.18	(Liu et al., 2019)
239	2009–2011	Greece	1.6–3	none	2.1	–	–	(Myridakis et al., 2015)
30	2004–2008	Spain	4	sg	4.2	–	–	(Casas et al., 2011)
145	2011–2012	Slovenia	6–11	none	2.39	–	–	(Snoj Tratnik et al., 2019)
70	2011–2012	Denmark	6–11	none	1.7	–	–	(Frederiksen et al., 2013b)
409	2013–2014	USA	6–11	none	1.4	0.3	0.3	(CDC, 2009)
246	**2018**	**Slovenia**	**6–9**	**sg**	**1.9**	**0.085**	**< LOQ**	**This study**
462	2013–2014	USA	12–19	none	1.2	0.4	0.4	(CDC, 2009)
166	2012–2013	Brazil	11–14	none	1.64	<LOD	<LOD	(Rocha et al., 2018)
129	2006–2008	Denmark	6–21	24 h	1.73	–	–	(Frederiksen et al., 2013a)
246	**2018**	**Slovenia**	**11–15**	**sg**	**1.6**	**0.11**	**< LOQ**	**This study**

* None – unadjusted, sg – specific gravity adjusted, 24 h – 24 h composite sample, no adjustment needed.
** Combined data for several EU member countries.

established for BPA and TCS, and measure to 100 μg/L and 2200 μg/L, respectively. Thus, in this study noone exceeded HBM I value for BPA and TCS, implicating that by current standards the children and adolescents are not at risk for adverse health effects posed by BPA and TCS individually.

3.4. Correlations among EDCs

Pearson correlation coefficient was calculated among levels of EDCs in urine (Fig. 1). Only coefficients with significance level lower than 0.1 were retained in the analysis. Compounds were in general not correlated with each other, however a strong correlation was found between MeP and PrP (0.79, $p = 2.2e{-}16$) in samples of children. The correlation was not observed in the samples from adolescents.

Individuals with urinary MeP level above 300 μg/L were found to have also high levels of PrP. However, the opposite was not true, i.e. not all individuals with high level of PrP were found to have high level of MeP. This correlation between MeP and PrP has been previously described in literature. (Casas et al., 2011; Kim et al., 2018; Lu et al., 2019) It has been reported, that PrP is used together with MeP in certain products, as their combination yields stronger antimicrobial activity. (Kim et al., 2018; Lu et al., 2019).

Except for MeP and PrP, EDCs were not strongly correlated with each other, which indicates that there is no connection between specific sources of exposure for these chemicals. However, the rate of metabolism, individual physiology and genetics influence the urinary levels of EDCs and can reduce the significance of potentially existing correlations.

3.5. Exposure patterns

Exposure patterns varied among children and adolescents. Differences were observed for BPF ($GM_{children} = 0.11$ μg/L versus $GM_{adolescents} = 0.17$ μg/L, $p = 0.02$) and EtP ($GM_{children} = 2.7$ μg/L versus $GM_{adolescents} = 4.4$ μg/L, $p = 0.02$). Eventhough quantification rates were well below 50%, differences were observed also for BPS ($GM_{children} = 0.30$ μg/L versus $GM_{adolescents} = 0.36$ μg/L, $p = 0.07$) and TCS ($GM_{children} = 0.19$ μg/L versus $GM_{adolescents} = 0.31$ μg/L, $p = 0.04$). Higher levels of EtP and TCS in adolescents reflect increased use of personal care products in the older group. Differences were observed also between sexes, where significantly higher urinary levels of MeP were found in samples from girls than boys ($GM_{girls} = 7.5$ μg/L versus $GM_{boys} = 3.3$ μg/L, $p = 4e{-}4$), which likewise implicates higher use of certain personal care products by girls. The difference was pronounced within younger age

Table 4
Comparison of median urinary concentrations of parabens and TCS in children and adolescents from other studies.

N	Sampling year	Age of participants (yrs)	Country	Adjustment*	MeP (μg/L)	EtP (μg/L)	PrP (μg/L)	iPrP (μg/L)	BuP (μg/L)	iBuP (μg/L)	BzP (μg/L)	TCS (μg/L)	Reference
134	2012–2013	6 – 10	Brazil	none	27.4	0.36	2.61	–	<LOQ	–	–	14.1	(Rocha et al., 2018)
96	2015	3–6	China	sg	3.02	0.20	0.38	–	0.11	–	0.03	–	(Lu et al., 2019)
239	2009–2011	1.6–3	Greece	none	17.10	1.50	0.90	<LOD	<LOD	<LOD	–	–	(Myridakis et al., 2015)
30	2004–2008	4	Spain	sg	150	8.10	21.50	–	1.20	–	–	1.2	(Casas et al., 2011)
93	2005–2007	3–8	USA	none	36	–	3.20	–	<LOD	–	–	6.6	(Philippat et al., 2015)
129	2006–2008	6–21	Denmark	24 h	–	–	–	–	–	–	–	1.45	(Frederiksen et al., 2013a)
70	2011–2012	6–11	Denmark	none	3.0	0.40	0.92	<LOD	1.40	<LOD	<LOD	0.46	(Frederiksen et al., 2013b)
409	2013–2014	6–11	USA	none	22.7	<LOD	2.40	–	<LOD	–	–	6.1	(CDC, 2009)
246	**2018**	**6–9**	**Slovenia**	**sg**	**5.4**	**2.5**	**<LOQ**	**<LOQ**	**<LOQ**	**<LOQ**	**<LOQ**	**<LOQ**	**This study**
462	2013–2014	12–19	USA	none	32.0	<LOD	3.10	–	<LOD	–	–	6.0	(CDC, 2009)
48	2015	7–12	Korea	sg	7.8	24.6	<LOD	–	<LOD	–	–	<LOD	(Kim et al., 2018)
166	2012–2013	11–14	Brazil	none	48.5	0.44	3.30	–	<LOQ	–	–	121.5	(Rocha et al., 2018)
246	**2018**	**11–15**	**Slovenia**	**sg**	**7.2**	**6.0**	**<LOQ**	**<LOQ**	**<LOQ**	**<LOQ**	**<LOQ**	**<LOQ**	**This study**

* none – unadjusted, sg – specific gravity adjusted, 24 h – 24 h composite sample.

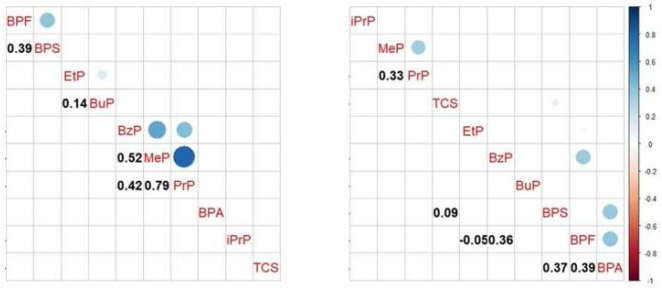

Fig. 1. Pearson correlation coefficients among bisphenols, parabens and TCS for children (left) and adolescents (right). Only correlations with p-value < 0.10 are presented in the plot.

group ($GM_{girls} = 7.1$ μg/L versus $GM_{boys} = 3.3$ μg/L, $p = 0.01$), as well as among adolescents ($GM_{girls} = 8.3$ μg/L versus $GM_{boys} = 3.3$ μg/L, $p = 0.02$).

Exposure of individuals was furthermore location-dependent for certain EDCs. Statistically significant higher levels of BPF ($GM_{L1} = 0.19$ μg/L, $GM_{L2} = 0.10$ μg/L, $GM_{L3} = 0.064$, $p = 2e\text{-}5$) and EtP ($GM_{L1} = 4.1$ μg/L, $GM_{L2} = 2.8$ μg/L, $GM_{L3} = 2.1$ μg/L, $p = 0.03$) were detected in samples from L1. Even though all three locations sampled in this study are in very close geographical proximity, individuals attending schools at L1 had higher urinary levels of these EDCs than other locations, regardless of gender and age.

3.6. Determinants of exposure

Along with measuring biomarkers of exposure and researching exposure patterns, questionnaire data that participants provided during sampling was analysed to obtain information on potential sources of exposure. For increased statistical power, statistical models were constructed on the whole population.

Determinants of exposure were studied by LR and PLSR. Model and validation parameters for LR and PLSR are presented in Table 5. For LR models, R^2 (coefficient of determination), F-statistics and p-value were used to check model validity. Valid LR models were constructed for all four EDCs, with p-value lower than 0.1. PLSR models were checked for overfitting by permutation test (N = 100). Valid PLSR models were constructed for BPA, BPF and MeP, with permuted parameters (R2Y and Q2) being lower than those of the test model. Permutation plots are presented in Fig. SI-5. Number of principal components (PCs) was 2 for all three models. No valid PLSR models were constructed for EtP. LR and PLSR models showed several significant variables, which were regarded as candidates for exposure determinants. To validate each determinant candidate, urinary levels of EDCs were sorted into two groups, according to the reported relationship with the determinant. For example, perfume use was connected to MeP urinary levels, therefore the difference in urinary MeP levels between perfume users and non-users was validated using Student's *t*-test. Groupings for each determinant are presented in Table 6. Only candidates with p-value ≤ 0.15 were accepted as determinants.

Food was expected to be found associated with BPA levels, being the main source of BPA exposure in humans, as it migrates from epoxy coatings of cans, as well as from polycarbonate plastic in contact with food and drink. (Geens et al., 2012) Canned food compared to non-canned food contains much higher levels of BPA. (Lorber et al., 2015) Previously reported association of canned food consumption with higher

Table 5
LR and PLSR parameters of the constructed models.

	LR						PLSR						
EDC	R^2	F	Model p-value	Variable	Coefficient	Variable p-value	PCs	R2Y (cum)	Q2 (cum)	R2Y (perm)	Q2 (perm)	Coefficient	VIP-score
BPA	0.29	4.5	1.90E-06				2	0.238	0.139	0.0028	−0.098		
				pastry	0.48	0.013						0.12	1.02
				butter	0.33	0.014						0.14	1.2
				sun. oil	0.055	0.0070						0.11	1.0
				tea	−0.046	0.013						−0.12	0.90
				milk	−0.032	0.026						−0.14	0.95
				vit. drink	−0.26	0.007						−0.13	0.93
BPF	0.38	5.9	2.60E-06				2	0.446	0.13	0.050	−0.147		
				cream	0.11	0.034						0.11	1.1
				fruit cooked	0.22	0.00024						0.11	0.90
				can. veget.	0.10	0.021						0.07	0.94
MeP	0.44	6.7	2.82E-09				2	0.558	0.013	0.25	−0.21		
				perfume	29	0.00045						0.09	1.1
				lipstick	1.22	0.011						0.15	1.5
				juice	−8.22	1.1e-05						−0.008	0.57
EtP	0.080	2.6	0.039										
				deodorant	7.19	0.0010							
				fruit cooked	−2.2	0.046							

Table 6
Parameters of groups formed for validation of the determinants of the exposure, with t-statistics and p-values of the group difference.

BPA	Group	Butter1	Butter2	Sf. Oil1	Sf. Oil2	Pastry1	Pastry2	Tea1	Tea2	Milk1	Milk2	Drink1	Drink2
	Condition*	< 3	≥ 3	< 4	≥ 4	< 3	≥ 3	< 8	≥ 8	< 7	≥ 7	< 6	≥ 6
	N	58	188	133	113	186	60	212	34	133	113	221	25
	Min(μg/L)	0.67	0.15	0.15	0.49	0.15	3.0	0.15	0.57	0.57	0.15	0.15	0.48
	Median(μg/L)	1.8	1.9	1.7	2.1	1.5	5.3	2.0	1.5	2.1	1.2	1.9	1.4
	GM(μg/L)	1.9	2.3	1.9	2.2	1.5	6.1	2.1	1.6	2.3	1.8	2.1	1.5
	Max(μg/L)	9.9	40	19	40	3.0	40	40	7.4	40	20	40	6.3
	t-value	−2.5		−1.7		−1.8		2.6		2.3		3.1	
	p-value	0.014		0.095		0.080		0.0098		0.020		0.0024	
BPF	Group	Cream1	Cream2	Fruit1	Fruit2	Veg.1	Veg.2						
	Condition*	< 4	≥ 4	< 3	≥ 3	< 4	≥ 4						
	N	78	168	122	124	129	117						
	Min(μg/L)	0.015	0.015	0.015	0.015	0.015	0.015						
	Median(μg/L)	0.089	0.099	0.088	0.11	0.082	0.11						
	GM(μg/L)	0.11	0.14	0.10	0.16	0.11	0.16						
	Max(μg/L)	3.4	11	7.2	11	7.2	11						
	t-value	−2.5		−1.5		−1.5							
	p-value	0.013		0.13		0.13							
MeP	Group	Lipstick1	Lipstick2	Perfume1	Perfume2	Juice1	Juice2						
	Condition*	1	0	1	0	< 4	≥ 4						
	N	79	46	78	47	81	44						
	Min(μg/L)	0.25	0.25	0.25	0.25	0.25	0.25						
	Median(μg/L)	10	4.2	9.8	4.8	9.5	6.7						
	GM(μg/L)	11	3.8	10	4.4	8.0	6.6						
	Max(μg/L)	310	109	310	110	310	91						
	t-value	2.6		2.7		2.1							
	p-value	0.011		0.0092		0.033							

* Condition represents a number of average portions of food or drink item consumed per week. Average portions were: 2 table spoons for butter, sunflower oil and cream, 1 piece for pastry, 2.5 dcl for tea, milk and vitamin drink, and 150 g for canned fruit and canned vegetables. In the case of cosmetic item, 1 represents to 'use', and 0 to 'never use'. Sf. Oil – sunflower oil, Drink – vitamin drink, Fruit – canned fruit, Veg. – canned vegetables, GM - geometric mean.

levels of BPA in urine (Snoj Tratnik et al., 2019) was, however, not observed in this study. On the other hand, we observed significant association of fatty foods, such as butter ($p = 0.014$), sunflower oil ($p = 0.095$) and pastry (0.080) with BPA. As BPA is relatively non-polar (log $P = 3.32$) (Staples et al., 1998), it is expected to distribute into fat. Similarly, several studies have found non-polar contaminants, such as phthalates present in high concentrations in fatty foods, such as animal fat and vegetable oil. (Husøy et al., 2019) Furthermore, BPA levels were negatively correlated with consuming more polar food items such as tea ($p = 0.0098$), milk ($p = 0.020$) and vitamin drink ($p = 0.0024$). In contrast to BPA, BPF has not been widely reported in canned food, even though it is also used in production of epoxy resins and plastic, which is in direct contact with food. (Lorber et al., 2015) Here, consumption of cream ($p = 0.010$), local cooked fruit ($p = 0.13$) and canned vegetables ($p = 0.13$) were positively associated with urinary BPF levels. No non-dietary variables were associated with BPF levels.

Use of cosmetics was associated with higher urinary MeP levels, specifically the use of lipstick ($p = 0.011$) and perfume ($p = 0.0092$). Parabens are widely used in cosmetics, (Kizhedath et al., 2019) thus the association of most abundant paraben with lipstick and perfume is not surprising. The most likely route of exposure in the case of lipstick is oral, since individuals ingest a part of the product. Dermal absorption or

inhalation are highly probable in perfume users. The association between MeP and perfume use has also been previously reported in literature. (Braun et al., 2014) MeP has also been reported to be associated with other personal care products, such as body lotion (Larsson et al., 2014) and skin care and hair products. (Kim et al., 2018) Consumption of juice ($p = 0.033$) was found to negatively correlate with MeP.

A valid LR model was built for EtP and use of deodorant and cooked fruit were associated with it, however due to a poor model fit ($R^2 = 0.080$), associations were not further researched.

Associations discovered in this study using LR and PLSR might be scarce and should be interpreted with caution as questionnaires were more focused on obtaining data on monthly food consumption, other dietary habits and cosmetics use rather than intake directly prior to sample collection. As EDCs included in this study are rapidly cleared from the body (the majority of EDCs are excreted within 24-hours), detailed and more extensive associations might have been missed.

3.7. *UGT2B15 genotyping*

Genotyping of participants' DNA samples for a polymorphic *UGT2B15* gene was performed in order to find associations between presence of the SNP in *UGT2B15* gene and levels of EDC biomarkers in urine. *In-vitro* tests have shown that the BPA glucuronidation rate of 85Tyr UGT2B15 variant (A allele) can be decreased down to 14% to that of the variant with 85Asp (C allele). (Hanioka et al., 2011) Thus, the presence of the SNP can indicate susceptible individuals to EDC adverse effects.

231 (94%) participants were successfully genotyped for UGT2B15 SNP. Allele frequency was in accordance with Hardy-Weinberg equilibrium ($\chi^2 = 0.19$, $p = 0.66$). The frequency of the variant allele was 0.46 in the studied population. Genotype representation is given in Table 7, while location-wise representations and frequencies are presented in SI-9. Genotypes were equally represented by both sexes, however, younger group represented slightly larger percentage of individuals with AC and CC genotype. ANOVA tests were performed to check for significant differences in urinary EDC levels among genotypes. The population was further stratified to check for the influence of sex and age on the analysis, which are significant in the developing population, however the results of stratified population were generally not different than that of the whole population (see table SI-5).

Although the main reason for selecting *UGT2B15* SNP was the reported affinity of UGT2B15 isoform towards BPA and the effect of Tyr to Asp substitution on enzyme activity, no associations were found between this SNP and urinary BPA levels. It should be noted, that the connection, reported by Hanioka et al., 2011 was established on *in-vitro* tests. However, recently reported *in-vivo* tests showed no correlation between serum BPA and the SNP. (Luo et al., 2020) No associations were similarly found for BPF, however different UGT isozyme, i.e. UGT1A10, was found to be most active towards this compound. (Gramec Skledar et al., 2015) In addition, the sulphate, not the glucuronide conjugate, was reported as the main BPF metabolite, (Cabaton et al., 2006) so the absence of this association is not surprising.

Significant associations with *UGT2B15* SNP were, however, found for EtP in the whole population and for MeP in individuals who reported the perfume use ($N = 131$), being a determinant of exposure for MeP (see Section 3.5). Due to higher levels of MeP ($GM_{AA} = 6.6$ μg/L, $GM_{AC} = 6.5$ μg/L, $GM_{CC} = 4.6$ μg/L) and EtP ($GM_{AA} = 0.59$ μg/L, $GM_{AC} = 0.87$ μg/L, $GM_{CC} = 0.33$ μg/L) the samples from individuals with AA and AC genotype were grouped together and compared to the levels of the CC group using Student's *t*-test. Individuals with AA/AC genotype had significantly higher urinary levels of MeP ($GM_{AA/AC} = 7.9$ μg/L versus $GM_{CC} = 3.7$ μg/L, $p = 0.0324$) and EtP ($GM_{AA/AC} = 2.0$ μg/L versus $GM_{CC} = 1.1$ μg/L, $p = 0.000643$). Results of t-tests are presented in table SI-6.

Table 7
Number of participants with AA, AC and CC genotype.

Genotype	N(%)	Sex	N(%)	Age(y)	N(%)
AA	66(29)	F	36(55)	6–9	35(53)
		M	30(45)	11–15	31(47)
AC	118(51)	F	58(49)	6–9	73(62)
		M	60(51)	11–15	45(38)
CC	47(20)	F	23(49)	6–9	33(70)
		M	24(51)	11–15	14(30)

Although parabens are actively glucuronidated by a wide variety of UGT isozymes, including UGT1A1, UGT1A8, UGT1A9, UGT2BT, UGT2B17 and UGT2B15 (Abbas et al., 2010), these results indicate a significant role of the latter isozyme, UGT2B15 in MeP and EtP glucuronidation, and more exactly, the presence of SNP in *UGT2B15* gene on urinary MeP and EtP levels. The results indicate SNP rs1902023 as a potential biomarker of susceptibility to adverse effects posed by MeP and EtP. However, further research is required to determine if the SNP presence is connected to higher or lower susceptibility.

Many individuals were exposed to a number of EDCs simultaneously. The selection of EDCs in this study is not exhaustive, yet the data shows significant load of the studied children and adolescents. EDCs interfere with normal bodily functions through a number of mechanisms, while individual's genetics play the role in the susceptibility to their adverse health effects. Although levels of EDCs found in this study are low and do not exceed currently available HBM I values, several studies showed that individual compounds may have no toxicological effect, however, when present concurrently, they can induce significant synergistic adverse effects. (Rajapakse et al., 2002; Silva et al., 2002) Besides, it is important to consider that this exposure is long-term, and can thus induce chronic or sub-chronic alterations in molecular mechanisms, yielding significant adverse effects in the next generations. (Brehm and Flaws, 2019; Xin et al., 2015) This issue certainly rises concern about the safety of currently manufactured personal care products, food packaging, cosmetics, etc., being the main sources of exposure to a number of distinct EDCs. This is especially concerning when exposure occurs in vulnerable populations, such as children and adolescents with an intensively developing and maturing reproductive system.

4. Conclusions

This study presents data on exposure of 246 Slovenian children and adolescents to bisphenols, parabens and triclosan, all of them known EDCs. Urinary levels found in Slovenian population were comparable to the levels found across the world, but with generally lower levels of PrP and TCS and higher levels of EtP. Exposure was not uniform throughout the population, with adolescents having higher urinary levels of BPF and MeP and girls of MeP, when compared to boys. Exposure turned out to be location dependent, as samples from individuals in Goričko contained higher levels of BPF and EtP than in other locations. Furthermore, several determinants of exposure have been implied. BPA was significantly associated with high fat food items, while MeP with personal care products, such as lipstick and perfume. UGT2B15 has been found to play a role in MeP and EtP metabolism. Furthermore, the association of urinary MeP and EtP levels with the presence of SNP in *UGT2B15* gene indicates the SNP rs1902023 as potential biomarker or susceptibility to MeP and EtP-related adverse health effects. Children and adolescents were exposed to a variety of EDCs, and even though the urinary levels were relatively low and below the current adverse effect levels for individual EDCs, cumulative and mixture effects of several concurrently present toxicants on health should be further considered.

5. Author statement

Žiga Tkalec: Conceptualization, Chemical analysis, Data processing and evaluation, Results interpretation, Writing - Original Draft, Writing - Review & Editing;

Tina Kosjek: Supervision, conceptualization, Writing - Review & Editing;
Janja Snoj Tratnik: Project administration and organisation, Sampling, Writing - Review & Editing, SNP genotyping;
Anja Stajnko: Writing - Review & Editing;
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Marianthi Sykiotou: Sample preparation;
Darja Mazej: Project administration and organisation, Sampling;
Milena Horvat: Project administration and organisation, Funding acquisition, Resources;

Declaration of Competing Interest

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

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2020.106172.

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3.3.2 Exposure of men and lactating women to environmental phenols, phthalates and DINCH

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This manuscript describes the exposure of Slovenian men and lactating women to environmental phenols, phthalates and the phthalate alternative DINCH [120]. The formal analysis and writing of the paper were conducted by Agneta Annika Runkel, whereas my contribution was the optimization of the analytical method for the analysis of environmental phenols.

