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Target and non-target screening of biomarkers in wastewater: towards a unique analytical methodology for sample preparation†

Gauthier Bernier-Turpin, *[ab] Thomas Thiebault, [b] Fabrice Alliot, [b] Emmanuelle Mebold, [c] Sabrina Guérin-Rechdaoui, [d] Marcos Oliveira, [d] Julien Le Roux [a] and Régis Moilleron [a]

This study aims to optimize a single preparation methodology based on solid-phase extraction (SPE) that could fit both target and non-target screening of organic biomarkers in raw wastewater, allowing the cross-comparison of results obtained from a same dataset. The efficiency of SPE sorbents used alone (HLB) or in combination in a multilayer cartridge was evaluated based on (i) the extraction recovery and matrix effect in environmental samples (surface water and wastewater) for a list of biomarkers (pharmaceuticals, licit and illicit drugs, artificial sweeteners, isoprostanes, polyphenols) and (ii) a number of detected features and their intensity in HRMS. The selected method uses a combination of three SPE sorbents mixed together (HLB, X-AW and X-CW) and seems to take full advantage of each, providing satisfactory validation parameters (recovery, instrumental limit of detection, linearity range and limit of quantification) over a large range of physico-chemical properties while ensuring promising results for non-target screening applications. Of the 65 targeted compounds, nearly all of them (47) were detected in wastewater influent samples with concentration above the limit of quantification, while at the same time over 10 000 features were recorded according to the high resolution mass spectrometry (HRMS) fingerprint, holding out the promise that a common protocol for these two analyses, with their very contrasting constraints and objectives, is possible.

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Introduction

Wastewater treatment plants (WWTPs) play a crucial role in the environmental fate of water bodies, acting as the boundary between diverse anthropogenic contaminants present in sewage water and the natural receiving environment. The increasing variety of organic chemicals discharged through sewer systems, driven by diversified consumption patterns, exerts an increasing pressure on the environment. Among these contaminants, drugs (both licit and illicit) are particularly concerning due to their poor removal during wastewater treatment processes. The growing number of emerging pollutants increases the complexity of monitoring efforts. Monitoring directives such as the Water Framework Directive (2000/60/CE) or the Urban Wastewater Directive (91/271/CEE) struggle to address this broad diversity. While some substances of concern have been included in these regulations, a gap remains between the monitored compounds and the diversity of potential targets. Recent advancements in the field of high-resolution mass spectrometry (HRMS) offer a promising solution by providing a comprehensive overview of contamination within a sample and enabling the characterization and identification of unknown pollutants.

Wastewater monitoring is therefore essential in order to ensure continuous observation between the urban network and receiving surface water bodies. Additionally, because of its direct connection with urban metabolism, wastewater has emerged as a valuable source of information. The so-called monitoring, namely, wastewater-based epidemiology (WBE) has become a well-documented and powerful tool in order to assess consumption and/or exposure patterns within a given population. Following the first studies that focused on illicit drug monitoring,[1] this method proved to be a near real-time and low-cost method that could be applied to a broad list of targeted compounds within a narrow temporal–spatial framework. While drugs in general have been widely discussed, whether illicit[2,3] or licit, such as alcohol,[4,5] tobacco or coffee,[6–8] WBE studies have also been extended to broader topics, such as assessing disease prevalence using pharmaceutical products as proxy.[9–12]

[a]Leesu - Univ Paris Est Creteil, Ecole des Ponts, Creteil, F-94010, France. E-mail: gauthier.bernier-turpin@u-pec.fr
[b]METIS, Sorbonne Univ, CNRS, EPHE, PSL Univ, UMR 7619, F-75005 Paris, France
[c]OSU-EFLUVE, PRAMMICS Platform, UPEC, CNRS, F-94010 Créteil, France
[d]SIAAP, Innovation Department, FR-92700 Colombes, France

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Recent studies have explored both current and potential future applications of wastewater-based epidemiology (WBE), expanding the range of targeted biomarkers to include new topics such as dietary intake, oxidative stress, pesticides, flame retardants, and demographic markers.[13–15] In addition, the recent developments in the field of high-resolution mass spectrometry (HRMS) have opened up a new potential to broaden WBE applications,[16] either to elucidate suitable WBE markers[17] or to obtain wastewater fingerprints that could be representative of population health or socio-economic status.[18]

As WBE studies are often focused on a specific subject (*i.e.*, the consumption of drugs or pharmaceuticals), the physico-chemical properties of targeted compounds are often in the same range, resulting in a single dedicated and optimized preparation and analytical protocol. Targeting a wide range of uses (from commonly studied illicit drugs to potential innovative dietary or demographic markers) can be tricky, as it also broadens the range of chemical properties of the targeted compounds. Therefore, one of the main drawbacks of a wide range of chemical properties is the need to make compromises that often reduce the analysis sensitivity to preserve sufficient specificity,[19] which adds to the difficulty of finding a robust and efficient analytical method that does not require multiple chromatographic columns/mobile phases and/or sample preparation.[20] These problems might be tackled using the increasing diversity of available materials, such as versatile solid-phase extraction (SPE) sorbents[21] or polar reversed phase columns, which enable better retention of polar compounds than the conventional reversed phase column.[22]

The common workflow for target screening methods usually relies on SPE for sample clean-up and analyte pre-concentration. Separation and detection of analytes are mainly achieved by reversed phase liquid chromatography coupled to tandem mass spectrometry (RPLC-MS2) with a triple quadrupole mass spectrometer (QqQ). This workflow generally allows performing sensitive, selective and rapid analysis of moderately polar compounds in complex matrices such as raw wastewater.

Because of their ability to cover a wide range of physico-chemical properties, universal phases such as the hydrophilic–lipophilic balanced resin (HLB) are the main choice for multi-residue methods.[23–25] SPE is also used for HRMS purpose, but while a common phase such as HLB can generally be chosen, there has been a change in the type of sorbent that should be used to perform such an analysis, and the emergence of multilayer cartridges (*i.e.*, universal phases combined with ion-exchange phases) seems to provide more exhaustive results, particularly for polar ionized compounds.[26–28] Indeed, while the HLB sorbent enables enrichment through hydrophobic interactions, ion-exchange materials can interact with charged compounds, therefore providing an additional type of interaction. Since most of our targeted compounds are either positively or negatively charged (Table S1†), the addition of those materials is relevant. Thus, Strata X-CW and X-AW were selected to retain cationic and anionic compounds, respectively.

In light of the aforementioned context, the aim of this work was to develop an analytical method that relies on a single sample preparation step and would meet the following requirements:

Enable the quantitative analysis using LC-MS2 of a list of WBE biomarkers covering a wide range of both uses and chemical properties ($\log D$ at pH 7 ranging from −4.5 to 4.4); a non-target screening (NTS) analysis based on HRMS that will serve as a tool to monitor wastewater in order to identify new suitable markers in WBE or potential trends over time in the observed fingerprints; the present work was oriented based on the existing literature, so that a single sorbent (HLB phase) protocol (hereafter named the "HLB protocol") was compared with two multi-layered cartridge protocols ("HAC and MC protocol", with 3 and 4 sorbents, respectively, HLB, X-AW, X-CW and HLB and X-AW, X-CW, and ENV$^+$) for their ability to, respectively, retain both our list of targeted compounds and a high amount of HRMS features in order to meet our two requirements.

Finally, the selected protocol was applied to a series of environmental samples (*i.e.*, wastewater influent) in order to collect a first set of data for future WBE applications.

Materials and methods

Chemicals and materials

Reference standards' suppliers and CAS number are provided in the ESI (Table S1†). All products were purchased in high purity grade (at least >93%). Isotopically labelled internal standards (ILISs) were 4-methyl-benzotriazole-d4 (4-MBZ-d4), 5-hydroxyindoleacetic acid-d5 (5-HIAA-d5), acesulfame-d4 (ACS-d4), acetaminophen-d4 (ACT-d4), amisulpride-d5 (AMI-d5), benzoylecgonine-d3 (BZE-d3), caffeine-$^{13}C_3$ (CAF-$^{13}C_3$), carbamazepine-$^{13}C_6$ (CBZ-$^{13}C_6$), 3-carboxy-4-methyl-5-propyl-2-furanpropionic acid-d3 (CMPF-d3), cotinine-d3 (COT-d3), diclofenac-d4 (DIC-d4), fexofenadine-$^{13}C_6$ (FEX$^{13}C_6$), fluoxetine-d6 (FLX-d6), ibuprofen-d3 (IBU-d3), irbesartan-d6 (IRB-d6), metformin-d6 (MET-d6), oxazepam-d5 (OXZ-d5), propranolol-d7 (PRO-d7), sulfamethoxazole-$^{13}C_6$ (SMX-$^{13}C_6$), 11-nor-9-carboxy-Δ9-tetrahydrocannabinol-d3 (THC-COOH-d3), tramadol-^{13}C-d3 (TRA^{13}C-d3), and trimethoprim-d3 (TRI-d3). All ILISs were diluted together to form a working mix at 1 mg L^{-1}.

