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Applicability of an on-line solid-phase extraction liquid chromatography – tandem mass spectrometry for the wastewater-based assessment of human exposure to chemicals from personal care and household products

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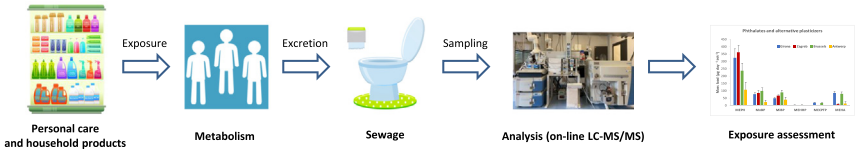
HIGHLIGHTS

- Biomarkers of human exposure to personal care and household products were studied.
- A fully automated method for their determination in wastewater was developed.
- More than 20 biomarkers were detected in the wastewater of 4 European cities.
- Exposure to selected chemicals was assessed by wastewater-based epidemiology.
- Safe reference values were exceeded for several substances.

GRAPHICAL ABSTRACT



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ABSTRACT

Wastewater-based epidemiology (WBE) can be a useful complementary approach to assess human exposure to potentially harmful chemicals, including those from personal care and household products. In this work, a fully automated multiresidue method, based on on-line solid-phase extraction liquid chromatography – tandem mass spectrometry, was developed for the determination of 27 biomarkers of human exposure to selected chemicals from personal care and household products, including parabens, UV filters, phthalates and alternative plasticizers, phosphorous flame retardants/plasticizers (PFRs), and bisphenols. These biomarkers include both the parent compounds and their human metabolites. In addition, two oxidative stress biomarkers, 8-epi-prostaglandin F2α and 4-hydroxy nonenal mercapturic acid, were also considered in the study. The method was carefully optimized to tackle the challenges of analyzing compounds with different physico-chemical properties in a highly complex raw wastewater matrix, while model experiments were performed to investigate filtration losses and analyte stability. The applicability of the developed method was tested by analyzing raw wastewater from four European cities: Antwerp, Brussels (Belgium), Girona (Spain), and Zagreb (Croatia). Twenty-one biomarkers (10 parent compounds and 11 metabolites) were detected in all analyzed wastewater samples. The parent compounds with the highest mass loads were PFRs, parabens, and bisphenol S, while phthalate monoesters were the most prominent metabolites. The mass loads of most compounds were quite similar across cities, but geographic differences were observed for some biomarkers, such as metabolites of phthalates and alternative plasticizers. Exposure was then assessed for seven substances for which quantitative urinary excretion

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data are known. Our results indicate that safe reference values were exceeded for several contaminants, including butylated phthalates, bisphenol A, and tris(2-butoxyethyl) phosphate, particularly for toddlers. With this relatively simple method, which requires less sample manipulation, it is possible to promptly identify and monitor exposure to harmful chemicals at the population level using the WBE approach.

1. Introduction

Personal care and household products, including cosmetics, plastics, clothing, furniture, electronics, paints, lubricants, and others, contain numerous chemical compounds, such as parabens, UV filters, plasticizers, flame retardants, bisphenols, etc. (Senta et al., 2020). Human exposure to these potentially harmful substances can be assessed by human biomonitoring (HBM) studies, which involve the analysis of specific biomarkers (parent compounds and/or their metabolites) in biological matrices, such as serum, urine, hair, and human milk (Vorkamp et al., 2021). This approach, which involves complex cohort studies, is costly and requires ethical approval. In addition, HBM studies often target specific populations (e.g., children, pregnant women, or workers occupationally exposed to harmful substances). Even when the general population is monitored, the number of subjects is always limited, making it difficult to extrapolate results from individuals to the population level and to monitor temporal trends over time.

Wastewater-based epidemiology (WBE) is a relatively new approach to obtaining valuable population-level epidemiological information. WBE involves the analysis of biomarkers in sewage, which can be considered a diluted, pooled urine (and feces) sample from the entire population connected to a given sewer network. It is similar to HBM in terms of the biomarkers analyzed, which are, in fact, often the same as in HBM studies. The main advantage and key feature of WBE is the possibility to obtain near real-time data with a finer temporal resolution and, in most cases, without major ethical issues (Senta et al., 2020). Nowadays, WBE is routinely used to assess people's lifestyle habits, especially illicit drug use (González-Mariño et al., 2020), but it can also be extended to public health surveillance, including SARS-CoV-2 virus prevalence, dietary habits, and exposure to food and environmental contaminants (Gracia-Lor et al., 2017; Vitale et al., 2021). However, this approach also has some limitations, including its aggregate nature and the fact that it cannot be used to obtain individual exposure/consumption data, as is common in molecular epidemiology. Therefore, its true potential lies in the complementarity to conventional methods rather than in obtaining epidemiological data alone. For example, WBE could be used in parallel with pan-European HBM studies to monitor population exposure to environmental contaminants (Gilles et al., 2022), as has already been implemented for illicit drugs (González-Mariño et al., 2020).

Exposure to several classes of chemicals from personal care and household products has already been studied using the WBE approach, including phosphorous flame retardants/plasticizers (PFRs) (Been et al., 2017, 2018; Castro et al., 2019), phthalates (Du et al., 2018; González-Mariño et al., 2017, 2021; Tang et al., 2020), plasticizers (Estévez-Danta et al., 2021), bisphenol A (BPA) (Lopardo et al., 2019), and endocrine disrupting chemicals (Lopardo et al., 2018). All of these studies involved analysis of selected metabolites to assess human exposure to their parent compounds. In addition, several related studies estimated the per capita input (mass load) of the parent substances themselves, such as parabens (Karthikraj et al., 2017; Wang and Kannan, 2016) and UV filters (O'Malley et al., 2019). Because of their additional sources in sewage (besides human metabolism), parent compounds are not reliable WBE biomarkers for exposure assessment, at least not in quantitative terms. However, their determination in WBE studies may be warranted in some cases, e.g., for substances for which no or limited human metabolism data are available or for substances whose metabolites are not commercially available as reference standards. In addition to studies that focused on environmental contaminants, few WBE studies also investigated endogenous biomarkers related to human health, such as oxidative stress biomarkers (Ryu et al., 2015; Sims et al., 2019) or human-specific mitochondrial DNA (Yang et al., 2017). Their levels in wastewater may be associated with the exposure to harmful chemicals, including those from personal care and household products. However, most of the WBE studies performed so far, although quite promising, focus exclusively on one specific group, while there is no multiresidue analytical method with representatives of multiple compound classes. Moreover, previous studies have used off-line extraction techniques, mainly solid-phase extraction (SPE), while the potential of fully automated on-line techniques, which are faster and require minimal sample manipulation, has not yet been explored.

Therefore, the aim of this study is to develop a fully automated multiresidue method, based on on-line solid phase extraction and liquid chromatography – tandem mass spectrometry (LC-MS/MS), for the determination of 27 biomarkers of chemicals from different classes of personal care and household products, including parabens, UV filters, phthalates and alternative plasticizers, PFRs and bisphenols, as well as two oxidative stress biomarkers. The method applicability is demonstrated by determining the mass loads of selected compounds in four European cities, and assessing population exposure to the substances for which quantitative excretion data are known. To the authors' knowledge, this is the first WBE study to include major representatives of multiple chemical classes, and the first to investigate the applicability of a fully automated on-line SPE LC-MS/MS technique in the WBE context.