In this study, urinary concentrations of methyl paraben (MeP), ethyl paraben (EtP), propyl paraben (PrP), iso-propyl paraben (iPrP), butyl paraben (BuP), iso-butyl paraben (iBuP), benzyl paraben (BzP), triclosan (TCS), bisphenol A (BPA), bisphenol S (BPS), and bisphenol F (BPF) were determined in urine using GC-MS/MS, while the analysis of phthalate metabolites, MEHP, 5oxo-MEHP, 5OH-MEHP, 5cx-MEPP, MEP, MiBP, MnBP, MBzP, cx-MINP, OH-MIDP, OH-MINCH, oxo-MINCH, and OH-MINP was conducted using UHPLC-MS/MS. After the quantification, the concentrations were evaluated by statistical analyses, followed by simplified risk assessment procedure. Along with all phthalate metabolites and DINCH, the most frequently detected compounds were BPA, MeP and EtP. Women and men exhibited sex-specific differences, while the connection with questionnaire data showed associations that indicate specific determinants of exposure for some of the chemicals. Risk assessment approach showed that no risk can be expected for the studied population at the determined exposure levels.

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Exposure of men and lactating women to environmental phenols, phthalates, and DINCH

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HIGHLIGHTS

- Endocrine disrupting chemicals were detected at high frequencies.
- Concentrations were low compared to the literature.
- The combined hazard quotient is near 1.
- Concentrations of phthalates and phenols are decreasing between 2008 and 2014.
- Concentrations of DINCH are increasing between 2008 and 2014.

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ABSTRACT

Phthalates and 1,2-Cyclohexane dicarboxylic acid diisononyl ester (DINCH), bisphenols (BPs), parabens (PBs), and triclosan (TCS) are high-production-volume chemicals of pseudo-persistence that are concerning for the environment and human health. This study aims to assess the exposure to 10 phthalates, DINCH, and environmental phenols (3 BPs, 7 PBs, and TCS) of Slovenian men (n = 548) and lactating primiparous women (n = 536). We observed urinary concentrations comparable to studies from other countries and significant differences among the sub-populations. In our study, men had significantly higher levels of phthalates, DINCH, and BPs, whereas the concentrations of PBs in urine were significantly higher in women. The most significant determinant of exposure was the area of residence and the year of sampling (2008–2014) that mirrors trends in the market. Participants from urban or industrialized sampling locations had higher levels of almost all monitored analytes compared to rural locations. In an attempt to assess the risk of the population, hazard quotient (HQ) values were calculated for individual compounds and the chemical mixture. Individual analytes do not seem to pose a risk to the studied population at current exposure levels, whereas the HQ value of the chemical mixture is near the threshold of 1 which would indicate a higher risk. We conclude that greater emphasis on the risk resulting from cumulative exposure to chemical mixtures and additional studies are needed to estimate the exposure of susceptible populations, such as children.

Abbreviations: ACN, acetonitrile; ADI, acceptable daily intake; BBP, Butylbenzyl phthalate; BE, biomonitoring equivalent; BPA, bisphenol A; BPF, bisphenol F; BPS, bisphenol S; BuP, butyl paraben; BzP, benzyl paraben; cx-MINP, monocarboxy-isononyl phthalate; DBP, Di-n-butyl phthalate; DCM, dichloromethane; DIBP, Diisobutyl phthalate; DINCH, 1,2-Cyclohexane dicarboxylic acid diisononyl ester; DiNP, di-*iso*-nonyl phthalate; EDI, estimated daily intake; EtAc, ethyl acetate; EtP, ethyl paraben; HQ, hazard quotient; iBuP, iso-butyl paraben; iPrP, iso-propyl paraben; MBzP, mono-benzyl phthalate; MCHP, mono-cyclohexyl phthalate; McxEPP, mono (2-ethyl-5- carboxypentyl) phthalate; MEHP, mono (2-ethylhexyl) phthalate; MeOH, methanol; MEP, monoethyl phthalate; MiBP, mono-isobutyl phthalate; MnBP, mono-n-butyl phthalate; MnOP, mono-n-octoyl phthalate; MnPeP, mono-n-pentyl phthalate; MOE, margin of exposure; MP, methyl paraben; MSTFA, *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide; NOAEL, no adverse effect level; OH-MEHP, mono (2-ethyl-5-hydroxyhexyl) phthalate; OH-MIDP, monohydroxy isodecyl phthalate; OH-MINCH, cyclohexane-1,2-dicarboxylic acid-mono (hydroxyl – isononyl) ester; OH-MINP, monohydroxy-isononyl phthalate; oxo-MEHP, mono (2-ethyl-5-oxohexyl) phthalate; oxo-MINCH, cyclohexane-1,2-dicarboxylic acid-mono (oxo-isononyl) ester; PrP, propyl paraben; P95, 95th percentile; TCS, triclosan; TDI, tolerable daily intake.

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1. Introduction

Bisphenols (BPs), parabens (PBs), triclosan (TCS), phthalates, and DINCH are synthetic chemicals that are employed in many consumer products and widely measured in environmental samples. Exposure to these contaminants is causing increasing global concern due to their ubiquitous presence in biological and environmental matrices, suspected adverse health effects, and the inevitable contact between individuals and these chemicals (Husøy et al., 2019). Despite their short half-life (3–18h) and fluctuating concentrations in the body, these chemicals can be measured at high detection rates in urine of the general population at any given time (Koch and Calafat, 2009).

BPs, PBs, and TCS can be categorized as "environmental phenols" due to their structural similarities. BPs are employed in the manufacture of polycarbonate plastics and epoxy resins and are found in many products, such as cans, food packaging, dental fillings, and thermal paper (Koch and Calafat, 2009; Lehmler et al., 2018). Among them, bisphenol A (BPA) is produced and applied in the largest volumes but finds increasing replacement by related BPs with assumed lesser endocrine activity, such as bisphenol S (BPS) and bisphenol F (BPF). However, recent studies evaluating the endocrine disruptive effects of alternative BPs report comparable or exceeding effects in humans (Pelch et al., 2019). For humans, dietary ingestion is the most important route of exposure (Lehmler et al., 2018). In the body, BPs undergo rapid metabolism and excretion mainly as the more hydrophilic sulphates and glucuronides. PBs are utilized as preservatives in dietary products, personal care products (PCPs), and medical products. Chemicals such as methyl paraben (MP), ethyl paraben (EtP), propyl paraben (PrP), and butyl paraben (BuP) are the most commonly employed representatives of these chemicals, while chemicals such as iso-propyl paraben (iPrP), isobutyl paraben (iBuP), and benzyl paraben (BzP) are less applied (Honda et al., 2018; Moos et al., 2015). Due to the variety of applications, PBs can enter the human body via ingestion, inhalation, and dermal absorption, where they are rapidly metabolized and mainly excreted as β-D-glucuronide and sulphate within 1–7h (Frederiksen et al., 2014). Although no acute toxicity is observed, studies suggest potential endocrine-disrupting properties of these chemicals (Boberg et al., 2010; Nowak et al., 2018; Witorsch and Thomas, 2010). TCS is a synthetic broad-spectrum antibacterial agent with various applications in PCPs (Weatherly and Gosse, 2017). Accordingly, human exposure occurs mainly via dermal absorption and ingestion. Despite its lipophilic character, the bioaccumulative potential of TCS is assumed to be insignificant due to its rapid metabolism and excretion as glucuronide and sulphate conjugates via urine within 48 h after exposure (Frederiksen et al., 2014). Studies investigating the potential health effects of TCS often report conflicting results that often suggest estrogenic and androgenic activity in mammals as well as the potential to trigger antibiotic resistance (Goodman et al., 2018; McNamara and Levy, 2016; Wang and Tian, 2015).

Phthalates are high-production-volume synthetic chemicals with numerous applications. High molecular weight (HMW) phthalates are widely employed as softening agents in plastics, such as polyvinyl chloride (PVC) and food packaging materials, while low-molecular-weight (LMW) phthalates find additional applications in PCPs, solvents, pesticide formulations, paints, and lubricants (Berger et al., 2019). Due to their presence in food contact materials, the main pathway of exposure to HMW phthalates is via ingestion, while LMW phthalates often enter the body via dermal absorption and inhalation (Wormuth et al., 2006). The metabolic fate of phthalates is highly dependent on the chain length. Hydrolytic monoesters of the parent diesters are rapidly formed, and HMW phthalates can be further oxidized to secondary metabolites followed by excretion as mainly glucuronide conjugates via urine within 48 h after exposure. In 2015, four phthalates (DEHP, DBP, BBP, DIBP) have been restricted in the European Union because of their suspected endocrine activity (Koch et al., 2017). Whether other phthalates have negative health impacts in humans remains a controversial topic in the literature, but accumulating evidence suggest endocrine activity with endpoints, such as male fertility impairment, adverse child neurodevelopment, and increased levels of follicle-stimulating hormone (FSH) (Koch et al, 2007a, 2017; Koch and Calafat, 2009). As such, alternatives to traditional phthalate plasticizers have been introduced to the market, among which, DINCH had the largest market share in 2012 (Bui et al., 2016). DINCH was introduced to the market in 2002 and is structurally similar to di-*iso*-nonyl phthalate (DiNP) (Bui et al., 2016; Urbancova et al., 2019). Therefore, it finds the most applications in medical devices, food packaging materials, and toys. As are phthalates, DINCH is not bound to the matrix in which it is applied and can therefore leach into the environment. Upon entering the human body mainly via ingestion, but also inhalation, DINCH undergoes rapid metabolism and is excreted either free or in the form of conjugates with glucuronic acid (Urbancova et al., 2019; Völkel et al., 2016). To date, it is assumed that neither DINCH nor its metabolites cause adverse health effects in humans and frequent monitoring is carried out mainly in response to the increased market share and to obtain more data on its behaviour in humans (Schütze et al., 2017; Urbancova et al., 2019). Therefore, we included DINCH in this monitoring study to provide information of DINCH exposure of the Slovenian population.

Human biomonitoring (HBM) is an important tool in estimating the exposure of populations to potentially harmful chemicals. The human body is especially sensitive to the effects of endocrine-disrupting chemicals during critical developmental stages. Pregnant and lactating women are among the most susceptible groups (Koch and Calafat, 2009). Within the first national HBM project, 536 primiparous women and 548 men were recruited from 12 regions in Slovenia during two sampling campaigns (2008–2009 and 2011–2014) to assess the exposure of men and lactating women to harmful chemicals (Runkel et al., 2021; Snoj Tratnik et al., 2019a). Exposure to trace elements and persistent organic pollutants has been estimated in our previous studies and is described elsewhere (Runkel et al., 2021; Snoj Tratnik et al., 2019a). The present study exploited available samples and data for further assessment of exposure, including the above-mentioned chemicals.

2. Material and methods

2.1. Study population and design

A detailed description of the study population and design have been previously published (Snoj Tratnik et al., 2019b). In the original study, a total of 536 lactating women and 548 men between the ages of 18 and 49 have been recruited from the 12 statistical regions of Slovenia covering rural, urban, and industrial environments. Out of all participants, data on phthalate and DINCH metabolites, BPs, PBs, and TCS was obtained for 304 women and 299 men. As such, the population included in this study represents a subset of the original parent study. The sampling regions are visualized in Fig. 1. We categorized the regions based on air pollution levels as a source of phthalate exposure (Quintana-Belmares et al., 2018), which deviates from the original study design presented by Snoj Tratnik et al. (2019a). Urban areas (Ljubljana, Maribor, and Koper) and regions with particulate matter (PM_{10}) values exceeding 50 μg/m^3 for more than 35 days per year (Jesenice, Celje, and Zasavje) were categorized as "polluted", while rural areas not exceeding this threshold were categorized as "rural". Data on air pollution for 2010 were obtained from annually published reports by the Slovenian Environmental Agency (Republika Slovenije, 2010). The regions Ljubljana, Kočevje and Cerknica, and Bela Krajina were selected within the pilot study (2008–2009), whereas other regions were included at a subsequent stage during the follow-up project (2011–2014). Participants were recruited via maternity hospitals, maternity classes, and gynaecologists and could withdraw from the study at any time. Each participant provided a random spot urine sample collected in a previously distributed

100 mL polypropylene (PP) urine collection cup. The samples were further aliquoted into 2 or 5 mL PP cryovials and stored at −20 °C from the sampling period (2008–2014) until analysis (2019–2020). A general questionnaire that covers information on lifestyle, diet, residence, occupation, and health was included in the sampling campaign. Population characteristics are provided in Table S1. The National Medical Ethics Committee of the Republic of Slovenia granted approval of the pilot study (number of accordance 42/12/07) and the follow-up study (number of accordance 53/07/09). Additional ethical approval was obtained for the use of biobanked samples (number of accordance 0120–431/2018/4). All participants were asked to provide informed written consent to allow the use of biobanked samples. Positive answers were obtained from 56% of the participants in the initial study, which revealed some demographic trends. The average age of those that gave permission was slightly higher (GM 29.9) than the age of those who did not (GM 28.9). Among the participants with a university degree, 66 % gave permission, whereas among those with a lower level of education only 49 % gave permission. Among participants from the rural sampling locations, 56 % gave permission, whereas 57 % from the urban/-industrialized sampling locations gave permission.

SG was measured on a PAL-10 S refractometer, and calculation of SG-adjusted concentrations was based on the method described by (Suwazono et al., (2005)).

2.2. Laboratory analysis of bisphenols, parabens, and triclosan

A detailed description of the analytical methods has been published elsewhere (Tkalec et al., 2021). Briefly, metabolites were deconjugated over night at 37 °C using *β*-glucuronidase/arylsulfatase (*Helix Pomatia*, type H-2) at a concentration of 200 U/mL. Next, 1 mL of sample spiked with internal standard was extracted on an Oasis HLB (60 mg/1 mL) 96-well plate that was previously conditioned and equilibrated with 1 mL DCM, 1 mL EtAc, 1 mL MeOH, and 1 mL water. The sample was washed with 1 mL of 20 % MeOH in water and eluted with 1.8 mL of EtAc/MeOH (1:1). Eluates were evaporated under nitrogen stream until dryness and reconstituted in 100 μL ACN. Samples were derivatized with MSTFA at 50 °C for 1 h. Afterwards, samples were cleaned on two Strata Si (100 mg/1 mL) 96-well plates that were previously conditioned with 1 mL DCM and 2 mL EtAc. To achieve sample cleaning through two plates, one Strata Si plate was mounted on top of the other plate, and loaded samples were eluted with DCM. Eluates were concentrated under nitrogen stream to an approximate volume of 100 μL and transferred to inserts for analysis.

Samples were analysed on an Agilent 7890 B gas chromatograph coupled to a triple quadrupole mass analyzer Agilent 7000. Chromatographic separation was achieved on a DB5-MS UI column (30 m, 0.25 mm, 0.25 μm) using helium as a carrier gas (1 mL/min). The temperature program is described as follows: 120 °C (2 min), ramped at 16 °C/min until 300 °C, and 300 °C (12 min). The injector temperature was set to 270 °C. Injections were performed in splitless mode at an injection volume of 2 μL. Nitrogen was utilized as collision gas at 1.5 mL/min. Three transitions per molecule were employed in multiple reaction monitoring mode for compound detection. The most intensive transitions were used for quantification, and the second transition was used for qualification.

Method validation was performed using method linearity, range, accuracy error, repeatability, reproducibility, limit of detection, and limit of quantification (LOQ). Reproducibility was evaluated using the relative standard deviation of spiked urine (quality control, QC) samples (5 ng/mL). We did observe fluctuations in QC samples for iPrP that exceed the acceptable 20 % of deviation. The presented results should be regarded as indicatory. Other compounds did not exceed the deviation of 20 %. The LOQs are listed as follows BPA: 0.25 ng/mL, BPF: 0.25 ng/mL, BPS: 0.5 ng/mL, MP: 0.5 ng/mL, EtP: 0.5 ng/mL, iPrP: 0.5 ng/mL, PrP: 1 ng/mL, iBuP: 1 ng/mL, BuP: 1 ng/mL, TCS: 0.25 ng/mL, and BzP: 0.25 ng/mL.

The laboratory for organic analysis at the Jožef Stefan Institute is a successful participant of the ICI-GEQUAS rounds (inter-laboratory comparison investigation) organized within the European project HBM4EU.

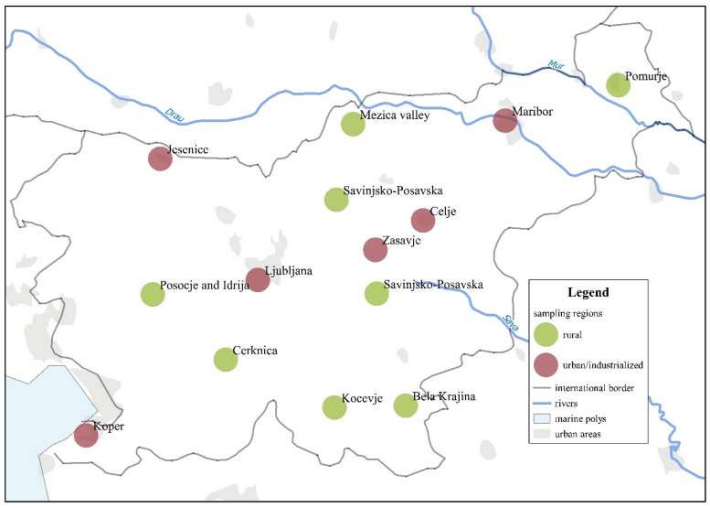

Fig. 1. Sampling regions (Natural Earth quick start for QGIS). Green circles represent rural sampling locations. Urban and/or industrialized areas are indicated by red circles. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

2.3. Laboratory analysis of phthalate metabolites

Urine samples were sent to the VITO NV laboratory in Belgium for analysis. A total of 13 phthalate metabolites (MEP, MBzP, MiBP, MnBP, MCHP, MnPeP, MEHP, OH-MEHP, oxo-MEHP, McxEPP, MnOP, cx-MINP, OH-MIDP, and OH-MINP) and 2 metabolites of DINCH (OH-MINCH and oxo-MINCH) were analysed. Briefly, 1 mL of urine sample were deconjugated using β-glucuronidase in ammonium acetate buffer solution and analysed after direct injection with ultraperformance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS). Samples were analysed in batches of 20, each batch containing one procedure blank, one QC sample (water), one spiked QC sample (urine), one sample analysed in duplicate, and one independent control sample (G-EQUAS). Recovery of the QC samples in water and urine were within 20 % of deviation for all analytes. Repeatability of all analytes, as controlled by duplicate samples, was within 20 % of variation. The reported LOQs are listed as follows: oxo-MEHP, cx-MINP, McxEPP, MnOP, MnPeP, OH-MEHP, OH-MiDP, OH-MINCH, OH-MINP, and oxo-MINCH: 0.1 ng/mL; MBzP and MCHP: 0.2 ng/mL; MEP, MiBP, and MnBP: 0.5 ng/mL; and MEHP: 0.8 ng/mL.

Vito is a successful participant of the ICI-GEQUAS rounds (inter-laboratory comparison investigation) organized within the European project HBM4EU.

2.4. Statistical analyses

Statistical analysis was performed on specific gravity (SG)-adjusted concentrations to overcome the urine dilution effect. Concentrations < LOQ were replaced with the value of LOQ/2 for each analyte. All statistical analyses have been carried out in the statistical software R version 3.6.2, and only analytes with detection frequencies greater than 30% were included in the advanced statistical analysis and modelling. The correlations among the analytes were investigated in a correlation matrix using Spearman's rank correlation coefficient and Benjamini-Hochberg adjustment using ggstatsplot (Patil, 2018) after the Shapiro-Wilk test revealed a non-normal distribution of data. Questionnaire data on dietary habits were categorized into 7 categories representing increasing consumption frequency averaged for one year. In response to the uneven distribution over the categories, we re-categorized as consumers and non-consumers and individually explored associations for all analytes. The correlations between continuous variables, such as age and body mass index (BMI) and analyte concentrations were investigated using Spearman's rank correlation coefficient. Following this approach, significantly associated confounders were included in the regression modelling and Akaike information criterion (AIC) analysis was performed to optimize the model parameters. Challenges occurring in this step of the data analysis could be attributed to the unreliability (diet) and unavailability (personal care products) of questionnaire data, which was originally optimized for persistent pollutants and trace elements. An attempt to utilize linear regression modelling (LRM) resulted in unsatisfactory diagnostic plots in response to a lack of appropriate confounders. We attempted to overcome these limitations by categorizing analyte concentrations into quartiles and performed ordinal logistic regression (OLR) analysis. The utilization of quartiles in contrast to continuous variables reduces the effect of extreme values and, thus, decreases the uncertainty related to the model outcome. Therefore, as confounders were used only parameters that can either influence physiology (age, BMI, intake of alcohol, smoking, and intake of supplements) or exposure (residential environment, level of education, year of sampling, and occupational exposure). Directed Acyclic Graphs (DAGs) analysis (Textor et al., 2016) confirmed the inclusion of age, alcohol consumption, education, and smoking into the model (data not presented). However, as the AIC analysis highlighted the importance of year of sampling, smoking, intake of supplements, occupation, these variables were chosen as confounders as well. All OLRs were confirmed in LRM that showed the same trends, however under unsatisfactory model performance. Therefore, the results from LRM are not presented or discussed in this study.

The models were separately applied to men and women as metabolism differs between the sexes and can additionally be influenced by pregnancy (Koh et al., 2014; Waxman and Holloway, 2009). The dataset was split into train data (70%) and test data (30%). The train data were employed to build the models for each analyte, and model performance was evaluated using the test data set. Model parameters were optimized based on the p-values for each confounder and the overall prediction error obtained when predicting the results for the test dataset. High prediction errors can be attributed to a lack of sufficient confounders. The trends of the obtained results were confirmed with linear regression modelling, but as the errors are high, any conclusions based on these results have large uncertainties. The authors are aware of this limitation.

