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Identification of biomarkers in wastewater-based epidemiology: Main approaches and analytical methods

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ABSTRACT

Wastewater-based epidemiology (WBE) has become popular to estimate the use of drugs of abuse and recently to establish the incidence of CoVID 19 in large cities. However, its possibilities have been expanded recently as a technique that allows to establish a fingerprint of the characteristics of a city, such as state of health/disease, healthy/unhealthy living habits, exposure to different types of contaminants, etc. with respect to other cities. This has been thanks to the identification of human biomarkers as well as to the fingerprinting and profiling of the characteristics of the wastewater catchment that determine these circumstances. The purpose of this review is to analyze the different methodological schemes that have been developed to perform this biomarker identification as well as the most characteristic analytical techniques in each scheme, their advantages and disadvantages and the knowledge gaps identified. We also discussed the future scope for development.

1. Introduction

The theoretical idea of wastewater-based epidemiology (WBE) was first described by Daughton in 2001 [1]. Since then, the concept was put into practice first through the estimation of the consumption of drugs of abuse from the concentrations present in wastewater and then, it was enriched through the estimation of the consumption of other substances or even the exposure to different pollutants, such as pesticides or phthalates [2–4]. The prevalence of some bacteria and virus diseases has also been checked with this technique [5–7]. At this point, the role that WBE has played in the recent popular monitoring of the Sars-CoV-2 spread in populations from their wastewater cannot be skipped over [8–12]. Almost 20 years later, Daughton [13] again proved to be a true visionary of the possibilities of WBE to establish a fingerprint of a community's exposure to toxic substances, its health status and/or its lifestyle by establishing the concept of Sewage Chemical-Information Mining (SCIM). This concept involves the monitoring of sewage for the information that resides in natural and anthropogenic chemicals present in sewers as a result of the everyday actions, activities, and behaviors of humans. SCIM implies a broader application of WBE that can establish authentic fingerprints and profiles of cities, including not only consumption aspects but also health and socio-economic aspects. Currently, the transition towards sustainability of European Cities is a reality that needs to lead with climate neutrality, safe and healthy food systems and water optimization keeping an appropriate live status. WBE can play a pivotal role helping through the fingerprinting and profiling to study in a global way the habits and the needs of its citizens to fix them in the most suitable way. In fact, already many scientists are looking for mining the chemical information on urban wastewater [14–20].

Logically, to fully develop, WBE needs to evolve and find solutions to certain gaps and shortcomings remaining on the approach [3,4,21–23]. As can be deduced from the previous paragraphs, WBE is completely multidisciplinary, including analysts, epidemiologists, microbiologists, sociologists, chemical engineers, economists, health specialists, etc. [24]. However, it is also true that it has a very strong analytical component, which we consider relevant and of special interest for the topic covered by this journal [3,4].

In WBE the ability to move from theory to practice correlates with the ability to identify biomarkers that attain estimation of drug use, leisure habits, health, disease, exposure, etc … [15,25–27]. This analytical field is dominated by liquid

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chromatography-mass spectrometry (LC-MS) techniques since the target molecules belongs to the excreted human metabolites that tend to be more polar than parent compounds [3]. However, there are many different instruments and workflows being crucial to have methodologies (or analytical schemes for detection and elucidation) that achieve determination of these biomarkers appropriately, and with well-validated analytical methods capable of quantifying. This is challenging, since wastewater has a complex and highly variable chemical composition, making it difficult to analyze due to the high number of interferences. Nowadays, there are also many approaches to detect viruses based on any type of DNA or RNA techniques, but we have considered that this is other important field that deserves its own review and have focus only on biomarkers of the human being.

In recent years there has been a tremendous methodological effort to identify biomarkers in wastewater, in fact there are many essential reviews that have this sole objective [15,25–27]. However, there is a lack of a critical review that outlines the different types of analytical protocols following a systematic approach that includes a description of the methods and techniques applied in each case. This review focuses on closing that void, classifying approaches used to identify biomarkers in wastewater and offering a critical discussion of new trends and future directions of analytical methodologies applied. This review also provides information on the major technological advancements in methodologies employed for quantitative biomarkers based WBE research. This is intended to be a critical rather than a comprehensive review, so a selection of the most relevant recently published work in terms of instrumental and methodological aspects, and applications is presented. The number of studies in this field is enormous, so representative papers published since 2017 are discussed. A few papers that have represented a milestone within WBE outside that time range are also included.

2. Classification and characteristics of the different approaches to identify biomarkers

The different approaches that exist to identify biomarkers can be classified as those used to analyze proteins in (i) top-down and (ii) bottom-up approaches (schematized in Fig. 1). The formers study the metabolism of the substances in the human being, mostly through the information reported in human biomonitoring (HBM) studies, then, identify which of the resulting substances can be an appropriate biomarker to be identified in wastewater. Contrarily to what happens in the proteomic field, the top-down approach is the most well developed in WBE. This approach requires the study of the human metabolism, identification of the substances excreted by urine, selection of those representative excreted in a high percentage, development of an analytical method able to determine them, study of their stability in wastewater and then, finally their assessment in wastewater. There are many remarkable and well conducted reviews that treat the topic of potential biomarkers to be used in the WBE for different estimations [15,24–26]. These reviews categorized biomarkers depending a little bit on the authors criterion but in all cases consumption, exposure, health/disease and lifestyle/consumption are main drivers of these classifications. Studies focus on consumption of mostly illicit drugs [19,23,28–41] but also food [42,43], artificial sweeteners [44,45], alcohol [46–51], caffeine [52,53], nicotine [47,51–53] and/or tobacco [54–58], new psychoactive substances [39,59–63], opioids [18,64,65], pharmaceuticals [22,34,53,66–72] and personal care products [34] are the most common. These studies are the most elaborated, as especially in the case of drugs of abuse, work has been ongoing since 2005 and there has been a major effort by many research groups to collaborate many times disinterestedly to systematize the methodology and address the drawbacks.