2. Materials and methods

2.1. Chemicals and materials

All target compounds are listed with their abbreviations in Table 1. MeP, EtP, PrP, BzP, TBOEP, TCIPP, BPS, EtP-$^{13}C_6$, and TPhP-d_{15} were purchased from Sigma-Aldrich (Germany), MeP-d_4 from CDN Isotopes, and MEHA from SynCan (Canada). PGF2α, PGF2α-d_4, 4-HNA, 4-HNA-d_3, and 3-OH-EtP-$^{13}C_3$ were obtained from Santa Cruz Biotechnology (Germany), while EHPhP and OH-DPhP were custom synthesized by Dr. Vladimir Belov (Max Planck Institute, Göttingen, Germany). All other target compounds were purchased from Toronto Research Chemicals (Canada). Individual stock solutions were prepared in either methanol or acetonitrile, at a concentration of 1–2 mg mL^{-1}, with the exception of MEHA (5 mg mL^{-1}), while few compounds were already purchased as solutions. These include EtP-$^{13}C_6$ (0.05 mg mL^{-1}), 4-HNA (10 mg mL^{-1}), PGF2α-d_4 (0.1 mg mL^{-1}), and 4-HNA-d_3 (0.5 mg mL^{-1}). All stock solutions were stored at −20 °C. Mixed intermediate solutions were prepared by diluting the stock solutions with methanol and stored at 4 °C. Standard solutions, used for calibration and model experiments, were prepared by diluting the intermediate solutions with 0.1 % acetic acid before each analytical batch.

Hypersil GOLD aQ (20 × 2.1 mm; 12 μm) HPLC column was purchased from CromLab (Spain), while Luna Omega C18 (100 × 2.1 mm; 1.6 μm) and Kinetex Biphenyl (50 × 2.1 mm; 2.6 μm) columns were purchased from Phenomenex (Spain), along with the corresponding pre-columns.

LC-MS grade solvents (water, methanol, and acetonitrile) were purchased from Fisher Scientific (Canada). Ultrapure water for the model experiments was prepared using the Milli-Q-Advantage system (Millipore Ibérica S.A., Spain). Formic acid (ACS reagent) was purchased from Merck, HPLC-grade acetic acid from Panreac Química (Spain), while ammonium acetate and ammonium fluoride were supplied by VWR Chemicals (Spain).

Glass fiber GF/B (47 mm; 1.0 μm) and syringe filters (25 mm; 0.45 μm) were purchased from Whatman (USA). Cellulose acetate filters (47 mm;

Table 1
List of chemicals and their human metabolites included in the study.

Class	Parent compound	Metabolite
Parabens	Methylparaben (MeP)	Methyl 3,4-dihydroxybenzoate (3-OH-MeP)
	Ethylparaben (EtP)	Ethyl 3,4-dihydroxybenzoate (3-OH-EtP)
	Propylparaben (PrP)	–
	n-Butylparaben (BuP)	–
	Benzylparaben (BzP)	–
UV filters	Benzophenone-3 (BP-3)	Benzophenone-1 (BP-1)[a]
Phthalates	*Diethyl phthalate (DEP)*[b]	Monoethyl phthalate (MEPH)
	Di-n-butylphthalate (DnBP)[b]	Mono-*n*-butyl phthalate (MnBP)
	Di-iso-butylphthalate (DiBP)[b]	Mono-*iso*-butyl phthalate (MiBP)
	Di(2-ethylhexyl) phthalate (DEHP)[b]	Mono(2-ethyl-5-hydroxyhexyl) phthalate (MEHHP)
	Di(2-ethylhexyl) terephthalate (DEHTP)[b]	Mono(2-ethyl-5-carboxypentyl) terephthalate (MECPTP)
Alternative plasticizers	*Diisononyl-cyclohexane-1,2-dicarboxylate (DINCH)*[b]	Cyclohexane-1,2-dicarboxylate-mono-(7-hydroxy-4-methyl) octyl ester (OH-MINCH)
	Bis(2-ethylhexyl) adipate (DEHA)[b]	Mono(2-ethylhexyl) adipate (MEHA)
Phosphorus flame retardants/plasticizers	2-Ethylhexyldiphenyl phosphate (EHDPhP)	2-Ethylhexyl phenyl phosphate (EHPhP)
	Tris(2-butoxyethyl) phosphate (TBOEP)	Bis(2-butoxyethyl) 2-hydroxyethyl phosphate (BBOEHEP)
	Triphenyl phosphate (TPhP)	Diphenyl phosphate (DPhP)[a]
		4-Hydroxyphenyl phenyl phosphate (OH-DPhP)
	Tris(2-chloroisopropyl) phosphate (TCIPP)	–
	Tris(2-chloroethyl) phosphate (TCEP)	–
Bisphenols	*Bisphenol A (BPA)*[b]	Bisphenol A sulfate (BPA-SO_4)
	Bisphenol S (BPS)	–
Oxidative stress biomarkers	–	8-epi-Prostaglandin F2α (PGF2α)
	–	4-Hydroxy nonenal mercapturic acid (4-HNA)

[a] Also used as parent compounds.
[b] Parent compounds in italic are not included in the analytical method.

0.45 μm) were purchased from Allcrom (Spain) and regenerated cellulose syringe filters (15 mm, 0.2 μm) from Phenomenex. All other filter types, tested during method development, including nylon (47 mm; 0.20 μm) and PVDF (47 mm; 0.45 μm) membrane filters, mixed cellulose ester filters (47 mm; 0.22 μm) and PVDF syringe filters (33 mm; 0.45 μm), were supplied by Sigma-Aldrich.

SPE cartridges (Oasis HLB and MAX), used for the development of an off-line SPE method, were purchased from Waters (USA).

2.2. Sample collection and preparation

Twenty-four-hour composite raw wastewater (RW) samples were collected at the inlet of wastewater treatment plants (WWTPs) in four European cities – Antwerp, Brussels (Belgium), Girona (Spain), and Zagreb (Croatia). Sampling campaigns were conducted on 7 consecutive days in spring 2021, except in Girona, where 5 samples were collected (the weekend was not included). Full details, including sampling dates, composite sample type, wastewater flow, and population served by the WWTPs, can be found in the Supplementary Material (Table S1). After collection, samples were immediately frozen and stored at −20 °C until analysis.

After thawing, 30-mL aliquots were transferred to Falcon tubes and centrifuged at 3500 rpm for 10 min. The supernatants (20 mL) were then collected and 10 μL of the internal standard mixture (1 ng μL^{-1}) was added. The samples (supernatants) were then acidified to pH 3 with acetic acid (approximately 200 μL for 20-mL supernatants) and filtered through the regenerated cellulose filters for further sample processing (on-line SPE) and instrumental analysis (LC-MS/MS), which is discussed in Section 2.3.1. The step-by-step sample processing procedure is presented in the Supplementary Material.

Off-line SPE performance was tested using drinking water spiked with target compounds at a concentration of 1 μg L^{-1} ($n = 4$). Oasis HLB cartridges were tested at the initial sample pH (~ pH 7) and at pH 3 (acidified with formic acid), while MAX cartridges were tested only at the initial pH. Both cartridges were conditioned with 6 mL of methanol and 6 mL of ultrapure water, while 6 mL of 0.1 % formic acid was additionally added on HLB cartridges when acidified samples were extracted. 100-mL samples were passed through the cartridges at a flow rate of approximately 5 mL min^{-1}. After washing with 6 mL of ultrapure water and drying under vacuum for 30 min, cartridges were eluted with 5 mL of methanol. MAX cartridges were additionally eluted with 5 mL of 2 % formic acid in methanol and the extracts from these cartridges were combined. The extracts were then evaporated to dryness and the residue was dissolved in 1 mL of 0.1 % acetic acid for instrumental analysis.