2.5. Exposure and risk assessment

To assess the risk of the target population to the selected compounds, we closely followed the approach presented by Sanchis et al., (2020). Risk assessment was attempted only for compounds with detection frequencies greater than 30 %. We estimated the risk of the population by calculating the hazard quotient (HQ) using reference values available in the literature, such as biomonitoring equivalents (BEs) and no-observed-adverse-effect levels (NOAEL), or by calculating the estimated daily intake (EDI) of the respective compound. An HQ > 1 indicates risk and is calculated with Equation (1) (EFSA, 2013):

$$HQ = (P95)/(BE) \tag{1}$$

To assess the hazard to mixtures, the HQ can be calculated for combined exposure if BE values are available (Equation (2)) (Porras et al., 2020):

$$HQ_{combined} = \left(\frac{P95_{compound\ 1}}{BE_{compound\ 1}}\right) + \left(\frac{P95_{compound\ 2}}{BE_{compound\ 2}}\right) + \left(\frac{P95_{compound\ 3}}{BE_{compound\ 3}}\right) + compound\ n \tag{2}$$

In the absence of BE values, the ratio between the calculated EDI and the acceptable daily intake (ADI) can be utilized to estimate the HQ using Equation (3):

$$HQ = (EDI)/(ADI) \tag{3}$$

The EDI of phenols can hereby be calculated as follows (Equation (4)):

$$EDI\left(\frac{\frac{mg}{kgbw}}{day}\right) = \left(P95\left(\frac{mg}{L}\right) \times V_{urine}\left(\frac{L}{day}\right)\right) \Big/ \left(F \times BW\ (kg)\right) \tag{4}$$

whereas the EDI calculation of phthalates should follow Equation (5):

$$EDI\left(\frac{\frac{mg}{kgbw}}{day}\right) = \left[\left(\frac{\mathrm{P95}(\mu g/\mathrm{L})}{MW_m\left(\frac{g}{\mathrm{mol}}\right)} \times \frac{MW_P\left(\frac{g}{mol}\right) \times V_{urine}\left(\frac{L}{day}\right)}{F \times BW\ (kg)}\right)\right] \Big/ 1000 \tag{5}$$

where P95 is the 95th percentile of each compound; V_{urine} is the total urine volume excreted in 24h (1.7 L/day for men and 1.6 L/day for women (Aylward et al., 2009a)); F is the urinary excretion factor of the

compound; MW_m and MW_p represent the molecular weight of the metabolite and parent compound, respectively, and BW is the mean body weight of the population in kg (women: 63.7; men: 83.4).

For DEHP, we estimated the EDI by including the sum of all measured metabolites (MEHP (m1), OH-MEHP (m2), oxo-MEHP (m3), and McxEPP (m4)) as follows (Equation (6)):

$$EDI\left(\frac{\frac{mg}{kg_{BW}}}{day}\right) = \left[\left(\left(\frac{P95_{m1}\left(\frac{ng}{L}\right)}{MW_{m1}\left(\frac{g}{mol}\right)}\right) + \left(\frac{P95_{m2}\left(\frac{ng}{L}\right)}{MW_{m2}\left(\frac{g}{mol}\right)}\right) + (m3) + (m4)\right) \times \left(\frac{MW_P\left(\frac{g}{mol}\right) \times V_{urine}\left(\frac{L}{day}\right)}{\Sigma F_{m1,\ m2,\ m3,\ m4} \times BW\ (Kg)}\right)\right] \Big/ 1000 \quad (6)$$

Apel et al., (2020) suggest ΣF = 0.471 for all four included metabolites.

For compounds with no available BE or ADI in the literature, it is possible to estimate the risk using the margin of exposure (MOE), which indicates low risk if the value exceeds 10,000 (EFSA, 2005). The MOE can be calculated as follows (Equation (7)):

$$MOE = (NOAEL)/(EDI) \quad (7)$$

An overview over the applied parameters for different analytes is presented in Table 1. The excretion factor for BPS and BPF is assumed to be the same as that for BPA, as the metabolic pathway of all three compounds is assumed to be very similar. HQ calculation is performed on a worst-case-scenario basis, meaning that the highest values of a given range of EDI or ADI were included (applied to ΣEtP + MP and BzP) to obtain the HQ.

3. Results and discussion

3.1. Levels of phenols and metabolites of phthalates and DINCH in urine samples

We analysed a total of 11 environmental phenols, 14 phthalate metabolites, and 2 metabolites of the alternative plasticizer DINCH in urine

Table 1
Parameters for the calculation of estimated daily intake (EDI), hazard quotient (HQ), and margin of exposure (MOE). No parameters are available for BPF, OH-MIDP, and OH-MINP (italic).

Compounds	MW (g/mol)	P95 F (mg/L)	P95 M (mg/L)	F		BE (mg/L)		ADI ($mg/kg_{bw}/d$)		NOAEL ($mg/kg_{bw}/d$)	
BPA	228.3	0.011	0.017	100	Krishnan et al. (2010a)	2	Krishnan et al. (2010a)				
BPS	250.3	0.002	0.0008	100						10	Khmiri et al., (2020)
BPF	350.4	0.002	0.008	100							
MP	152.2	0.185	0.294	17.4	Moos et al., (2017)[b]			10[b]	EFSA, (2004)		
EtP	166.2	0.068	0.058	13.7	Moos et al. (2017)			10[b]	EFSA (2004)		
iPrP	180.2	0.002	0.001	10.2	Moos et al. (2017)			0.02	Moos et al., (2017)		
PrP	180.2	0.029	0.042	9.7	Moos et al. (2017)			0.1	Honda et al., (2018)		
iBuP	194.2	0.001	0.0004	6.8	Moos et al. (2017)			0.02	Moos et al., (2017)		
BuP	194.2	0.003	0.003	5.6	Moos et al. (2017)			0.02	Moos et al. (2017)		
BzP	228.2	0.0004	0.0004	100–1							
TCS	289.5	0.002	0.003	54	Apel et al., (2017)	2.6	Krishnan et al. (2010b)				
MEHP	278.3	0.009	0.016	47.1[c]	Apel et al., (2020)	1[c]	Aylward et al. (2009a)				
Oxo-MEHP	292.3	0.016	0.021	47.1[c]	Apel et al. (2020)	1[c]	Aylward et al. (2009a)				
OH-MEHP	294.3	0.024	0.035	47.1[c]	Apel et al. (2020)	1[c]	Aylward et al. (2009a)				
McxEPP	308.3	0.034	0.035	47.1[c]	Apel et al. (2020)	1[c]	Aylward et al. (2009a)				
MEP	194.2	0.227	0.947	69	Koch and Calafat, (2009)	18	Aylward et al. (2009b)				
MBzP	256.3	0.015	0.021	73	Koch and Calafat (2009)	12	Aylward et al. (2009b)				
MnBP	222.2	0.043	0.071	69	Koch and Calafat (2009)	0.2	Aylward et al. (2009b)				
MiBP	222.2	0.088	0.124	69	Koch and Calafat (2009)	0.19	Porras et al., (2020)				
cx-MINP	322.6	0.008	0.013	9.1	Koch and Calafat (2009)	0.03	Porras et al. (2020)				
OH-MIDP	322.4	0.002	0.004	100–1							
OH-MINP	308.4			18.4	Koch and Calafat, (2009)						
OH-MINCH	314.4	0.013	0.022	10.73	Kasper-Sonnenberg et al., (2019)			1[a]	Kasper-Sonnenberg et al., (2019)		
Oxo-MINCH	312	0.006	0.012	2.03	Kasper-Sonnenberg et al. (2019)			1[a]	Kasper-Sonnenberg et al. (2019)		

[a] TDI.
[b] Sum of EtP and MP.
[c] Sum of DEHP metabolites

Table 2
Descriptive statistics of specific gravity corrected concentrations, sample size, and LOQs. Lines in italic represent analytes with detection rates <30 %, which were excluded from the statistical analysis.

		<LOQ %	GM	Median	Range	LOQ (ng/mL)
BPF	*women*	*73*	*<LOQ*	*<LOQ*	*<LOQ-47.7*	0.25
	men	59	0.37	0.25	0.08–129	
BPA	women	8	2.41	2.52	0.12–60.6	0.25
	men	2	3.81	3.70	0.17–59.8	
BPS	*women*	*90*	*<LOQ*	*<LOQ*	*<LOQ-30.7*	0.5
	men	*86*	*<LOQ*	*<LOQ*	*<LOQ-1.93*	
MP	women	2	20.7	21.5	0.18–1523	0.5
	men	6	12.5	13.6	0.19–1086	
EtP	women	16	5.86	6.71	0.13–145	0.5
	men	20	4.32	5.26	0.15–564	
iPrP	*women*	*79*	*<LOQ*	*<LOQ*	*<LOQ-76.1*	0.5
	men	*84*	*<LOQ*	*<LOQ*	*<LOQ-30.5*	
PrP	women	24	1.73	1.75	0.07–327	0.25
	men	40	0.97	0.58	0.07–286	
iBuP	*women*	*95*	*<LOQ*	*<LOQ*	*<LOQ-5.76*	1
	men	*98*	*<LOQ*	*<LOQ*	*<LOQ-2.47*	
BuP	women	46	0.41	0.37	0.06–9.21	0.25
	men	59	0.29	0.22	0.08–43.2	
BzP	women	49	0.17	0.17	0.05–0.76	0.25
	men	*71*	*<LOQ*	*<LOQ*	*<LOQ-1.44*	
TCS	*women*	*70*	*<LOQ*	*<LOQ*	*<LOQ-15.9*	0.1
	men	*73*	*<LOQ*	*<LOQ*	*<LOQ-43.7*	
MEHP	women	25	1.98	2.05	0.26–33.5	0.8
	men	5	4.29	4.34	0.49–85.3	
Oxo-MEHP	women	1	3.89	3.80	0.20–35.3	0.1
	men	1	5.23	5.48	0.08–71.3	
OH-MEHP	women	0	5.82	5.53	0.20–51.5	0.1
	men	0	9.11	8.72	0.82–115	
McxEPP	women	1	7.75	7.07	0.12–97.6	0.1
	men	0	9.34	9.43	1.42–112	
MEP	women	0	29.9	27.9	1.02–1062	0.5
	men	0	65.0	55.7	1.21–5033	
MBzP	women	12	2.23	2.67	0.09–66.4	0.2
	men	4	3.6	3.76	0.10–256	
MIBP	women	2	19.8	19.7	0.34–266	0.5
	men	1	26.0	25.4	0.40–363	
MnBP	women	0	11.4	11.4	1.05–106	0.5
	men	1	16.3	15.0	0.40–268	
cx-MINP	women	1	1.99	1.85	0.15–44.2	0.1
	men	0	2.90	2.82	0.52–94.3	
OH_MidP	women	23	0.37	0.37	0.04–72.3	0.1
	men	8	0.81	0.77	0.04–83.5	
OH-MINCH	women	20	0.66	0.49	0.04–297	0.1
	men	10	1.08	0.87	0.04–720	
Oxo-MINCH	women	39	0.32	0.23	0.03–84.5	0.1
	men	21	0.56	0.48	0.04–254	
OH-MINP	women	0	2.21	2.26	0.67–11.7	0.1
	men	0	3.66	3.03	0.54–114	

samples of men and lactating women. Among the phenols, BPS, iPrP, iBuP, and TCS were detected in <30 % of all samples, with the detection rate as low as 3% for iBuP. Other phenols could be detected at rates ranging from 96% (MP) to 34% (BPF). Detection rates were generally higher for phthalate and DINCH metabolites. OH-MEHP, McxEPP, MEP, MnBP, and OH-MINP could be detected in 100% of all samples, whereas detection rates of other metabolites ranged between 99% (oxo-MEHP, MIBP, cx-MINP) and 70% (oxo-MINCH). MnOP, MnPeP, and MCHP were detected in <2% of all samples. Descriptive statistics for SG-corrected urinary concentrations and LOQs are presented in Table 2, whereas information on unadjusted and creatine-adjusted levels are provided in the supplementary material (Table S2 and S3). Results for MnOP, MCHP, and MnPeP are not presented due to their low detection rates. Men have significantly ($p \leq 0.01$) higher levels of BPA and BPF, as well as all phthalate and DINCH metabolites, while urinary concentrations of MP, EtP, iPrP, PrP, and BuP were significantly higher in women ($p \leq 0.04$). Similar trends have been observed in other studies (Husøy et al., 2019), while a study on phthalate exposure of the general Slovene population (DEMOCOPHES) did not observe large differences (Runkel et al., 2020). The authors attributed higher paraben levels in women to a larger number of applications and a higher diversity of PCPs compared to men, but our study lacks the data to confirm or reject this hypothesis.

Furthermore, there is increasing evidence that the activity of drug metabolizing enzymes is significantly altered during pregnancy (Isoherranen and Thummel, 2013; Koh et al., 2014). As our female study population consists of primiparous women, it cannot be excluded that the observed differences could at least partially be attributed to the physiological state. The levels of BzP did not differ significantly between genders, but the lower detection rate in men (29%) versus women (51%) might bias the results. The urinary concentrations of phenols and phthalates and the differences between men and women are illustrated in Fig. 2.

Fig. 3 presents the associations among analytes in urine of men and women. All observed correlations were positive; however more correlations were significant in the samples of women. For both genders, the associations among phthalates were the strongest, with Spearman's rho mostly greater than 0.5, whereas among BPs rho was mostly less than 0.4. Lesser correlations among BPs and PBs have been reported by other studies (Husøy et al., 2019; Sakhi et al., 2018) and attributed to differences in the exposure pathway. Exposure to PBs occurs mostly via PCPs, while the main pathway of exposure to BPs is diet. The same route is shared with HMW phthalates, which explains the observed correlation patterns in both our study and other studies. The results show a relatively strong correlation between MP and PrP (rho of 0.74 and 0.41 in women and men, respectively), suggesting a common exposure source. To increase the preservative effect, different PBs are often applied in combination, and strong correlations between PrP and MP have been observed in similar studies from Slovenia and other countries (Sakhi et al., 2018; Tkalec et al., 2021). Less correlations were significant in men, but the observed trends are similar between men and women. MEP was correlated weaker with other phthalates compared to other metabolites, suggesting different industrial applications for this compound. Metabolites of the same parent compound were correlated the strongest, confirming their relationship. Similar conclusions have been derived in other studies (Porras et al., 2020).

3.2. Comparison with the literature

Concentrations of phthalate metabolites vary substantially throughout the literature (Wang et al., 2019), with very low concentrations for **MEP** being reported in a study on men and women from Germany (13.5 ng/mL) (Koch et al., 2017) and concentrations of 246 ng/mL being reported in pregnant women from Spain (Valvi et al., 2015); the latter exceeding concentrations observed in an occupationally exposed population (201 ng/mL) from Slovakia (Kolena et al., 2017). The concentrations observed in this study (median: 27.9 and 55.7 ng/mL SG for women and men, respectively) are among the lower values reported in the literature and are comparable to concentrations measured in Japan (21.4 ng/mL) (Itoh et al., 2009), Italy (61.0 ng/mL and 73.2 ng/mL for women and men, respectively) (Tranfo et al., 2013), Belgium (37.6 ng/mL, adults) (Dewalque et al., 2014), China (28.2 ng/mL, men) (Zhang et al., 2016), and the Czech Republic (56.7 ng/mL, women) (Černá et al., 2015). DEP is a LMW phthalate that does not underlie any regulations and finds wide applications in e.g. fragrances.

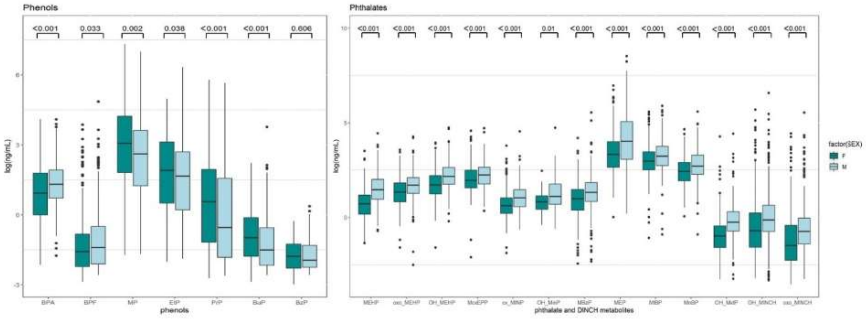

Fig. 2. Log transformed urinary concentrations of phenols, phthalates, DINCH (ng/mL) grouped by sex (F = women and M = men). Significance obtained using Wilcoxon test and indicated by p-values.

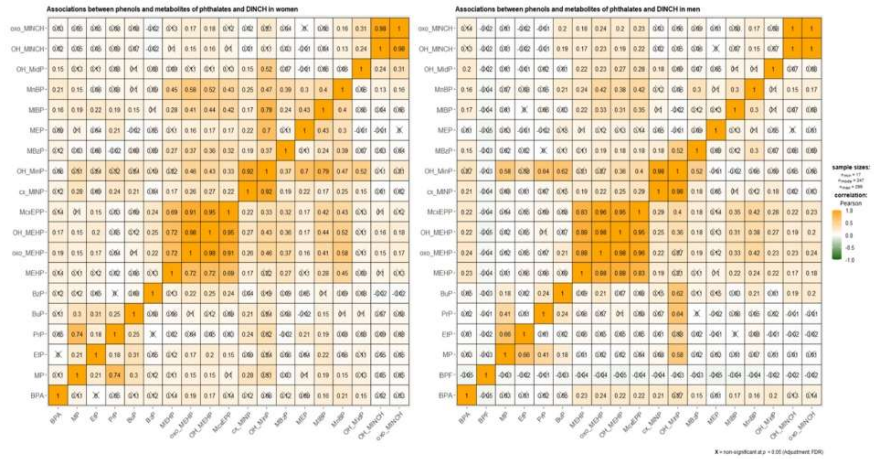

Fig. 3. Spearman's rank correlation of phthalate and DINCH metabolites and environmental phenols in women (left) and men (right). Non-significant correlations (p value < 0.05) are crossed out.

As such, exposure largely depends on personal lifestyles. In a study from 2011 including Slovenian women and men the observed concentrations of MEP in urine were more than twice as high in women (median: 50.2 ng/mL SG) and slightly higher in men (median: 39.7 ng/mL SG) (Runkel et al., 2020) compared to the present study. Differences among women from other countries as well as from Slovenia could be attributed to the temporarily altered physiological stage during pregnancy and lactation or to altered behaviour. Unfortunately, our dataset does not allow the confirmation or rejection of this hypothesis. The determined concentrations in men are comparable to those observed in the DEMOCOPHES study. As such, differences to other countries can most likely be attributed to differences in lifestyle and/or the respective market. Other metabolites, such as **MnBP**, exhibit less variation among populations, but differences still apply even within one country. In the DEMOCOPHES study, urinary levels were 25.3 ng/mL SG for women and 29.4 ng/mL SG for men (median) (Runkel et al., 2020). However, in the present study, the respective values were 11.4 ng/mL SG and 15 ng/mL SG. Similarly, three studies from Germany report MnBP concentrations of 181 ng/mL, 19.6 ng/mL, 8.0 ng/mL, and 16.4 ng/mL for adults (Göen et al., 2011; Koch et al, 2003, 2017bib_Koch_et_al_2017bib_Koch_et_al_2003), whereas values of 11.8 ng/mL, 31.3 ng/mL, 49.6 ng/mL, 27.1 ng/mL, and 13.7 ng/mL were reported from Australia (Heffernan et al., 2016), Belgium (Dewalque et al., 2014), China (Guo et al., 2011a), Spain (Valvi et al., 2015), and the USA (Wenzel et al., 2018). The wide range among MnBP concentrations furthermore reflects the importance of adequate questionnaire data. The included studies were selected based on the time period in order not to bias the results. As such, we can only hypothesize about lifestyle differences or physiological alterations that can influence urinary metabolite levels. Levels of **MiBP** in urine from Belgium (24.3 ng/mL) (Dewalque et al., 2014) and Germany (25.5 ng/mL and 20.3 ng/mL) (Göen et al., 2011; Koch et al., 2017) in 2011 are comparable to this study (19.7 ng/mL SG for women and 25.4 ng/mL SG for men), whereas three studies from the USA (Colacino et al., 2010; Ferguson et al., 2015; Wenzel et al., 2018) observe lower concentrations in their populations (4.9 ng/mL, 7.57 ng/mL, and 11.3 ng/mL, respectively) Other countries, however, observe higher concentrations. As

such, studies from China report median concentrations of 55.6 ng/mL (young adults) (Gao et al., 2016) and 51.7 ng/mL and 70.2 ng/mL for women and men, respectively (Guo et al., 2011b) and a study from Germany reports urinary MiBP levels in women of 43.0 ng/mL (Kasper-Sonnenberg et al., 2012) Kolena et al., (2017) included occupationally exposed hairdressers (mostly female) and a non-occupationally exposed control group from Slovakia in their study. The hairdressers had median concentrations of 40.3 ng/mL MiBP in their urine, whereas the values for the control group were 24.2 ng/mL. These results highlight that exposure to phthalates is highly influenced by behaviour and obtained metabolite concentrations can vary among countries, but also within a state. The latter is demonstrated by the MiBP levels for women and men that were determined in the DEMOCOPHES study (38.9 ng/mL SG and 47.3 ng/mL SG, respectively) that were twice as high as in the here presented population. The observed concentrations of **ΣDEHP** metabolites in Slovenia are low compared with other studies (24.3 ng/mL SG and 36.9 ng/mL SG for women and men, respectively); in comparison, the values for women and men from the DEMOCOPHES study were 24.3 ng/mL SG and 36.9 ng/mL SG, respectively (calculated based on the results published by Runkel et al., (2020)). Values of a magnitude comparable to those observed in this study were only reported by two studies from Germany (Koch et al., 2017), Belgium (Dewalque et al., 2014), and Slovakia (Kolena et al., 2017) with calculated ΣDEHP levels of 29.2 ng/mL, 26.3 ng/mL, and 26.9 ng/mL. However, a study from Kuwait reports a median of 180.4 ng/mL (Guo et al., 2011a) and the Slovakian study (Kolena et al., 2017) included occupationally exposed hairdressers with urinary levels of 31.4 ng/mL that were only slightly above the here observed levels. These results demonstrate the differences in the market, with DEHP being widely restricted in Europe, as well as the application of the chemical in plastic materials rather than PCPs; the latter being seen by the concentrations obtained by Kolena et al., (2017). Levels of DINCH metabolites in Slovenia are comparable to those obtained by a German study (Schütze et al., 2012) that determined **oxo-MINCH** and **OH-MINCH** at concentrations of 0.22 ng/mL and 0.36 ng/mL in non-occupationally exposed adults. In this study, urinary concentrations of both metabolites in women were 0.23 ng/mL SG and 0.49 ng/mL SG, respectively, whereas the levels for men were 0.48 ng/mL SG and 0.87 ng/mL SG. Unfortunately, the levels of DINCH metabolites were not determined in the DEMOCOPHES study and also other studies rarely report these concentrations for samples obtained between 2008 and 2014. However, DINCH is becoming a more frequently applied plasticizer (Bui et al., 2016) and more and more studies include its metabolites in their analyses. Metabolites of DINP were observed at comparable concentrations by two German studies that report comparable median values of 2.5 ng/mL and 5.0 ng/mL (Koch et al., 2007b) for **OH-MINP** and **cx-MINP**, respectively and 5.6 ng/mL for OH-MINP (Fromme et al., 2007). However, the geometric means reported by (Koch et al., (2007b)) are up to 5 times higher than in our study. The comparable levels observed among the studies could suggest that exposure to DINP underlies less variations than other compounds. A possible reason for this might be the restriction of this compound und REACH (ECHA, 2012), but our dataset lacks the necessary information to evaluate this hypothesis. Similarily, concentrations of the BBzP metabolite **MBzP** observed by (Koch et al., (2007b)) are higher (5.6 ng/mL) than those observed in this study (2.67 ng/mL SG for women and 3.76 ng/mL SG for men). However, the results from the DEMOCOPHES study (5.5 ng/mL SG for women and 5.95 ng/mL SG for men) are comparable to the German study, whereas (Campbell et al., 2018) report slightly higher values (8.0 ng/mL for women and 6.4 ng/mL for men). Higher levels of MBzP in women compared to men have been often reported in the literature and studies suggest a higher exposure of women through PCPs (Berger et al., 2019; Wormuth et al., 2006).