One of the areas that is increasing is the evaluation of human exposure [20,26] reflected on a high number of studies regarding exposure to pesticides [73–76], mycotoxins [77], bisphenol A and its analogues [78], personal care and household products [26], phthalates [79], organophosphorus flame retardants and plasticizers [14,80]. Some of these studies are highly useful reviews of the known pharmacokinetics datasets [26,73] that help in the identification of proper biomarkers and highlights the limitation to overcome in this type of studies that is share with consumption studies —many of the biomarkers used are not exclusive to humans and can therefore sometimes come from other sources, making it difficult to assess exposure.

Other studies focus on heath/disease status, such as stress [42,43,57,81,82], hepatitis B [83], diabetes mellitus [84], Gout [85] or just in endogenous human biomarkers (such as catecholamines [86] and others [87]). Studies on disease have a major flaw since are

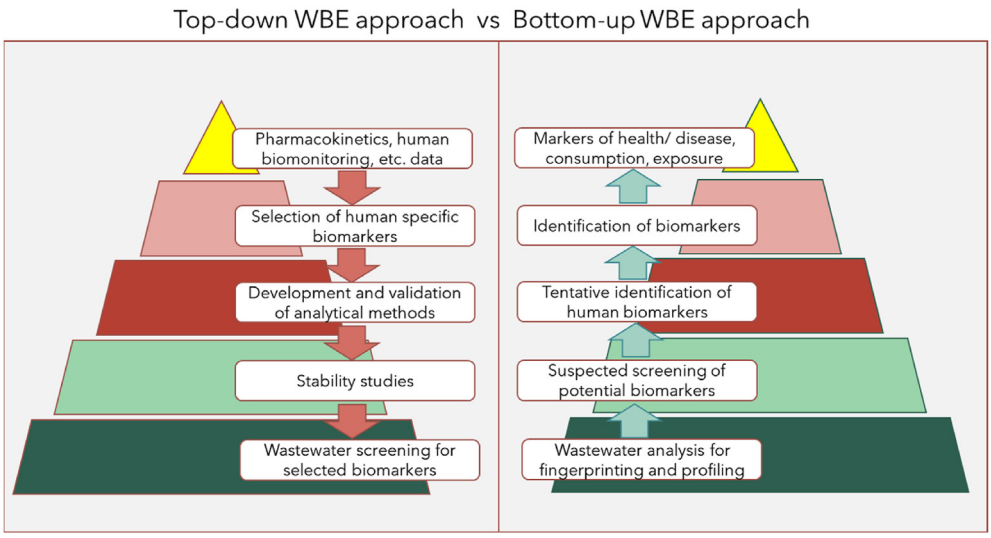

Fig. 1. Comparison of the top-down and bottom-up schemes for WBE.

Table 1
Recent application of top-down schemes to determine biomarkers in WBE.

Substances	Biomarkers	Extraction of wastewater	Determination	LODs (ng/L)	Stability[a]	Ref.
Consumption						
Illicit drugs	Methamphetamine	SERS: 200 mL acidified at pH 2 SPE cationic AFFINISEP and elution with 2 mL MeOH LC-MS: 200 mL MCX SPE and elution with 4% NH_4OH in MeOH. FEV: 200 μL	SERS-active sensor: with Au@Ag core-shell in form of glass paper LC-QqQ-MS/MS (standard procedure not specified)	–	Appropriate	[28]
Illicit drugs	Methamphetamine/amphetamine	200 mL acidified at pH 2 MCX SPE and elution with MeOH and 4% NH_4OH in MeOH. FEV: 225 μL	GC-MS (Derivatization TFA) HP-1 column (0.25 mm i.d., 0.25 μm)	2.86–3.36	Appropriate	[30]
Illicit drugs and NPSs	36 illicit drugs 40 NPs	100 mL (acidified at pH 2 for NPSs) Oasis HLB (Oasis MCX for NPSs) SPE and elution with MeOH and 5% NH_4OH in MeOH. FEV: 1 mL	UHPLC-QqQ-MS/MS ESI (+) Force Biphenyl® column (100 mm × 2.1 mm i.d. × 1.8 μm) and H_2O 0.1% FA and MeOH at 0.2 mL/min	<10	Appropriate	[32]
Illicit drugs	BE, METH, AMP, THC, and THCCOOH	125 mL Oasis HLB SPE and elution with MeOH:AcN. FEV: 1 mL	UHPLC-QqQ-MS/MS ESI (+) Zorbax SB-C18 (30 × 2.1 mm, 3.5 μm) and H_2O 0.1% FA and AcN at 0.2 mL/min	<10	Appropriate	[33]
Illicit drugs, pharmaceuticals and PPCPs	30 illicit drugs and PPCPs, along with their metabolites	Passive samplers (MPTs of Strata-X and Strata-X in agarose) directly in the influent. FEV: 1 mL Composite samples: 100 mL SPE on Strata X and eluted with MeOH. FEV: 1 mL	LC-QqQ-MS/MS (QTRAP) ESI (±) Phenomenex Biphenyl (50 × 2.1 mm, 2.6 μm) and H_2O 0.1% FA and MeOH at 0.3 mL/min	<10	Cocaine only detected in MPTs	[34]
Illicit drugs and antibiotics	15 illicit drugs and 18 antibiotics	Thin film passive samplers [three types of resin gels (HLB, XAD 18, and XDA-1)] directly in the influent. FEV: 1 mL. SPE: 100 mL (adjusted to pH 2 and EDTA) SPE on Oasis HLB or MCX and eluted with MeOH. FEV: 1 mL	LC-QqQ-MS/MS ESI (+) ZORBAX XDB-C18 column (150 × 4.6 i.d., 3.5 μm) and H_2O 0.4% FA and 10 mM NH_4HCO_2 and MeOH at 0.2 mL/min	<10	Appropriate	[102]
Illicit drugs and pharmaceuticals	29 compounds	250 mL adjusted at pH 2 for Oasis MCX and 6 for HLB Two SPE Oasis MCX (11 compounds) and Oasis HLB (18 compounds) and elution with 2% of NH_3 MeOH (MCX) or 5% of NH_4OH MeOH (HLB). FEV: 1 mL	LC-QqQ-MS/MS ESI (+) Analytical column not reported and H_2O 0.1% FA and AcN at 0.2 mL/min	0.66–13.9	Appropriate	[35]
Illicit drugs	benzoylecgonine, 6-acetylmorphime, amphetamine, methamphetamine, MDMA, and 11-nor-9-carboxy-Δ9-tetrahydrocannabinol	5 mL of 50-fold diluted Online SPE PLRPs cartridges and elution with the mobile phase	LC-QqQ-MS/MS (QTRAP) ESI (+) Purospher Star RP-18 end-capped column (125 × 2.0 mm, 5 μm) and H_2O 20 mL FA/NH_4HCO_2 (pH 3.8) and AcN at 0.3 mL/min		Appropriate	[37]
Illicit drugs	Amphetamine, its metabolites and degradation products	50 mL of water adjusted or not at pH 2 SPE Oasis MCX and MeOH. FEV:0.2 mL	LC-QqQ-MS/MS ESI (+) Phenomenex Luna HILIC (150 mm × 3 mm, 5 μm) or CHIRALPAK® CBH HPLC column (amphetamine) (100 × 2 mm, 5 μm) and H_2O and 1 mM AA and MeOH	–	Appropriate	[95]
Illicit drugs	amphetamine, methamphetamine, 3,4-methylenedioxymethamphetamine and cocaine benzoylecgonine and 11-	Several methods: -Strata X SPE and elution with MeOH -Online SPE PLRPs cartridges -Oasis MCX SPE and elution with 5%	Several methods based on LC-QqQ-MS/MS, ESI (+) Enantiomeric method: Lux AMP	<10	Appropriate	[38]