2.3. Instrumental analysis

2.3.1. On-line solid-phase extraction and chromatographic separation

The final method, used for method validation, model experiments, and analysis of real samples, employed on-line SPE ultrahigh-performance liquid chromatography – tandem mass spectrometry (UHPLC-MS/MS). In the preliminary experiments, as well as in the experiments to determine the relative recovery of the on-line SPE method, off-line SPE – UHPLC-MS/MS was also used. On-line preconcentration and chromatographic separation were performed automatically using the EQuan MAX™ system, consisting of a PAL autosampler (CTC Analysis) and two quaternary pumps: a loading pump (Accela™ 600) and an eluting pump (Accela™ 1250), both from Thermo Fisher Scientific (USA), connected to a TSQ Vantage triple quadrupole mass spectrometer, equipped with an electrospray ionization source (Thermo Fisher Scientific). Two LC columns were used for the on-line SPE UHPLC-MS/MS method: Hypersil GOLD aQ (20 × 2.1 mm; 12 μm) for analyte preconcentration and Kinetex Biphenyl (50 × 2.1 mm; 2.6 μm) for chromatographic separation. A Luna Omega C18 column (100 × 2.1 mm; 1.7 μm) was also tested in the preliminary experiments for separation of the target compounds. During method development, different solvents and gradient programs were tested for both preconcentration and separation. In the final method, LC–MS grade water and methanol were used for the preconcentration step. Preconcentration and separation columns were connected via a three-valve switching device unit with a six-port valve, used to control the loading and elution of the columns. During the loading step, the sample (1 mL) was transferred to the preconcentration column at a flow rate of 1.2 mL min^{-1}. After 60 s, when this process was completed, the switching valve was activated, which started the transfer of analytes from the preconcentration column to the separation column. This step took 300 s and 360 s for the target compounds analyzed in negative and positive ionization polarity, respectively. Upon completion of the transfer, the preconcentration column was rinsed at the lower flow rate (0.4 mL min^{-1}) and then returned to the initial conditions for the next sample. Further details on the EQuan MAX™ system can be found elsewhere (Gorga et al., 2013).

The flow rate through the separation column was maintained at 0.4 mL min^{-1}. For most analytes, optimal separation was achieved using 0.1 % acetic acid and methanol as eluents. These compounds were analyzed by Method 1 (negative ionization polarity) and Method 3 (positive ionization polarity), while some compounds ionized in negative polarity were analyzed by Method 2, using ultrapure water and methanol as eluents. The total analysis time, including analyte loading, transfer, and separation, as well as columns cleaning and re-equilibration, was 18 min for Methods 1 and 2, and 15 min for Method 3. The on-line SPE – LC conditions for all three methods can be found in Table 2. Method parameters are also listed in Supplementary Material (Tables S3–S5).

2.3.2. Mass spectrometry

Both negative and positive ionization polarities were used for target compound analysis. The spray voltage was 3500 V in positive and 2500 V in negative ionization polarity, while the other parameters were the same in both ionization modes. The capillary temperature was 300 °C, the vaporizer temperature 350 °C, while the sheath, auxiliary, and ion sweep gas pressures were 40, 20, and 0.5 arbitrary units, respectively. Detection and quantification were performed in multiple reaction monitoring (MRM) mode. Two characteristic transitions were selected for each analyte, while one transition was used for internal standards. The precursor ions were (de)protonated molecular ions, i.e., $[M + H]^+$ or $[M - H]^-$, while the two most abundant fragments (one in the case of internal standards) were generally selected as product ions. Quantification was based on the first transition, while the second transition was used for confirmation along with the ratio of the two transitions. Collision energy and S-lens offset were optimized for each target compound individually by infusing their standard solutions at a concentration of 4 μg mL^{-1}. The selected transitions and the optimized S-lens offset and collision energies, are listed in Table 3, along with the method used for each compound.

2.4. Method validation

Method validation included several parameters evaluated with either ultrapure water or real RW matrix. Instrumental parameters, including linearity, instrumental detection limit (IDL), intraday and interday repeatability, as well as relative recovery of the on-line SPE method, were determined using ultrapure water as the matrix. Method parameters, including method detection limit (MDL), method quantification limit (MQL), process efficiency, trueness, and precision were evaluated using RW.

Linearity was determined from 10-point internal standard calibration curves obtained by injecting standard solutions, prepared in a solvent (0.1 % acetic acid), containing analytes in a concentration range of 5 ng L^{-1} to 5 μg L^{-1} and internal standards at a fixed concentration of 0.5 μg L^{-1}.

IDLs were assessed from the lowest point of the calibration curve. They were calculated as concentrations giving a signal-to-noise ratio (S/N) of 3. MDLs and MQLs were determined as concentrations of analytes in RW giving a *S/N* of 3 and 10, respectively. For the compounds that were present in real samples, MDLs and MQLs were estimated based on their *S/N* in these samples. For the compounds that could not be detected in any of the analyzed samples, they were determined by spiking RW samples with low analyte concentrations.

Relative recovery, process efficiency, trueness, precision, as well as intraday and interday repeatability, were determined in the model experiments performed in quadruplicate. The instrumental parameters (relative recovery, intraday and interday repeatability) were determined at two concentration levels – 0.1 μg L^{-1} and 1 μg L^{-1}, while the method parameters (process efficiency, trueness, and precision) were determined at a concentration level of 1 μg L^{-1}. The individual concentration of the internal standards was 0.5 μg L^{-1} in all experiments.

Intraday and interday repeatability, expressed as relative standard deviation (RSD), were determined by analyzing the mixture containing the target compounds (analytes and internal standards) on the same day (intraday repeatability) and on four consecutive days (interday repeatability).

Relative SPE recovery evaluates the performance of the on-line SPE methods, i.e., the efficiency of analyte transfer to the on-line system (Farré et al., 2016; Čelić et al., 2017). It was determined by injecting the same amount of target compounds in ultrapure water (1 and 0.1 ng of analytes and 0.5 ng of internal standards) onto the analytical column using the off-line and on-line LC-MS/MS methods. Relative recovery was then calculated using the following equation:

$$\text{Relative SPE recovery } (\%) : [(A_{on-line}/AIS_{on-line})/(A_{off-line}/AIS_{off-line})]^* 100$$

where $A_{on\text{-}line}$ and $A_{off\text{-}line}$ represent the analyte responses in on-line and off-line modes, respectively, while $AIS_{on\text{-}line}$ and $AIS_{off\text{-}line}$ represent the internal standard responses in the two modes.

Process efficiency was determined by comparing the average response of the analytes in spiked RW (A_{spiked}) with their average response in the standard solution of the same concentration (A_{std}), taking into account the analytes already present in the original RW sample (A_{orig}):

$$\text{Process efficiency } (\%) = \left[\left(A_{spiked} - A_{orig}\right)/A_{std}\right]^* 100$$

Method trueness was determined using the following equation:

$$\text{Trueness } (\%) = [(c_2 - c_1)/c_0]^* 100$$

Table 2
On-line SPE – LC conditions.

Time (min)	Method 1 + Method 2			Time (min)	Method 1		Method 2		Method 3						
	Pump 1: load				*Pump 2: elute*[a]		*Pump 2: elute*[a]		Time (min)	*Pump 1: load*			Time (min)	*Pump 2: elute*[a]	
	Flow (mL min^{-1})	Solvent A (%)	Solvent C (%)		Solvent B (%)	Solvent C (%)	Solvent A (%)	Solvent C (%)		Flow (mL min^{-1})	Solvent A (%)	Solvent C (%)		Solvent B (%)	Solvent C (%)
	1.2	95	5	0.0	70	30	80	20	0.0	1.2	80	20	0.0	60	40
0.0															
	1.2	95	5	1.5	70	30	80	20	1.0	1.2	80	20	1.0	60	40
1.0															
	0.4	50	50	12.0	30	70	30	70	2.5	0.4	50	50	11.0	0	100
2.5															
	0.4	0	100	14.0	0	100	0	100	5.0	0.4	0	100	13.0	0	100
5.0															
	0.4	0	100	16.0	0	100	0	100	11.0	0.4	0	100	13.5	60	40
15.0															
	0.8	95	5	16.5	70	30	80	20	13.5	0.8	80	20	15.0	60	40
16.0															
	1.2	95	5	18.0	70	30	80	20	15.0	1.2	80	20	–	–	–
18.0															

[a] Flow rate of the pump 2 was 0.4 mL min^{-1} during the whole chromatographic run; Solvent A: water; Solvent B: 0.1 % acetic acid; Solvent C: methanol.