TCS is generally detected in urine at low frequencies and in varying concentrations (Wang and Tian, 2015). Wang and Tian (2015) summarize the results from 10 studies on TCS exposure and report detection frequencies between 0 % in Belgium and 76 % in China, which illustrates the differences in TCS exposure among populations. As such, the reported maximum values for adults of 2580 ng/mL from Greece (Asimakopoulos et al., 2014), of 3790 ng/mL from USA (Calafat et al., 2008), of 232 ng/mL (women) and 109 ng/mL (men) from China (Engel et al., 2014), of 1586 ng/mL from Denmark (Frederiksen et al., 2013), of 3158 ng/mL from Korea (Kim et al., 2011), of 2388 ng/mL, 2780 ng/mL and 2690 ng/mL from USA (Koeppe et al., 2013; Meeker et al., 2013), of 1630 ng/mL from Germany, and 599 ng/mL from Belgium (Pirard et al., 2012), are higher than those observed in Slovenia (15.9 ng/mL SG for women and 43.7 ng/mL SG for men). The detection frequencies of 30 % (women) and 27 % (men) fall in the range of those summarized by Wang and Tian (2015). TCS is used as an antimicrobial in PCPs, such as soaps and tooth paste. However, its application varies among products and countries and the intensity and frequency of application is highly consumer dependent. As such, exposure underlies large variations and our dataset lacks the necessary data to determine the sources of exposure in this population. Among frequently monitored **PBs**, MP, EtP, and PrP are detected at the highest frequencies and concentrations. Moos et al., (2015) report median MP levels of 32–50.8 ng/mL, 1–2.2 ng/mL EtP, and 2.2–5.7 ng/mL PrP, as well as iPrP and iBuP medians < LOQ for the German population in the years 2008, 2009, and 2012. MP and PrP concentrations are higher than in the present population (MP: 21.5 ng/mL SG for women and 13.6 ng/mL SG for men; PrP: 1.75 ng/mL SG for women and 0.58 ng/mL SG for men), whereas EtP, iPrP, and iBuP are comparable (EtP: 6.71 ng/mL SG for women and 5.26 ng/mL SG for men; iPrP and iBuP: < LOQ for men and women). We found that the values observed for MP and PrP in the present study are widely comparable to concentrations reported from several Asian countries (Honda et al., 2018) and generally much lower than levels observed in the USA (MP: 39.0 ng/mL; PrP: 4.70 ng/mL) (CDC, 2017), Korea (MP: 112 ng/mL and PrP: 47.4 ng/mL) (Honda et al., 2018), and Germany (MP: 42.6 ng/mL and PrP: 2.2 ng/mL) (Moos et al., 2015). However, concentrations of EtP in Slovenia are higher in comparison to China (2.74 ng/mL), India (0.25 ng/mL), Japan (0.53 ng/mL), Kuwait (0.68 ng/mL), Saudi Arabia (0.19 ng/mL), Vietnam (0.26 ng/mL), Greece (0.76 ng/mL), and USA (0.30 ng/mL) (Honda et al., 2018). BzP is one of the lesser monitored PBs, and studies observe generally low detection frequencies (Frederiksen et al., 2014) and low concentrations that are comparable with the present study (0.17 ng/mL SG in women and < LOQ in men). The results from other studies demonstrate how differences in the market and culture can influence exposure, especially if it occurs through PCPs. PBs are common additives in many products and the individual lifestyle largely determines how much a person is exposed. However, this hypothesis is in need of confirmation from other studies, as our questionnaire did not cover data on PCPs. Among the **BPs** in this study, BPA is detected at the highest frequencies and concentrations (2.52 ng/mL SG in women and 3.70 ng/mL SG in men), whereas the alternatives BPF and BPS are present in urine samples at much lower levels (BPF: < LOQ in women and 0.25 ng/mL SG in men; BPS: < LOQ in women and men). Similar trends have been observed in women from other studies from Greece (BPA: 1.2 ng/mL) (Myridakis et al., 2015) and Norway (BPA: 3.08 ng/mL SG; BPF: <LOQ; BPS: 0.13 ng/mL SG). It is known that BPA as a high-production-volume chemical is ubiquitous in the environment and that humans are widely exposed to it (Lehmler et al., 2018). However, its reputation as an endocrine disrupting chemical lead to the replacement of BPA with alternatives such as BPS and BPF that are currently produced and applied in much lesser quantities compared to BPA (Pelch et al., 2019). Therefore, the trends observed in this study and by others can be explained by the dynamics of the global market.

3.3. Determinants of exposure to phthalates, DINCH, and phenols

The demographic characteristics of the study population that were included in the models are presented in Table S1. As we observed

Table 3
Results of ordinal logistic regression modelling and suggested determinants of exposure. Model results were separately obtained for men (M) and women (F). Listed are the results for each confounder included in the separate models. Blank values indicate that this confounder was not included in this model. Included confounders: occupational exposure, education, age, year of sampling, BMI, supplements, smoking, alcohol, and residence; training dataset: 70 % and test dataset: 30 %. The values before brackets represent beta coefficients, whereas the p value is given after the slash and significant p values are indicated in bold (beta/p-value).

	Age	BMI	Residence/R	Education/U	Year	Exposure/Yes	Supplements	Alcohol	Smoking	error
					F					
MEHP		−0.8/0.3			−316/**<0.0001**		−0.4/0.2			**0.64**
Oxo_MEHP	−1.8/0.07				−569/**<0.0001**	0.2/0.58				**0.64**
OH_MEHP			−1.0/**0.0002**	−0.4/0.14	−692/**<0.0001**		−0.3/0.3	−0.09/0.6		**0.61**
McxEPP	−1.1/0.3		−0.6/**0.02**		−860/**<0.0001**			−0.3/0.07		**0.64**
Cx_MINP		1.2/0.12			−366/**<0.0001**	−0.3/0.4				**0.72**
MBzP	−2.8/**0.007**	3.0/**0.0005**	−0.6/**0.04**		−518/**<0.0001**					**0.64**
MEP		1.4/0.08	−0.6/**0.02**		−569/**<0.0001**					**0.67**
MIBP			−0.6/**0.01**		−567/**<0.0001**	−0.3/0.6				**0.65**
MnBP		1.0/0.3	−7.1/**0.01**		−1396/**<0.0001**	−0.6/0.1	−2.8/0.4			**0.63**
OH_MidP			−0.5/**0.05**	−0.7/**0.01**	−126/**<0.0001**		−0.5/0.1			**0.67**
OH_MINCH			−0.7/**0.01**		59.4/**<0.0001**		−0.7/**0.03**			**0.65**
OH_MinP	−5.0/**0.03**				210/**<0.0001**					**0.64**
Oxo_MINCH			−0.7/**0.008**		94.4/**<0.0001**	0.3/0.5		−0.3/0.1		**0.65**
BPA		2.0/**0.03**	−1.0/**0.0009**	−0.3/0.3	495/**<0.0001**			−0.2/0.3		**0.59**
MP					−542/**<0.0001**			0.5/**0.01**		**0.67**
EtP			−0.3/0.3	0.5/0.07	−303/**<0.0001**			0.3/0.1		**0.66**
PrP				−0.6/0.07	−586/**<0.0001**		0.7/0.06	0.3/0.2		**0.70**
BuP			−0.6/**0.05**	0.7/**0.02**	−640/**<0.0001**					**0.59**
BzP				1.0/**0.001**	181/**<0.0001**					**0.61**
					M					
MEHP		−2.3/**0.02**			−78.6/**<0.0001**				−1.1/0.07	**0.70**
Oxo_MEHP					−261/**<0.0001**		0.5/0.09			**0.68**
OH_MEHP		−1.0/0.4			−359/**<0.0001**				−1.5/**0.01**	**0.62**
McxEPP					−567/**<0.0001**	0.2/0.5	−0.1/0.7	−0.3/0.2	−1.8/**0.004**	**0.62**
Cx_MINP	1.4/0.06			−0.2/0.49	- 408/**<0.0001**	0.5/0.13		−0.3/0.12	−2.1/**0.0005**	**0.67**
MBzP					−318/**<0.0001**				−7.4/0.2	**0.68**
MEP		−1.0/0.3			−51.9/**<0.0001**	0.4/0.3		0.2/0.4		**0.64**
MIBP		0.6/0.6		0.5/0.09	−59.6/**<0.0001**				−0.5/0.4	**0.69**
MnBP		−2.4/**0.02**	−0.3/0.38		−332/**<0.0001**		−0.2/0.59			**0.61**
OH_MidP	1.1/0.1				−298/**<0.0001**				−1.0/0.08	**0.68**
OH_MINCH	−1.0/0.2			0.5/**0.05**	184/**<0.0001**					**0.67**
Oxo_MINCH	−1.2/0.1			0.5/**0.04**	273/**<0.0001**		0.3/0.3			**0.69**
BPF		−0.9/0.4		−0.09/0.7	53.1/**<0.0001**					**0.68**
BPA			−1.5/**<0.0001**		12.8/**<0.0001**					**0.53**
MP	2.2/**0.02**	−1.8/0.09	−0.4/0.2		−380/**<0.0001**			0.2/0.3		**0.65**
EtP		−3.6/**0.002**	0.04/0.9		−18.4/**<0.0001**			0.6/**0.02**		**0.62**
PrP	1.2/0.2	−2.5/**0.03**	0.6/**0.05**	−0.3/0.4	−569/**<0.0001**	0.9/**0.02**				**0.63**
BuP			−1.1/**0.0003**		373/**<0.0001**	−0.4/0.3	0.6/0.06			**0.58**

significant differences between men and women, OLR models were presented separately for each gender (Table 3).

Interesting time trends in exposure could be observed. While the concentrations in traditional phthalate plasticizers are significantly decreasing between 2008 and 2014 in this population (M = men and F = women), levels of DINCH metabolites are increasing significantly, mirroring trends in production (Bui et al., 2016). Concentrations of BPF and BPA follow an increasing trend, whereas concentrations of PBs are mostly decreasing, with the exception of BuP in men and BzP in women. All observed time trends in the model are significant ($p < 0.0001$). HBM studies from Europe report a decline in urinary phthalate metabolite concentrations between 2011 and 2016 (Tranfo et al., 2018) and a 67% decline in DEHP metabolites in the USA (Wang et al., 2019). Similar trends were previously observed for phenols, which has been linked to the negative image of these chemicals in the media (Sakhi et al., 2018). MP concentrations in men were positively associated with age (coef: 2.2, $p = 0.02$), whereas other PBs and BPs did not show any significant trend with age. Among phthalate metabolites, we observed significantly decreasing concentrations (F:MBzP and F:OH-MINP F) with age (coef: 2.8, $p = 0.007$ and coef: 5.0, $p = 0.03$, respectively). As this study lacks application frequencies of PCPs and detailed data on dietary habits as a confounder, these results should be carefully considered. The model results are conflicting regarding the influence of BMI on contaminant concentrations. Despite an often significant impact on the model, no distinct trend is visible and more information on the participants' lifestyles would be needed to increase the certainty of the results. Significantly negative associations were observed between BMI and MEHP, MnBP, EtP, and PrP in men (coef: 2.3, $p = 0.02$; coef: 2.4, $p = 0.02$; coef: 3.6, $p = 0.002$; coef: 2.5, $p = 0.03$, respectively), whereas positive correlations were observed between BMI and MBzP, and between BMI and BPA in women (coef: 3.0, $p = 0.0005$ and coef: 2.0, $p = 0.03$, respectively). Participants from urban/industrialized sampling regions had significantly higher concentrations of almost all phthalate metabolites, BPs, and PBs in urine with the exception of PrP in men's urine, which were significantly higher in rural locations (coef from −0.6 to −7.1, $p < 0.05$). The lack of sufficient data limits the possibilities of discussion here, but reported higher concentrations of these compounds in urban air (Rakkestad et al., 2007; Rudel and Perovich, 2009) are a possible explanation. Participants with a university degree had significantly higher ($0.05 > p > 0.001$) levels of some compounds (M: OH-MINCH, coef: 0.5; M: oxo-MINCH, coef: 0.5; F: BuP, coef: 0.7; F:BzP, coef: 1.0), whereas concentrations of OH-MIDP were significantly higher in women with lower education (coef: 0.7, $p = 0.01$). Negative associations between metabolite concentrations of HMW phthalates and the level of education was observed also in the DEMOCOPHES study that included Slovenian men and women (Runkel et al., 2020), whereas the LMW phthalates (excluding MEP) were significantly higher in participants with a university degree. It was discussed that higher education might be associated with a higher awareness towards harmful food contaminants, such as HMW phthalates, while the occupation associated

with a higher level of education might require more a frequent application of PCPs, such as make-up. However, the results in this study are inconclusive and cannot confirm the trends observed in DEMOCOPHES. The inclusion of occupational exposure (self-reported exposure to solvents, lubricants, plasticizers, adhesives, and paints) and dietary supplement intake was beneficial for lowering the prediction error, but the results were mostly non-significant. Men that reported occupational exposure had significantly higher concentrations of PrP (coef: 0.9, $p = 0.02$) and the intake of supplements was significantly negatively associated with concentrations of OH-MINCH in women (coef: 0.7, $p = 0.03$). However, the trend was not repeatable for oxo-MINCH. Alcohol intake was significantly associated with elevated levels of MP in women (coef: 0.5, $p = 0.01$) and with elevated levels of EtP in men (coef: 0.6, $p = 0.02$). In men, smoking was associated with lower levels of cx-MINP (coef: 2.1, $p = 0.0005$), McxEPP (coef: 1.8, $p = 0.004$), and OH-MEHP (coef: 1.5, $p = 0.01$). Alcohol has been associated with a negative impact on hepatic drug-metabolizing enzymes that would decrease the elimination rates of contaminants in the human body (Miyashita et al., 2015). Smoking, on the other hand, has been found to induce cytochrome P450 enzymes involved in phase I metabolism of phthalates, which would lead to faster elimination of pollutants from the body (Flesch-Janys, 1996; Miyashita et al., 2015). It needs to be highlighted that the obtained prediction errors in this study are very high. Therefore, the results are in urgent need of verification and should be considered indicatory.

3.4. Estimated daily intake and risk assessment for men and lactating women

The calculated EDIs for men and women are presented in Table 4. As the calculation of HQs is based on the 95th percentile, so are the EDIs. Therefore, this should be considered when comparing the EDIs from this study with other publications. To allow an overview, a few studies from Europe (Norway and Germany) with supposed comparable exposure and Asia (Taiwan) with supposed higher exposure were selected and presented here. The EDIs in Slovenia are comparable or slightly lower than those reported in other studies. Sakhi et al., (2018) report EDIs of 0.056, 0.024, 0.032, 0.00027, and 0.0076 $mg/kg_{bw}/day$ for MP, EtP, PrP, BPA, and TCS, respectively, in Norwegian mothers. With the exception that the EDIs of BPA in Slovenian women from this study are slightly higher (0.0007 $mg/kg_{bw}/day$), the intakes of other compounds are lower than those reported by Sakhi et al., (2018). We obtained an EDI of 0.006 $mg/kg_{bw}/day$ for the sum of DEHP metabolites for men and women, which is slightly lower than the value reported for a population in Taiwan (0.008 $\mu g/kg_{bw}/day$) and substantially lower than the EDI of 0.021 $mg/kg_{bw}/day$ reported for the adult general population in Germany (95th percentile) (Koch et al., 2006). At this point it is worth noting that different urinary adjustment methods might introduce uncertainty to the exposure assessment as observed and discussed by Runkel et al., (2020). Other reasons for variations in EDIs among countries might lie in the exposure itself (lifestyle and market) or differences in the calculation of intake values, e.g. the use of different excretion factors or by presenting the 95th percentile instead of the geometric mean as it was done in this study.

The results of the HQ calculation are presented in Table 4. No analyte exceeds the value of 1 in men or women, and the MOE obtained for BPS exceeds 10,000, which corresponds to a no-risk scenario. The highest HQ was obtained for PrP (0.08 and 0.09) for women and men, respectively. To assess the risk of cumulative exposure to chemical mixtures, the HQ for all analytes was estimated. While the value for phenols is near 0.6 in both men and women, the risk resulting from phthalate exposure is lower (HQs: 0.15 and 0.19 for women and men, respectively). The combined HQ of phenols and phthalates does not exceed but approaches the limit of 1 in both populations (0.74 and 0.76 for women and men, respectively), which might cause concern due to the unknown impact of other chemicals not addressed in this study. Similar results were reported for BPA, BPS, BPF, and PBs in the framework of a Norwegian HBM study. Sanchis et al., (2020) report EDIs and HQs of comparable magnitude for lactating women ($EDI_{EtP\ +\ MP}$ 0.01–0.04 $mg/kg_{bw}/day$, HQ_{BPA} 0.005 and $HQ_{EtP\ +\ MP}$ 0.004). The authors conclude that current exposure levels do not imply a risk for mothers; however, HQs were evaluated only for individual analytes, while the HQ for the mixture has not been evaluated. A Finnish study evaluating a non-occupationally exposed population reports individual HQs higher than those obtained

Table 4
Presentation of Estimated Daily Intakes (EDI), Hazard Quotients (HQ), and Margins of Exposure (MOE) for women (F) and men (M). Cells were left blank if the calculation of HQs was not possible (BPS, BPF, BzP, OH-MIDP) because no biological equivalent (BE) values were available for these compounds. The availability of a no-adverse-effect-level (NOAEL) value for BPS allowed the calculation of the MOE for this compound.

Compounds	EDI F ($mg/kg_{bw}/d$)	EDI M ($mg/kg_{bw}/d$)	HQ F	HQ M	MOE F	MOE M
BPA	0.0007	0.0008	0.006	0.009		
BPS	0.0001–0.01	0.00004–0.004			199063	613235
BPF	*0.0002–0.02*	*0.0006–0.06*				
MP	0.04	0.05	0.004	0.004		
EtP	0.02	0.01				
iPrP	0.0009	0.0004	0.03	0.01		
PrP	0.01	0.02	0.08	0.09		
iBuP	0.0008	0.0002	0.02	0.006		
BuP	0.002	0.002	0.07	0.06		
BzP	0.00002–0.002	0.00001–0.002				
TCS	0.002	0.003	0.001	0.001		
ΣDEHP	0.006	0.006	0.006	0.006		
MEP	0.009	0.03	0.0005	0.002		
MBzP	0.0006	0.001	0.00005	0.00006		
MnBP	0.002	0.003	0.01	0.01		
MiBP	0.004	0.005	0.021	0.02		
cx-MINP	0.003	0.004	0.10	0.13		
OH-MIDP	*0.0001–0.07*	*0.0001–0.11*				
OH-MINCH	0.004	0.006	0.004	0.006		
Oxo-MINCH	*0.010*	*0.02*	0.01	0.02		
phenols			**0.59**	**0.57**		
phthalates			**0.15**	**0.19**		
total			**0.74**	**0.76**		

in the present study, and the HQ of the mixture exceeds the threshold of 1 (1.41–1.46) (Porras et al., 2020). Relatively high concentrations of MnBP (0.059 mg/g creatinine) compared to other European and US populations (CDC, 2019; Tranfo et al., 2018) as well as the present population (0,047 mg/g creatinine) substantially contributed to the high HQ. The monitoring of HQs in populations exposed to endocrine disruptors is an important measure in risk assessment. Exposure to mixtures is a controversial topic, as they can act either synergistic or antagonistic and the dose-response relationship in the human body is rarely linear (Siroux et al., 2016). Studies report that exposure to phthalates at a critical developmental stage can lead to an impaired development of psychomotoric skills (Polanska et al., 2014) and neurological disorders, such as attention deficit syndrome, hyperactivity or lower intelligent quotients (Ejaredar et al., 2015). A recent study by Papaioannou et al., (2021) estimated by utilization of multi-omics analyses how exposure to phthalate mixtures disturbs the urea cycle and choline metabolism. Findings like this highlight the need of frequent risk monitoring of populations as for instance through the calculation of HQs.

4. Study limitations

This study is part of the first national HBM program in Slovenia, which was originally designed for the monitoring of trace elements and persistent organic pollutants. The included questionnaire was optimized accordingly and lacks many details necessary for exposure assessment to non-persistent chemicals. This represents the largest limitation of this study. In an attempt to overcome this limitation, ordinal logistic regression modelling was performed, and the results were validated using prediction modelling. The obtained high prediction errors highlight the need for an adapted questionnaire, and the results should be considered indicatory. In future studies, first morning urine samples should be used instead of random spot samples as this limits the inter-individual variation between exposure and sampling.

5. Conclusion

This study evaluated the exposure of men and lactating women in Slovenia to phthalates, BPs, PBs, and TCS. We observed significantly higher concentrations of phthalates, DINCH, and BPs in men, whereas the levels of most PBs were significantly higher in women. We observed differences in exposure based on the area of residence (urban/industrial > rural) and urinary concentrations that were comparable to several other studies. Additionally, we assessed the risk of the population via HQs and conclude that while HQ values are sufficiently low, the HQ of the chemical mixture is near the threshold value of 1. Considering that many compounds were not included in this study, it is likely that the HQ of a larger chemical mixture will exceed 1. Additional studies that address a wider range of chemicals and potentially other susceptible populations, such as children, are needed.

Credit author statement

Agneta Runkel: Conceptualization, Methodology, Formal analysis, Investigation, Writing-Original draft, visualization, Tina Kosjek: supervision, writing - review & editing, Žiga Tkalec: laboratory supervision, writing - review & editing, Darja Mazej: project administration, writing – review & editing, Janja Snoj Tratnik: project administration, writing – review & editing, Milena Horvat: project administration, supervision, funding acquisition.

Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2021.131858.