(*continued on next page*)

Table 1 (*continued*)

Substances	Biomarkers	Extraction of wastewater	Determination	LODs (ng/L)	Stability[a]	Ref.
	nor-9-carboxy-Δ9-tetrahydrocannabinol and several NPSs	NH_3 in MeOH. (Different sample and extract volume	(150 × 3 mm, 3 μm) and H2O 5% NH_3 and MeOH at 0.4 mL/min			
Illicit drugs	58 licit and illicit compounds	Wastewater volume not reported. SPE in Tecan Cerex Trace-B and elution with DCM:isopropyl alcohol:NH_4OH. FEV: 100 μL	LC-QqQ-MS/MS ESI (+). Two instruments: 1.- Restek Raptor Biphenyl (50 × 2.1 mm, 2.7 μ) and H_2O 2 mM AF+0.1% FA and MeOH 2.- Cortecs C18 HPLC column (2.1 × 100 mm; 2.7 μm) and H_2O 2 mM AF+0.1% FA and MeOH	<10	Appropriate	[40]
Illicit drugs	amphetamine, cocaine benzoylecgonine and 11-nor-9-carboxy-Δ9-tetrahydrocannabinol	50 mL Oasis HLB SPE and eluted with 5 mL of MeOH. FEV: 500 μL	LC-QqQ-MS/MS ESI (+) Allure® PFPP (50 mm × 2.1 mm, 5 μm) and H_2O 2 mM AF and MeOH at 0.4 mL/min	<10	Appropriate	[41]
Illicit drugs	Methamphetamine, cocaine	Direct injection of 20 μL of water filtered with 0.22 μm microporous membrane	Colorimetric biosensor based on hybridation of DNA to nanoparticles (non-aggregated noble metal nanoparticles (AuNPs and Au@Ag)	1–3	Appropriate	[98]
Opiods	Human metabolites of heroin, fentanyl, codeine, morphine, hydrocodone, hydromorphone, oxycodone, oxymorphone, tramadol, methadone, buprenorphine, and naloxone	10 L over 24 h period Robotic sampler: In-situ Oasis HLB extraction of composite samples and elution in the laboratory with MeOH. FEV: not specified	LC-QqQ-MS/MS (no information on the separation and detection conditions)	–	–	[18]
Opiods	19 Opiods	100 mL HLB Oasis SPE and elution with 2 mL of 2% FA in MeOH. FEV: 150 μL	LC-QqQ-MS/MS ESI (+) Atlantis® T3 column (150 × 2.1 mm, 3 μm) and H_2O and MeOH 0.1% FA at 0.275 mL/min	1–100	Buprenorphine, EDDP, fentanyl and normorphine are not benchtop stable	[64]
Opiods, antidepressants and stimulants	Cocaine, methamphetamine, amphetamine, MDMA, cannabis, heroin, ketamine, and fentanyl	110 mL acidified at pH 2 Oasis MCX SPE and elution with MeOH- FEV: 0.4 mL	LC-LTQ-Orbitrap-MS ESI (+) with selection of precursor ions for MS^2 LC-Separation not reported	14–24	Appropriate	[65]
NPSs	Areca alkaloid	50 mL filtered Cleanert PCX SPE and elution with 5% $NH_3 \cdot H_2O$ in MeOH. FEV: 0.5 mL	LC-QqQ-MS/MS ESI (+) Phenomenex Luna HILIC column (150 × 3 mm, 5 μm) and H_2O 0.1% Ac.Ac. And can at 0.3 mL/min	0.1–30	In 24 h decrease 20% (tested for several time intervals and temperatures)	[61]
NPSs	Multiclass illicit drugs (cocainoids, opiates, amphetamines, cannabinoids) and psychoactive pharmaceuticals (anxiolytics, hypnotics, antipsychotics, antidepressants, antiparkinsonian	200 mLadicified at pH 2 Oasis MCX SPE and elution with 6 mL MeOH (0.1% FA) Oasis HLB SPE and elution with 6 mL MeOH (0.5% FA) FEV: 1 mL	LC-QqQ-MS/MS ESI (+) BEH C18 (100 × 2.1 mm, 1.7 μm) and H_2O and MeOH 0.1% FA at 0.3 mL/min	0.8–9.4	Appropriate	[39]
NPSs	MDMA, MDA, cocaine and methamphetamine and three NPSs: ethylone, mephedrone and N-ethylpentylone	100 mL acidified at pH 4-5 Mixed mode UCT XRADH 506 SPE and elution with 6 mL of DCM: isopropanol:NH_3 FEV: 100 μL	LC-QqQ-MS/MS ESI (+) Kinetex Biphenyl column (150 × 2.1 mm, 1.7 μm) and H_2O and MeOH 0.1% FA at 0.3 mL/min	0.2–300	14 days at pH 2	[63]
Tobacco	Nicotine metabolites: -cotinine -trans-3′-hydroxycotinine	Filtered through a glass filter 0.22 μm GHP filters, 1.15 mL adjusted to pH 5 and treated with 0.15 mL β-glucuronidase and injected 10 μL	LC-QqQ-MS/MS ESI (+) Fusion-RP (100 × 2.0 mm, 4 μm) and H_2O and MeOH 5 mm NH_4Ac. At 0.4 mL/min	400–1000	Appropriate	[54]
Tobacco	Anabasine, anatabine, and nicotine	Direct injection: 1 mL adjusted to pH 2 filtered through 0.22 μm and injected 50 μL	LC-QqQ-MS/MS ESI (+) Gemini NX-C18 column (3 μm, 110 Å,	–	Appropriate	[55]