Table 3
MRM parameters and method used for the determination of target compounds included in the study.

Analyte	Internal standard	Polarity	Precursor ion (*m/z*)	S-Lens	Product ion 1 (*m/z*)	CE 1 (eV)	Product ion 2 (*m/z*)	CE 2 (eV)	Method
MeP	MeP-d_4	−	151.1	70	92.0	23	136.0	16	1
3-OH-MeP	3-OH-EtP-$^{13}C_3$	−	167.1	67	108.1	22	152.1	16	1
EtP	EtP-d_4	−	165.0	78	92.1	24	137.1	16	1
3-OH-EtP	3-OH-EtP-$^{13}C_3$	−	181.0	75	108.1	23	153.1	17	1
PrP	EtP-d_4	−	179.0	87	92.1	24	136.1	17	1
BuP	EtP-d_4	−	193.1	77	92.1	26	136.1	19	1
BzP	EtP-d_4	−	227.0	88	92.1	25	136.1	16	1
BP-3	BP-3-d_3	+	229.1	81	151.0	19	77.1	34	3
BP-1	BP-1-d_5	+	215.1	75	137.0	19	81.1	33	3
MEPH	MEPH-d_4	−	193.1	47	77.2	19	121.1	14	2
MnBP	MEHHP-d_4	−	221.1	48	77.2	18	177.1	12	1
MiBP	MEHHP-d_4	−	221.1	48	77.2	18	134.1	15	1
MEHHP	MEHHP-d_4	−	293.1	76	145.2	16	121.1	21	1
MECPTP	MECPTP-d_4	−	307.1	76	165.0	16	121.0	26	1
OH-MINCH	OH-MINCH-d_8	−	313.2	77	153.1	18	109.2	29	2
MEHA	OH-MINCH-d_8	−	257.1	67	83.2	17	127.1	13	2
EHDPhP	TPhP-d_{15}	+	363.2	49	251.0	14	77.1	40	3
EHPhP	–[a]	−	285.1	108	93.2	42	79.1	26	1
TBOEP	TPhP-d_{15}	+	399.0	121	199.0	13	299.2	10	3
BBOHEP	TPhP-d_{15}	+	343.2	75	243.1	11	99.0	32	3
TPhP	TPhP-d_{15}	+	327.1	137	152.0	34	77.1	36	3
DPhP	DPhP-d_{10}	−	248.9	103	93.1	38	155.0	23	2
OH-DPhP	DPhP-d_{10}	−	265.0	112	93.2	41	108.1	46	2
TCIPP	TPhP-d_{15}	+	327.0	87	98.9	26	80.9	55	3
TCEP	TPhP-d_{15}	+	287.0	89	98.9	23	225.0	12	3
BPA-SO_4	BPA-SO_4-d_6	−	307.0	93	227.2	26	212.2	33	1
BPS	BPS-d_8	−	249.0	92	108.0	29	92.0	40	1
PGF2α	PGF2α-d_4	−	353.2	110	193.2	27	291.0	22	1
4-HNA	4-HNA-d_3	−	318.1	48	171.1	22	189.1	15	2
MeP-d_4	–	−	154.9	70	96.0	28	–	–	1
3-OH-EtP-$^{13}C_3$	–	−	184.0	79	108.0	24	–	–	1
EtP-$^{13}C_6$	–	−	171.0	77	98.0	25	–	–	1
BP-3-d_3	–	+	232.1	95	154.0	17	–	–	3
BP-1-d_5	–	+	220.1	81	137.0	20	–	–	3
MEPH-d_4	–	−	197.1	49	81.2	19	–	–	2
MEHHP-d_4	–	−	297.2	76	145.2	16	–	–	1
OH-MINCH-d_8	–	−	321.3	74	161.2	19	–	–	2
MECPTP-d_4	–	−	311.2	78	169.0	16	–	–	1
TPhP-d_{15}	–	+	342.2	137	160.1	43	–	–	3
DPhP-d_{10}	–	−	259.1	102	98.1	35	–	–	2
BPA-SO_4-d_6	–	−	313.2	83	233.1	26	–	–	1
BPS-d_8	–	−	257.0	103	112.1	34	–	–	1
PGF2α-d_4	–	−	357.2	108	197.2	27	–	–	1
4-HNA-d_3	–	−	321.1	53	174.1	22	–	–	2

[a] External calibration

where c_0, c_1 and c_2 represent the nominal spiked concentration, the average concentration measured in the original (non-spiked) RW sample, and the average concentration measured in the spiked RW sample, respectively. Method precision (repeatability) was determined in the same experiment by calculating the relative standard deviation (RSD) of the analysis of spiked samples.

2.5. Filtration losses

Filtration losses were determined in a model experiment, performed in duplicate, with ultrapure water spiked with analytes (1 μg L^{-1}), filtered through different filter types (including 47 mm filters and syringe filters), and then analyzed using the developed analytical methods.

2.6. Analyte stability

Analyte stability was determined in two separate experiments performed in duplicate. In the first experiment, the RW sample was spiked only with the target metabolites (2 μg L^{-1}) and stored in the dark at 4 °C and room temperature (~22 °C). Aliquots were taken immediately after spiking (T_0) and after 2, 4, 6, 8, and 24 h, and analyzed by the developed methods. The setup of the second experiment was the same, but this time RW was spiked only with the parent compounds.

2.7. Estimation of human exposure

Population-normalized mass loads of the target compounds were calculated by multiplying their concentrations by the daily wastewater flow rates and dividing by the population served by the WWTPs:

$$\text{Mass load}\left(\mu g\ day^{-1}\ inh^{-1}\right) = \left[c\left(\mu g\ L^{-1}\right) x\ \text{flow rate}\left(L\ day^{-1}\right)\right]/\text{number of inhabitants}$$

Exposure was then assessed for the compounds with known quantitative excretion data (i.e., excretion fractions), using the correction factors (CFs) listed in Table S2:

$$\text{Exposure}\left(\mu g\ day^{-1}\ inh^{-1}\right) = \text{mass load}\left(\mu g\ day^{-1}\ inh^{-1}\right) x\ CF$$

$$CF = \left(M_{parent}/M_{metabolite}\right)/\text{molarexcretion fraction}$$

where M_{parent} / $M_{metabolite}$ is the molar ratio of parent compound and metabolite, while the molar excretion factor is the (weighted) average molar fraction of the parent compound excreted in the form of the target metabolite. For phthalates and DEHTP, the average molar excretion factors have already been determined in previous WBE studies (Estévez-Danta et al., 2021; González-Mariño et al., 2017). For the TBOEP and BPA, they

were calculated considering the excretion fractions determined in human metabolism studies (Table S2).

3. Results

3.1. Method development and optimization

During initial method development, a Luna C18 HPLC column was used to separate the target compounds. However, this column could not completely separate the two isomers of monobutyl phthalate (MnBP and MiBP), which separation was achieved on a Kinetex Biphenyl column. Since the separation of the other analytes was quite similar on both columns, the Kinetex Biphenyl was selected as the analytical column in the final method. This column was also used in the offline SPE LC-MS/MS method. It is noteworthy that a pre-column should always be used when analyzing wastewater samples. This is important to avoid deterioration of the peak shapes and possible clogging of the analytical column, which was occasionally observed, even with the filtered samples.