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3.3.3 Contaminants of emerging concern in urine: A review of the analytical methods for determining diisocyanates, benzotriazoles, benzothiazoles, UV-filters, isothiazolinones, musks and non-phthalate plasticizers

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This manuscript was prepared in collaboration with Agneta Annika Runkel, with whom the work was equally split. In order to review methods used for quantification of CECs, we reviewed literature on analytical methods for selected groups of compounds from 2010 onwards. We searched for compounds that are currently understudied and not yet commonly implemented in existing HBM schemes. We identified isocyanates, benzotriazoles, pyrrolidones, ultra-violet (UV) filters, the antimicrobials, methylchloroisothiazolinone and methylisothiazolinone (MCI, MI), fragrances, and non-phthalate plasticizers. With the emergence of these chemicals on the market, there is a need for new sample preparation procedures and analytical methods to enable rapid monitoring of CECs. This literature showed that to extract, concentrate, and purify the samples, LLE and SPE were most commonly used and several new sample preparation procedures were applied, such as solid-supported liquid extraction and low-volume procedures. Prior to extraction, enzymatic hydrolysis was most commonly used to degrade phase II metabolites. Analytes were most commonly separated using HPLC and UHPLC, followed by GC for more non-polar, volatile CEC. Prevalently, tandem mass spectrometry (MS) was used to detect compounds, offering high selectivity and sensitivity. Due to widespread use of these analytical platforms, inclusion of these CECs into current HBM frames can be swiftly made. This will enable accurate and rapid monitoring, with this literature-reviewed collection of analytical and sample preparation procedures serving as a base for any group, wishing to include these CECs into their monitoring routines. This manuscript has been submitted to the Trends in Analytical Chemistry journal.

Contaminants of emerging concern in urine: a review of analytical methods for determining diisocyanates, benzotriazoles, benzothiazoles, 4-methylbenzylidene camphor, isothiazolinones, musks and non-phthalate plasticizers

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Abstract

Several human biomonitoring (HBM) frameworks have emerged over recent years to assess the exposure load of populations to harmful substances. In contrast to currently prioritised substances, i.e. phthalates, mercury, lead, and PBDE flame retardants, many contaminants of emerging concern (CEC) are under-researched but, based on their toxicity, are potential candidates for inclusion in future HBM campaigns. This review provides an overview of the state-of-the-art sample preparation techniques and analytical methods for the determination of isocyanates, benzotriazoles, pyrrolidones, ultra-violet filter 4-methylbenzylidene camphor (MBC), the antimicrobials methylchloroisothiazolinone and methylisothiazolinone (MCI, MI), fragrances, and non-phthalate plasticizers in urine, a common, readily available, non-invasive matrix suitable for large-cohort HBM studies.

Abbreviations

1-DEHTM - 1,4-di-(2-ethylhexyl) trimellitate, 1H-BTR - 1H-benzotriazole, 1-MEHTM - 1-mono-(2-ethylhexyl) trimellitate, 1OH-BTR - 1-hydroxybenzotriazole, 2-amino-BTH - 2-amino benzothiazole, 2cx-1-MMHTM - 1-mono-(2-carboxymethylhexyl) trimellitate, 2cx-2-MMHTM - 2-mono-(2-carboxymethylhexyl) trimellitate, 2cx-MMHTP - mono-(2-ethyl-2-carboxymethylhexyl) terephthalate, 2-DEHTM - 2,4-di-(2-ethylhexyl) trimellitate, 2-HESI - 2-hydroxy-N-ethylsuccinimine, 2-MEHTM - 2-mono-(2-ethylhexyl) trimellitate, 2-MeS-BTH - 2-methylthio benzothiazole, 2-NMSI - 2-hydroxy-N-methylsuccinime, 2-OH-BTH - 2-hydroxy benzothiazole, 2-SCNMeS-BTH - 2-thiocyanomethylthio benzothiazole, 2-SH-BTH - 2-mercapto benzothiazole, 3cx-MnPrA - mono-3-carboxy-n-propyl adipate,
3-NPA - 3-nitrophthalic acid anhydride, 4-Me-1H-BTR - 4-methyl-1H-benzotriazole, 4-MEHTM - 4-mono-(2-ethylhexyl) trimellitate, 4TDA - 2,4-toluenediamine, 5,6-diMe-1H-BTr - 5,6-dimethyl-1H-benzotriazole, 5-Cl-1H-BTR - 5-chloro-1H-benzotriazole, 5cx-1-MEPTM - 1-mono-(2-ethyl-carboxypentyl) trimellitate, 5cx-2-MEPTM - 2-mono-(2-ethyl-carboxypentyl) trimellitate, 5cx-MEHTP - 1-mono-(2-ethyl-5-carboxyhexyl) terephthalate, 5cx-MEPA - mono-5-carboxy-2-ethylpentyl adipate,
5-HNEP - 5-hydroxy-N-ethyl-2-pyrrolidone, 5-HNMP - 5-hydroxy-N-methyl-2-pyrrolidone,
5-Me-1H-BTR - 5-methyl-1h-benzotriazole, 5OH-1-MEHTM - 1-mono-(2-ethyl-5-hydroxyhexyl) trimellitate, 5OH-2-MEHTM - 2-mono-(2-ethyl-5-hydroxyhexyl) trimellitate, 5OH-MEHA - mono-2-ethyl-5-hydroxyhexyl adipate, 5OH-MEHTP - 1-mono-(2-ethyl-5-hydroxyhexyl) terephthalate,
5oxo-1-MEHTM - 1-mono-(2-ethyl-5-oxohexyl) trimellitate, 5oxo-2-MEHTM - 2-mono-(2-ethyl-5-oxohexyl) trimellitate, 5oxo-MEHA - mono-2-ethyl-5-oxohexyl adipate, 5oxo-MEHTP - 1-mono-(2-ethyl-5-oxohexyl) terephthalate, 6TDA - 2,6-toluenediamine, 7-HC - 7-hydroxycitronellal,
7-HCA - 7-hydroxycitronellic acid, AA - acetic acid, ACN - acetonitrile, ADBI - celestolide, AHTN - tonalide, AT - acetone, ATBC - acetyltributyl citrate, ATII - traesolide, CBC - 3-(4'-carboxybenzylidene) camphor, DEE - diethyl ether, DEHA - di-2-ethylhexyl adipate, DEHTP - di-(2ethylhexyl) terephthalate, DMAP - 4-dimethylaminopyridine, DMSO - dimethyl sulfoxide,
DnBA - di-n-butyl adipate, FA - formic acid, FD - fluorescence detection, FR - flow rate, HDI - 1,6-hexamethylene diisocyanate, HESI - heated electrospray ionization, HHCB - galaxolide, IPA - isopropanol, MA - musk ambrette, MBC - 3-(4-methylbenzylidene) camphor,
MCI - methylchloroisothiazolinone, MDA - methylene diphenyldianiline, MDI - methylene diphenyl diisocyanate, MEHA - mono-2-ethylhexyl adipate, MEHTP - mono-(2ethylhexyl) terephthalate, MI - methylisothiazolinone, MK - musk ketone, MnBPA - mono-n-butyl adipate, MX - musk xylene,
NDA - 1,5-naphthalenediamine, NEP - N-ethyl-2-pyrrolidone, NMMA - N-methylmalonamic acid, NMP - N-methyl-2-pyrrolidone, PBC - 4-(1-pyrene)butanoyl chloride, PLE - Pressurized liquid extraction,
PP - polypropylene, RT - room temperature, SPE - solid phase extraction, TAHI - trisaminohexyl isocyanurate, TBBA - tert-butyl benzoic acid, TBHA - tert-butyl hippuric acid, TEHTM - tri-(2-ethylhexyl) trimellitate, TTR - tolyl benzotriazole, UFLC – ultra-fast liquid chromatography, XTR - xylyl benzotriazole,

1. Introduction

With the development of new materials, consumer, and personal care products, new chemicals are emerging on the market and in the environment. The occurrence of new state-of-the-art instrumentations for high-resolution mass spectrometry coupled to separation techniques allows the detection of previously overlooked contaminants. Additionally, the knowledge on the toxic effects of known compounds is growing. Due to increasing evidence on the adverse health effects, many new and existing contaminants are becoming of emerging concern (CECs). However, they are currently still under-researched in routine monitoring schemes, such as the National Health and Nutrition Examination Survey (NHANES), human biomonitoring for Europe (HBM4EU), and the German Environmental Survey (GerES) (Yadav et al., 2021).

Human biomonitoring (HBM) measures potentially harmful chemicals and their metabolites in human samples in response to exposure to identify exposure sources, understand chemical risk, and ensure policy informing and efficiency of reduction strategies (Ganzleben et al., 2017). As such, it measures internal exposure that reflects the actual chemical uptake from oral, dermal and inhalation exposure pathways (Vorkamp et al., 2021). Blood and urine are the most commonly investigated matrices. Blood is an optimal matrix, as is in contact with all tissues in the body, allowing the partitioning of compounds into every organ. However, the disadvantage of using blood is the invasiveness of sample collection, making it less suitable for specific populations, such as children and infants. In contrast, urine can be non-invasively collected as a 24-hour composite or, more commonly, as a spot sample. The main disadvantage of collecting urine is the varying degrees of dilution due to an individual's hydration status, albeit adjustment methods like normalization to creatinine, specific gravity, and osmolarity can circumvent this issue (Esteban and Castaño, 2009). Although HBM schemes include many such chemicals, there are groups of potentially harmful compounds yet to be included. These include, for example, pyrrolidones, isocyanates, benzotriazoles, benzothiazoles, ultra-violet (UV) filters, antimicrobials (MCI, MI), fragrances, and non-phthalate plasticizers where evidence for their adverse health effects is mounting. Due to the lack of analytical methodologies reported in the literature for the compounds mentioned above (Salthammer, 2020 and Kolossa-Gehring, 2017), we reviewed the analytical methods used for their quantification in human urine to compile the data that could be used for future HBM studies.

2. Groups of under-researched CECs

Isocyanates are raw materials used to manufacture polyurethane foams, adhesives and paints, which are products commonly used in everyday life (Lépine et al., 2020). The most common isocyanates are MDI, 4TDI, 6TDI, NDI, PPDI and HDI. Once in the body, isocyanates are hydrolyzed to their corresponding amines, namely MDA, 4TDA, 6TDA, NDA, PPDA, HDA, which are used as biomarkers of exposure (BoE). Due to their high reactivity, they can form protein adducts that, after protein degradation, are excreted in urine as conjugates, particularly with glutathione (GSH) (Sabbioni et al., 2010). Several acute and chronic effects have been reported connected to isocyanate exposure, such as irritation of eyes, skin, mucous membranes and respiratory system, asthma and reduced lung function (Lépine et al., 2020). A study on the long-term effects of one-time high-dose exposure reported the occurrence of

diseases such as diabetes, hypertension, cancer, reproductive outcomes and respiratory/orthopedic/general morbidity in the exposed population (Ganguly et al., 2018).

Benzotriazoles (BTRs) are high production volume chemicals used as corrosion inhibitors and UV stabilizers for various photography and liquid coolant applications. **Benzothiazoles** (BTHs) are also used as corrosion inhibition, industrial biocides, and vulcanisation accelerators in rubber production (Asimakopoulos et al., 2013b; Maceira et al., 2018; Naccarato et al., 2014). In the human body, they are biotransformed into more hydrophilic metabolites that are excreted as glucuronidated and sulphated compounds (Asimakopoulos et al., 2013b). Although data on their toxicological effects are limited, available evidence suggests that they can adversely affect the liver and kidney (Naccarato et al., 2014), while BTH is a suspected carcinogen (X. Li et al., 2018).

Pyrrolidones (N-methylpyrrolidone-NMP and N-ethylpyrrolidone-NEP) are aprotic polar solvents used in the petrochemical and microelectronics industries for producing dyes, biocides, and cosmetics (Schmied-Tobies et al., 2021). Due to their volatility, the main route of exposure is inhalation, although ingestion and dermal absorption have also been reported (Suzuki et al., 2009). Their main metabolites are 5-HNMP, 2-NMSI, 5-HNEP, 2-HESI, which are used as BoE. Pyrrolidones are skin, eye and respiratory tract irritants and exhibit reproductive effects (Substance Information - ECHA).

Since phthalate-based plasticizers have been under criticism due to their adverse health effects, **phthalate alternatives** have entered the market with a constantly increasing share (Bui et al., 2016). Phthalate alternatives find applications as softeners in plastics, additives in personal care products, and industrial materials. Although many have been well-studied, e.g., diisononyl ester of cyclohexane-1,2-dicarboxylic acid (DINCH), others such as tri-(2-ethylhexyl) trimellitate (TEHTM), di-2-ethylhexyl adipate (DEHA), di-n-butyl adipate (DnBA), and di-(2-ethylhexyl) terephthalate (DEHTP) were studied less. Most of these are alternatives for the traditional phthalate plasticizer di-2-ethylhexyl phthalate (DEHP), while DnBA is an alternative for di-n-butyl phthalate (DnBP). The toxicity and migration rates of these alternatives are significantly lower than of traditional phthalates; however, leaching into the surrounding media still occurs with DnBA being restricted to low temperature applications due to high migration rates. This compound is often used in high concentrations in personal care products (PCPs), solvents, and cleaning products. It exhibits very low toxicity to humans with a set no-observed-effect level (NOEL) of 300 mg/kg bw (Ringbeck et al., 2020); however, estimating human exposure to DnBA is a challenge due to the lack of exposure data, intake rate estimates and data on health effects. For instance, there is currently no data on the potential endocrine disruptive capacity of DnBA (Bui et al., 2016). Monitoring is therefore needed as a preventive measure due to the increasing exposure of the population (Bui et al., 2016).

DEHA is a high production volume adipate, and human exposure is continuously increasing (Bui et al., 2016). Animal studies show evidence of adverse health outcomes in rats following exposure to high doses of DEHA (Silva et al., 2013). DEHTP is a structural isomer of DEHP that has not shown any of the reported adverse health effects of DEHP with a reported NOEL of 500 mg/kg bw/d. It is produced in quantities similar to DINCH in the European Union (>10 000 t/year). It has been frequently detected in household dust and indoor air, and humans are

widely exposed to this compound (Bui et al., 2016). TEHTM is a tri-ester structurally similar to DEHP but has lower toxicity. It is, however, classified as a GHS Category 2 reproductive toxicant, a Category 3 specific target organ toxicant, and is relatively environmentally persistent (half-life 16-60 days). As such, TEHTM has a set derived no effect level (DNEL) of 1.13 mg/kg bw/d within the ECHA registration (Höllerer et al., 2018a).

These phthalate alternatives fall under the high molecular weight compounds (> 6 C atoms in the side chain) and undergo similar metabolic pathways in humans. The tri-ester compound TEHTM can form three diester isomers during hydrolysis, followed by further hydrolysis to monoesters. TEHTM, DEHA, DnBA, and DEHTP also undergo biotransformation to secondary metabolites containing hydroxy, oxo, or carboxy groups on the side chain. We included them in the list of phthalate alternatives due to their expected increasing market share, limited exposure data, and limited toxicological data.

Organic UV filters are a group of compounds capable of filtering UV radiation due to their degree of conjugation and are used in personal care products (PCPs) such as sunscreens and other cosmetic products to protect the skin. They can be roughly classified into benzophenone (BP), cinnamate, crylene, camphor, and salicylate derivatives. The latter consists of other organic UV filters that include compounds that do not fall into the abovementioned groups (Huang et al., 2021). For example, although organic UV filters are highly studied in HBM, an exception is the camphor derivative 4-methylbenzylidene camphor (MBC). MBC is currently detected at relatively low concentrations (<LOD - 13.93 ng/mL) and frequencies, with most studies focusing on aquatic systems, where it is known to be highly lipophilic and accumulates in the fatty tissue of biota (Ao et al., 2018b; Huang et al., 2021). The metabolic pathway of MBC in humans has been studied after dermal application. 3-(4′-carboxybenzylidene)camphor (CBC) and four isomers of 3-(4′-carboxybenzylidene)hydroxycamphor (CBC-OH) were identified as the main metabolites of MBC and are the main BoE (León-González et al., 2013).

Human exposure to MBC can result from either direct dermal application or ingestion of contaminated food and drinks, for instance, fish consumption or tap water (Li et al., 2019). In terms of risk health concerns associated with MBC have increased due to its suspected endocrine activity, while in vitro studies suggest that MBC can enhance the process of apoptosis and hence directly affect nerve cells (Broniowska et al., 2016). Other studies report adverse effects on the immune system (Ao et al., 2018a), and it has been suggested that MBC facilitates the migration of human breast cancer cells (Alamer and Darbre, 2018). Studies investigating the toxicological profile of MCB report mild endocrine disruptive effects on the thyroid gland and the potential to delay tissue growth and placenta formation during early pregnancy (HBM4EU, 2020)

Exposure to **fragrances** is widespread as they find applications in personal care products and scented non-cosmetic products. The main pathways of exposure are inhalation and dermal absorption. The most common health endpoint of fragrances is an allergic response, with some studies reporting endocrine disruptive effects (Dodson et al., 2012). Musk xylene (MX) and musk ketone (MK) are nitro musks, which have now been replaced due to their estrogenic activity and neural toxicity (Taylor et al., 2014) by polycyclic musks, such as

celestolide (ADBI), traseolide (ATII), galaxolide (HHCB), and tonalide (AHTN). MX is classified under REACH as a chemical of high concern, and a warning about its use has been placed on MK (Taylor et al., 2014). Polycyclic musks are under scrutiny because of their persistence and adverse health effects, such as estrogenic activity. Along with musks, other synthetic fragrances of concern include Lysmeral with the known metabolites lysmerol, lysmerylic acid, hydroxylysmerylic acid, TBHA, and TBBA (Scherer et al., 2017) and 7-hydroxycitronellal (7-HC) with the main metabolite 7-hydroxycitronellic acid (7-HCA). Known health endpoints of 7-HC are skin irritation and skin sensitization (Stoeckelhuber et al., 2018), whereas endocrine disruptive effects are suspected (Scherer et al., 2021).

Frequently used antimicrobials in personal care products are **isothiazole and its derivatives**. The most extensively used among them are based on isothiazolinone, many of which are restricted for personal care product (PCP) applications. The knowledge of their potential adverse health effects has been emerging only recently. Methylchloroisothiazolinone and methylisothiazolinone **(MCI/MI)** are often used in combination with each other to enhance the overall antimicrobial effect and find application in preserving solutions in PCPs and industrial products. Human exposure is mainly the result of dermal applications. Both MCI and MI undergo rapid biotransformation in the human body with a mean half-life of 3.6 h. The main biomarker of MCI/MI exposure is N-methylmalonamic acid (NMMA), whereas acetylamino([3-(methylamino)-1-(methylthio)-3-oxopropyl]thio)acetic acid (M-21) is a minor metabolite with an excretion fraction of between 10 % and 23 % in humans (Schettgen et al., 2021b). MCI/MI are broad-spectrum antimicrobials with high efficiency at trace concentrations (Silva et al., 2020). Over recent years, concerns over their sensitization potential and allergic dermatitis, and other adverse health outcomes such as cytotoxicity have been rising, leading to an interest in monitoring these compounds (Castanedo-Tardana and Zug, 2013; Park and Seong, 2020). Of the two, MCI has a higher potential toward the health endpoints mentioned above (Silva et al., 2020).

2. Occurrence of selected CECs in urine

Most of the publications included in this review verified their respective method by implementation in HBM studies of varying scales, thereby confirming the occurrence of CECs in human urine (Table 1). Most of the studied CECs (fragrances, BTR and BTH, antimicrobials, pyrrolidones, and diisocyanates) are frequently detected in urine, whereas the UV filter MBC was rarely detected and, if detected at all, it was in trace amounts (Ao et al., 2018b; Frederiksen et al., 2017; Leng and Gries, 2017). The included non-phthalate plasticizers, on the other hand, are detected often and in relatively high concentrations, with a maximum concentration of 1165 ng/mL of OH-MEHTP (Bastiaensen et al., 2020). Therefore, it is clear that the presumably non-exposed general population is frequently exposed to most of the included CECs, which highlights the need to include these compounds in routing HBM studies.

Table 1: Occurrence of CECs in urine

Compound group	Analyte	Median (ng/ml)	Mean (ng/mL)	Range (ng/mL)	population	Reference
Fragrances	ADBI			n.d.		