		SPE clean-up: 1 mL adjusted to pH 2 filtered through 0.22 μm RC, HLB SPE to retain impurities, and injected 50 μL of the cleaned water.	50 × 2 mm) and H_2O with NH_3/NH_4 Ac. (pH 10) and MeOH at 0.4 mL/min			
Tobacco	cotinine, hydroxycotinine	1 mL adjusted to pH 2, filtered through 0.22 μm RC, HLB SPE to retain impurities, and injected 50 μL of the cleaned water.	LC-QqQ-MS/MS ESI (+) Gemini NX-C18 column (3 μm, 110 Å, 50 × 2 mm) and H_2O with NH_3/NH_4 Ac. (pH 10) and MeOH at 0.4 mL/min	–	Appropriate	[56]
Nicotine	cotinine *trans*-3′-hydroxycotinine	Direct injection: 1 mL adjusted to pH 2 filtered through 0.22 μm and injected 50 μL SPE clean-up: 1 mL adjusted to pH 2 2 filtered through 0.22 μm RC HLB SPE to retain impurities, and injected 50 μL of the cleaned water.	LC-QqQ-MS/MS ESI (+) Phenomenex Kinetex Biphenyl (50 × 2 mm, 2.6 μm) and H_2O and MeOH 0.1% FA at 0.3 mL/min	100 500	Appropriate	[47]
Nicotine	cotinine *trans*-3′-hydroxycotinine	3 mL pH near 7 HLB SPE and elution with MeOH. FEV: 100 μL	LC-QqQ-MS/MS (QTRAP) ESI (+) Separation not described	–	Appropriate	[52]
Nicotine	Nicotine Cotinine	3 mL pH near 7 PEP-2 SPE and elution with methanol. FEV: 100 μL	GC-MS (derivatization with MBTFA HP-1 (30 m × 0.25 mm × 0.25 μm)	253	Appropriate	[53]
Nicotine	Cotinine Nicotine	50 mL pH near 7 HLB SPE and elution with MeOH. FEV: 500 μL	LC-QqQ-MS/MS ESI (+) Allure PFPP (50 × 2.1 mm, 5 μm) and H_2O 0.1% NH_4F. and MeOH at 0.4 mL/min	300	Appropriate	[51]
Caffeine	1,7-dimethyluric acid	3 mL pH near 7 HLB SPE and elution with MeOH. FEV: 100 μL	LC-QqQ-MS/MS (QTRAP) ESI (+) Separation not described	–	Appropriate	[52]
Caffeine	Caffeine	3 mL pH near 7 PEP-2 SPE and elution with methanol. FEV: 100 μL	GC-MS (derivatization with MBTA) HP-1 (30 m × 0.25 mm × 0.25 μm)	253	Appropriate	[53]
Alcohol	Ethyl sulphate	Centrifuged at 3500 rpm for 10 min, 190 μL filtered through 0.22 μm and 10 μL injected.	LC-QqQ-MS/MS ESI (−) RESTEK (100 × 2.1 mm, 2.7 μm) and H_2O 0.1% FA and AcN at 0.3 mL/min	300	Appropriate	[46]
Alcohol	Ethyl sulphate	Centrifuged at 3500 rpm for 10 min, 1 mL filtered through 0.22 μm and 10 μL injected.	LC-QqQ-MS/MS ESI (−) Polar-RP (50 × 2.1 mm, 1.7 μm) and H_2O 0.1% FA and MeOH at 0.3 mL/min	1000	Appropriate	[47]
Alcohol	Ethyl sulphate	Centrifuged at 3500 rpm for 10 min, 190 μL filtered through 0.22 μm and 10 μL injected.	LC-QqQ-MS/MS ESI (−) Atlantis T3 (2.1 mm × 150 mm, 3 μm) and H_2O 0.1% Ac.Ac. And AcN at 0.180 mL/min	1000	Appropriate	[49]
Alcohol	Ethyl sulphate	Centrifuged at 3500 rpm for 10 min, 190 μL filtered through 0.22 μm and 10 μL injected.	LC-QqQ-MS/MS ESI (−) Allure PFPP (50 × 2.1 mm, 5 μm) and H_2O 0.1% NH_4F. and MeOH at 0.4 mL/min	300	Appropriate	[51]
Artificial sweeteners	cyclamate, acesulfame, sucralose, saccharin, aspartame, neotame and neohesperidin dihydrochalcone	Filtration through RC 0.22 μm and injection of 6 μL.	LC-QqQ-MS/MS ESI (+) Luna Omega Polar C18 100 Å (50 × 2.1 mm, 1.6 μm) and H_2O 0.1% FA and AcN at 0.3 mL/min	50–950	Appropriate	[44]
Artificial sweeteners	Sucralosa acesulfame	Direct injection, sample treatment prior to injection, not specified, volume injected not specified	LC-QqQ-MS/MS ESI (+) Other conditions not specified	2–20	Appropriate	[45]