Methanol was used as the organic modifier because chromatographic separation was better compared with acetonitrile. Several mobile phase additives (formic acid, acetic acid, ammonium acetate, ammonium fluoride) were also tested and the optimal results were obtained with acetic acid. The addition of acid to the mobile phase is very common in LC-MS, to enhance the sensitivity of analytes ionized in positive polarity. Indeed, signal intensity of these compounds increased up to five times when 0.1 % acetic acid was used instead of ultrapure water. The acidified mobile phase also improved the separation and peak shapes of several target compounds analyzed in negative ionization polarity, such as phthalate monoesters (MnBP, MiBP and MEHHP), EHPhP, MECPTP, and BPA-SO_4. However, at the same time, the addition of acid led to a decrease in signal intensity in negative polarity, as well as a deterioration of the peaks for some compounds, especially DPhP and OH-DPhP. Since it was not feasible to develop a single method that would allow reliable simultaneous determination of all target compounds, with rather different physico-chemical properties, it was decided to use two separate methods for the analysis of compounds ionized in negative polarity. The MRM chromatogram of the standard mixture, under the final chromatographic conditions for all three methods, is presented in Supplementary Material (Fig. S1).

With respect to sample concentration, the performance of off-line SPE was investigated in the initial phase of method development, using two types of SPE cartridges – HLB and MAX. The sample volume and the volume of the final extract were 100 mL and 1 mL, respectively. Therefore, injection of 10 μL of extract is equivalent to direct injection of 1 mL of sample. HLB was tested because this polymeric reversed-phase sorbent can extract a wide range of organic compounds with a fairly simple, generic protocol, while MAX, a mixed-mode anion exchange polymeric sorbent with higher selectivity for acidic compounds, was tested because most target substances have acidic properties. In addition, the MAX sorbent has already been successfully used for the extraction of several compounds included in this study, such as metabolites of phthalates and alternative plasticizers (Estévez-Danta et al., 2021; González-Mariño et al., 2017). HLB cartridges were tested without pH adjustment, which is common for this type of sorbent, and at pH 3, to investigate whether the retention of acidic compounds could be improved by the reversed-phase mechanism. MAX cartridges were tested only at initial pH (~7.5) because they can retain acidic compounds without pH adjustment. Recovery of several analytes, including parent parabens, most phthalates (MnBP, MiBP, MEHHP), TBOEP, BBOEHEP, BP-1, OH-MINCH, MEHA, and BPS was generally high (>70 %) with all protocols used. However, this was not the case for the remaining compounds (Fig. S2), and none of the tested procedures were able to extract all target compounds simultaneously. Therefore, to avoid the use of multiple extraction protocols, which would be cumbersome and time consuming, the next step was to test the on-line LC-MS/MS.

The Hypersil GOLD aQ column was used as a preconcentration column for the on-line LC-MS/MS method. This column was selected because it has already been used to retain some endocrine disrupting chemicals included in this study, such as parent parabens and PFRs (Gorga et al., 2013). To achieve high recoveries, several parameters had to be optimized during method development, including sample pH and volume, mobile phase composition and flow rates, transfer time, and elution time.

As for the mobile phase composition during sample loading (preconcentration), different combinations of water/methanol and 0.1 % acetic acid/methanol were tested. In the final methods, water/methanol mixtures were used: 95/5 (*v/v*) in negative (Methods 1 and 2) and 80/20 (*v/v*) in positive ionization polarity (Method 3). The flow rate was set at 1.2 mL min^{-1}, and the transfer time of 60 s allowed complete transfer of 1-mL sample from the loop to the preconcentration column. During sample transfer from the preconcentration column to the analytical column, the methanol percentage was gradually increased to elute less polar analytes (Table 2). Complete elution of all target compounds was achieved in 300 s for Methods 1 and 2, and in 360 s for Method 3.

Preliminary experiments with ultrapure water and wastewater showed that the signal intensity of several analytes was higher at pH 3 than at the initial pH (~7). Therefore, all samples were acidified to pH 3 prior to injection. In the initial phase of method development, the sample volume was set at 2 mL. However, when RW samples were injected, the signal intensity of most analytes decreased substantially, which could be due to lower extraction recovery and/or higher matrix effect in RW. However, in on-line SPE, the matrix effect cannot be evaluated separately, which is why process efficiency, a parameter combining analyte recovery and matrix effect, is determined (Farré et al., 2016). For some of the compounds included in this study, the process efficiency was even lower than 5 %, which is probably related to the extremely high matrix effect in RW. Indeed, when lower sample volumes were injected (0.5–1.5 mL), the process efficiency increased substantially. However, reducing the injection volume affects the method sensitivity, which was already relatively low for some compounds. Therefore, as a compromise, 1 mL was finally selected as the injection volume. Since the method efficiency was still low for several compounds, other ways to improve it were also investigated. For example, the elution time can also influence the matrix effect (Čelić et al., 2017). However, in our study, this influence was negligible. Another possibility investigated was to increase the overall analysis time by employing a slower gradient program, which would reduce the amount of interferences co-eluting with the target substances. Indeed, this approach notably increased the process efficiency, although it still remained relatively low for several compounds.

3.2. Filtration losses

In the initial phase of method development, the performance of 47 mm filters made of different materials was tested. The results, presented in Fig. S3, indicate that filtration losses were very high for some compounds (up to 100 %), especially on nylon filters. Losses on cellulose filters were generally lower, but still above 90 % for EHDPhP, BP-3, and TPhP. Although glass-fiber and PVDF filters performed better, the loss of EHDPhP was still high, especially on PVDF filters (96 %). Furthermore, the signal intensity of some compounds, such as 3-OH-MeP and 3-OH-EtP, unexpectedly increased after filtration. Therefore, to avoid the problems with filtration losses and signal variability, it was decided to omit the first filtration step and perform centrifugation instead.

However, the final filtration step (before sample injection) could not be avoided because residual particles could clog the preconcentration and/or analytical column. Considering the results of the filtration experiment with 47 mm filters, three types of syringe filters were tested. Although losses were generally lower with syringe filters than with 47 mm filters, for few compounds they were still ≥70 % in some cases (Fig. S4). The most problematic compound was again EHDPhP, with losses of 99 % on both glass-fiber and PVDF filters, while losses on regenerated cellulose syringe filters were lowest for almost all analytes. In fact, only three compounds had losses above 10 % on these filters – BuP (13 %), TPhP (20 %), and EHDPhP (45 %). Therefore, in the final analytical procedure, all samples were filtered using regenerated cellulose syringe filters prior to injection.

3.3. Method validation

The method validation parameters are listed in Table 4. Method linearity was acceptable in all cases, with correlation coefficients (r^2) above 0.99. The IDLs, determined in the solvent, were in the low ng L^{-1} range (mostly <5 ng L^{-1}). The relative recovery of the on-line SPE method was generally between 80 % and 120 % at both concentration levels (1 μg L^{-1} and 0.1 μg L^{-1}). In a few cases, the recovery was outside this range, mostly due to the use of non-ideal internal standards for some compounds. Intraday repeatability was always ≤10 %, while interday repeatability was ≤20 % in almost all cases.

For parameters determined in the real matrix (RW), process efficiency was relatively low (<50 %) for several compounds. As mentioned earlier, this was mainly due to the pronounced matrix effect. However, the method trueness was acceptable in most cases, except for BzP (25 %), TCIPP (16 %), and TBOEP (158 %), which is again a consequence of the use of non-ideal internal standards. Therefore, the results for these compounds should be considered semi-quantitative. In contrast, the method precision was very good (≤5 %) for all analytes.

MDLs were generally in the low ng L^{-1} range. However, there were few exceptions with MDLs between 10 and 30 ng L^{-1}, while MQLs ranged from 0.7 to 100 ng L^{-1}. Although these values are generally higher than in some previous WBE studies using off-line SPE for the determination of biomarkers of phthalates (González-Mariño et al., 2017), PFRs (Been et al., 2017; Castro et al., 2019), and plasticizers (Estévez-Danta et al., 2021), they were still acceptable in most cases because the concentrations of most target compounds were well above the MQLs, allowing their reliable determination.