Mobile phase components (acetonitrile and formic acid) were of LC-MS grade. The cartridges used for SPE validation were Oasis HLB (200 mg, 6 mL, Waters, Guyancourt, France), Strata X-AW and X-CW (Phenomenex, Le Pecq, France) and Isolute ENV$^+$ (Biotage, Glamorgen, UK). Pure water (PW) used for method development was supplied by a Veolia pure water system (Veolia, Lane End, UK).

Sample collection and treatment

Surface water (SW) used for method development consisted of a grab sample taken from the Seine River at Austerlitz (Paris, France) in 1 L aluminium bottles, while the influent wastewater (IWW) samples consisted of 24 hour composite urban wastewater samples collected at the Seine Centre wastewater treatment plant (WWTP) (Colombes, France) in 1 L amber glass

bottles. Samples were kept frozen at −18 °C to limit degradation during storage.

Prior to analysis, samples were thawed and filtered through 110 mm GF/F glass fiber filters (Whatman, Fontenay-sous-Bois, France) with a nominal cut size of 0.7 μm to remove particulate matter. Next, the filtered samples were adjusted to pH 7 using 1% phosphoric acid to ensure sample stabilization and reproducibility between all samples.

Solid phase extraction

Solid phase extraction (SPE) of samples was carried out using a Supelco Visiprep SPE Manifold 24-port model connected to a vacuum system for the conditioning, loading, and elution steps.

For the HLB protocol, SPE was carried out as follows: cartridges (Oasis HLB, 200 mg, 6 mL) were conditioned with 2 × 5 mL of methanol (MeOH) and 2 × 5 mL of ultrapure water. 50 mL of sample were spiked with 100 μL of a 1 ng μL^{-1} (100 ng) ILIS mix and loaded onto the cartridges while ensuring a slow flow rate, then left to dry for 30 min under vacuum before eluting with 2 × 5 mL of MeOH.

The MC protocol consists of an Oasis HLB cartridge (200 mg, 6 mL) manually filled with an additional layer containing 100 mg of Strata-X-AW, 100 mg of Strata-X-CW and 150 mg of Isolute ENV$^+$ sorbents mixed together.[27]

The HAC protocol comprised the same initial layer (200 mg of Oasis HLB), while the additional layer contained only 100 mg of Strata-X-AW and 100 mg of Strata-X-CW.

SPE with both MC and HAC protocols was carried out as described for the HLB protocol, with the exception of the elution step. In these protocols, a two-step elution consisting of 6 mL of ethyl acetate/MeOH with 1.5% ammonia, followed by 3 mL of ethyl acetate/MeOH with 2% of formic acid was used due to the specificities of ion-exchange materials.

All SPE extracts were then evaporated using an EZ-2 rotary evaporator (Genevac Ltd, Ipswich, UK), followed by a gentle nitrogen stream until the vial meniscus (almost dryness, residual volume of ≈100 μL). Reconstitution was carried out with 2 × 175 μL of an 80/20 PW/MeOH mixture. The reconstituted extracts were then transferred to 0.2 μm PTFE tube filters for a 10 min centrifugation at 5000 rpm. Samples were then placed in 2 mL amber glass vials and 50 μL of the 80/20 PW/MeOH mixture was used to rinse centrifugal filters. Samples were then stored at −18 °C until injection. The concentration factor of the sample preparation method was 100.

Liquid chromatography/tandem mass spectrometry (LC-MS^2)

An Agilent 1260 infinity II (Agilent Technologies, Massy, France) equipped with a degasser, a thermostatic column compartment, a binary high-pressure gradient pump and an autosampler module was used for LC analysis. Preliminary investigations on several chromatographic conditions including various LC columns, injection volume/flow rates and mobile phase compositions/gradients were conducted in order to optimize compound separation. Best performances were achieved by injecting 10 μL of the extract on an X Select T3 (Waters® 2.1 mm × 100 mm, 3.5 μm) equipped with a guard column (C18, 2.1 mm, Phenomenex) and warmed at 40 °C. Mobile phases consisted of 0.1% v/v formic acid in ultrapure water (A) and 0.1% v/v formic acid in acetonitrile (ACN) (B) at a flow rate of 0.7 mL min^{-1}.

The final gradient was as follows: 0 to 0.5 min: 90% of A, between 0.5 and 6 min: from 90 to 0% of A, 6–7.5 min: 0% A, 7.5–9 min: 0–90% A, 9–11 min: 90% A. Including the final equilibration step, the total runtime was 13.5 min. The chosen flow rate and mobile phase composition make this protocol a fast and cost effective method useful for routine applications on a large number of samples.

Detection and quantification were carried out using an Agilent ULTIVO triple quadrupole mass spectrometer equipped with an electrospray ionization source (ESI, Jetstream, Agilent) operating in polarity-switching mode, enabling positive and negative ionizations to be obtained in a single run. Nitrogen (99.9%, Air Liquide, Paris, France) was used as the collision gas, while nitrogen was used as the nebulizing gas (7 L min^{-1}, nebulizer pressure 15 psi) and was produced by a nitrogen generator (Olympia N70-1, Gengaz, France). The gas temperature was 300 °C and capillary voltage was 4000 V. Full list of source parameters are available in ESI data (Table S2†).

Compounds' MS^2 parameters such as collision energy, fragment voltage and the resulting multiple reaction monitoring (MRM) transitions were optimized by direct infusion of standard solutions using Optimizer software (Agilent) and adjusted using the literature when necessary. Signal acquisition was performed using the dynamic MRM (dMRM) mode, with the most abundant transition used for quantification, while at least one another was used for qualification confirmation. Data processing was conducted with Mass Hunter Workstation Quantitative Analysis (Version 10.0) software (Agilent).

UPLC-HRMS

Samples were analyzed using a Vion UPLC-IMS-QTOF (Waters) equipped with an ACQUITY UPLC BEH C18 (2.1 × 100 mm, 1.7 μm) column and the corresponding pre-column. The mobile phases were PW water + 0.1% formic acid (A) and ACN + 0.1% formic acid (B) following the gradient described in the ESI (Table S3†), for a total runtime of 34 min. Analyses were performed in positive mode (ESI$^+$) with screening between 50 and 1000 *m*/*z*.

To ensure data quality, several steps were implemented: prior to each analysis, a quality reference standard, consisting of 9 components (acetaminophen, caffeine, leucine enkephalin, reserpine, sulfadimethoxine, sulfaguanidine, serfenadine, val-tyr-val, and verapamil), was injected five times to check the system performance by calculating the mass error deviation, mean peak width and CCS (collision cross section) error for those compounds. If the mass error deviation was greater than 2 ppm, the CCS error deviation was higher than 2% or the mean peak width was greater than 3.0 seconds, system calibration was conducted. The same procedure was repeated until the expected conditions were met. Each sampling sequence began with two

Table 1 Multiple Reaction Monitoring (MRM) features for targeted compounds, with Isotopically Labelled Internal Standards (ILIS), Fragmentor (Frag.), Retention Time (RT), Collision Energy (CE), and instrumental detection limit (IDL)[a]