(*continued on next page*)

Table 1 (*continued*)

Substances	Biomarkers	Extraction of wastewater	Determination	LODs (ng/L)	Stability[a]	Ref.
Pharmaceuticals	168 pharmaceuticals and metabolites	50 mL treated with EDTA, prepared in duplicated and adjusted at pH 2 and 7 Waters Oasis HLB, Agela Cleanert PEP-2, and CNW Poly-Sery HLB SPE were tested and elution with 4 mL of MeOH. FEV: 0.5 mL	LC-QqQ-MS/MS (QTRAP) ESI (±) Phenomenex C18 column (50 × 3.0 mm, 2.6 μm) and H_2O 0.1% FA and AcN at 0.3 mL/min	0.1 (for 162)	Appropriate	[22]
Pharmaceuticals	Citalopram, enalapril, losartan, Ramipril, N-desmethylcitalopram, Enalaprilat, EXP-3174, Ramiprilat	25 mL acidified at pH 2 Oasis MCX SPE and elution with MeOH and 2% NH_3 MeOH. FEV: 0.2 mL	LC-QqQ-MS/MS (QTRAP) ESI (+) Kinetex C18 column (150 × 3.0 mm, 2.6 μm) and H_2O 0.1% FA and MeOH at 0.2 mL/min	0.6–9.3	Appropriate	[66]
Pharmaceuticals	Trimethoprim, nalixidic acid, chloramphenicol, erythromycin, clarithromycin, roxithromycin, clindamycin, lincomycin, sulfadiazine, sulfamethoxazole	100 mL filtrated, added of EDTA, L-ascorbic and acidified at pH 4 Oasis HLB SPE and elution with MeOH/H_2O. FEV: 0.5 mL	LC-QqQ-MS/MS ESI (+) Agilent XDB C18 column (150 × 3.0 mm, 3.5 μm) and H_2O 0.1% FA and MeOH at 0.2 mL/min	<10	Appropriate (tested for several time intervals and temperatures)	[67]
Pharmaceuticals	NSAIDs	100 mL without treatment) Oasis HLB SPE and elution with 6 mL of MeOH. FEV: 0.5 mL	LC-QqQ-MS/MS (QTRAP) ESI (−) Athena C18 column (250 × 4.6 mm × 5 μm) and H_2O 5 mM NH_4Ac/Ac.Ac. And MeOH at 0.2 mL/min	0.05–0.1	Appropriate	[68]
Pharmaceuticals	Pharmaceuticals and their metabolites	50 mL added of EDTA Oasis HLB SPE and elution with 6 mL of MeOH. FEV: 1 mL	LC-QqQ-MS/MS (QTRAP) ESI (±) ESI(+): Acquity HSS T3 (50 × 2.1 mm, 1.8 μm) and H_2O 5 mM NH_4F/FA and MeOH at 0.2 mL/min ESI (−):Acquity BEH C18 column (50 × 2.1 mm, 1.7 μm) and H_2O 5 mM NH_3/NH_4Ac (pH 8.3) and MeOH at 0.2 mL/min	0.24–128	Appropriate	[70]
Pharmaceuticals	11 Benzodiazepines and 3 transformation products	1 L of water without treatment (determined in the particulated matter after retains it in a 0.45 μm glass filter and extracted the analytes in acidified MeOH) Oasis HLB SPE and elution with MeOH/ethyl acetate/DCM. FEV: 1 mL	LC-QqQ-MS/MS ESI (+) Eclipse Plus C18 column (2.1 × 50 mm, 1.8 μm) and H_2O 5 mM FA/NH_4Ac and AcN at 0.2 mL/min	0.038–0.916	Appropriate	[71]
Exposure to contaminants						
Organophosphorus flame retardants	Metabolites of OPFRs	100 mL acidified at pH 4–5, Bond-Elut C18 SPE and elution with MeOH. FEV: 0.2 mL	LC-QqQ-MS/MS ESI (±) by automatic switching Phenomenex Kinetex Biphenyl reversed phase column (2.1 × 100 mm, 2.6 μm) and H_2O 5 mM NH_4Ac and MeOH at 0.2 mL/min	0.8–15.4	Appropriate (tested for several time intervals and temperatures)	[14]
Plasticizers	Phthalate, terephthalate and di-iso-nonyl cyclohexane-1,2-dicarboxylate metabolites	100 mL Oasis MAX SPE and elution with 5 mL of 2% FA in MeOH. FEV: 0.1 mL	LC-QqQ-MS/MS Raptor Biphenyl column (150 × 2.1 mm, 1.8 μm) and H_2O 0.1% AA and AcN at 0.3 mL/min	0.08–4.4	Appropriate	[80]
Pesticides (Triazines, and pyrethroids)	Urinary metabolites	100 mL acidified at pH 7–7.5 Oasis HLB and elution with MeOH. FEV: 0.1 mL	LC-QqQ-MS/MS ESI (+) XSELECT™ CSH™ C18 (2.1 × 100 mm, 2.5 μm) and H_2O 0.1% AA and AcN at 0.3 mL/min	0.99–15.36	Appropriate	[75]
Pesticides (Triazines, organophosphorus and pyrethroids)	Urinary metabolites	Alkyl phosphate compounds DEP, DETP, DMP and DMTP were analyzed by direct injection of 4 μL after centrifugation of 180 μL	LC-QqQ-MS/MS ESI (+) XSELECT™ CSH™ C18 (2.1 × 100 mm,	0.99–15.36 (SPE) 35–790 (DI)	Appropriate	[74]