3.4. Analyte stability

The stability of target biomarkers is an important issue in WBE. Stability experiments were performed separately for the parent compounds and metabolites, to minimize the influence of possible formation of metabolites from parent compounds in the RW (although this could not be completely excluded because some parent compounds were already present in the original RW sample). The temperature of 4 °C was selected as the usual temperature during the collection of 24-h composite wastewater samples. Stability at ~22 °C was assessed to determine if the samples could be collected/stored at room temperature.

The results of the first stability experiment suggest that the target compounds are fairly stable at 4 °C in RW (Fig. S5). The residual percentage after 24 h was mostly ≥75 %, except for the parent parabens, which showed a clear decreasing trend depending on the size of the alkyl group (MeP – 75 %; EtP – 73 %; PrP – 67 %; BuP – 57 %; BzP – 41). Therefore, 24-h composite sampling at 4 °C seems to be suitable for most analytes.

The lowest stability at room temperature was again observed for the parent parabens (Fig. S6). For example, the residual percentage of BuP and BzP after 24 h was only 22 % and 3 %, respectively. However, the stability of several other biomarkers, including phthalate monoesters, MECPTP, MEHA, EHDPhP, and 4-HNA, was also lower than at 4 °C. Although prolonged storage at room temperature should be avoided, degradation of most biomarkers in wastewater is not very extensive (<15 %) up to 8 h.

These results are generally consistent with stability data reported in previous WBE studies (Been et al., 2017; Estévez-Danta et al., 2021; González-Mariño et al., 2017). The most notable exception is 4-HNA. The stability of this oxidative stress biomarker at room temperature was lower than in the previous study at a slightly lower temperature of 17 °C (Sims et al., 2019). Interestingly, our study confirmed the findings of Been et al., who reported a greater decrease in analyte response for OH-DPhP at 4 °C compared with 20 °C (Been et al., 2017). However, that study also suggested that PFR metabolites are not extensively formed from the corresponding parent substances. Yet, in our study, a concentration increase was observed for some metabolites in RW spiked with the parent compounds,

Table 4
Method validation parameters.

Compound	Instrumental parameters (ultrapure water)								Method parameters (wastewater)				
	Linearity (r^2)	IDL (ng L^{-1})	Relative recovery (%)		Repeatability (%)				MDL (ng L^{-1})	MQL (ng L^{-1})	1 μg L^{-1}		
					Intraday		Interday				Process efficiency (%)	Trueness (%)	Precision (%)
			0.1 μg L^{-1}	1 μg L^{-1}	0.1 μg L^{-1}	1 μg L^{-1}	0.1 μg L^{-1}	1 μg L^{-1}					
MeP	0.9991	4	95	110	3	1	14	8	30	100	44	125	3
3-OH-MeP	0.9983	6	84	92	12	4	2	9	20	60	26	72	4
EtP	0.9986	1	90	95	3	1	7	8	10	30	40	83	1
3-OH-EtP	0.9988	4	83	98	7	2	6	8	5	20	74	95	3
PrP	0.9984	1	109	115	1	1	8	8	3	10	49	102	1
BuP	0.9976	0.8	60	74	8	2	20	11	2	5	38	86	5
BzP	0.9983	0.3	83	88	2	1	9	6	1	3	11	25	2
BP-3	0.9979	0.5	108	89	3	1	10	10	1	3	25	88	2
BP-1	0.9989	1	99	93	2	1	7	9	3	10	22	72	1
MEPH	0.9972	2	117	120	4	6	10	10	15	50	26	88	4
MnBP	0.9973	3	114	109	6	2	9	12	3	10	61	82	3
MiBP	0.9983	3	110	106	3	1	9	13	4	12	64	85	3
MEHHP	0.9972	5	84	115	3	1	14	9	6	20	65	88	4
MECPTP	0.9986	0.4	93	104	2	1	10	7	1	4	30	98	1
OH-MINCH	0.9987	0.1	94	104	1	2	10	11	10	35	31	100	3
MEHA	0.9941	0.1	97	91	5	2	5	10	0.2	0.7	28	94	3
EHDPhP	0.9906	1	57	61	4	2	23	26	3	10	27	66	3
EHPhP	0.9974	0.2	93	89	4	1	3	4	0.3	1	103	103	1
TBOEP	0.9978	0.08	100	109	2	2	16	22	5	20	80	158	1
BBOHEP	0.9987	0.8	127	82	2	1	15	13	6	20	49	131	1
TPhP	0.9988	0.3	108	91	3	2	16	7	0.5	2	28	73	1
DPhP	0.9981	0.03	104	107	3	4	7	10	0.3	1	152	117	1
OH-DPhP	0.9974	0.1	102	111	5	10	10	5	5	20	66	51	3
TCIPP	0.9941	1	66	134	3	1	17	8	20	70	10	16	2
TCEP	0.9956	8	155	118	1	2	16	9	10	40	30	79	5
BPA-SO4	0.9983	0.4	88	98	1	1	11	7	3	10	113	81	2
BPS	0.9988	1	91	102	2	1	8	8	20	60	34	87	1
PGF2α	0.9994	5	100	109	4	1	10	4	15	60	28	100	5
4-HNA	0.9987	0.6	96	103	5	4	4	14	10	30	89	92	2

especially at room temperature. These include EHPhP (from EHDPhP) and DPhP (from TPhP and possibly other PFRs), as well as BPA-SO_4 (from BPA present in the original sample).

Finally, it should be pointed out that these stability experiments were not designed to mimic conditions in real sewer systems, which are more complex and include the effects of biofilms. In fact, a recent study by He et al. suggests that substantial hydrolysis of parent phthalates to their monoesters, as well as further degradation of phthalate monoesters, occurs in sewers, even in the absence of biofilms (He et al., 2021). For example, in addition to human phase I metabolism, MnBP can also be formed from DnBP by bacteria, such as *Agrobacterium* sp. strain JDC-49 (Wu et al., 2011). Therefore, (bio)degradation in the sewer system may affect exposure estimates and increase the overall uncertainty of the WBE approach for assessing exposure to phthalates (and possibly some other substances).

3.5. Occurrence of target compounds in wastewater

Twenty-one biomarkers (10 parent compounds and 11 metabolites) were detected in all analyzed wastewater samples, while the phthalate metabolite MEHHP was also regularly detected, except in the samples collected in Antwerp. Their concentrations can be found in Tables S6 and S7, while the population-normalized mass loads are presented in Fig. 1.

The mass loads of the parent parabens decreased in the following order: MeP > EtP > PrP > BuP, while BzP was not detected in any sample. These values reflect their use, but also recent restrictions in the EU, including the ban of BzP from cosmetic products sold on the EU market and additional limitations for PrP and BuP (European Commission, 2014). Nevertheless, the mass loads of parent parabens determined in this study were generally higher than those determined in the U.S. (Wang and Kannan, 2016) and China (Karthikraj et al., 2017), although these studies included analytes present in suspended particulate matter. Protocatechuates 3-OH-MeP and 3-OH-EtP, metabolites of MeP and EtP, were not detected, although some studies reported that their mass loads, in both urine (Wang and Kannan, 2013) and wastewater (Karthikraj et al., 2017; Wang and Kannan, 2016) may be similar or even higher than those of the corresponding parent parabens (especially in the case of EtP). However, this is not consistent with the results of the human metabolism study (Moos et al., 2016), which suggests that the molar excretion fraction of parent MeP (16.8 %) is much higher than that of 3-OH-MeP (only 0.1 %). These conflicting results are difficult to explain and should be addressed in future studies.