	ILIS	RT (min)	Pol.	[M–H]	MRM 1	Frag. (V)	CE 1 (V)	MRM 2	CE 2 (V)	Linearity range (μg L^{-1})	R^2	IDL (ng mL^{-1})
Antibiotics												
Clarithromycin	SMX-$^{13}C_6$	3.86	+	749.0	83.0	170	61	157.8	25	1–1000	0.999	29
Sulfamethoxazole	SMX-$^{13}C_6$	3.07	+	254.2	64.9	100	53	107.9	21	1–1000	0.998	121
Trimethoprim	TRI-d3	1.95	+	291.3	230.0	150	21	261.1	25	1–1000	0.999	60
Analgesics & anti-inflammatories												
Acetaminophen	ACT-d4	0.89	+	152.1	110.1	51	13	65.1	33	1–10000	0.999	59
Diclofenac	DIC-d4	5.09	+	297.1	215.0	90	33	251.1	6	1–1000	0.999	469
Ibuprofen	IBU-d3	5.18	+	207.3	161.1	110	5	119.1	22	1–1000	0.999	118
Ketoprofen	IBU-d3	4.44	+	255.3	105.0	100	21	76.7	53	1–1000	0.999	42
β-Blockers												
Metoprolol	TRI-d3	2.67	+	268.3	72.1	120	21	77.0	69	1–1000	0.999	64
Propranolol	PRO-d7	3.23	+	260.3	56.1	110	33	73.9	21	1–1000	0.999	14
Salbutamol	TRI-d3	0.62	+	240.3	148.1	90	17	222.1	5	1–1000	0.997	12
Anticonvulsants												
Carbamazepine	CBZ-$^{13}C_6$	3.85	+	237.2	194.0	101	17	165.1	55	1–1000	0.999	23
Gabapentin	CBZ-$^{13}C_6$	1.07	+	172.2	154.0	90	9	55.0	25	1–1000	0.995	26
Antidepressants												
Amisulpride	AMI-d5	2.30	+	370.5	242.0	140	25	195.9	45	1–1000	0.998	18
Citalopram	FEX$^{13}C_6$	3.45	+	325.4	108.9	130	29	83.0	80	1–1000	0.994	10
Fluoxetine	FLX-d6	3.85	+	310.3	43.9	90	9	148.2	5	1–1000	0.998	3
Venlafaxine	FEX$^{13}C_6$	3.08	+	278.4	58.0	100	17	260.1	5	1–1000	0.997	8
Antidiabetics												
Glibenclamide	CBZ-$^{13}C_6$	5.09	+	495.0	370.1	110	9	169.0	49	1–1000	0.997	50
Metformin	MET-d6	0.44	+	130.0	60.0	80	9	71.1	21	1–10000	0.999	10
Antihistamines												
Cetirizine	FEX$^{13}C_6$	3.82	+	389.9	166.0	110	73	201.0	17	1–1000	0.993	23
Desloratadine	FEX$^{13}C_6$	2.78	+	311.8	260.0	150	21	295.0	17	1–1000	0.999	130
Fexofenadine	FEX$^{13}C_6$	3.76	+	502.7	467.3	180	29	171.0	41	1–1000	0.999	146
Hormones												
Levonorgesterel	IBU-d3	4.97	+	313.4	109.0	120	65	91.0	53	1–1000	0.997	49
Anti-hypertensive												
Candesartan	IRB-d6	4.12	+	441.4	263.0	110	9	423.1	5	1–1000	0.999	4
Irbesartan	IRB-d6	3.96	+	429.5	207.0	130	21	195.1	21	1–1000	0.998	1
Stimulants												
Anhydroecgonine methyl ester (AEME)	AMI-d5	0.57	+	182.2	118.0	110	21	91.0	29	1–1000	0.993	10
Benzoylecgonine	BZE-d3	2.45	+	290.3	168.0	120	17	76.9	65	1–1000	0.999	7
Cocaine	BZE-d3	2.88	+	304.3	182.1	120	17	82.0	33	1–1000	0.999	9
Hallucinogens												
MDMA	BZE-d3	1.81	+	194.2	163.0	80	9	105.1	25	1–1000	0.999	19
Opioids												
6-Acetylmorphine	TRI-d3	1.5	+	328.0	165.1	150	49	211.0	25	10–1000	0.999	703
Codeine	TRI-d3	1.06	+	300.3	127.9	150	73	215.1	25	10–1000	0.999	315
EDDP ☆	TRA^{13}C-d3	3.59	+	278.4	234.1	160	33	249.1	25	1–1000	0.982	3
Methadone	TRA^{13}C-d3	3.82	+	310.3	265.0	110	13	104.9	29	1–1000	0.998	28
Morphine	TRA^{13}C-d3	4.59	+	286.3	151.9	150	69	128.1	73	20–1000	0.999	995
Naloxone	TRA^{13}C-d3	0.96	+	328.4	310.2	120	17	211.9	45	1–1000	0.999	23
Tramadol	TRA^{13}C-d3	2.69	+	264.3	57.9	100	17	41.9	80	1–1000	0.999	12

Table 1 (*Contd.*)

	ILIS	RT (min)	Pol.	[M–H]	MRM 1	Frag. (V)	CE 1 (V)	MRM 2	CE 2 (V)	Linearity range (μg L^{-1})	R^2	IDL (ng mL^{-1})
Psychiatric drugs												
Diazepam	OXZ-d5	4.59	+	285.7	194.2	70	37	223.2	29	1–1000	0.993	27
Oxazepam	OXZ-d5	3.98	+	287.7	242.1	60	25	104.0	45	1–1000	0.996	36
Cannabinoid												
THC-COOH	THC-COOH-d3	5.82	+	345.4	327.1	120	13	299.1	17	1–10 000	0.999	207
Coffee												
Paraxanthine ☆	CAF-$^{13}C_3$	0.87	+	181.2	123.8	70	17	—	—	1–4000	0.998	49
Caffeine	CAF-$^{13}C_3$	1.60	+	195.2	137.9	110	17	42.0	41	1–10 000	0.999	231
Tobacco												
Cotinine	COT-d3	0.54	+	177.2	79.9	110	25	98.1	21	1–4000	0.999	27
Nicotine	COT-d3	0.53	+	163.2	130.1	100	21	132.0	13	1–10 000	0.991	380
Alcohol												
Ethyl sulfate ☆	ACS-d4	0.50	—	125.1	97.1	90	17	80.0	37	1–4000	0.990	28.0
Artificial sweeteners												
Acesulfame	ACS-d4	0.73	—	162.1	81.9	80	13	77.9	41	1–4000	0.999	32
Saccharin	ACT-d4	1.02	—	182.2	105.9	110	17	41.8	37	1–4000	0.999	57
Dietary intake												
2PY ☆	CMPF-d3	0.9	+	153.0	109.9	110	21	—	—	10–10 000	0.995	4412
αCEHC ☆	CMPF-d3	4.25	—	277.3	233.2	130	13	162.9	25	10–1000	0.972	1719
4-Pyridoxic acid ☆	COT-d3	0.55	—	182.0	138.0	80	13	—	—	20–10 000	0.998	1954
CMPF	CMPF-d3	4.10	—	239.2	150.9	90	17	195.1	9	1–4000	0.998	29
Daidzein	CMPF-d3	3.42	—	253.2	131.9	160	45	222.9	37	1–1000	0.994	566
Enterodiol	CMPF-d3	3.45	—	301.0	253.2	150	25	271.1	25	1–1000	0.991	83
Enterolactone	CMPF-d3	3.96	—	297.0	253.1	160	21	106.9	29	1–1000	0.999	380
Oxidative stress												
8-Iso-prostaglandin F2α	CMPF-d3	3.85	—	353.5	193.0	170	25	291.2	21	1–1000	0.990	207
Dinor-11β-prostaglandin F2α	CMPF-d3	3.48	—	325.4	237.0	130	9	42.8	25	1–1000	0.993	811
Prostaglandin E2	CMPF-d3	4.10	—	351.0	333.1	170	9	315.1	9	1–1000	0.991	217
Demographic marker												
5-HIAA	5-HIAA-d5	1.58	+	192.2	146.0	84	14	91.0	42	1–10 000	0.996	115
Anti-corrosive												
5-Methyl-1*H*-benzotriazole	4-MBZ-d4	2.94	+	134.1	79.0	110	17	76.9	29	1–1000	0.999	6
Mycotoxin												
Deoxynivalenol	PRO-d7	1.22	+	297.3	249.0	90	5	77.1	77	4–1000	0.999	27

[a] Results marked with a star (☆) should be considered with caution.

blank injections to wash and balance the column. Each sample was then injected in randomized triplicates to minimize the effect of instrumental deviation. For data processing purposes, only features detected in each replicate of a given sample were taken into account. Further details about this method can be found elsewhere.[28]

HRMS data were acquired and pre-processed using UNIFI software (Waters). After peak detection, grouping of mass spectra and isotopes, and alignment, each detected feature was given a unique identifier (ID) composed of its *m*/*z*, retention time, and drift time. The data were then exported as a csv table containing each feature ID and its respective peak area in each sample for further processing with the R software and an in-house R-Shiny application.

Method performance and validation for target screening

Instrumental performance (LC-MS^2). The linearity of the method was assessed using linear regression on an 8-point calibration curve based on the relative response (analyte peak area/ILIS peak area) of standard solutions in methanol with analyte concentrations ranging from 1 to 1000 μg L^{-1}. These values refer to a range of 0.01 to 10 μg L^{-1} in the initial sample,

taking into account the concentration factor induced by the preparation process. Additional calibration points (2000, 4000 and 10 000 μg L^{-1}) have been added for target compounds whose environmental matrix concentrations (especially in IWW) could exceed the calibration range (see the linearity range column in Table 1).

The instrumental limit of detection (IDL) was expressed as the minimum amount of analytes injected on the column giving a signal-to-noise ratio (S/N) of 3. It was calculated based on the chromatogram of the lowest level used in the calibration curve.

Quantification and method validation. In order to determine analyte recovery for each matrix investigated (PW, SW and IWW), triplicates were prepared by spiking samples at 2000 ng L^{-1} with a mix of the targeted analytes. At the same time, blank samples (only spiked with ILISs) were prepared in order to subtract the possible response value induced by targeted analytes already present in environmental samples. Absolute recoveries were expressed as the ratio between the mean area in spiked samples (corrected by the blank value) and the area of a standard sample injected at the same concentration directly on the column (eqn (1)).

$$\mathrm{Rec_{Abs}}(\%) = \frac{\left(A_{\text{spiked replicates}} - A_{\text{unspiked}}\right)}{A_{\text{standard}}} \times 100 \quad (1)$$

The potential suppression/enhancement of signal in the environmental sample (ES) due to the matrix effect (ME) was estimated based on the signal intensity evolution of the spiked amount in sample compared to that of pure water (PW) (eqn (2)):

$$\mathrm{ME}(\%) = \left(\frac{A_{\text{ES+spiking}} - A_{\text{ESblank}}}{A_{\text{PWspiked}} - 1}\right) \times 100 \quad (2)$$

where $A_{\text{ES+spiking}}$ is the response (based on the peak area) of the spiked replicates in environmental samples (SW or IWW), A_{ESblank} is the response of the unspiked sample in the same matrix and A_{PWspiked} is the response of the spiked samples in pure water.