		Others: 100 mL Oasis HLB and elution with MeOH. FEV: 0.1 mL	2.5 μm) and H_2O 0.1% AA and AcN at 0.3 mL/min			
Pesticides (Triazines, organophosphorus and pyrethroids)	Urinary metabolites	Alkyl phosphate compounds DEP, DETP, DMP and DMTP were analyzed by direct injection of 4 μL after centrifugation of 180 μL Others: 100 mL Oasis HLB and elution with MeOH. FEV: 0.1 mL	LC-QqQ-MS/MS ESI (+) XSELECT™ CSH™ C18 (2.1 × 100 mm, 2.5 μm) and H_2O 0.1% AA and AcN at 0.3 mL/min	0.99–15.36 (SPE) 35–790 (DI)	Appropriate	[76]
Phthalate plasticizers	Phthalate metabolites identified from HBM data	100 mL acidified at pH 2 Oasis HLB Prime and elution 2 mL with MeOH. FEV: 1 mL	UHPLC-QqQ-MS/MS ESI (−) Luna Phenyl-Hexyl column (150 × 2 mm, 3 μm) and H_2O 0.1% AA and AcN at 0.2 mL/min	<10	Appropriate	[79]
Mycotoxins	11 specific biomarkers	25 mL acidified at pH 2.5–3 Oasis HLB Prime and elution 3 mL with MeOH. FEV: 0.1 mL	LC-QqQ-MS/MS XSelect CSH C18 (2.1 × 100 mm, 2.5 μm) and H_2O 0.1% FA and AcN at 180 μL/min	05–98	Decrease of 3AcDON, T2-toxin, B-ZEN in 24 h	[77]
Prevalence of disease						
Hepatitis B	Lamivudine	50 mL adjusted at pH 2 MCX Prime SPE and elution with 5 mL 5% NH_3–MeOH. FEV: 0.2 mL	LC-QqQ-MS ESI (+) ZORBAX Eclipse Plus C18 (50 × 2.1 mm, 1.8 μm) and H_2O 0.1% FA and AcN at 0.3 mL/min		Appropriate (at 27°C and 4°C degradation could be 15%)	[83]
Food and stress	30 biomarkers, 22 not previously assessed	Filtered (not indicated the pore size or characteristics of the filter) acidified at pH 2 and injected (not indicated the μL injected)	LC-QqQ-MS/MS (Q TRAP) Kinetex Biphenyl (2.1 × 50 mm, 2.6 μm) and H_2O 0.1% AA and MeOH at 0.3 mL/min	0.01–0.45	γCEHC, phloretin and 3MeHis degraded Anserine, PC, ClY, BrY, 8OHdG and 8OHG formed	[42]
Stress metabolites	8-iso-prostaglandin F2α, its metabolite dinor-11β-Prostaglandin F2α and Prostaglandin E2	Filtered (not indicated the pore size or characteristics of the filter) acidified at pH 2 and injected (not indicated the μL injected)	LC-QqQ-MS/MS (QTRAP) ESI (±) Kinetex Biphenyl (2.1 × 50 mm, 2.6 μm) and H_2O 0.1% AA and MeOH at 0.3 mL/min	0.03–0.43	Appropriate (tested for several time intervals and temperatures)	[81]
Stress	hydroxynonenal–mercapturic acid, 8-iso-prostglandin F2beta, 8-nitroguanine and 8-hydroxy-2-deoxyguanosine	100 mL without any adjustment Oasis HLB SPE and elution with 4 mL of MeOH. FEV: 0.5 mL	LC-QqQ-MS/MS ESI (±) BEH C18 column (150 × 1.0 mm, 1.7 μm) and H_2O:MeOH with 1 mM NH_4F (for ESI-) or H_2O:MeOH with 0.1% FA (for ESI+) at 0.04 mL/min	1.3–3.0	Appropriate (tested for several time intervals and temperatures)	[82]
Stress	8-iso-prostaglandin F2α	100 mL with β-glucuronidase 8-isoprostane affinity sorbent SPE and elution with eicosanoid affinity column elution solution. FEV: 0.1. mL	LC-QqQ-MS/MS ESI (±) BEH C18 column (150 × 1.0 mm, 1.7 μm) and H_2O:MeOH with 1 mM NH_4F (for ESI-) or H_2O:MeOH with 0.1% FA (for ESI+) at 0.04 mL/min	1.3	Not reported	[57]
Diabetes	Metformin	5 mL adjusted at pH 7 Strata X–CW SPE and elution MeOH + AcN and 1% FA. FEV: 0.4 mL	GC-MS (derivatization with MBTA) HP-1 (30 m × 0.25 mm × 0.25 μm)	253	Appropriate	[84]
Gout (Allopurinol)	Oxypurinol	Filtered with RC filters of 0.22 μm. Injection volume not specified.	LC-QqQ-MS/MS (QTRAP) ESI (+) Hydro RP 80 Å LC (150 × 3 mm, 4 μm) and H_2O 0.1% FA and MeOH at 0.45 mL/min	–	Appropriate	[85]
Catecholamine	homovanillic acid and vanillylmandelic acid	LLE: 10 mL + NaCl + ethyl acetate. FEV: 0.2 mL	LC-QqQ-MS/MS ESI(+) Phenomenex ® Kinetex ™ F5 column (100 × 2.1 mm, 1.7 μm) and H_2O 10 mM	–	Appropriate (tested for several time intervals and temperatures)	[86]

(*continued on next page*)

Table 1 (*continued*)