The mass loads of parabens were comparable in all 4 cities. In fact, their average total loads were similar in Antwerp, Brussels, and Zagreb (417–461 μg day^{-1} inh^{-1}). Only in Girona the average total load was notably higher (716 μg day^{-1} inh^{-1}). This is mostly due to MeP loads, which were approximately 2.5 times higher than in the other cities. This could be related to the fact that the samples from Girona were collected in late spring (end of May), when the use of sunscreens could be higher. Indeed, the mass loads of UV filters, especially BP-1, were notably higher in Girona than in the other cities. In general, their mass loads were comparable with those determined in the U.S. (Wang and Kannan, 2017) and Australia (O'Malley et al., 2019).

The mass loads of phthalate metabolites were lower than those determined in Australia (Tang et al., 2020). However, their concentrations were in very good agreement with those reported for 13 Spanish cities (González-Mariño et al., 2021). Mass loads of phthalate metabolites were consistently lower in Antwerp than in the other three cities included in this study. In all cities, the mass loads of MEPH were the highest and those of MEHHP the lowest. Interesting results were obtained for MECPTP and MEHA, metabolites of the alternative plasticizers DEHTP

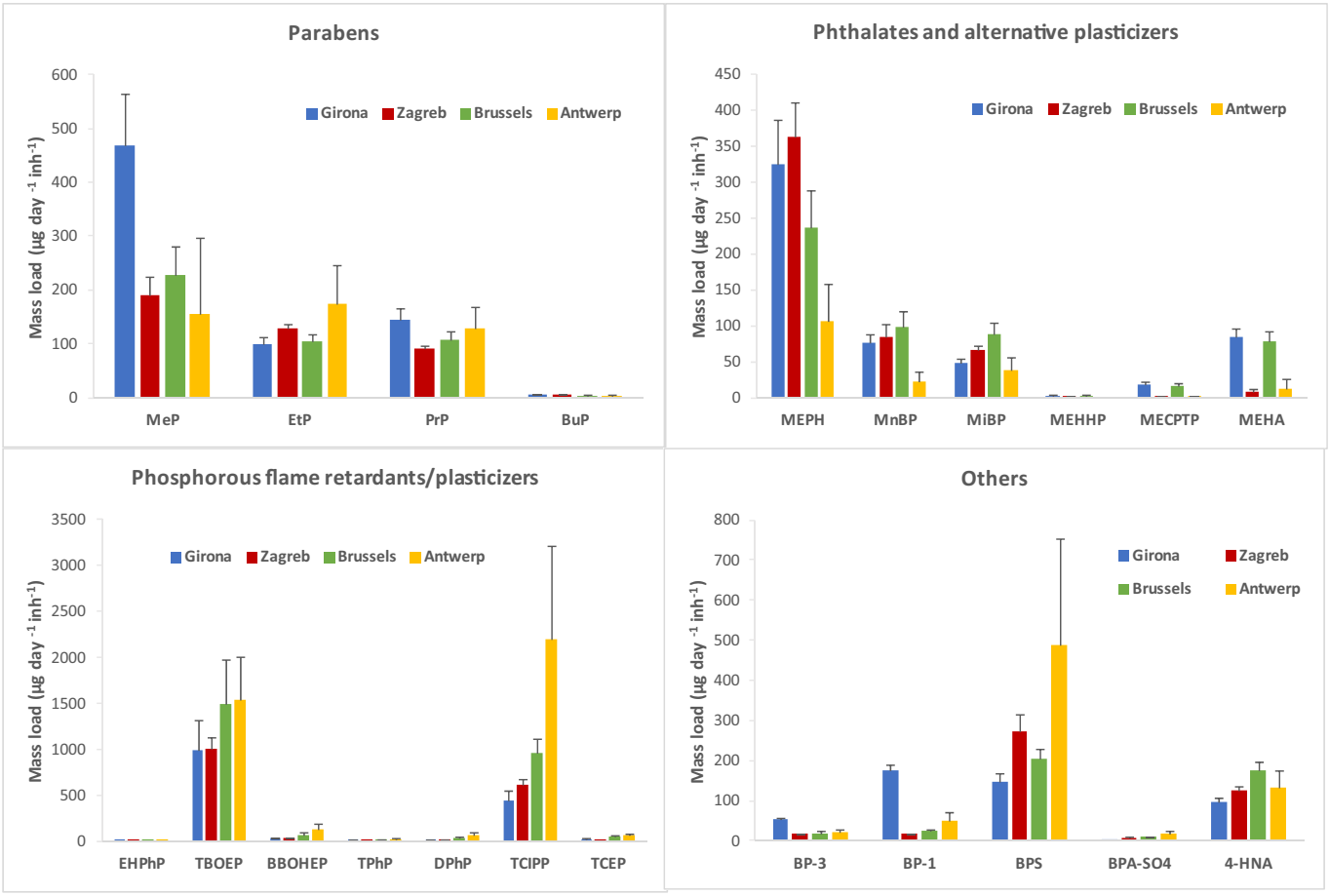

Fig. 1. The population-normalized mass loads of the target compounds in RW of the selected cities.

and DEHA, respectively. Their average mass loads were approximately an order of magnitude higher in Girona and Brussels than in Antwerp and Zagreb, indicating substantial differences in population exposure. MECPTP concentrations were lower than those determined in Santiago de Compostela (Spain) (Estévez-Danta et al., 2021), while, to our knowledge, MEHA was detected in wastewater for the first time.

The mass loads of the parent PFRs were similar to those determined in Australia (O'Brien et al., 2015), but lower than in the U.S. study (Kim et al., 2017). Mass loads of DPhP were similar and mass loads of BBOEHEP slightly lower than in the WBE studies by Been et al. (Been et al., 2017, 2018), while OH-DPhP was not detected, which is also consistent with these studies. However, the average mass loads of EHPhP in our study (0.7–3.9 μg day^{-1} inh^{-1}) were consistently lower, even in Antwerp and Brussels, which were included in one of the studies (Been et al., 2018). Decreasing mass loads of EHPhP were already observed in Antwerp between 2013 and 2016, suggesting possible changes in exposure to EHDPhP. This parent PFR was also included in our study, but could not be detected in any sample. However, as pointed out by Been et al., 2018, EHPhP might have other sources (apart from human excretion), which could also explain the discrepancy between studies. In almost all cases, mass loads of PFRs (both parent compounds and metabolites) were higher in Belgian cities, especially Antwerp, suggesting higher exposure to PFRs.

The mass loads of BPS were an order of magnitude higher than in the U.S. (Xue and Kannan, 2019). Its concentrations were also higher than in wastewater of Slovenian cities in most cases (Česen et al., 2018). Unfortunately, the human metabolism of BPS is not fully elucidated (Mas et al., 2021), and not all reference standards of its metabolites are commercially available. Concentrations of BPA-SO_4, a metabolite of BPA, were much lower than in the study by Lopardo et al. (Lopardo et al., 2019). These results may indicate a gradual replacement of BPA by analogous bisphenols (Gramec Skledar and Peterlin Mašič, 2016). The highest mass loads of both BPS and BPA-SO_4 were determined in Brussels, while their loads were lowest in Girona.

As for oxidative stress biomarkers, PGF2α could not be detected in any sample, which could be explained by the significant presence of its glucuronide conjugate in wastewater. In most cases, glucuronide conjugates rapidly hydrolyze in wastewater by the action of β-glucuronidase enzymes of fecal bacteria, and, therefore, a deconjugation step is usually omitted (Been et al., 2017; González-Mariño et al., 2017). However, it seems that β-glucuronidase treatment should be included in the sample preparation step for PGF2α analysis (Ryu et al., 2015). Even then, the total concentration of this biomarker in wastewater of that study (around 20 ng L^{-1}) would likely be lower than its MQL in the present study. Ryu et al. were able to lower MQLs by using immunoaffinity clean-up to remove interfering components in the sample matrix. However, these specific procedures are generally incompatible with the multiresidue analytical methods which include compounds from different groups. The other oxidative stress biomarker, 4-HNA, was detected in all samples, and its average mass loads decreased in the following order: Brussels > Antwerp > Zagreb > Girona. The average mass load in Brussels was almost two times higher than in Girona, indicating possible differences in oxidative stress levels in the population scale. However, even in Girona, the average mass load was twofold higher than in the study conducted in the UK (Sims et al., 2019). Therefore, further studies including additional oxidative stress biomarkers and a larger number of samples are needed to confirm these preliminary findings.