ME values above 100% indicate signal enhancement in the environmental matrix, while values below 100% mean signal suppression.

Relative recoveries ($\mathrm{REC_{ILIS}}$) were used in order to compensate for low SPE recoveries and signal suppression in order to maintain correct quantification of all compounds. Where possible, each analyte was corrected using its own labelled ILIS. Due to the large number of targeted analytes in this method, it was impossible to use an ILIS specifically dedicated to each compound. Surrogate ILISs were chosen in order to best match the response and the retention time of the corresponding targeted analytes (eqn (3)).

$$\mathrm{Rec_{ILIS}}(\%) = \frac{\left(A_{\text{spiked replicates}} - A_{\text{unspiked}}\right)}{A_{\text{ILIS}}} \quad (3)$$

Precision was evaluated as the relative standard deviation (RSD) of replicate measurements, and the limit of quantification in sample was estimated as the concentration giving a signal-to-noise ratio of 10 taking into account the spiked amount and the initial concentration in the environmental sample.

Each set of analysis included the injection of a methanol sample to assess contamination, followed by the complete set of calibration standard samples. The blank (pure water spiked with ILISs and subjected to the sample preparation process), methanol and standard samples were randomly injected during the sequence to ensure instrumental and method performance over time.

Results and discussion

Choice of markers for target screening

The initial list of targeted compounds comprised suitable or potential WBE markers that were selected based on the literature. Briefly, it covers a broad range of pharmaceutical products (antibiotics, analgesics, anti-inflammatories, β-blockers, anticonvulsants, antidepressants, antidiabetics, antihistamines, anti-hypertensives and one synthetic progestogen) and drugs, including illicit (stimulants, hallucinogens, opioids, psychiatric drugs, and cannabinoids) and licit (coffee, tobacco, and alcohol) ones. The list also covered more recent markers that are still considered as explorative (*e.g.*, dietary intake or oxidative stress exposure). Additional information concerning the selected compounds are available in ESI data (Table S1†).

Instrumental performance of LC-MS^2 analysis

The protonated [M + H^+] or deprotonated [M − H^-] molecular ion was chosen as the parent ion, and every compound was monitored using at least 2 transitions except for 2-PY, 4-pyridoxic acid and paraxanthine where only one transition was available. Therefore, results obtained for these compounds should only be considered as semi-quantitative (☆).

Otherwise, the stability of the ratio between the two transitions (MRM1/MRM2) was used as an additional criterion to ensure correct identification. Most of the compounds showed better ionization in ESI^+ mode. THC-COOH showed a similar response in both polarities, but since only one transition was available in ESI^- it was analyzed in ESI^+ where both quantification and qualification m/z could be measured.

Among all tested chromatographic columns, the X Select T3 (Waters® 2.1 mm × 100 mm, 3.5 μm) showed better peak shapes for a majority of compounds (Fig. 1). The initial mobile phase gradient (90% of 0.1% v/v formic acid in PW between 0 and 0.5 min) was set in order to promote the retention of the most polar analytes on the column. The mobile phase flow rate was adjusted in order to provide a better peak shape for the majority of compounds while ensuring a short runtime (all compounds eluted in 6 min). These conditions enabled the elution of the majority of the targeted compounds including some of the most polar ones (*i.e.*, metformin, with a log D of −3.36 at pH 7, and acesulfame, with a log D of −2.77 at pH 7). Nevertheless, some compounds (anserine, carnosine, lamotrigine, proline betaine and TMAO) still showed insufficient retention in those conditions and were therefore discarded in

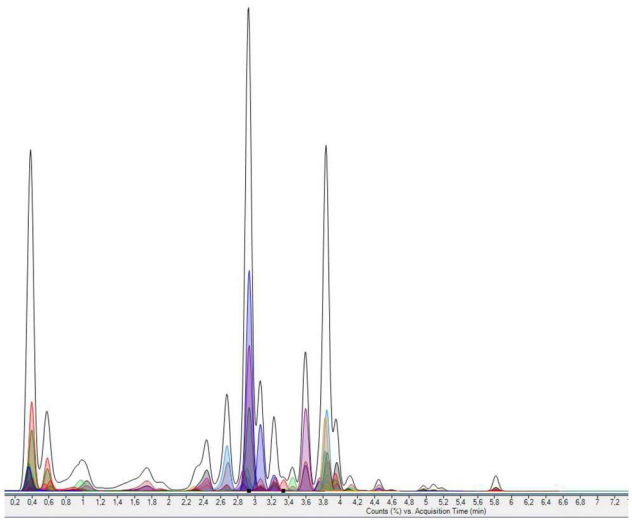

Fig. 1 Total ion chromatogram (TIC) with MRM transitions of all targeted analytes for a calibration standard solution at 200 μg L^{-1}.

the present work. Those compounds should undergo specific analysis with appropriate chromatographic conditions more adapted to their physico-chemical properties.

All targeted compounds showed acceptable linearity (>0.99) in the studied range, except for EDDP ($R^2 = 0.982$) and αCEHC ($R^2 = 0.972$). Such values can be considered inadequate for quantification purposes since calculated concentrations will be subject to higher variability. In the case of EDDP, the R^2 value is lowered due to high concentration standards, and expected environmental concentrations (see the Environmental application section) fall within the linear region of the calibration curve. However, the results for these two compounds should be interpreted with caution (☆).

The instrumental limit of detection (IDL) ranged from 1 ng mL^{-1} (irbesartan) to 4412 ng mL^{-1} (2-PY) injected on the column, with the majority of compounds showing values around 100 ng mL^{-1}. High IDL values are attributed to the low intensity of the recorded signal due to the lack of an intense transition resulting from poor fragmentation.

Evaluation of SPE procedures

Suitability for target screening. The structure of the HLB polymeric sorbent allows for both hydrophilic and lipophilic retention mechanisms, making it suitable for the extraction of a wide range of chemicals. Thus, it has found widespread application in multi-component protocols.

However, it has already shown a limited ability to retain highly polar compounds such as the anti-diabetic metformin.[29] These low recoveries can be overcome when dealing with highly concentrated compounds (several μg L^{-1}), but they still present an issue in terms of measurement uncertainty or when dealing with less concentrated compounds.

Absolute recoveries calculated for the three protocols (HLB, MC and HAC) in each matrix (PW, SW, and IWW) are listed in ESI data (Table S4†).

Among all targeted compounds, ethyl sulfate (EtS) showed poor recovery (<1%) across all protocols and matrices studied. EtS is typically measured using direct injection after centrifugation of the sample.[4] Briefly, HLB showed the best overall recoveries, especially for the studied PPCP's and drugs with 20 compounds falling within 70–120% (*i.e.*, described as the satisfactory range) in PW, compared with 16 for the MC protocol and 17 for the HAC protocol. Signal enhancement resulting in recoveries above 120% was more pronounced for HLB (11 compounds) than for MC and HAC (4 compounds each).

Very low recoveries (<20%) on the HLB protocol were observed for three compounds (acesulfame, gabapentin and metformin), showing values which can be deemed problematic, with a minimum recovery of 8% for the anti-diabetic drug (metformin).

The low affinity of these three compounds with the HLB sorbent can be explained by their hydrophilic properties, resulting in poor retention on such materials intended for reversed-phase interactions during analyte enrichment. Moreover, all three compounds have ionizable functions (see Table S1†), leading to non-neutral speciation at pH 7. As a result, ionic interactions are possible with the ion-exchange materials present in the two multilayer protocols, leading to improved SPE enrichment for these protocols (up to 30% for metformin).

Considering the two ion-exchange protocols, both showed poor recoveries for deoxynivalenol (around 10%). Individually, paraxanthine did not exceed the 20% criterion for HAC (19%), while THC-COOH showed really poor recovery in the MC protocol (5%). Otherwise little distinction could be made between both the HAC and MC protocol in terms of recoveries which was expected based on other studies that already compared the efficiency of each sorbent used alone.[30]

Strong signal suppression was observed in environmental samples, especially in IWW showing drastic decreases of absolute recovery values for a large majority of targeted compounds (Table 2). The presence of many impurities in the matrix can indeed impact the recovery of the analytes either by reducing the ability of the SPE sorbent to retain the targeted compounds or by limiting their ionization efficiency in the source.

Matrix effects in environmental samples have already been widely discussed in mass spectrometry, and the addition of ILISs in order to compensate for those losses is a common practice for quantitative analysis when dealing with highly charged matrices such as wastewater. ILIS correction significantly improved the quantification and precision for a majority of compounds, achieving recoveries within or near the range of compliance (70–120%) and with RSD values below 20%. In addition, several compounds failed to meet this REC_{ILIS} criterion in IWW only because of their high concentration in the unspiked environmental sample, which made the spiked amount too low, causing difficulty in discriminating the unspiked sample from the spiked replicates (see [a] in Table 2).