Substances	Biomarkers	Extraction of wastewater	Determination	LODs (ng/L)	Stability[a]	Ref.
Population biomarkers	4-Pyridoxic acid Cotinine 3′-hydroxycotinine 1,4-methylimidazole acetic acid	50 mL adjusted at pH 2 Oasis PRIME MCX SPE and elution with 4 mL 5% NH_3–MeOH, FEV: not specified	NH_4Ac and ACN:MeOH 0.01% FA at 0.3 mL/min LC-QqQ-MS/MS ESI(+) ZORBAX Eclipse Plus C18 column (50 × 2.1 mm, 1.8 μm) and H_2O 0.1% FA and AcN at 0.45 mL/min	2.5–250	Appropriate (tested for several time intervals and temperatures)	[87]

3-AcDON: 3-acetyl-deoxynivalenol; 3MeHis: 3-methylhistidine; 8OHdG: 8-hydroxyguanosine; 8-OHG: 8-hydroxyguanosine; αCEHC: α-carboxyethyl hydrochroman; β-ZEN: beta-zearalenol: AA: acetic acid; AcN: acetonitrile; AMP: amphetamine; BE: benzoyl ecgnonine methyl ester; BrY: bromotyrosine; CIY: chlorotyrosine; DI: direct injection DCM: dichlormethane; ESI: electrospay ionization; FA: formic acid; GC-MS: gas chromatography-mass spectrometry; FEV: final extract volumen; HBM: human biomonitoring; LC-QqQ-MS/MS: liquid chromatography triple quadrupole tándem mass spectrometry; LLE: liquid; MBTFA: N-Methyl-bis (trifluoroacetamide); MDA: 3,4-Methylenedioxyamphetamine; MDMA: 3,4-metilendioxi-metanfetamina or ecstasy; MeOH: methanol; METH: methadone; MPTs: microplastic polyethylene tubes; NH_4Ac: ammonium acetate; NPSs: new psychoactive substances; NSAID: non steroidal antiinflammatories; OPFRs: organophosphorus flame retardants; PC: propionylcarnitine; PPCPs: pharmaceuticals and personal care products; QTRAP: quadrupole linear ion trap; RC: re-generated cellulose; SERS: surface-enhanced Raman spectroscopy; SPE: solid-phase extraction; TFA: trifluoracetic acid; THC: s delta-9-tetrahydrocannabinol; THCCOOH: 11-nor-9-carboxy- 9-tetrahydrocannabinol; UHPLC-QqQ-MS/MS: ultra high-performance liquid chromatography tándem mass spectrometry.

[a] Appropriate: analyte's losses were <25% after 1 day at 4°C and 3 month at −20°C.

based on determining a drug or its metabolite used in their treatment. This, in the case of chronic diseases, or diseases of difficult diagnosis, leads to leave aside a part of the population untreated or undiagnosed, whose identification would be one of the objectives of the WBE not yet reached.

The other approach that could be considered as a bottom-up approach is based on the non-target analysis of wastewater and the identification of substances derived from humans and the study of their ability to be used as biomarkers. This analytical scheme identifies the different substances (biomarkers, their transformation products and other compounds) present in wastewater after a difficult and long analytical process. Due to the complexity of wastewater, high amount of organic matter, different types of human and biota metabolites, degradation products, etc., this approach has been much less used than the top-down approach. However, it could offer interesting and extensive information about substances still unknown that can be pivotal to make reality the possibilities of WBE.

Nowadays, the possibilities of the bottom-up approach to detect different biomarkers have already been quite explored. Several bottom-up studies are involved in the estimation of community-wide exposure to bisphenol A [88], searching of proteins [89], cancer [65] and multi-chemical exposure [90] markers, as well as investigation of (bio)transformation products [91,92] and new psychoactive substances [93,94]. All these studies highlight the difficulty of using this system in wastewater, mainly because many of the biomarkers are not major compounds in the matrix. However, this analytical strategy could become a successful, fast and an economically advantageous tool for the screening of biomarkers in wastewater after performing many improvements still needed.

2.1. Analytical technique employed in the top-down approaches

Table 1 summarizes the analytical methods using the top-down approach. Most of them included a sample preparation step. Two classical approaches direct injection (after filtering, centrifuging or any other type of mechanical or physical operation) and solid-phase extraction (SPE) [using from the classical hydrophilic lipophilic balance (HLB) phases to ionic exchangers, passing through mixed mode or home-made prepared cartridges mixing several phases] are the most common. The use of either method depends on a combination of two factors (i) the ability of the biomarker to be retained in a cartridge and (ii) the concentration of biomarker found in wastewater. For example, illicit drugs and pharmaceuticals are usually extracted by SPE [32,34,37,95] while alcohol (ethyl sulphate) is determined by direct injection [46,47,49,51] (see Table 1 for more exhaustive examples).

There is one important development in extraction that deserves to be mentioned. The passive sampling at sewage treatment plants, which has proven to be advantageous. McKay et al. [34] developed, calibrated and validated a microporous polyethylene tubes (MPT) passive sampler for quantitative estimation of illicit drugs and pharmaceuticals and personal care products (PPCPs) by in-situ deployment in wastewater influent. Liu et al. [69] evaluated diffusive gradients in thin-film passive samplers for 15 illicit drugs and 18 antibiotics using three types of resin gels (HLB, XAD 18, and XDA-1). Both types of samplers were capable to establish the concentrations of target biomarkers in wastewater influents showing as promising devices for providing essential monitoring data for WBE. However, passive samplers are not without their disadvantages, such as to retain a limited number of compounds, little robustness (affected by environmental conditions, biofouling, etc.) and particularly the need to in-field calibrate them, which is why their use is still in an early stage.