	AHMI			1.38 - 2.65		(Chen et al., 2018a)
	ATII			n.d.		
	HHCB			0.48 - 2.09		
	AHTN			0.14 - 0.57		
	MX			n.d.		
	MK			n.d. - 1.49		
	Lysmerol	0.8	2.25 ±6.14	0.1 - 38.7		(Pluym et al., 2016)*
	Lysmerylic acid	0.8	1.25 ± 1.59	0.02 - 9.2		
	TBBA	19.6	28.7 ± 34.3	5.3 - 188.9		
	OH-lysmerylic acid	0.06	0.76 ± 1.46	0.01 - 8.2		
	7-HCA	15.1	34.4 ± 85.2	4.9 - 557.0		(Stoeckelhuber et al., 2017)*
BTRs & BTHs	1H-BTR		1.23	<LLOQ - 5.67	males	(Asimakopoulos et al., 2013a)*
			1.63	0.85–3.16	females	
	1-OH-BTR		-	-	males	
			-	-	females	
	TTR		0.70	<LLOQ - 2.14	males	
			0.72	<LLOQ–3.37	females	
	XTR		0.39	<LLOQ - 2.39	males	
			-	3.69	females	
	5-Cl-1H-BTR		-	-	males	
			-	-	females	
	BTH		3.51	<LLOQ - 16.7	males	
			5.59	<LLOQ–34.3	females	
	2-OH-BTH		1.55	<LLOQ	males	
			2.40	<LLOQ–6.00	females	
	2-Me-S-BTH		-	-	males	
			-	-	females	
	2-amino-BTH		0.11	<LLOQ - 0.30	males	
			0.17	<LLOQ–0.25	females	
	2-SCNMeS-BTH		-	-	males	
			-	-	females	
	MTB (total)	<1	<1	<1 - 10.8	Non-exposed	(Gries et al., 2015)
		4527	3958	567 - 6210	exposed	
	MTB (free)	70	69	<1–137	exposed	
	1-OH-BTR	0.78	1.13	<LOQ-1.13		(Li et al., 2017)
	1H-BTR	2.45	2.4	<LOQ-4.12		
	TTR	2.38	2.46	<LOQ-4.79		
	5-Cl-BTR	n.d.	n.d.	n.d.		
	XTR	2.38	2.46	<LOQ-4.79		
	BTH	-	-	1.74		
	2-OH-BTH	-	-	0.38		
	2-amino-BTH	0.26	0.28	<LOQ-0.68		
	2-MeS-BTH	n.d.	n.d.	n.d.		
	2-SCNMeS-BTH	n.d.	n.d.	n.d.		
	1H-BTR	1.09	1.31	<LOD - 3.86	Pregnant women	(X. Li et al., 2018)
	1-OH-BTR	0.21	0.25	<LOD - 1.39		
	TTR	0.22	0.27	<LOD - 0.90		
	5-Cl-1H-BTR	0.015	0.023	<LOD - 0.096		
	BTH	1.13	1.49	0.016 - 3.91		
	2-OH-BTH	<LOD	<LOD	<LOD		
	1-H-BTR		0.10	? - 36		(Zhou et al., 2018)#
	1-OH-BTR		0.32	? - 330		
	XTR		0.04	? - 18		

	TTR		0.036	? - 6.1		
	BTH		1.5	? - 53		
	2-OH-BTH		0.28	? - 160		
	2-MeS-BTH		0.30	? - 15		
	2-NH_2-BTH		0.017	? - 8.3		
	2-SCNMeS-BTH		<LOD	? - 0.66		
	5-Cl-H-BTR		<LOD	? - 0.53		
Antimicrobials	NMMA	3.6		? - 7.5	Non-exposed males	(Schettgen et al., 2017)*
		2.9		? - 11.8	Non-exposed females	
	M-12	0.57*		0.20–1.79*	Non-exposed	(Schettgen et al., 2021b)
	NMMA	2.7		0.9–9.6		
Diisocyanates	MDA			0.1 - 0.2*		(Henriks-Eckerman et al., 2015)
	MDA		3.30	2.0 - 4.0	Factory workers	(Mirmohammadi et al., 2013)*
	HDA			0.06 - 5.96		(Robbins et al., 2018)
	TAHI			<LOD - 1.99		
	MDA			<LOD - 15.18		(Sun et al., 2018)
	6TDA			<LOD - 8.46		
	4TDA			<LOD - 2.35		
Pyrrolidones	5-HNMP	0.39			exposed	(Haufroid et al., 2014)*
	2-HMSI	0.56				
	5-HNMP	0.09			Non-exposed	
	2-HMSI	0.23				
	5-HNMP	69.5		? - 620.0	Non-exposed	(Schindler et al., 2012)
	2-HMSI	63.5		? - 256.2		
	5-HNEP	<15.0		? - 769.3		
	2-HESI	<5.0		? - 310.8		
UV filters	MBC	2.46	0.65	<LOD - 13.93		(Ao et al., 2018b)*
	MBC	-		<LOD	Children/adolescents	(Frederiksen et al., 2017)
	CBC			<LOD	Non-exposed	(Leng and Gries, 2017)
	CBC-OH			<LOD		
Non-phthalate plasticizers	1-MEHTM	0.10	0.52	<LOQ - 13.1		(Pinguet et al., 2019)
	2-MEHTM	0.47	25.6	<LOQ - 925		
	4-MEHTM	0.04	0.19	<LOQ - 4.14		
	1-MEHTM		455			(Höllerer et al., 2018b)
	2-MEHTM		1629			
	4-MEHTM		16.0			
	5OH-1-MEHTM		308			
	2OH-2-MEHTM		256			
	5oxo-1-MEHTM		81.4			
	5oxo-2-MEHTM		60.7			

5cx-1-MEPTM		338			
5cx-2-MEPTM		46.6			
2cx-2-MMHTM		<LOD			
2cx-1-MMHTM		14.6			
1-DEHTM	<LOQ		<LOQ - 0.89		(Bastiaensen et al., 2020)
2-DEHTM	<LOQ		<LOQ - 0.91		
MEHA	0.08	0.38	<LOQ - 7.92		(Pinguet et al., 2019)
5OH-MEHA	<LOQ		<LOQ - 0.07	Pregnant women	(Nehring et al., 2019)
5oxo-MEHA	<LOQ		<LOQ - 0.13		
5cx-MEHA	<LOQ		<LOQ - 0.24		
5OH-MEHA	<LOQ		<LOQ	adults	
5oxo-MEHA	<LOQ		<LOQ		
5cx-MEHA	<LOQ		<LOQ - 0.24		
MEHA	0.02		0.02 - 3.18		(Bastiaensen et al., 2020)
OH-MEHA	0.92		0.07 - 2.94		
MnBA	<LOQ		? - 0.18		(Ringbeck et al., 2020)
3OH-MnBA	<LOQ		<LOQ		
3cx-MnBA	2.54		? - 78.3		
MEHTP	<LOQ	0.21	<LOQ - 9.90		(Pinguet et al., 2019)
MEHTP	0.02		0.02 - 50.8		(Bastiaensen et al., 2020)
OH-MEHTP	0.51		0.07 - 1165		

**creatinine adjustment*

#SG adjustment

3. Sample preparation and analytical methods for the determination of CECs in urine

Once in the human body, many compounds undergo phase I and II metabolism involving esterases, lipases, and enzymes of the cytochrome P450 family (phase I). Phase II metabolism occurs mainly in the liver, although these enzymes are expressed to a lower degree also in other tissues such as kidneys and intestine. The main phase II reactions are glucuronidation, sulfation and glutathione conjugation (James, 2021). In order to measure total CECs (free and conjugated), enzymatic deconjugation is usually applied, using enzymes like β-glucuronidase/arylsulfatase or acid/base hydrolysis (Glauser et al., 2014).

Sample clean-up and extraction procedures are needed to remove unwanted matrix constituents, reduce matrix effects and eliminate interferences. Furthermore, during extraction, the sample is usually concentrated to detect low levels of BoEs at concentrations significant for HBM. Solid phase extraction (SPE) is a commonly used method for preparing biological samples (Bocato et al., 2019). Compared to LLE, it offers higher selectivity due to the availability of variously functionalized sorbents for the retention of analytes of choice with a wide range of physico-chemical properties. The availability of SPE in 96-well format offers an adaptation to high-throughput sample preparation workflows, which is beneficial in HBM since large cohorts are analyzed. However, it requires extensive parameter optimization.

Along with common extraction methods, other more specific ones were used, such as solid-supported liquid-liquid extraction (SSLE) on microporous diatomite cartridges to extract fragrances (Liu et al., 2015), ultrasound-assisted emulsification microextraction (USAEME) with tetrachloromethane for the extraction of polycyclic and nitro musks (Chen et al., 2018a) or solid-phase microextraction (SPME) for extraction of BTRs and BTHs using polyacrylate fibre (Naccarato et al., 2014).

After extraction that often also includes preconcentration step, the most commonly applied separation methods include LC or GC, but other approaches have been used (see below). Due to the high degree of molecular functionalization and suitable polarity, most analysts use reversed-phase liquid chromatography (LC). High and ultra-performance LC (HPLC and UPLC) do not require high volatility and thermal stability and are suitable for analysing polar matrices, such as urine. For volatile fragrances, gas chromatography (GC) is the method of choice. Gas chromatography can also separate semi and non-volatile compounds by using an appropriate derivatization step, such as silylation.

For detection, LC and GC are hyphenated with mass spectrometry (MS) in most cases. MS enables increased sensitivity and selectivity, particularly when using tandem MS (MS2). Compared to MS, which is performed by following single compound-specific ion (single ion monitoring, SIM), MS2 follows multiple reactions (multiple reaction monitoring, MRM), which offers higher selectivity and specificity, which is especially important in the analysis of complex samples, such as urine. This need for specificity is reflected in the methods reported here, as CECs were detected almost exclusively in MRM mode. Besides MS, other approaches are suitable for detecting certain compounds. In one case, HPLC with fluorescence detection was used to detect BoEs to diisocyanates (Sun et al., 2018). This required fluorescence derivatization using 4-(1-pyrene)butanoyl chloride (PBC) (Sun et al., 2018). Compared to the other methods using GC NCI-SIM (Mirmohammadi et al., 2013), UPLC with MRM detection (Bhandari et al., 2016; Lépine et al., 2019a, 2020), and nano-UPLC with MRM detection (Robbins et al., 2018) described for the detection of MDA, this approach yields a ten times lower LOQ (0.001 ng/mL), while not requiring any complex sample preparation procedures (offline-SPE).

3.1 Fragrances

For most fragrances, no deconjugation was performed before sample extraction and clean-up. The majority of nitro and polycyclic musks lack the functional groups for conjugation, whereas, for lysmerol, deconjugation was achieved enzymatically using β-glucuronidase.

The reviewed methods for extracting fragrances from urine adopt an LLE approach using between 0.8 and 1 mL of sample volume. Due to the inherent volatility of fragrance compounds, they were mainly analysed using GC methods, except for lysmeral and 7-HCA, where both GC and LC-based methods have been applied.

For lysmeral and 7-HCA, a derivatization step is required to improve their volatility, whereas polycyclic and nitro musks are sufficiently volatile to be analyzed *via* GC with no further adaptations. The two methods for detecting polycyclic musks in urine (Chen et al., 2018a; Liu et al., 2015) yielded comparable results in terms of LOQ. However, regarding LOQs, method by Liu et al. (2015) using a solid-supported liquid-liquid extraction (SSLE)

316 approach yields slightly better results (Table 2) compared to using ultrasound-assisted
317 emulsification microextraction (USAEME) as suggested by Chen et al. (2018).
318 To determine lysmeral and 7-HC BoEs, two UPLC-based methods using ESI-MRM
319 detection are presented (Pluym et al., 2016; Stoeckelhuber et al., 2017). Separation was
320 achieved on a 100 mm C18 analytical column with a binary-solvent mixture. To detect BoEs
321 of lysmeral, MeOH and an aqueous solution of 5mM ammonium acetate with 0.025%
322 ammonium hydroxide at a pH of 9.2 were used as mobile phases (Pluym et al., 2016). The
323 method for the determination of 7-HCA included 0.1 % FA in ACN and aqueous 0.1 % FA.
324 Both methods report low LOQs for the studied compounds (≤ 0.5 ng/mL).
325
326 3.2 **Benzotriazoles and benzothiazoles**
327
328 To determine benzotriazoles and benzothiazoles, they are first deconjugated using β-
329 glucuronidase and then extracted using LLE (Asimakopoulos et al., 2013a; Li et al., 2017; Zhou
330 et al., 2018) or SPE (Asimakopoulos et al., 2013a; C. Li et al., 2018) and online-SPE (Gries et
331 al., 2015). Online-SPE requires the lowest sample volume (0.5 mL), whereas the presented LLE
332 methods require between 0.6 and 1 mL of sample and the SPE methods between 1.33 and 2
333 mL. The most commonly used sorbent for SPE was mixed-mode Oasis HLB (Waters). The
334 separation method of choice is LC. The SPME method by Naccarato et al. (2014) yields the
335 highest LOQs (0.4 - 4.9 ng/mL); however, it is also the only GC approach among the reported
336 methods. Therefore, the high LOQ cannot be attributed to the sample preparation procedure
337 alone. The analytes were separated using a 30m TR-5MS column and detected by MS in MRM
338 mode. Although detecting BTRs and BTHs using GC is possible, the more complex sample
339 preparation procedure and the relatively high LOQs compared to the LC-based methods suggest
340 that GC separation is less suitable for these compounds. The online-SPE method included only
341 one compound, 2SH-BTH, and achieved comparable LOQ and has the advantage of increased
342 efficiency (time) and sample handling compared to offline approaches.
343 For BTRs and BTHs, four out of five methods (see Table 2) used an LC-ESI-MS2 but
344 with different columns. Asimakopoulos et al. (2013a) used a 150 mm Zorbax SB aq column,
345 whereas Zhou et al. (2018) and Li et al. (2017) used a 100 mm C18 column, Gries et al. (2015)
346 used a 50 mm C8, and Li et al., (2018) used a BEH shield RP18 column, and from that it is
347 evident that C18-based columns are the most commonly used to achieve the optimal separation.
348 All presented methods use a binary mixture of FA in water in a concentration range of 0.01 -
349 1% FA and all achieved comparably low LOQs (Table 2). Due to the similarity in the separation
350 and detection methods, it is most likely that slight differences in achieved LOQs are attributed
351 to the differences in sample preparation.
352
353 3.3 **Antimicrobials**
354
355 Only two methods describe the determination of BoE of MI/MCI exposure in urine
356 using both LLE (Schettgen et al., 2017) and SPE (Schettgen et al., 2021b). The LLE approach
357 includes an additional derivatization step with pentafluorobenzyl bromide to increase
358 lipophilicity. This step is omitted in the SPE procedure (Schettgen et al., 2017). However, the
359 SPE method requires 0.5 mL of urine, whereas LLE yields better results (LOQ 0.5 ng/mL for

NMMA) with 0.1 mL of sample, which is particularly important when sample volume is limited. The SPE method achieves an equally low LOQ (0.2 ng/mL); however, the analyte, in this case, is a different BoE, a mercapturic acid metabolite of MI/MCI, named M12, which means that a direct comparison of the methods is not possible. However, both methods achieve low LOQs, require a small sample volume, and do not require complex procedures or installations in the laboratory.

To determine MI/MCI BoE M-12, Schettgen et al. (2021) used a LC-ESI-MRM method achieving separation on a 150 mm C18 column using a tertiary solvent system of water (pH 2.5, adjusted with FA), MeOH and ACN. Together with SPE, the authors obtained LOQ of 0.2 ng/mL, which is suitable for HBM studies (Table 2). Schettgen et al. (2017) presented a GC-based method with MRM detection for MI/MCI BoE NMMA based on a HP-5-MS 60m column. After LLE and derivatization with PFBBr, the method achieved a LOQ of 0.5 ng/mL, suitable for HBM studies (Table 2).

3.4 Diisocyanates

In the case of diisocyanates, deconjugation is achieved using mineral acid hydrolysis. LLE is the most commonly applied method for the extraction of diisocyanates (Henriks-Eckerman et al., 2015; Lépine et al., 2019a; Mirmohammadi et al., 2013; Robbins et al., 2018), whereas SPE is likewise used, although less often (Bhandari et al., 2016; Lépine et al., 2020; Sun et al., 2018) with cation exchange sorbents being the most used. Large differences in the required sample volume can be observed, as SPE methods required volume in a microliter range while LLE required larger volumes (1 and 2 mL). The exception is the method of Lépine et al. (2019a), where the authors use LLE with a volume of 250 µL. Only one method used prior derivatization step using acetic anhydride (Lépine et al., 2019b). (Lépine et al., 2019). By far, the lowest LOQ (0.001 ng/mL for MDA) was achieved by Sun et al. (2018), applied SPE followed by derivatization with 4-(1-pyrene)butanoyl chloride. There is no difference in achieved LOQs by LLE and SPE, albeit micro-SPE using a 96-well plate is more feasible for implementation in large-scale HBM schemes.

Most of the included LC methods involve UPLC, followed by HPLC and nano-UPLC with C18 phase the most commonly used for separation. Three methods apply ESI-MRM detection (Lépine et al., 2019a, 2020; Robbins et al., 2018) whereas Sun et al. (2018) applied FLD detection and Bhandari et al. (2016) used APCI(+)MRM detection. Three methods describe separation on a 100mm or 150mm C18 column (Bhandari et al., 2016; Robbins et al., 2018; Sun et al., 2018), whereas two studies used a 50 mm TSS T3 column (Lépine et al., 2019a, 2020). Sun et al. (2018) achieved the lowest LOQ (0.001 ng/mL) for MDA but did require a derivatization step, which is omitted by Bhandari et al. (2016) while achieving a bit higher, but equally satisfactory low LOQ as well (0.03 ng/mL) for MDA (Table 2). Other LC-based methods either require a derivatization step while achieving higher LOQs (Lépine et al., 2020) or higher LOQ while omitting the derivatization (Lépine et al., 2019a). The method presented by Robbins et al. (2018) does not include MDA but achieves a LOQ of 0.03 ng/mL for TAHI.

Henriks-Eckerman et al and Mirmohammadi et al.used GC to determine MDA. Mirmohammadi et al. (2013) only briefly describe their method using NCI-SIM detection and

separation on a BP-5 column and do not report LOQ, whereas Henriks-Eckerman et al. (2015) achieved a LOQ of 0.1 ng/mL using a 25 m HP-5 column for separation and NCI-MS-SIM detection. Despite being suitable for HBM, the latter method requires a higher sample volume (1 mL) and requires derivatization with HFBA, leading to higher LOQs (see Table 2) than the LC-based methods.

3.5 **Pyrrolidones**

As a pre-treatment step, enzymatic deconjugation using β-glucuronidase is performed. The samples were either minimally prepared with the dilute-and-shoot approach (Bhandari et al., 2019; Haufroid et al., 2014; Suzuki et al., 2009) or extracted with SPE (Schindler et al., 2012). Based on the LOD/LOQ, required sample volume, and overall sample preparation time, the dilute-and-shoot approach seems to yield better results. Only one study (Schindler et al., 2012) included a derivatization step in their sample preparation to adapt the compounds for GC analysis. Applying derivatization, however, did not lead to lower LOQ.

Two methods are presented for the determination of NMP BoEs using LC-ESI-MRM. Haufroid et al. (2014) and Suzuki et al. (2009) achieved compound separation on a 100 mm C18 column using aqueous 0.1% FA and ACN as mobile phase. The second method separated compounds on a 100 mm HSS-PFP column using 5 mM aqueous ammonium formate and MeOH as mobile phase (Bhandari et al., 2019).

Schindler et al. (2012) present a method for determining NMP and NEP using cooled injection GC and separation on a 60 m DB-35 MS column. Their method achieves LOQs (5 - 20 ng/mL) significantly higher than those achieved with LC-based methods (Bhandari et al., 2019; Haufroid et al., 2014; Suzuki et al., 2009). The LOQs obtained in this method might be too high for HBM, where the analytes are often present in trace concentrations (Table 1) and suggest that LC is a better approach for determining these compounds.

3.6 Methylbenzylidene camphor (MBC)

Following enzymatic deconjugation, the most commonly applied sample preparation method for MBC is offline SPE (Ao et al., 2018b; León-González et al., 2013), followed by online SPE (Frederiksen et al., 2017; Krause et al., 2017; Leng and Gries, 2017). Janjua et al. (2008) used urine lyophilization with reconstitution before analysis. Among those methods, online SPE requires the lowest sample volume (500 µL) for analysis, whereas other methods require a minimum of 2mL. Additionally, the method presented by Leng and Gries (2017) yields the lowest LOQ (0.15 ng/mL for CBC), whereas a LOQ of 0.9 ng/mL (Frederiksen et al., 2017; Krause et al., 2017) is the second-lowest achieved showing that online-SPE is the most suitable sample preparation method for determining these compounds. Only one study (Ao et al., 2018b) included a derivatization step necessary for the instrumental analysis (GC); however, this does not increase the LOQ.

Four methods describe the detection of MBC and BoEs of MBC using LC and most commonly C18 phase. While most studies achieve separation on a 50 - 250 mm C18 column (Janjua et al., 2008; Leng and Gries, 2017; León-González et al., 2013), Frederiksen et al.

(2017) and Krause et al. (2017) used a 50 mm TurboFlow Cyclone P and a 50 mm hypersil Gold aQ column. Classical ESI-MRM detection is used by León-González et al. (2013) and Leng and Gries (2017), whereas Janjua et al. (2008) applied UV detection, specifically SPD-6 UVD, whereas Frederiksen et al. (2017) and Krause et al. (2017) chose APCI-MRM. Different mobile phase compositions are presented in the included studies. A binary mixture of aqueous 0.1 % FA and 0.1 % FA in ACN:MeOH (1:1) is used by León-González et al., whereas Frederiksen et al. (2017) and Krause et al. (2017) use a tertiary system without specifying the solvents. Isocratic elution with MeOH/water (88:12) is used in one study (Janjua et al., 2008), whereas Leng and Gries (2017) do not specify their mobile phases.

Krause et al. (2017) achieved by far the lowest LOQ for MBC (0.87 ng/mL), whereas the LOQs of the other studies range between 2.9 and 6 ng/mL for the same compound. Leng and Gries (2017) also achieved low LOQs, but for different BoEs: CBC (0.15 ng/mL) and CBC-OH (0.3 ng/mL), which makes a comparison with other approaches difficult. Since Leng and Gries's (2017) method involves time-saving online extraction with minimal sample handling and no derivatization, this method seems to be the most efficient one for the determination of MBC. Ao et al. (2018a) method for detecting MBC involves derivatisation with BSTFA-TMCS followed by GC (30 m with TSQ Quantum XLS Column), with MS (MRM) detection. The obtained LOQ (3.5 ng/mL) are higher than that (0.9 ng/mL) obtained by Frederiksen et al. (2017) and Krause et al. (2017), making GC slightly less advantageous.

3.7 Non-phthalate plasticizers

Following enzymatic deconjugation, extraction methods for BoEs of **TEHTM** all involve SPE. The methods of Höllerer et al. (2018b) and Pinguet et al. (2019) include online-SPE, whereas Bastiaensen et al. (2020) and Been et al. (2019) describe an offline version. Only Pinguet et al. (2019) use 50 µL of the sample, whereas the other methods require 1 mL. In terms of LOQs, Pinguet et al.'s (2019) method gave the best results for mono-esters. In their method, Bastiaensen et al. (2020) and Been et al. (2019) determined di-esters at trace concentrations (LOQ 0.1 ng/mL). Although Höllerer et al.'s (2018b) method had the highest LOQ, they also had the most analytes (n = 11) of interest in HBM studies. From this work, it is clear that online-SPE achieves the best results for determining TEHTM metabolites, although the inclusion of too many compounds can increase the LOQ and researchers have to make a compromise between the costs and benefits of each method based on their research question.

Three online-SPE methods and one offline SPE method are published for determining **DEHA** BoEs. None of the presented methods requires a derivatization step for LC analysis. The offline procedure (Bastiaensen et al., 2020; Been et al., 2019) requires a much higher sample volume compared to the online-SPE methods (1mL vs 50-300µL); however, it yields the highest LOQ (0.15 ng/mL for MEHA and OH-MEHA). The online-SPE methods by Nehring et al. (2019) and Pinguet et al. (2019) yield comparable results regarding LOQs. However, Pinguet et al. (2019) determined only MEHA, whereas Nehring et al. (2019) included OH-MEHA, oxo-MEHA, and cx-MEHA. Ringbeck et al. (2020) describe a method for the determination of DnBA BoEs (MnBA, 3OH-MnBA, and cx-MnPrA) using online-SPE and report low LOQs (0.05 - 0.5 ng/mL). From this, it is evident that all of the presented methods are suitable for implementation in HBM studies, although Nehring et al.'s (2019) method has

advantages in terms of preparation procedure (online), LOQ (comparable with others), and the number of analytes included (n=3).

For determining terephthalates, namely, **DEHTP** BoEs, an online-SPE based method (Pinguet et al., 2019) and an offline-SPE based method (Bastiaensen et al., 2020; Been et al., 2019) were reported. The offline method requires a much larger sample (1mL) than the online method (50µL), and none of the procedures requires derivatization. Although there are significant differences in sample preparation, the online method requires minimal sample handling compared to offline SPE. The benefits of this method are visible in terms of achieving lower LOQs; the online-SPE-based method achieved LOQ for MEHTP of 0.018 ng/mL, whereas the offline method achieved a LOQ of 0.1 ng/mL for the same analyte.

TEHTM BoEs were determined exclusively with LC-based methods using ESI-MRM detection (Bastiaensen et al., 2020; Been et al., 2019; Höllerer et al., 2018b; Pinguet et al., 2019). While two of the methods chose a more traditional HPLC approach to determine BoEs of TEHTM (Bastiaensen et al., 2020; Been et al., 2019; Höllerer et al., 2018b), Pinguet et al. (2019) utilized and UFLC instrumental setup for analysis.