For compounds where recovery correction was not sufficient due to the lack of appropriate ILISs, a factor was applied in order to avoid over/underestimation and reach an accurate concentration, based on the fact that the low RSD values still suggested a proper correction of the analyte signal by the

Table 2 Corrected recoveries (Rec ILIS in % ± RSD) and the matrix effect (ME) measured on absolute response for the three matrices and the three protocols[b]

		Pure water ($n = 3$)			Surface water ($n = 3$)						IWW ($n = 3$)					
		HLB	HAC	MC	HLB		HAC		MC		HLB		HAC		MC	
	ILIS	Rec ILIS (±RSD)	Rec ILIS (±RSD)	Rec ILIS (±RSD)	Rec ILIS (±RSD)	ME (%)	Rec ILIS (±RSD)	ME (%)	Rec ILIS (±RSD)	ME (%)	Rec ILIS (±RSD)	ME (%)	Rec ILIS (±RSD)	ME (%)	Rec ILIS (±RSD)	ME (%)
Antibiotics																
Clarithromycin	SMX-$^{13}C_6$	46 ± 30	85 ± 4	97 ± 10	104 ± 24	181	76 ± 26	76	91 ± 5	90	153 ± 14	139	152 ± 3	77	147 ± 4	72
Sulfamethoxazole	SMX-$^{13}C_6$	101 ± 0	104 ± 2	102 ± 2	101 ± 3	80	104 ± 5	85	99 ± 0	93	98 ± 1	41	96 ± 5	40	89 ± 1	42
Trimethoprim	TRI-d3	101 ± 3	100 ± 1	104 ± 0	101 ± 0	98	105 ± 7	108	99 ± 3	109	110 ± 2	78	103 ± 7	84	102 ± 2	77
Analgesics & anti-inflammatories																
Acetaminophen	ACT-d4	120 ± 3	110 ± 5	110 ± 2	124 ± 0	98	102 ± 3	105	109 ± 2	102	[a]	[a]	[a]	[a]	[a]	[a]
Diclofenac	DIC-d4	116 ± 4	125 ± 2	119 ± 4	120 ± 5	103	123 ± 9	83	115 ± 2	96	135 ± 3	63	118 ± 9	47	120 ± 8	50
Ibuprofen	IBU-d3	109 ± 6	114 ± 6	115 ± 2	114 ± 5	83	114 ± 10	91	113 ± 2	102	111 ± 7	32	94 ± 10	22	83 ± 4	27
Ketoprofen	IBU-d3	99 ± 4	117 ± 1	108 ± 6	110 ± 3	88	115 ± 12	90	112 ± 1	107	173 ± 1	45	188 ± 12	41	165 ± 3	47
β-Blockers																
Metoprolol	TRI-d3	72 ± 6	93 ± 2	90 ± 1	72 ± 3	98	83 ± 3	91	80 ± 2	101	74 ± 3	73	68 ± 7	60	71 ± 3	61
Propranolol	PRO-d7	92 ± 4	101 ± 1	102 ± 3	95 ± 4	129	99 ± 15	78	95 ± 3	85	96 ± 1	91	100 ± 15	61	90 ± 2	65
Salbutamol	TRI-d3	160 ± 1	77 ± 4	75 ± 6	130 ± 1	80	71 ± 5	94	65 ± 2	98	141 ± 9	62	61 ± 9	64	62.4 ± 3	64
Anticonvulsants																
Carbamazepine	CBZ-$^{13}C_6$	104 ± 3	109 ± 4	107 ± 3	106 ± 3	101	109 ± 9	90	106 ± 0	98	109 ± 1	56	102 ± 9	47	99 ± 2	49
Gabapentin	CBZ-$^{13}C_6$	18 ± 19	47 ± 5	47 ± 1	14 ± 8	74	38 ± 9	72	41 ± 1	85	20 ± 1	66	55 ± 9	50	58 ± 2	74
Antidepressants																
Amisulpride	AMI-d5	101 ± 6	108 ± 3	107 ± 2	106 ± 2	118	107 ± 5	142	101 ± 2	126	105 ± 2	85	98 ± 5	97	101 ± 4	88
Citalopram	FEX$^{13}C_6$	74 ± 22	115 ± 9	100 ± 11	94 ± 18	121	108 ± 12	81	95 ± 5	93	120 ± 6	107	114 ± 9	83	104 ± 6	81
Fluoxetine	FLX-d6	97 ± 6	102 ± 2	99 ± 4	87 ± 1	67	92 ± 14	21	92 ± 6	31	103 ± 10	58	96 ± 14	41	96 ± 6	74
Venlafaxine	FEX$^{13}C_6$	125 ± 8	137 ± 11	116 ± 14	133 ± 5	114	161 ± 10	101	124 ± 10	105	135 ± 3	80	105 ± 5	63	103 ± 7	69
Antidiabetics																
Glibenclamide	CBZ-$^{13}C_6$	62 ± 21	67 ± 19	62 ± 8	84 ± 3	136	39 ± 12	52	50 ± 18	80	121 ± 6	106	106 ± 12	80	90 ± 3	75
Metformin	MET-d6	90 ± 12	104 ± 2	105 ± 2	120 ± 37	41	105 ± 3	116	102 ± 3	101	[a]	[a]	[a]	[a]	[a]	[a]
Antihistamines																
Cetirizine	FEX$^{13}C_6$	128 ± 10	133 ± 11	129 ± 16	126 ± 8	106	125 ± 9	82	112 ± 16	85	114 ± 10	66	97 ± 9	61	89 ± 9	54
Desloratadine	FEX$^{13}C_6$	48 ± 17	81 ± 7	75 ± 16	67 ± 31	145	73 ± 22	77	65 ± 5	85	84 ± 8	127	78 ± 22	81	75 ± 5	79
Fexofenadine	FEX$^{13}C_6$	117 ± 14	156 ± 21	123 ± 22	132 ± 15	122	138 ± 8	78	112 ± 22	88	132 ± 21	85	124 ± 8	66	117 ± 24	73
Hormones																
Levonorgesterel	IBU-d3	49 ± 28	79 ± 6	86 ± 3	100 ± 15	160	64 ± 14	74	81 ± 9	98	274 ± 5	137	261 ± 14	83	216 ± 1	74%
Anti-hypertensive																
Candesartan	IRB-d6	143 ± 7	139 ± 7	127 ± 9	106 ± 7	87	152 ± 9	97	113 ± 5	99	142 ± 1	71	117 ± 9	68	110 ± 6	65%
Irbesartan	IRB-d6	106 ± 3	114 ± 2	112 ± 7	110 ± 5	129	110 ± 11	87	100 ± 2	102	121 ± 3	91	96 ± 11	69	97 ± 4	66%

Table 2 (*Contd.*)