Figs. S7–S11 show the daily variations in the mass loads for the cities where samples were collected for seven consecutive days (Antwerp, Brussels, and Zagreb). In general, no clear trends were observed for most compounds. A similar pattern was observed for most biomarkers in Antwerp, with the highest mass loads on Tuesday and/or Saturday. However, this could be related to differences in flow rates, which were much more pronounced in Antwerp than in Brussels and Zagreb. Therefore, further sampling campaigns should investigate the possible daily variations, as well as the temporal variability at other time scales, such as seasonal differences.

3.6. Estimation of human exposure to selected chemicals

Population exposure could be assessed for those compounds for which quantitative excretion data (i.e., molar excretion fractions) (Table S2) are known, and whose biomarkers (human metabolites) could be determined in the analyzed samples (Tables S6–S7). These include phthalates, DEHTP, TBOEP, and BPA. Population exposure for selected compounds was compared with the Oral Reference Dosis (RfD) provided by the U.S. Environmental Protection Agency (EPA) and/or the Tolerable Daily Intake (TDI) set by the European Food Safety Authority (EFSA) (Table 5). Among the phthalates, the highest exposure was determined for DEP, followed by DnBP and DiBP, while the exposure to DEHP was the lowest. These results are in good agreement with the WBE studies conducted in Spain (Estévez-Danta et al., 2021; González-Mariño et al., 2017, 2021). Exposure to DnBP and DiBP exceeded safe reference values based on the TDI in several cities in Spain (González-Mariño et al., 2021). This was

Table 5
Estimated human exposure to selected chemicals.

	Exposure (average ± SD) / (μg day^{-1} $inhabitant^{-1}$)						
	DEP	DnBP	DiBP	DEHP	DEHTP	TBOEP	BPA
Girona	540 ± 101	140 ± 20	84 ± 10	25 ± 5	188 ± 27	465 ± 85	43 ± 9
Zagreb	603 ± 78	153 ± 34	118 ± 11	24 ± 4	13 ± 6	395 ± 60	202 ± 25
Brussels	392 ± 88	180 ± 41	154 ± 31	28 ± 8	164 ± 28	1018 ± 325	242 ± 30
Antwerp	178 ± 86	43 ± 24	66 ± 34	–	16 ± 15	1783 ± 830	407 ± 183
RfD (μg kg(BW)$^{-1}$ day^{-1})	800[a]	100[a]	100[a]	20[a]	200[b]	15[c]/50[d]	50[e]
TDI (μg kg(BW)$^{-1}$ day^{-1})	–	10[a]	10[a]	50[a]	–	–	4[f]
Safe reference value for adults (μg day^{-1})	56640[g]	7080[g] 708[h]	7080[g] 708[h]	1416[g] 3540[h]	14160[g]	1062[g] 3540[g]	3540[g] 283[h]
Safe reference value for toddlers (μg day^{-1})	9200[g]	1150[g] 115[h]	1150[g] 115[h]	230[g] 575[h]	2300[g]	173[g] 575[g]	575[g] 46[h]

RfD - Oral Reference Dosis; TDI - Tolerable Daily Intake; Weight for adults and toddlers is considered 70.8 kg and 11.5 kg, respectively (González-Mariño et al., 2017).
[a] González-Mariño et al., 2017.
[b] Toxicity Review for Di-2-ethylhexyl Terephthalate (DEHT), University of Cincinnati, 2018.
[c] Bastiansen et al., 2020. Environ. Int. 147, 106,368.
[d] Völkel et al., 2018. Arch. Toxicol. 92, 651–660.
[e] U.S. Environmental Protection Agency, Chemical Assessment Summary for Bisphenol A.
[f] Commission Regulation (EU) 2018/213 of 12 February 2018, Official Journal of the European Union.
[g] Based on RfD.
[h] Based on TDI.

also the case in our study for both butylated phthalates in Zagreb and Brussels, and for DnBP in Girona, while the safe reference values for DEP and DEHP were not exceeded. The exposure pattern was rather different in a Chinese study (Du et al., 2018), indicating geographic differences in exposure to phthalates. However, the study conducted in Australia suggests that urinary excretion may not be the main source of phthalate monoesters in wastewater, especially short-chain monoesters (Tang et al., 2020), which was recently confirmed by He et al. (He et al., 2021). As mentioned in Section 3.4, this could affect the overall reliability of WBE for assessing human exposure to phthalates, at least in quantitative terms.

Exposure to DEHTP was lower than that determined in the recent WBE study in Spain, with an average value of 524 μg day^{-1} inh^{-1} (Estévez-Danta et al., 2021). The difference was particularly pronounced for Zagreb and Antwerp, where the average exposure to DEHTP was below 20 μg day^{-1} inh^{-1}, an order of magnitude lower than in Brussels and Girona. However, all exposure estimates determined in this study are well below the RfD values, both for adults and toddlers.

On the contrary, when the lower RfD value (15 μg $kg(BW)^{-1}$ day^{-1}) is used for the calculation, the exposure to TBOEP exceeds the safe reference values for toddlers in all cities. Exposure was also higher than the safe reference value for adults in Antwerp and very close to this value in Brussels.

Finally, exposure to BPA also exceeded the safe reference values based on the TDI in several cases – for adults in Antwerp and for toddlers in all cities, except Girona. Lopardo et al. also reported increased exposure to BPA in some cases (Lopardo et al., 2019). However, these exposure estimates are highly dependent on the excretion factor used for the calculation. Therefore, additional studies are needed to refine the excretion factor of BPA-SO_4 and increase the accuracy of the WBE assessment of exposure to BPA.

It should be noted that the exposure values estimated in this study may be influenced by several uncertainties of the WBE approach, including sampling, chemical analysis, biomarker stability, consumption/exposure back-calculation, and population size assessment. Some of these uncertainties can be minimized by applying "best practice requirements", as proposed for illicit drugs (Castiglioni et al., 2013). However, other uncertainties, such as the specificity and stability of biomarkers in real sewer systems, are more difficult to address. Even then, WBE is limited to population-level exposure data, whereas calculation of individual exposure may be more accurate using traditional epidemiological methods.

4. Conclusions

The developed method allows multiresidue determination of biomarkers of human exposure to selected substances from personal care and household products in RW. Although not as sensitive as some previously published methods that focus on one specific class of compounds, it is relatively simple, rapid, and requires minimal sample manipulation. The suitability of the method for WBE was demonstrated by analyzing wastewater from four European cities, quantifying approximately 20 biomarkers. In some cases, differences in mass loads were found between cities, indicating different exposure to some environmental contaminants, such as phthalates and alternative plasticizers. Furthermore, exposure to some substances, such as butylated phthalates, BPA, and TBOEP, exceeded safe reference values, especially for toddlers. Overall, the method is sufficiently reliable to detect differences between populations, but estimation of "true" exposure at the individual level could be more accurate using traditional clinical methods.

CRediT authorship contribution statement

Ivan Senta: Conceptualization, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft. **Sara Rodríguez-Mozaz:** Conceptualization, Writing – review & editing. **Lluís Corominas:** Conceptualization, Writing – review & editing. **Adrian Covaci:** Writing – review & editing. **Mira Petrovic:** Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing.

Declaration of competing interest

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

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Sampling

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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.2022.157309.

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