Additionally, differences between the methods lie in the choice of the analytical column and the mobile phase. Biphenyl columns are used in two methods (Bastiaensen et al., 2020; Been et al., 2019; Höllerer et al., 2018b), whereas Pinguet et al. (2019) utilize a phenyl hexyl column with a binary solvent system (0.1 % AA/water, 0.1 % AA / ACN). The other studies also report using a binary system using a different solvent. Höllerer et al. (2018b) achieved good separation using 0.1 % FA in water and 0.1% FA in ACN, whereas (Bastiaensen et al., 2020; Been et al., 2019) used 0.1 % AA in water and ACN. Among the methods, UFLC gave the best results in terms of LOQ (0.01 - 0.04 ng/mL) and required sample volume (50 µL). Bastiaensen et al. (2020) and Been et al. (2019) methods achieved promising results as well (LOQ 0.1 ng/mL), although they targeted different BoEs.

For **adipates**, four LC-based methods are published, of which three target DEHA and one DnBA. All utilize LC-ESI-MRM, but (Pinguet et al., 2019) used ultra-fast liquid chromatography UFLC, whereas the other studies chose HPLC (Bastiaensen et al., 2020; Been et al., 2019; Nehring et al., 2019; Ringbeck et al., 2020). Three different types of columns were used, namely biphenyl (Bastiaensen et al., 2020; Been et al., 2019), phenyl hexyl (Pinguet et al., 2019), phenyl-X (Nehring et al., 2019), and C18 (Ringbeck et al., 2020). In all cases, the mobile consisted of a binary system of aqueous AA and AA in ACN, but Nehring et al. (2019) and Ringbeck et al. (2020) use 0.05 % AA, whereas the other studies use 0.1 % AA (Bastiaensen et al., 2020; Been et al., 2019; Pinguet et al., 2019). They all achieve comparable results for DEHA BoEs (LOQs 0.04 - 0.15) and are suitable for HBM (Table 1). The lowest LOQ, however, was achieved with only one analyte (MEHA) in the method (Pinguet et al., 2019), which makes the method presented by Nehring et al. (2019) more appealing for implementation in HBM. The method for the determination of DnBA BoEs achieved low LOQs (0.05 - 0.5 ng/mL), which is suitable for HBM as well (Table 1) (Ringbeck et al., 2020).

Two studies determined **DEHTP** BoEs. Pinguet et al. (2019) used a phenyl hexyl column and UFLC-ESI-MRM, while Bastiaensen et al. (2020) and Been et al. (2019) used biphenyl column and HPLC-ESI-dMRM. Both studies use the same binary solvent system (aqueous 0.1 % AA and 0.1 % AA in ACN). Pinguet et al. (2019) achieved a lower LOQ for MEHTP (0.018 ng/mL), whereas 0.1 ng/mL was achieved by (Bastiaensen et al., 2020; Been et

al., 2019). The latter also includes OH-MEHTP. A benefit to HBM of the UFLC approach is that it requires minimal sample (50 µL) and minimal sample handling (Pinguet et al., 2019).

538 *Table 2: Sample preparation procedures and analytical methods for the selected CECs*

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Analyte family	Compounds	BoE	Sample preparation	Analysis		LOQ (ng/mL)	Ref.
				Separation	MS		
Fragrances	Polycyclic musks: ADBI, ATII, HHCB, AHTN	ADBI, ATII, HHCB, AHTN	SSLE: 0.8 mL sample loaded on a cartridge, equilibrated 10 min, extracted with 8 ml HEX. Dried under N_2 and reconstituted in 80 µL of HEX.	GC: TG-5HT column (15 m × 0.25 mm × 0.1 µm), program: 70 °C 5 min, 70-190 °C at 30 °C/min, 190 °C 3 min, 190-300 °C at 30 °C/min, 300 °C 3 min	EI-MS2-MRM: TSQ Quantum (Thermo Fischer), EI 40 eV	0.107, 0.143, 0.250, 0.017, 0.103	(Liu et al., 2015)
	Polycyclic musks and nitro musks: ADBI, AHMI, ATII, HHCB, AHTN, MX, MK	ADBI, AHMI, ATII, HHCB, AHTN, MX, MK	USAEME: 1 mL mixed with 0.1 g NaCl and 50 µL of CCl_4, ultrasonicated for 1 min at 40 °C. Then centrifuged at 7000 rpm for 3 min. Extract collected and 10 µL analyzed.	GC: Instrument Varian 450 (Walnut Creek, CA, USA), column: DB-5MS (30 m × 0.25 mm × 1.0 µm), Injection: 10 µL, 80 °C for 2 min then 200 °C/min to 280 °C Program: 70 °C, 4 min, 70-190 °C, 30 °C/min, 190-196 °C, 1 °C/min, 196-280 °C, 120 °C/min, 120 °C, 2.3 min	EI-MS2-MRM: Varian 220 (Walnut Creek, CA, USA)	0.25, 0.1, 0.5, 0.1, 0.1, 0.1, 0.25	(Chen et al., 2018b)
	Lysmeral	TBBA, lysmerol, lysmerylic acid, hydroxy lysmerylic acid, TBHA	LLE: 1 mL of deconjugated urine (37 °C, 16 h) acidified and extracted with DCM. Dried and derivatized with 3-NPA in WA (80 °C, 30 min).	UPLC: Waters Acquity UPLC I-Class Column: Waters Acquity BEH C18 (100 mm × 2.1 mm, 1.7 µm) Mobile phases: A (MeOH), B (5 mM ammonium acetate with 0.025 % ammonium hydroxide, pH 9.2), flow 0.35 mL/min Gradient elution: 80 % B, 0-4 min, 80-55 % B, 4-10 min, 55-35 % B, 10-11 min, 35-0 % B, 11-11.1 min, 0 % B, 11.1-13 min, 0-80 % B, 13-13.1 min, 80 % B, 13.1-15 min	ESI(-)-MS2-MRM: Waters TQ-S Triple Quadrupole	0.42, 0.1, 0.36, 0.45, 0.39	(Pluym et al., 2016)
	7-HC	7-HCA	LLE: 1 mL urine deconjugated (37 °C, 3h), acidified and extracted with DCM. Dried and derivatized with 3-NPA (80 °C, 30 min).	UPLC: Waters Acquity UPLC I-Class Column: Waters Acquity BEH C18 (100 mm × 2.1 mm, 1.7 µm) Mobile phases: A (0.1 % FA / W), B (0.1 % FA / ACN), flow 0.5 mL/min Gradient elution: 80 % A, 0-1 min, 80-20 % a, 1-5 min, 20 % A, 5-5.5 min, 20-80 % A, 5.5-7 min	ESI(-)-MS2-MRM: Waters TQ-S Triple Quadrupole	0.5	(Stoeckelhuber et al., 2017)

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Benzotriazoles and benzothiazoles	1H-BTR , 1OH-BTR, TTR, XTR, 5-Cl-1H-BTR, BTH, 2-OH-BTH, 2-MeS-BTH, 2-amino-BTH, 2-SH-BTH, 2-SCNMeS-BTH	1H-BTR , 1OH-BTR, TTR, XTR, 5-Cl-1H-BTR, BTH, 2-OH-BTH, 2-MeS-BTH, 2-amino-BTH, 2-SH-BTH, 2-SCNMeS-BTH	SPE: 2 mL, deconjugation with β-glucuronidase (37 °C, 24 h), diluted, pH adjusted to 3, extracted on Oasis HLB 6 cc/200 mg with MeOH/ACN (1:1), dried and reconstituted in 200 µL MeOH/ACN (1:1) LLE: 600 µL extracted with 6 mL ACN/DCM (1:1), extract dried and reconstituted in 150 µL MeOH/ACN	HPLC: Agilent 1100 HPLC Column: Zorbax SB aq (150 × 2.1 mm, 3,5 µm), Mobile phases: A (0.1 % FA / W), B (ACN), flow 0.25 mL/min Gradient elution: 10-40 % B, 4.5 min, 40-100 % B, 11.5 min, 100 % B, 6.1 min, 10 % B, 7.1 min	ESI(+)-MS2-MRM:Applied Biosystems triple quadrupole	SPE: 0.50, 2.00, 0.20, 0.20, 0.50, 5.00, 2.50, 0.20, 0.20, 0.50 LLE: 1.70, 0.70, 0.70, 1.70, 0.70. 17.00, 3.50, 1.70, 0.70, 1.70	(Asimakopoulos et al., 2013a)
	1H-BTR , 1OH-BTR, TTR, XTR, 5-Cl-1H-BTR, BTH, 2-OH-BTH, 2-MeS-BTH, 2-amino-BTH, 2-SCNMeS-BTH	1H-BTR , 1OH-BTR, TTR, XTR, 5-Cl-1H-BTR, BTH, 2-OH-BTH, 2-MeS-BTH, 2-amino-BTH, 2-SCNMeS-BTH	LLE: 1 mL deconjugated with β-glucuronidase (37 °C, 24 h), extracted with 3 mL MTBE/EA (5/1), dried and reconstituted in 200 µL ACN/W (6/4)	UPLC: Dionex Ultimate 3000 UHPLC Column: Thermo Hypersil GOLD (100 × 2.1 mm, 1.9 µm) Mobile phases: A (0.01 % FA / W), B (ACN), flow 0.25 mL/min Gradient elution: 10-83 % B, 9.5 min, 83-100 %, 100 % B, 3 min, 10 % B	ESI(+)-MS2-MRM: Thermo Scientific TSQ Quantiva Triple Quadrupole	0.2, 11, 2, 7, 4, 9, 6, 11, 4, 14	(Li et al., 2017)

	1H-BTR , 1OH-BTR, TTR, XTR, 5-Cl-1H-BTR, BTH, 2-OH-BTH, 2-MeS-BTH, 2-amino-BTH, 2-SCNMeS-BTH	1H-BTR , 1OH-BTR, TTR, XTR, 5-Cl-1H-BTR, BTH, 2-OH-BTH, 2-MeS-BTH, 2-amino-BTH, 2-SCNMeS-BTH	LLE: 1 mL deconjugated with β-glucuronidase (37 °C, 24 h), extracted with 3 mL MTBE/EA (5/1), repeated twice, dried and reconstituted in 200 µL ACN/W (6/4)	UPLC: Waters Column: Acquity BEH C18 (100 × 2.1 mm, 1.7 µm) Mobile phases: A (0.01 % FA / W), B (ACN), flow 0.25 mL/min Gradient elution: 10 % B, 0.5 min, 10 % B, 9.5 min, 10-83 % B, 2 min, 100 % B, 2.8 min, 10 % B	ESI(+)-MS2-MSM: Waters Xevo TQ-XS Triple Quadrupole	0.03, 0.12, 0.06, 0.03, 0.03, 0.06, 0.03, 0.06, 0.03, 0.24	(Zhou et al., 2018)
	1H-BTR , 1OH-BTR, TTR, XTR, 5-Cl-1H-BTR, BTH, 2-OH-BTH, 2-MeS-BTH, 2-amino-BTH, 2-SCNMeS-BTH	1H-BTR , 1OH-BTR, TTR, XTR, 5-Cl-1H-BTR, BTH, 2-OH-BTH, 2-MeS-BTH, 2-amino-BTH, 2-SCNMeS-BTH	SPE: 2 mL deconjugated with β-glucuronidase (37 °C, 24 h) and extracted on Oasis HLB 6cc/200 mg with MeOH/ACN (1:1), dried and reconstituted in 200 µL MeOH/ACN (1:1)	UPLC: Waters Acquity Column: Waters Acquity BEH Shield RP18 (100 × 3 mm, 1.7 µm) Mobile phases: A (0.1 % FA / W), B (MeOH / ACN, 1:1), flow 0.4 mL/min Gradient elution: 10 % B, 0.5 min, 10-40 % B, 0.5-1.0 min, 40-100 % B, 1.0-2.0 min, 100 % B, 2.0-6.0 min, 100-10 %, 6.0-6.1 min, 10 % B, 6.0-7.0 min	ESI(+)-MS2-MSM: Waters Xevo TQ-S Triple Quadrupole	0.005-0.51	(X. Li et al., 2018)
	1H-BTR , 5,6-MeMeBTR, 4-Me-BTR, 5-Me-BTR, 5-Cl-1H-BTR, BTH, 2-Me-BTH, 2-OH-BTH, 2-MeS-BTH,	1H-BTR , 5,6-MeMeBTR, 4-Me-BTR, 5-Me-BTR, 5-Cl-1H-BTR, BTH, 2-Me-BTH, 2-OH-BTH, 2-MeS-BTH,	SPME: to 1.33 mL urine 0.5 g NaCl added, diluted, extraction by 85 µm polyacrylate fiber, direct immersion, 40 min, room T. Thermal desorption at 290 °C for 10 min	GC: Thermo Fisher TSQ Quantum Column: Thermo TR-5MS (30 m × 0.25 mm i.d., 0.25 µm) Program: 65 °C, 1 min, 65-240 °C, 10 °C/min, 240-290 °C, 50 °C/min, 290 °C, 3 min	EI-MS2-MRM: Thermo Fisher Triple Quadrupole Quantum	4.9, 3.1, 1.8, 2.7, 4.9, 0.91, 0.80, 0.45, 0.006, 0.41, 3.11	(Naccarato et al., 2014)

	2-amino-BTH, 2-SH-BTH	2-amino-BTH, 2-SH-BTH					
	SH-BTH	SH-BTH	Online SPE: 0.5 mL of urine deconjugated with β-glucuronidase (37 °C, overnight) and extracted on Waters Oasis HLB column (20 mm × 2.1 mm, 25 µm)	HPLC: Waters Alliance 2695 Column: Agilent Zorbax Eclipse XDB-C8 (50 mm × 4.6 mm, 5 µm) Mobile phases: A (W), B (1 % FA / W), C (ACN), flow rate 0.2 mL/min Gradient elution: 70 % A, 10 % B, 20 % C, 0-4 min, 70-0 % A, 10 % B, 20-90 % C, 4-5 min, 0 % A, 10 % B, 90 % C, 5-8 min, 0-70 % A, 10 % B, 90-20 % C, 8-8.5 min, 70 % A, 10 % B, 20 % C, 8.5-13 min	ESI(+)-MS2-MRM: Waters Quattro Ultima Triple Quadrupole	1	(Gries et al., 2015)
Antimicrobials	MI, MCI	NMMA	LLE: 100 µL of urine freeze-dried and redissolved in 1 mL of ACN. Derivatized with PFBBr (K_2CO_3, 60 °C, 16h) and extracted with 2x 1 mL of HEX. Dried and reconstituted in MePh.	GC: Agilent 7890 A Column: HP-5-MS (60 m × 0.25 mm i.d., 0.25 µm) Program: 90 °C, 1 min, 90-120 °C, 30 °C/min, 120 °C, 1min, 120-240 °C, 10 °C/min, 240-310 °C, 25 °C, 310 °C, 5 min	EI-MS2-MRM: Agilent 7000 Triple Quadrupole	0.5	(Schettgen et al., 2017)
	MI, MCI	M-12	SPE: 500 µL of urine mixed with 500 µL of 100 mM ammonium formate buffer (pH 2.5). Addition of 10 µL formic acid and 10 µL of D3-M-12 (1 µg/mL) in water) as an internal standard, Injecting 100 µL of this solution in the LC/MS/MS-system. Analyte enrichment and clean with Phenomenex Strata-X-column (20 × 2 mm; 20 µm) using water (pH 2.5) and methanol (90:10, v:v), flow rate of 0.5	LC system: Agilent Technologies 1200 In﬿nity series), column: Phenomenex C18(2), 150 4.6 mm, 3 mm, 100 ˚, Mobile phases: A: water pH 2.5 (adjusted with formic acid), B: MeOH, C: ACN, flow rate 0.5 mL/min, column switching technique: 90% A for 5 min for analyte enrichment, valve switching to analytical column: 75% A, 25% C at flow rate 0.3 mL/min	Sciex API 5500 LC/MS/MS, ESI+, detection mode: MRM	0.2	(Schettgen et al., 2021)

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			ml/min. Backflush on the analytical column [Phenomenex C18(2), 150 × 4.6 mm, 3 µm, 100 A], separation from interferences using a gradient of water (pH 2.5) and acetonitrile.				
Diisocyanates: MDI, 4TDI, 6TDI, NDI, PPDI, HDI	MDI	MDA	LLE: 2 mL of urine, deconjugated by sulfuric acid (100 °C, 90 min), neutralized, extracted with 4 mL DEE. Dried and reconstituted in 500 µL of MePh, derivatized by HFBA (55 °C, 60 min). Dried and reconstituted in 100 µL of MePh.	GC: type not given Column: BP-5 (dimensions not reported, 1 µm) Program: 150 °C, 1 min, 150-280 °C, 10 °C/min, 280 °C, 1.5 min	NCI-SIM: type not reported	Not given	(Mirmohammadi et al., 2013)
	MDI, 4TDI, 6TDI, NDI, PPDI	MDA, 4TDA, 6TDA, NDA, PPDA	SPE: 250 µL of urine deconjugated by HCl (80 °C, 4h), neutralized, extracted on Phenomenex Strata XC 30 mg/3mL with MeOH/IPA/NH_4OH 75/20/5. Evaporated and reconstituted in 250 µL of mixed buffer	UPLC: Waters Acquity UPLC Column; ACE Excel2 SuperC18 (dimensions not reported) Mobile phases: A (100 mM Ammonium acetate / w), B (100 mM ammonium acetate / ACN, 5/95), flow: 0.5 mL/min Gradient elution: 10 % B, 0-1 min, 10-30 % B, 1-2 min, 30-90 %, 2-2.5 min, 90-15 % B, 2.5-4 min	APCI(+)-MS2-sMRM: Sciex 5500 Triple Quadrupole	0.03, 0.10, 0.10, 0.10, 0.33	(Bhandari et al., 2016)
	MDI, 4TDI, 6TDI	MDA, 4TDA, 6TDA	SPE: 250 µL of urine deconjugated by HCl (80 °C, 4h), neutralized, extracted with Phenomenex Strata XC 30 mg/3mL. Evaporated and reconstituted in 250 µL of ACN and derivatized with PBC (DMAP, 80 °C, 20 min), neutralized with HCl.	HPLC: Agilent HP 1100 Column: Agilent SB C18 (4.6 × 150 mm, 3.5 µm), flow 1.0 ml/min Mobile phases: A (0.1 % FA in 5 % ACN/W), B (0.1 % FA in ACN) Gradient elution: 70-90 % B, 0-15 min, 90 % B, 15-20 min	FLD: Agilent HP 1000, excitation 330 nm, emission 475 nm	0.00105-0.00163	(Sun et al., 2018)

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	HDI	TAHI	LLE: 1 mL of urine deconjugated with 100 µL sulfuric acid (100 °C, 16 h), neutralized and extracted with DCM. Derivatized with acetic anhydride (55 °C, 16 h). Dried and reconstituted in 200 µL of ACN (0.1 % FA)	Nano-UPLC: Type not reported Column: Waters Symmetry C18 (100 mm × 100 µm, 3 µm) Mobile phases: A (0.1 % FA/W), B (0.1 % FA / ACN), flow: 0.6 µL/min Gradient elution: 95-10 % A, 17 min	ESI(+)-MS2-MRM: Type not reported	0.03 (LOD)	(Robbins et al., 2018)
	MDI	MDA	LLE: 190 µL of urine deconjugated with sulfuric acid (100 °C, 60 min) and neutralized and extracted with MePh. Dried and reconstituted in 200 µL of water.	UPLC: Waters Acquity Column: Waters Acquity HSS T3 (50 mm × 2.1 mm, 1.8 µm) Mobile phases: A (0.1 % ammonium acetate / W), B(0.1 % ammonium acetate / MeOH), flow 0.6 mL/min Gradient elution: 5 % B, 0.5 min, 5-90 %, 3 min, 90 %, 1 min	ESI(+)-MS2-MRM: Waters Xevo Triple Quadrupole	0.535	(Lépine et al., 2019a)
	MDI	MDA	LLE: 1 mL of urine deconjugated with sulfuric acid (100 °C, 16 h), neutralized and extracted with MePh. Dried and derivatized by HFBA. Dried and reconstituted in EA.	GC: Agilent 6890 A Column: HP-5 (25 m × 0.32 mm × 0.17 µm) Program: 50 °C, 1 min, 50-145 °C, 10 °C/min, 145-165 °C, 5 °C/min, 165-300 °C, 25 °C/min, 300 °C, 10 min	NCI-MS-SIM: Agilent 5973N	0.1	(Henriks-Eckerman et al., 2015)
	MDI, 4TDI, 6TDI, HDI	MDA, 4TDA, 6TDA, HDA	SPE: 190 µL of urine deconjugated with sulfuric acid (100 °C, 60 min) and neutralized and extracted with Waters MCX 30 mg / 1 ml. Evaporated and reconstituted in borate buffer. Derivatized with acetic anhydride (instantly, room T).	UPLC: Waters Acquity Column: Waters Acquity HSS T3 (50 mm × 2.1 mm, 1.8 µm) Mobile phases: A (0.1 % FA / W), B(0.1 % FA / MeOH), flow 0.6 mL/min Gradient elution: 2 % B, 1 min, 2-17 % B, 3 min, 17-40 % B, 1.5 min, 40-90 %, 0.5 min, 90 %, 1 min	ESI(+)-MS2-MRM: Waters Xevo Triple Quadrupole	1.33, 0.76, 1.24, 2.04	(Lépine et al., 2020)