		Pure water ($n = 3$)			Surface water ($n = 3$)						IWW ($n = 3$)					
		HLB	HAC	MC	HLB		HAC		MC		HLB		HAC		MC	
	ILIS	Rec ILIS (±RSD)	Rec ILIS (±RSD)	Rec ILIS (±RSD)	Rec ILIS (±RSD)	ME (%)	Rec ILIS (±RSD)	ME (%)	Rec ILIS (±RSD)	ME (%)	Rec ILIS (±RSD)	ME (%)	Rec ILIS (±RSD)	ME (%)	Rec ILIS (±RSD)	ME (%)
Stimulants																
AEME	AMI-d5	151 ± 6	152 ± 1	123 ± 4	120 ± 4	90	93 ± 6	87	85 ± 4	91	131 ± 15	69	59 ± 8	42	60 ± 6	44
Benzoylecgonine	BZE-d3	121 ± 4	124 ± 1	123 ± 3	134 ± 2	95	123 ± 5	94	120 ± 4	100	126 ± 1	65	110 ± 5	52	105 ± 1	63
Cocaine	BZE-d3	94 ± 9	107 ± 3	107 ± 3	81 ± 11	89	110 ± 9	109	106 ± 2	113	101 ± 1	87	106 ± 7	74	99 ± 1	71
Hallucinogens																
MDMA	BZE-d3	134 ± 6	164 ± 0	158 ± 3	139 ± 5	88	156 ± 7	91	151 ± 4	99	172 ± 3	74	159 ± 7	62	151 ± 2	63
Opioids																
6-Acetylmorphine	TRI-d3	129 ± 7	124 ± 1	122 ± 2	114 ± 4	87	126 ± 1	104	118 ± 1	110	177 ± 5	97	142 ± 8	94	140 ± 4	89
Codeine	TRI-d3	135 ± 8	130 ± 2	127 ± 3	148 ± 6	108	143 ± 2	112	127 ± 2	114	179 ± 5	97	125 ± 10	77	119 ± 4	76
EDDP ☆	TRA^{13}C-d3	69 ± 11	94 ± 7	66 ± 6	60 ± 27	81	83 ± 9	83	55 ± 4	94	68 ± 8	80	77 ± 9	62	68 ± 3	76
Methadone	TRA^{13}C-d3	47 ± 31	81 ± 10	76 ± 2	69 ± 16	105	61 ± 9	81	63 ± 3	93	53 ± 8	63	60 ± 9	56	60 ± 1	58
Morphine	TRA^{13}C-d3	55 ± 28	68 ± 21	70 ± 14	64 ± 13	119	52 ± 11	82	62 ± 13	99	59 ± 19	85	59 ± 11	67	50 ± 12	54
Naloxone	TRA^{13}C-d3	139 ± 7	130 ± 3	128 ± 3	148 ± 2	111	110 ± 8	89	112 ± 2	100	172 ± 4	99	128 ± 8	75	126 ± 1	74
Tramadol	TRA^{13}C-d3	101 ± 1	107 ± 2	107 ± 3	108 ± 2	111	110 ± 9	109	106 ± 0	11	105 ± 2	85	94 ± 9	66	92 ± 1	67
Psychiatric drugs																
Diazepam	OXZ-d5	112 ± 20	121 ± 17	120 ± 7	132 ± 10	141	115 ± 14	74	131 ± 14	97	199 ± 9	89	186 ± 14	65	176 ± 1	63
Oxazepam	OXZ-d5	131 ± 1	136 ± 7	134 ± 6	130 ± 3	118	141 ± 12	79	136 ± 4	90	157 ± 10	60	130 ± 12	40	138 ± 4	44
Cannabinoid																
THC-COOH	THC-COOH-d3	134 ± 4	156 ± 13	147 ± 11	127 ± 2	144	120 ± 4	30	149 ± 14	108	144 ± 1	100	132 ± 4	161	131 ± 1	249
Coffee																
Paraxanthine ☆	CAF-$^{13}C_3$	110 ± 18	76 ± 7	77 ± 4	129 ± 3	107	80 ± 3	108	80 ± 4	114	[a]	[a]	[a]	[a]	[a]	[a]
Caffeine	CAF-$^{13}C_3$	155 ± 5	159 ± 3	164 ± 6	156 ± 2	92	154 ± 4	100	149 ± 5	101	[a]	[a]	[a]	[a]	[a]	[a]
Tobacco																
Cotinine	COT-d3	120 ± 6	124 ± 2	127 ± 2	100 ± 32	67	124 ± 4	89	119 ± 2	85	112 ± 2	40	134 ± 4	14	90 ± 11	18
Nicotine	COT-d3	44 ± 30	62 ± 25	94 ± 26	46 ± 19	88	91 ± 7	126	72 ± 3	70	[a]	[a]	[a]	[a]	[a]	[a]
Alcohol																
Ethyl sulfate ☆	ACS-d4	2 ± 40	20 ± 2	11 ± 17	[a]	[a]	3 ± 11	17	3 ± 27	21	[a]	[a]	[a]	[a]	[a]	[a]
Artificial sweeteners																
Acesulfame	ACS-d4	106 ± 14	113 ± 6	113 ± 1	110 ± 5	142	112 ± 2	103	111 ± 7	99	[a]	[a]	[a]	[a]	[a]	[a]
Saccharin	ACT-d4	48 ± 34	130 ± 9	117 ± 11	88 ± 6	172	93 ± 8	69	80 ± 6	72	[a]	[a]	[a]	[a]	[a]	[a]

Table 2 (*Contd.*)

		Pure water ($n = 3$)			Surface water ($n = 3$)						IWW ($n = 3$)					
		HLB		HAC		MC		HLB		HAC		MC		HLB		HAC		MC	
	ILIS	Rec ILIS (±RSD)	Rec ILIS (±RSD)		Rec ILIS (±RSD)	Rec ILIS (±RSD)	ME (%)	Rec ILIS (±RSD)	ME (%)	Rec ILIS (±RSD)	ME (%)	Rec ILIS (±RSD)	ME (%)	Rec ILIS (±RSD)	ME (%)	Rec ILIS (±RSD)	ME (%)	Rec ILIS (±RSD)	ME (%)
Dietary intake																			
2PY ☆	CMPF-d3	108 ± 6	60 ± 15		62 ± 5	144 ± 27	89	71 ± 3	85	86 ± 20	126	[a]	[a]	[a]	[a]	[a]	[a]		
αCEHC ☆	CMPF-d3	38 ± 18	92 ± 71		78 ± 10	53 ± 13	96	50 ± 1	42	28 ± 110	32	76 ± 5	61	60 ± 1	13	54 ± 9	20		
4-Pyridoxic acid ☆	COT-d3	27 ± 18	61 ± 29		58 ± 33	73 ± 17	175	78 ± 6	183	93 ± 40	140	[a]	[a]	[a]	[a]	[a]	[a]		
CMPF	CMPF-d3	102 ± 2	109 ± 4		107 ± 3	103 ± 2	68	114 ± 4	79	108 ± 5	91	99 ± 7	29	95 ± 4	19	109 ± 4	30		
Daidzein	CMPF-d3	72 ± 22	65 ± 16		55 ± 8	73 ± 18	68	39 ± 31	45	41 ± 24	67	57 ± 9	23	51 ± 31	16	51 ± 22	27		
Enterodiol	CMPF-d3	127 ± 13	129 ± 8		135 ± 0	142 ± 24	74	98 ± 7	5	107 ± 8	72	84 ± 8	19	98 ± 7	18	90 ± 3	20		
Enterolactone	CMPF-d3	73 ± 29	83 ± 6		85 ± 3	99 ± 33	89	57 ± 5	51	68 ± 15	72	59 ± 7	26	84 ± 5	20	66 ± 5	22		
Oxidative stress																			
8-Iso-prostaglandin F2α	CMPF-d3	119 ± 6	91 ± 10		90 ± 0	112 ± 27	62	91 ± 8	76	95 ± 11	95	58 ± 20	14	74 ± 8	20	69 ± 23	22		
Dinor-11β-prostaglandin F2α	CMPF-d3	101 ± 3	85 ± 7		89 ± 3	97 ± 29	63	80 ± 16	72	75 ± 3	76	74 ± 16	21	95 ± 16	27	84 ± 12	28		
Prostaglandin E2	CMPF-d3	103 ± 6	68 ± 5		82 ± 9	120 ± 28	78	85 ± 4	94	90 ± 10	100	128 ± 6	36	102 ± 4	36	93 ± 3	34		
Demographic marker																			
5-HIAA	5-HIAA-d5	79 ± 1	87 ± 30		88 ± 4	87 ± 4	11	79 ± 4	80	62 ± 21	52	44 ± 12	6	119 ± 4	65	77 ± 1	88		
Anti-corrosive																			
5-Methyl-1*H*-benzotriazole	4-MBZ-d4	93 ± 3	117 ± 1		116 ± 0	98 ± 14	84	117 ± 6	91	112 ± 3	103	121 ± 0	81	112 ± 6	65	106 ± 2	66		
Mycotoxin																			
Deoxynivalenol	PRO-d7	74 ± 44	21 ± 15		23 ± 8	80 ± 12	150	43 ± 9	168	43 ± 9	171	69 ± 25	89	34 ± 33	101	28 ± 16	90		

[a] Not estimated due to the amount present in the "blank" sample. [b] Results marked with a star (☆) should be considered with caution.

selected ILIS. As mentioned earlier, recoveries for EtS remained notably low even after ILIS correction (≈20% in PW, <5% in SW and not estimated in IWW due to the amount in the blank sample), therefore limiting confidence in the calculation of environmental concentrations (☆).

Limits of quantification in SW were in the 0–100 ng L^{-1} range for most of the compounds, with values ranging from 0.3 to 344 ng L^{-1} for HLB, 0.5 to 882 ng L^{-1} for HAC and 0.2 to 1342 ng L^{-1} for MC. LOQs in IWW were slightly higher for all protocols, which was expected due to the growing complexity of the matrix. However, some LOQs were artificially overestimated (*e.g.*, from 25.6 ng L^{-1} in SW to 844.6 ng L^{-1} in IWW for acetaminophen in the HLB protocol) since some samples showed significantly high concentrations that were not counterbalanced by proportional S/N values. The LOQ in SW is therefore more appropriate for these compounds which will however be present in concentrations that easily exceed this limit. Therefore, the LOQ measured in IWW is probably much lower than the values mentioned here and should be much closer to the values obtained for SW. All LOQs are displayed in ESI data (Table S5†).

Suitability for non-target screening. Non-target screening (NTS) using HRMS has been developing since the 2010s and is often regarded as a tool with almost unlimited potential for characterizing pollution in aqueous environmental samples[31] and has been transposed to WBE applications for the identification of potential biomarkers.[17] Particular attention has been paid to sample preparation in order to avoid any loss of information during processing. Optimized methods have been developed to move away from typical target screening protocols, excluding the use of a single sorbent[32,33] in favor of more exhaustive protocols such as direct injection[34] or multi-layered cartridges.[35] Therefore, having assessed the ability of the two multilayer cartridge protocols to meet the criteria for routine targeted analysis, the potential of the HLB protocol regarding HRMS analysis was also evaluated. To this end, each sample was injected in triplicate in the UPLC-IMS-QTOF system. The list of markers detected in all samples was then exported for statistical analysis.

A simple data pre-processing workflow was implemented in order to estimate the performance of each protocol according to two main criteria: (i) the number of features measured for each protocol in the environmental matrix (a PW sample was also investigated in order to estimate contamination) and (ii) their total intensity (cumulative intensity of all markers).

First, a marker that was not detected in all replicates of the same sample was removed from this sample (intensity set to 0 for the three replicates). A marker with zero intensity values in all samples (*i.e.*, not detected in all three replicates of at least one sample) was discarded. Next, an intensity threshold value was set (1000) in order to reduce the number of markers originating from background contamination. Finally, all intensity values were normalized to mitigate signal loss observed during the sequence, which could introduce a bias in the total intensity values. Irbesartan-d6 was used for normalization as it showed similar recovery for all three protocols and is little affected by signal suppression in environmental matrices, hence its intensity should remain stable throughout the whole analytical sequence.

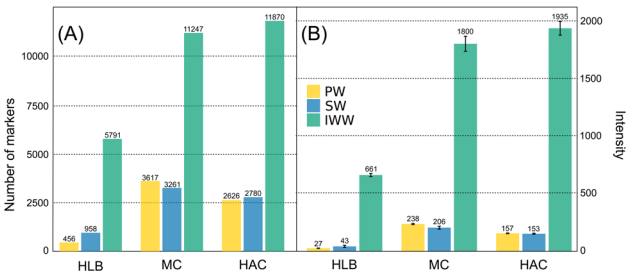

Fig. 2 Number of markers (A) and their total cumulative intensity (B) according to the three investigated protocols (HLB, MC, and HAC) on PW, SW and IWW.

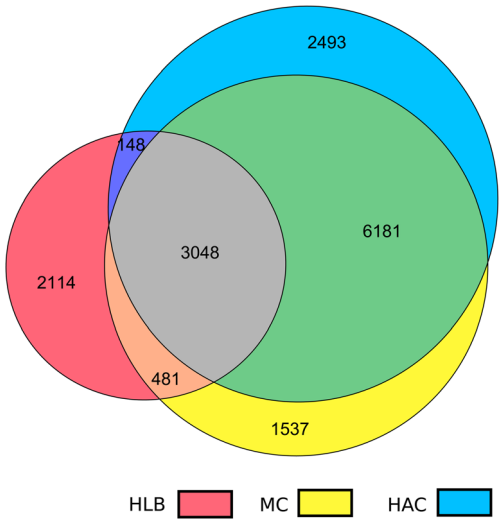

Fig. 3 Euler's diagram displaying specific and shared HRMS features retained by the three protocols on IWW.

As shown in Fig. 2, a first observation was that all three PW samples contained a non-negligible number of markers (≈450 for HLB and ≈2600 for HAC) and that for the MC protocol this number was even higher (≈3600) than the number of markers observed in surface water (≈3200). This could be explained by the small quantity (50 mL) of surface water used for protocol validation. Unsurprisingly, IWW presented a significantly higher number of markers for each protocol. Nevertheless, the HLB protocol retained only half of the number of markers (≈5800) compared to the two multilayered cartridges (≈11 200 for MC and up to 11 900 for HAC, Fig. 2). The same pattern occurred in terms of intensity, since both multilayered cartridge protocols showed up to twice the total intensity of HLB in IWW samples (Fig. 3). These results are in line with previous work made on the suitability of sample preparation methods for the HRMS objective,[28] which showed the limited number of markers retained by the HLB phase compared with a multilayered cartridge.

The distribution of markers between the three protocols was assessed in order to examine potential specificity of some

features in relation to a single protocol (Fig. 3). Interestingly, a significant number of markers (2114) were specific to the HLB protocol, while the two other protocols still contained the same amount of the HLB phase (200 mg). This indicates that the difference in extraction conditions (*i.e.*, elution solvents) had a significant influence on the number of recovered markers. This factor will be discussed in the following section.

As expected, the distribution of markers between HAC and MC seems to overlap, as highlighted by the proportion of shared features (≈80%) between both protocols. Since extraction conditions remain unchanged between both protocols, this suggests that the majority of the retained features was due to their common sorbents, namely, HLB, X-AW and X-CW. Only 1537 features were specific to the MC protocol and so can be attributed to a contribution of the ENV$^+$ sorbent.

Discussion. WBE monitoring studies necessitate routine analysis of samples over a potentially long period. Hence, ensuring the reproducibility of the results within a complex matrix over time is crucial to mitigate the impact of analytical variations on the interpretations issued from data collected.

All three protocols discussed herein benefit from the utilization of SPE as the sample preparation process, which offers several advantages, such as a pre-concentration factor that enables the detection of low levels of analytes, as well as a sample clean-up that reduces matrix interferences, thus enhancing reproducibility. The addition of ILISs also plays a crucial role in quality control of the method process, from preparation to analysis.

However, distinctions can be made with regard to the ability of the three protocols to retain the targeted analytes, as highlighted by the display of their absolute recoveries in IWW (Fig. 4).

First, this clearly shows that the HLB protocol provides higher recoveries for a wide range of moderately polar compounds compared to the two multilayer cartridge protocols. On the other hand, the use of multilayer cartridge protocols could present some benefits when considering absolute recoveries in IWW. Indeed, multilayer cartridges seem to reduce extreme values (*i.e.*, recoveries <20% and >150%) by providing sufficient enrichment and reducing signal enhancement.

Few differences in recoveries were observed between the HAC and MC protocols, suggesting that the ENV$^+$ sorbent provides limited enrichment for the investigated compounds. Additionally, the lack of a noticeable improvement in HRMS screening results (*e.g.*, similar intensities and number of markers) further indicates that the contribution of the ENV$^+$ cartridge remains unclear hereby. Nevertheless, the ENV$^+$ cartridge has already shown to be rather quite effective for both target and non-target screening,[36,37] even when used alone, with better specificity in the range of polar compounds,[28] but this specificity seems to be reduced when used in a mixed mode. The addition of an extra sorbent might be expected to bring further gains, but some also argue that increased complexity may simply introduce artefacts.[38] In light of these elements, and since HLB alone is of limited interest compared to multilayer cartridge protocols when it comes to performing HRMS screening, as has been highlighted in this work and elsewhere,[39] the HAC protocol was considered the most suitable to achieve our objectives. Nevertheless, the actual contribution of the ENV$^+$ sorbent should be investigated, for example, by identifying some of the features that are specific to this sorbent, especially in terms of physico-chemical properties. Finally, this work only focused on determining the ability of established protocols to meet our objective, and few optimizations were carried out, particularly concerning the SPE procedures. The elution solvent used for HLB (*i.e.*, methanol) is more polar than that used for the multilayer protocols (a mixture of methanol and ethyl acetate), therefore we can imagine that non-ionized polar analytes would have eluted better in the HLB protocol. This could explain not only the better recoveries of some targeted analytes but also the presence of specific HRMS features (Fig. 3 and 4). Finally, the absence of a washing step in the SPE procedure, explained by its incompatibility with NTS (loss of potential features with the washing solvent), was probably a drawback for target screening purposes, limiting the recovery and detection of some analytes due to a lower removal of possible interferents and greater matrix effects.

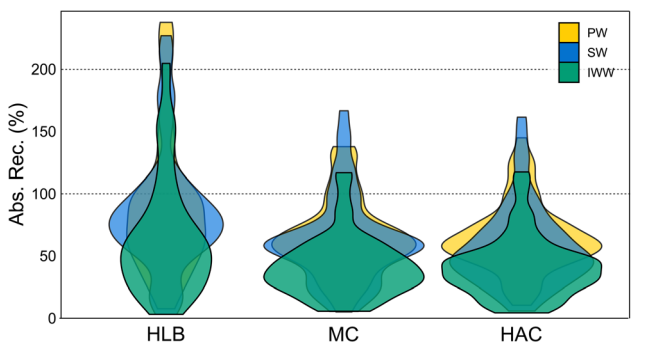

Fig. 4 Distribution of targeted compounds' absolute recoveries following each SPE protocol (HLB, MC, and HAC) on the three investigated matrices (IWW, PW, and SW).

Environmental application

The analytical scheme developed in this work was applied to a batch of IWW samples ($n = 20$) collected between 23-11-19 and 24-01-21 at the Seine Centre WWTP (Colombes, France). This WWTP drains sewerage from the city of Paris (population ≈ 2 300 000 inhabitants). The maximum and average concentrations of targeted compounds, together with some literature references, are shown in Table 3.