Pyrrolidones: NMP, NEP	NMP	5-HNMP, 2-NMSI	SPE: 600 µL urine extracted on Biotage Isolute ENV+ 100 mg / 1ml, eluted with EA/MeOH (4:1). Evaporated and derivatized with MTBSTFA (110 °C, 60 min) and diluted with EA.	Cooled injection GC: Agilent 7890 GC Column; DB-35MS (60 m × 0.25 mm i.d., 0.25 µm) Injector temperature program: 40 °C, 0.5 min, 40-240 °C, 120 °C/min, 240-260 °C, 600 °C/min, 600 °C, 10 min Column temperature program: 50 °C, 4 min, 50-90 °C, 25 °C/min, 90 °C, 1 min, 90-190 in, 190 min, 190-280 °C /min, 280 °C, 10 min	EI-MS: Agilent 5975	20, 5, 15, 5 (LOD)	(Schindler et al., 2012)
	NEP	5-HNEP, 2-HESI					
	NMP	5-HNMP, 2-NMSI	Dilute and shoot: 100 µL of urine diluted 10-fold with water	HPLC: Agilent Column: Agilent Zorbax Eclipse Plus C18 (100 mm × 4.6 mm, 3.5 µm) Mobile phases: A (0.1 % FA / W), B (ACN), 0.2 mL/min Gradient elution: 0-30 % B, 0-9 min, 30-100 % B, 9-9.5 min. 100 % B, 9.5-10.5 min	ESI(+)-MS2-MRM:Agilent 6460 Triple Quadrupole	0.2	(Haufroid et al., 2014), (Suzuki et al., 2009)
	NMP	5-HNMP	Dilute and shoot: Urine diluted 10-fold with 5 mM ammonium formate buffer	UPLC: Waters Acquity I-Class Column; Waters HSS-PFP (100 mm × 2.1 mm, 1.8 µm) Mobile phases: A (5 mM ammonium formate / w), B (MeOH), flow 0.4 ml/min Gradient elution: 2.5 % B, 0-0.6 min, 2.5-35 % B, 0.6-2.8 min, 35-80 %, 2.8-4.5 min, 80-2.5 % B, 4.5-5.5 min	ESI(+)-MS-MRM: Sciex Triple Quadrupole 5500	0.274 (LOD)	(Bhandari et al., 2019)
UV filters	MBC	MBC, CBC	SPE: 4 mL of deconjugated urine (β-glucuronidase, 37 °C, overnight) extracted on C18 (brand not reported) with acetone. Dried and reconstituted in W:MeOH:ACN (2:1:1)	UPLC: Waters Acquity Column: Waters Acquity BEH C18 (50 mm × 2.1 mm, 1.7 µm) Mobile phases: A (0.1 % FA / w), B (0.1 % FA / ACN:MeOH 1:1), flow 0.3 mL/min Gradient elution: 40 % B, 0-1 min, 40-100 % B, 1-1.1min, 100 % B, 2 min	ESI(±)-MS2-MRM: Waters Acquity TQD Triple Quadrupole MBC esi +, CBC esi-	6, 6 (LOD)	(León-González et al., 2013)
	MBC	MBC	SPE: 2 mL of deconjugated urine (β-glucuronidase, 37 °C, 12 h), centrifuged, diluted to 30	GC: Thermo Scietific TSQ Quantum XLS Column: RTX-5 (30 m × 0.25 mm, 0.25 µm)	EI-MS2-MRM: Thermo Scientific TSQ Quantum XLS	3.454	(Ao et al., 2018b)

			mL, extracted on Oasis HLB (200 mg, 6 mL) with EA. Dried and derivatized with BSTFA-TMCS (1 %) at (room T, 30 m).	Program: 40-200 °C, 15 °C/min, 200-280 °C, 8 °C/min, 280-320°C, 10 °C/min			
	MBC	MBC	On-line	HPLC: Aria TLX-1 Columns: TurboFlow on TurboFlow Cyclone P (50 × 0.5 mm) and Hypersil Gold aQ columns (50 × 4 mm) Mobile phases: A, B, C (not reported), flow 0.7 mL/min Gradient elution: 100 % A, 0-1.5 min, 41-32 % A, 59-68 % B, 2-5 min, 32-5 % A, 68-95 B, 5-6 min, 100 % C, 6-7.5 min, 0-5 % A, 0-95 % B, 7.5-8.5 min, 100 % A, 8.5-9.5 min	APCI(+)-MS2-MRM: Thermo Scientific TSQ Triple Quadrupole	0.87 (LOD)	(Krause et al., 2017), (Frederiksen et al., 2017)
	MBC	MBC	2 mL of urine lyophilized, resuspended in 1 mL of 90 % MeOH/W, centrifuged, and the Supernatant injected.	HPLC: Type not reported Column: Sephasil Peptide C18 (250 mm × 4.60 mm, 5 µm) Isocratic elution: 88:12 MeOH/W, flow 0.5 mL/min	UV: SPD-6 UVD	2.9 ng/mL (LOD)	(Janjua et al., 2008)
	MBC	CBC, CBC-OH	Online SPE: 0.5 mL urine deconjugated (β-glucuronidase, T and t not reported) and extracted on X Bridge C8 direct Connect HP (30 mm × 2.1 mm, 10 µm).	UPLC: Waters Acquity Column: Waters Acquity HHS C18 (150 mm × 2.1 mm, 1.8 µm) Mobile phases: Not reported Elution: Not reported	ESI (Polarity not reported)-MS2-MRM: Waters Xevo TSQ	0.15, 0.30	(Leng and Gries, 2017)
Non-phthalate plasticizers Trimellitate	TEHTM	1-MEHTM, 2-MEHTM, 4-MEHTM, 5OH-1-MEHTM, 5OH-2-MEHTM,	Online: 1 mL of urine deconjugated (β-glucuronidase, 37 °C, 2 h), centrifuged and injected on a restricted access material phase (Merck LiChrospher RP-18 ADS, 4 mm × 25 mm,	HPLC: Agilent 1100 Column: Restek Core-Shell Raptor Biphenyl (100 mm × 2.1 mm, 2.7 µm) Mobile phases: A (0.1 % FA / W), B (0.1 % FA / ACN), flow rate 0.3 mL/min Gradient elution: 0-2 % b, 0-2 min, 2-35 % B, 2-3 min, 35 % B, 3-8 min, 35-55 % B, 8-9 min, 55 %	ESI(-)-MS2-MRM: Sciex API 200 Triple Quadrupole	4.6, 1.0, 0.7, 2.6, 1.5, 1.1, 2.6, 1.6, 2.6, 1.7, 2.4	(Höllerer et al., 2018b)

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		5oxo-1-MEHTM, 5oxo-2-MEHTM, 5cx-1-MEPTM, 5cx-2-MEPTM, 2cx-2-MMHTM, 2cx-1-MMHTM	25 µm) with W/MeOH/FA 80/20/0.1 at 0.7 mL/min. Enrichment for 4 min.	B, 9-12 min, 55-80 % B, 12-16 min, 80 % B, 16-19 min, 80-2 % B, 19-20 min, 2 % B, 20-21 min			
	TEHTM	1-MEHTM, 2-MEHTM, 4-MEHTM	Online SPE: 50 µL of urine extracted on TurboFlow Cyclone column (50 mm × 0.5 mm) with 0.1 % AA / W and 0.1 % AA / ACN	UFLC: Shimadzu Prominence UFLC Column: Thermo Scientific Betasil phenyl/hexyl (100 mm × 3 mm, 3 µm) Mobile phases: A (0.1 % AA/W), B (0.1 % AA / ACN), flow 0.5 mL/min Gradient elution: 25 % B, 0-5 min, 25-55 % B, 5-9 min, 55-75 % B, 9-14 min, 75-99 % B, 14-15 min, 99 % B, 15-24 min, 99-25 % B, 24-24.1 min, 25 % B, 24.1-25 min	ESI(-)-MS2-MRM: Sciex QTrap 5500	0.012, 0.044, 0.012	(Pinguet et al., 2019)
	TEHTM	1-DEHTM, 2-DEHTM	SPE: 1 mL of deconjugated urine (β-glucuronidase, 37 °C, 90 min) extracted on Waters Oasis MAX 30 mg/3 mL with MeOH. Evaporated and reconstituted in ACN/W 1:1.	HPLC: Agilent 1290 LC Column: Phenomenex Kinetex biphenyl RP (100 mm × 2.1 mm, 2.6 µm) Mobile phases: A (0.1 % AA / W), B (0.1 % AA/ACN), flow rate 0.2 mL/min Gradient elution: 15-45 % B, 4 min, 15-100 % B, 10 min, 100-15 % B, 20 min, 15 % B, 10 min	ESI(-)-MS-dMRM: Agilent 6460 Triple Quadrupole	0.10, 0.10	(Bastiaensen et al., 2020), (Been et al., 2019)
Adipates	DEHA	MEHA	Online SPE: 50 µL of urine extracted on TurboFlow Cyclone column (50 mm × 0.5 mm) with 0.1 % AA / W and 0.1 % AA / ACN	UFLC: Shimadzu Prominence UFLC Column: Thermo Scientific Betasil phenyl/hexyl (100 mm × 3 mm, 3 µm) Mobile phases: A (0.1 % AA/W), B (0.1 % AA / ACN), flow 0.5 mL/min	ESI(-)-MS2-MRM: Sciex QTrap 5500	0.044	(Pinguet et al., 2019)

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				Gradient elution: 25 % B, 0-5 min, 25-55 % B, 5-9 min, 55-75 % B, 9-14 min, 75-99 % B, 14-15 min, 99 % B, 15-24 min, 99-25 % B, 24-24.1 min, 25 % B, 24.1-25 min			
	DEHA	MEHA, OH-MEHA	SPE: 1 mL of deconjugated urine (β-glucuronidase, 37 °C, 90 min) extracted on Waters Oasis MAX 30 mg/3 mL with MeOH. Evaporated and reconstituted in ACN/W 1:1.	HPLC: Agilent 1290 LC Column: Phenomenex Kinetex biphenyl RP (100 mm × 2.1 mm, 2.6 µm) Mobile phases: A (0.1 % AA / W), B (0.1 % AA/ACN), flow rate 0.2 mL/min Gradient elution: 15-45 % B, 4 min, 15-100 % B, 10 min, 100-15 % B, 20 min, 15 % B, 10 min	ESI(-)-MS-dMRM: Agilent 6460 Triple Quadrupole	0.15, 0.15	(Bastiaensen et al., 2020), (Been et al., 2019)
	DEHA	5OH-MEHA, 5oxo-MEHA, 5cx-MEPA	Online SPE: 300 µL of urine deconjugated (β-glucuronidase, 37 °C, 3 h) and extracted on Thermo Scientific TurboFlow Phenyl (50 mm × 0.5 mm).	HPLC: Agilent LC 1200 Column: Thermo Scientific Accucore Phenyl-X (150 × 3 mm, 2.6 µm) Mobile phases: A (0.05 % AA / W), B (0.05 % AA / ACN), flow rate 0.3 mL/min Gradient elution: 30 % B, 0-2.5 min, 30-40 % B, 2.5-4 min, 40-55 % B, 4-19 min, 55-95 % B, 19-20 min, 95 % B, 20-22 min, 95-30 % B, 22-23 min, 30 % B, 23-27 min	ESI(-)-MS2-MRM: AB Sciex Qtrap 5500	0.05, 0.1, 0.05	(Nehring et al., 2019)
	DnBA	MnBA, 3OH-MnBA, 3cx-MnPrA	Online SPE: 300 µL of urine deconjugated (β-glucuronidase, 37 °C, 2 h). Frozen and centrifuged. Samples extracted on Thermo Scientific TurboFlow Cyclone-P column (50 mm × 0.5 mm).	HPLC: Agilent 1260 Infinity II HPLC Column: Phenomenex Kinetex C18 (150 × 3 mm, 2.6 µm) Mobile phases: A (0.05 % AA / W), B (0.05 % AA / ACN), flow rate 0.4 mL/min Gradient elution: 12 % B, 0-3.5 min, 12-45 % B, 3.5-14 min, 45-95 % B, 14-15 min, 95 % B, 15-21 min, 95-12 %, 21-21.5 min, 12 % B, 21.5-29.5 min	ESI(-)-MS2-scheduled MRM: AB Sciex Triple Quadrupole 5500	0.1, 0.05, 0.5	(Ringbeck et al., 2020)
Terephthalates	DEHTP	MEHTP	Online SPE: 50 µL of urine extracted on TurboFlow Cyclone column (50 mm × 0.5	UFLC: Shimadzu Prominence UFLC Column: Thermo Scientific Betasil phenyl/hexyl (100 mm × 3 mm, 3 µm)	ESI(-)-MS2-MRM: Sciex QTrap 5500	0.018,	(Pinguet et al., 2019)

			mm) with 0.1 % AA / W and 0.1 % AA / ACN	Mobile phases: A (0.1 % AA/W), B (0.1 % AA / ACN), flow 0.5 mL/min Gradient elution: 25 % B, 0-5 min, 25-55 % B, 5-9 min, 55-75 % B, 9-14 min, 75-99 % B, 14-15 min, 99 % B, 15-24 min, 99-25 % B, 24-24.1 min, 25 % B, 24.1-25 min			
	DEHTP	MEHTP, OH-MEHTP	SPE: 1 mL of deconjugated urine (β-glucuronidase, 37 °C, 90 min) extracted on Waters Oasis MAX 30 mg/3 mL with MeOH. Evaporated and reconstituted in ACN/W 1:1.	HPLC: Agilent 1290 LC Column: Phenomenex Kinetex biphenyl RP (100 mm × 2.1 mm, 2.6 µm) Mobile phases: A (0.1 % AA / W), B (0.1 % AA/ACN), flow rate 0.2 mL/min Gradient elution: 15-45 % B, 4 min, 15-100 % B, 10 min, 100-15 % B, 20 min, 15 % B, 10 min	ESI(-)-MS-dMRM: Agilent 6460 Triple Quadrupole	0.10, 0.10	(Bastiaensen et al., 2020), (Been et al., 2019)

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4. Conclusions and future perspectives

In this review, we reviewed six groups of compounds that are overlooked in existing HBM schemes, but are good candidates from the point of view of recent increase of their detection urine and reports of toxicity. These compounds are present in urine at levels from several hundred ng/mL to below one ng/mL. This review summarizes the emerging techniques and current state-of-the-art analytical methods for inclusion into ongoing HBM schemes. The methods were assessed in terms of LOQ. Overall, the most common first step in sample handling is enzymatic hydrolysis, which hydrolyses phase II metabolites to parent compound or phase I metabolites. Accordingly, deconjugation was applied before analysis with an exception of diisocyanates, which were deconjugated using mineral acid hydrolysis and nitro musks, for analysis of which no deconjugation step was applied.

Samples preparation varied widely within compound groups. Online-SPE stood out regarding the low sample volume required, limited sample handling, and achieved LOQs. However, the main drawback is the specific setup, which might not be feasible for every laboratory, and offline SPE approaches were the most commonly used. Despite being more time-consuming than online SPE and costlier than LLE, SPE offers high flexibility due to the variety of available SPE sorbents out of which, general, wide polarity mixed mode sorbents were the most frequent, followed by ion exchange sorbents for diisocyanates and certain phthalate alternativesNew techniques, such as solid-supported liquid-liquid extraction and ultrasound-assisted emulsification microextraction represent new directions to achieve even lower detection limits and low sample volumes, which is essential in HBM studies, as sample amount is limited, particularly for vulnerable populations, such as infants and children.

Compounds were almost exclusively analyzed using separation techniques (GC and LC) coupled to mass spectrometry. Although both GC and LC can separate many compounds, LC-based approaches achieved better results in terms of the detection limits. Most of the studies chose LC. UPLC methods, achieving higher resolutions and reduced analytical times, are in rapid development to fill the analytical gap, for example, in the case of lysmeral, 7-HC, BTRs, BTHs and MBC. Furthermore, UFLC was used for the separation of non-phthalate pesticides. Used in almost every case, mass spectrometric detection showed the dominance of mass spectrometry in trace-level analyses in HBM, with triple quadrupole analyser dominating the scene, while quadrupole-linear ion-trap (QTrap) is following right behind. HRMS is currently scarcely used in targeted analysis of these compounds. Ionization was most commonly ESI with compound dependent polarity and, in some cases, APCI. The MS was operated almost exclusively using tandem mass spectrometry and MRM mode, ensuring high specificity and selectivity, which is important when dealing with complex samples like urine.

Various columns were used for GC separation depending on the compound type. Most studies used low polarity 5 % diphenyl / 95 % dimethylsiloxane based columns or equivalent for fragrances, MI and MCI, diisocyanates and MBC. Other columns phases, such as non-polar 5 % phenyl polysilphenylene-siloxane and mid polarity 35 % phenyl/methylpolysiloxane were used for BTRs and BTHs and pyrrolidones, respectively. Like in the case of LC-based methods, the compounds were commonly detected using tandem MS in MRM mode, using GC-specific EI ionization and less often, negative chemical ionization. Also, in the case of GC separation,

584 derivatization was often applied to achieve sufficient volatility. Often used reaction, silylation,
585 forming trimethylsilyl ethers was for here reviewed compounds likewise most commonly used,
586 followed by formation of pentrafluorobenzoyl and heptafluorobutyrate ethers and esters.
587 To improve the existing knowledge, there is a need for sensitive, specific and accurate
588 analytical methods to aid exposure assessment and inform health risk assessment. A necessary
589 step is the lowering of LOQs and thus improving the sensitivities of existing methods in order
590 to assess the exposure even at very low levels. The methods require to be fit-for-purpose and
591 therefore should comply to strict QA/QC procedures and report method validation procedures
592 in detail, including estimation of measurement uncertainty, which is so far rarely reported in
593 HBM studies. Furthermore, with advances in HRMS specificity and selectivity of targeted
594 analysis could be in future improved even further. Additionally, HRMS enables global-scale
595 non-targeted analysis, which informs on known and yet unknown chemicals present in the
596 urine, and new metabolites and biomarkers of effect could be identified and further aid in
597 understanding of compounds' toxicity and the health risks associated with the exposure.
598 Clearly, a certain amount of analytical techniques is available to analyze these six
599 groups of chemicals. Therefore, the optimal methods should be applied for various populations
600 to thoroughly assess their distribution and occurrence in a large population of people. To
601 improve cost- and time-efficiency, which is of immense importance in large cohort HBM
602 studies, one comprehensive multiresidue method would in ideal case be applied. However, with
603 analysis of such heterogeneous group of chemicals, this is clearly impossible, and compound
604 prioritization in compliance with the research question applied and based on that, most
605 appropriate method applied. Nevertheless, to improve the existing knowledge of potential
606 health risks associated with the exposure to here presented groups of chemicals, more studies
607 into their biological effects are needed to understand their associated risk properly, especially
608 when present as in real life scenario, simultaneously as a mixture.
609

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Chapter 4

Conclusions

Humans are exposed to a large number of environmental chemicals, many of which may show adverse effects on the human health. The main contribution of this doctoral work is in the new methodological approach for NTS and describing the exposure of children, which may be at risk from the ever-increasing load of potentially hazardous chemicals.

The novel contributions to the science were described in eight scientific publications, and were presented at five international conferences.

The main part of the thesis was the development of the non-targeted analytical procedure that would be applicable to large-scale HBM studies. This was achieved with SPE after the enzymatic hydrolysis of phase II xenobiotic metabolites. The separation of potential BoEs was achieved using UHPLC and the analysis by an HRMS instrument. Using pooled urine sample spiked with standards, covering wide polarity and mass range, optimal conditions for sample preparation and chromatographic separation were established. As standards were spiked at levels as expected for BoEs in real samples, data processing parameters were optimized in such a way as to ensure detection low-level BoEs. Identification of the standards using established identification procedures for NTS confirmed the method as fit-for-purpose. After being developed, the method was applied to a cohort of 200 children from Slovenia. In these samples we identified 76 BoEs to chemicals, including two restricted pesticides. This fact raises concern about the adverse health effects of children, which are exposed to a large number of distinct chemicals with various biological effects, particularly for those, which have been restricted due to high risk. Successfully developed and fit-for-purpose method and its productive application **confirmed** hypothesis **H1**: The developed NTA workflow is suitable for application in HBM, and hypothesis **H2**: The developed NTA workflow enables the identification of biomarkers of exposure in urine.

Following that, the workflow was adapted and applied to a study of environmental fate of a cytostatic drug imatinib, where we identified eight distinct TPs, out of which six were new to science, and one of them we detected in actual waste water in Slovenia. This was especially concerning as cytostatic drugs have no set threshold for their cytotoxic, carcinogenic and mutagenic activity. The ability to identify TPs using our in-house built protocol and finding the identified TP in real wastewater samples **confirmed** hypothesis **H3**: NTA data processing and identification protocols can be extended to other fields.

To cross-validate the results of NTS, a targeted investigation of selected EDCs was conducted on a part of the same population as analyzed with non-targeted screening. The EDCs included were bisphenols, parabens and triclosan. We found widespread exposure of children to these chemicals and found several determinants of exposure along with association between a SNP in *UGT2B15* gene with certain parabens, which indicate that some populations might be more susceptible to adverse health effects due to the exposure. Determination of bisphenols and parabens using both targeted and non-targeted screening

also **confirmed** the last hypothesis, **H4**: Biomarkers of exposure identified with NTA comply with the results of traditional, targeted HBM.

With its agnostic nature, NTS will in the future most likely become the starting point of HBM-based schemes. NTS can identify concerning exposures that should be further followed within the investigated population using specific and quantitative targeted methods. This is a cost-efficient and pragmatic alternative to currently employed approaches of initial monitoring with targeted methods. Along with identifying exposures to known concerning chemicals, NTS has the ability of identifying new chemicals with not yet known concern. As endogenous metabolites are simultaneously detected, NTS can identify potential metabolic perturbations and with that indicate potential health effects within the same run. As the result of ever-increasing computational innovations and advancements in data processing algorithms, the potential of NTS will in the future definitely increase even further. The processing, however, is largely dependent on the analyst and so are the results of NTS, therefore future work must involve efforts to produce transparent and harmonized NT workflows.

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Publications Related to the Thesis

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Biography

Education and research experience

- 2017 – Present: Doctoral study programme Ecotechnologies, Jožef Stefan International Postgraduate School
- July and August 2017: Researcher, Department of Organic Chemistry, Faculty of Chemistry and Chemical Technology, University of Ljubljana
- 2014 – 2017: MSc in Chemistry, Department of Organic Chemistry, Faculty of Chemistry and Chemical Technology, University of Ljubljana,
  Thesis title: Addition of -SCF_3 group to activated allenyl aryl ethers, Supervisor: Prof. Dr. Marjan Jereb
- 2011 – 2014: BSc in Chemistry, Department of Organic Chemistry, Faculty of Chemistry and Chemical Technology, University of Ljubljana,
  Thesis title: Synthesis of some ionic liquids, Supervisor: Prof. Dr. Marjan Jereb

Foreign experience

- 2019 (2 months): Research Centre for Toxic Compounds in the Environment (RECETOX), Masaryk University, Brno, Czech Republic
- 2018 (2 weeks): Metabolomics group, Food Quality and Nutrition, Fondazione Edmund Mach, San Michelle al'Adige, Italy

Fellowships, Scholarships and Grants

- 2017 - 2021: Young Researcher Grant, Slovenian Research Agency
- 2011 - 2014: Zois Scholarship

Research projects

- P1-0143 : Cycling of nutrients and contaminants in environment, mass balances and modelling of environmental processes, and risk analysis
- CRP V3-1722: Attempt of interpretation of the biomonitoring results with data on environmental pollution with emphasis on air pollution and elucidation of potential effects of the pollutants on inhabitant health
- HBM4EU WP16: Emerging substances
- HBM4EU WP09: Laboratory analysis and quality assurance

Awards

- Best poster award, TKALEC, Žiga, KOSJEK, Tina, CODLING, Garry, KLÁNOVÁ, Jana, HORVAT, Milena. Unexpected exposure of children: novel non-targeted screening workflow, 13th Jožef Stefan International Postgraduate School Students' Conference and 15th Young Researchers' Day of Chemistry, material science, biochemistry and environment, (CMBE day), 27th-28th May 2021, online

- Best poster award, TKALEC, Žiga, KOSJEK, Tina, CODLING, Garry, KLÁNOVÁ, Jana, HORVAT, Milena. Development of an analytical method for untargeted screening for organic contaminants in human urine. Exposome conference, March 5-6th, Mount Sinai Institute for Exposomic Research, New York City, New York, USA

Research interests: **Metabolomics, non-targeted screening, bioinformatics, exposomics, environmental health**