Among all targeted compounds, 11 of them were never detected (<LOD). For most of them, these results are consistent with other studies, as these compounds are rarely found and often fell below the limit of detection. For others, LOQs were too high to allow their quantification like morphine, which was never quantified (LOQ of 549 ng L^{-1}) despite its systematic detection (100% detection frequency, $n = 7$) in the influent of a UK WWTP at a mean concentration of 481 ng L^{-1} [20]. In contrast, glibenclamide was never detected despite a low LOD (3.5 ng L^{-1}) and reported concentrations up to 9800 ng L^{-1} in a previous study.[40] In addition, 5-methyl-1*H*-benzotriazole has an isomer, 4-methyl-1*H*-benzotriazole, with which it shares

Table 3 Concentration of targeted compounds measured in IWW from the Seine Centre WWTP (Paris, France), with Nb quant. – the number of quantification[a]

Compounds	LOQ (ng L^{-1})	Nb quant. $n = 20$	Min (ng L^{-1})	Max (ng L^{-1})	Average (ng L^{-1})	Averaged values (literature) (ng L^{-1})
Antibiotics						
Clarithromycin	34	20	95	260	177	1480 (ref. 10)[b]
Sulfamethoxazole	17	20	139	577	407	348 (ref. 45)
Trimethoprim	4	20	120	333	261	246 (ref. 45)
Analgesics & anti-inflammatories						
Acetaminophen	1359	20	53 424	143 274	110 541	58 860 (ref. 46)
Diclofenac	19	20	333	1154	911	131 (ref. 47)
Ibuprofen	569	20	2402	7794	5799	2265 (ref. 47)
Ketoprofen	123	20	389	1406	989	202 (ref. 47)
β-Blockers						
Metoprolol	31	19	<MDL	49	23	4 (ref. 47)
Propranolol	22	20	44	132	103	74 (ref. 45)
Salbutamol	1	20	3	8	6	6 (ref. 48)
Anticonvulsants						
Carbamazepine	8	20	122	229	189	72 (ref. 47)
Gabapentin	16	20	2089	6962	5397	13 170 (ref. 45)
Antidepressants						
Amisulpride	2	20	215	589	496	604 (ref. 49)
Citalopram	53	20	33	113	86	83 (ref. 10)[b]
Fluoxetine	5	20	15	37	27	86 (ref. 20)
Venlafaxine	7	20	213	580	464	618 (ref. 45)
Antidiabetics						
Glibenclamide	4	0	<MDL	<MDL	<MDL	9800 (ref. 40)
Metformin	309	20	30 698	94 497	77 309	110 000 (ref. 50)
Antihistamines						
Cetirizine	14	20	118	359	289	103 (ref. 51)
Desloratadine	2	0	<MDL	<MDL	<MDL	
Fexofenadine	41	20	204	488	392	180 (ref. 10)[b]
Hormones						
Levonorgesterel	877	0	<MDL	<MDL	<MDL	<LOD[52]
Anti-hypertensives						
Candesartan	10	20	111	360	270	1723 (ref. 45)
Irbesartan	1	20	838	2761	2370	1273 (ref. 45)
Stimulants						
Anhydroecgonine methyl ester (AEME)	3	20	6	14	10	<LOD[20]
Benzoylecgonine	1	20	615	2330	1727	2407 (ref. 46)
Cocaine	3	20	362	1255	838	264 (ref. 46)
Hallucinogens						
MDMA	1	20	56	603	271	39 (ref. 20)
Opioids						
6-Acetylmorphine	78	0	<MDL	<MDL	<MDL	22 (ref. 20)
Codeine	10	20	187	721	561	20 (ref. 46)
EDDP ☆	2	20	23	48	36	193 (ref. 20)
Methadone	1	20	13	40	22	88 (ref. 20)
Morphine	550	0	<MDL	<MDL	<MDL	481 (ref. 20)
Naloxone	9	0	<MDL	<MDL	<MDL	6 (ref. 46)
Tramadol	1	20	277	719	624	696 (ref. 45)

Table 3 (*Contd.*)

Compounds	LOQ (ng L^{-1})	Nb quant. $n = 20$	Min (ng L^{-1})	Max (ng L^{-1})	Average (ng L^{-1})	Averaged values (literature) (ng L^{-1})
Psychiatric drugs						
Diazepam	18	0	<MDL	<MDL	<MDL	<LOD[20]
Oxazepam	35	20	72	313	238	50 (ref. 20)
Cannabinoid						
THC-COOH	89	20	141	319	266	270 (ref. 53)
Coffee						
Paraxanthine ☆	137	20	5208	10 815	8293	24 300 (ref. 6)
Caffeine	393	20	24 840	71 768	54 786	25 300 (ref. 6)
Tobacco						
Cotinine	344	20	1094	2197	1794	1050 (ref. 6)
Nicotine	97	20	6313	18 178	11 813	2400 (ref. 6)
Alcohol						
Ethyl sulfate ☆	13	20	6876	19 703	11 965	19 200 (ref. 4)
Artificial sweeteners						
Acesulfame	37	20	7111	21 709	18 290	29 000 (ref. 44)
Saccharin	329	20	6797	24 135	18 850	12 000 (ref. 44)
Dietary intake						
B3-2PY ☆	15 229	20	9311	22 594	15 660	≈20 000 (ref. 54)
E-αCEHC ☆	446	15	<MDL	8389	5812	≈4000 (ref. 54)
4-Pyridoxic acid ☆	111	20	5418	23 485	16 641	≈2000 (ref. 54)
CMPF	31	20	7	2120	1089	<LOD[14]
Daidzein	259	20	1144	2498	1637	821 (ref. 55)
Enterodiol	20	20	169	477	335	≈500 (ref. 54)
Enterolactone	263	20	1906	6738	3756	≈9000 (ref. 54)
Oxidative stress						
8-Iso-prostaglandin F2α	120	0	<MDL	<MDL	<MDL	404 (ref. 56)
Dinor-11β-prostaglandin F2α	547	0	<MDL	<MDL	<MDL	<LOD[56]
Prostaglandin E2	714	0	<MDL	<MDL	<MDL	144 (ref. 56)
Demographic marker						
5-HIAA	66	20	502	5903	3035	9573 (ref. 57)
Anti-corrosive						
5-Methyl-1*H*-benzotriazole	2	20	24	65	47	11 788;[41] 120 (ref. 42)
Mycotoxin						
Deoxynivalenol	199	0	<MDL	<MDL	<MDL	35 (ref. 58)

[a] Results marked with a star (☆) should be considered with caution. [b] Median value instead of average.

a common retention time, as well as the precursor and product ions. Consequently, both compounds are often quantified together and referred to as TTR (tolytriazole).[26] The city of Athens exhibited TTR concentrations at 7735 and 15 841 ng L^{-1} [41], which are significantly higher than the 24–65 ng L^{-1} reported here. Meanwhile, another study conducted in Poland[42] showed levels of 5-methyl-1*H*-benzotriazole alone in a range (<LOD up to 380 ng L^{-1}) that is more in line with values obtained hereby. Apart from the specific cases mentioned above, all values measured in the Seine Centre WWTP influent were in good agreement with what has been observed in a previous study carried out on the same sampling site[43] or on other work (Table 3).

Conclusion

Given the fact that target and non-target screening have followed divergent paths regarding the types of sorbents employed in their respective workflow, this paper aims to bridge the gap between these approaches by promoting a unified methodology that would suit both. As target screening aims to be as sensitive and selective as possible, sample clean-up is often pushed to the maximum in order to retain only the desired features and limit possible interferents, while on the other hand NTS tends to be the most exhaustive possible.

In order to reconcile both approaches, this paper focused on the main existing methodology used for each and tried to develop the most suitable sample preparation that will fit the aforementioned requirements. The multilayer cartridge protocol, which has emerged as the optimal methodology for NTS in most of the current studies, has shown sufficient results to be adapted to a quantification method that covers a large range of targeted analytes in terms of physico-chemical properties while ensuring its principal objective in HRMS (*i.e.*, retaining a maximum of features). Further work on this protocol led to its simplification by removing one of the phase layers (ENV^+) without affecting the quantification method nor the information obtained in terms of HRMS features.

The developed methodology enables the quantification of a broad range of compounds, including pharmaceuticals, licit and illicit drugs, artificial sweeteners, isoprostanes, and polyphenols, while providing a set of HRMS data that can be further processed to identify potential unknowns, all with a single extract injected into both LC-MS^2 and LC-HRMS workflows, paving the way for cross-monitoring.

While primarily focused on future WBE applications, this article also provides insight into the monitoring of the targeted compounds in the aquatic environment. Given the poor elimination in wastewater treatment plants (WWTPs), these compounds tend to be ubiquitously present on other aqueous compartments, such as surface water or groundwater, as a result of contamination from wastewater discharge.[44]

Data availability

Requests to access the data should be directed to the corresponding author. HRMS data were processed using R Core Team; R: a language and environment for statistical computing; R Foundation for Statistical Computing: Vienna, Austria, 2009.

Author contributions

Conceptualization, G. B. T., T. T., and R. M.; data curation, G. B. T., F. A., E. M., and J. L. R.; formal analysis, G. B. T., F. A., E. M., and J. L. R.;, funding acquisition, T. T. and R. M.; investigation, G. B. T. and T. T.; methodology, G. B. T., F. A., and E. M.; supervision, T. T. and R. M.; resources, S. G. and M. O.; visualization, G. B. T., T. T., and J. L. R.; writing-original draft, G. B. T. and T. T.; writing-review & editing, G. B. T., T. T., F. A., E. M., J. L. R., and R. M.

Conflicts of interest

There are no conflicts to declare.

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