The detection, identification and quantification of biomarkers is carried out mostly by LC-MS using highly sensitive and quantitative triple quadrupole (QqQ) mass analysers (LC-QqQ-MS/MS) (see Table 1 for the appealing number of references). The linear ion trap is used in some of the references but always is used as a triple quad [22,34,37,42,52,66,68,70,81,85]. Most of the chromatography is a reverse phase chromatography that applies the ultra-high performance liquid chromatography (UHPLC) columns of 1.7 μm. The stationary phases are mostly C18 but as most of the analytes are very polar, there are other phases such as biphenyl [14,32,34,40,42,47,63,80,81] or pentafluoropropyl (PFP or F5) [41,51,86] also frequently used. The mobile phases are mostly water with formic or acetic acids or with volatile salts, such as ammonium formate, acetate, etc., methanol and acetonitrile. Most of the biomarkers are determined in positive ionization mode since basic or neutral molecules are most common (Table 1). However, there are a few biomarkers that are determined in negative mode [14,22,34,46,47,49,51,57,68,70,79,81,82], so, this mode cannot be dismissed.

Enantiomeric analysis has been used to distinguish human-eliminated drugs from those from a direct release (when the latter is suspected based on daily fluctuations in mass loading), particularly, for the amphetamic compounds [38,95]. This approach is more beneficial when the parent compound is used as human biomarker [95] but there are also cases of pharmaceuticals, which render enantiomerically pure substances where enantiomeric analysis is also useful [96].

Interestingly, some references report the determination of illicit drugs, pharmaceuticals, caffeine and metformin by GC-MS after derivatization with trifluoroacetic acid (TFA) [30] or methyl-bis (trifluoroacetamide) (MBTFA) [53,84]. This method is not commonly for biomarker profiling since most metabolites have polar groups and the derivatization step is mandatory. However, GC-MS showed high sensitivity and reproducibility highlighting its alternative character to LC [84] and derivatization could help to increase selectivity and specificity of the analysis.

Furthermore, the use of different types of (bio)sensors has also been reported to improve speed and efficiency, which has been the subject of several reviews that presented biosensors as an effective tool for WBE [28,29,97]. Furthermore, recently, Mao et al. [98] outlined a colorimetric method based on non-aggregated noble metal nanoparticles (AuNPs and Au@Ag) for determining illicit drugs. The biosensor consisted of DNA reporter probes, capture probes, and illicit drug-binding DNA aptamers (see Fig. 2). The absorbance intensity was correlated with the concentrations of illicit drugs, enabling their quantitation. These results proved that sensors have potential on estimating the consumption of illicit drugs for WBE. Mao et al. [28] reported a disposable paper sensor upon a surface-enhanced Raman spectroscopy (SERS) for the sensitive and selective detection of methamphetamine based upon the assembly of noble metal core-shell nanoparticles on a bespoke glassy nanofibrous electrospun paper matrix. Innovations like these represent the future of analytical technologies within WBE.

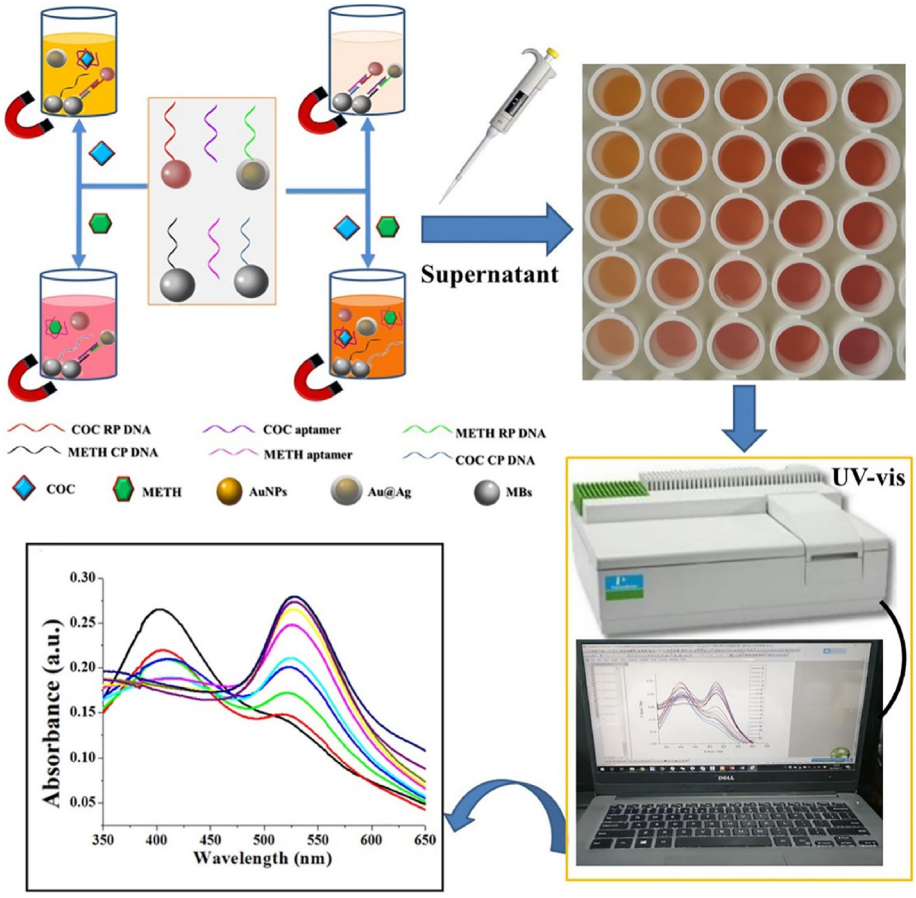

Fig. 2. Schematic illustration of colorimetric detection of METH and cocaine based on non-aggregated nanoparticles (C-RP: cocaine reporter probe, M-RP: methamphetamine reporter probe, M – CP, methamphetamine capture probe, and C-CP: cocaine capture probe). Reproduced from Ref. [98] with permissions of Elsevier.

2.2. Analytical techniques in the bottom-up approaches

Table 2 outlines the few studies that use bottom-up approaches, that mostly rely on liquid chromatography–HRMS. These studies have many common characteristics and a number of distinguishing features. The first common feature is that most studies propose a prior step of isolation and concentration of the analytes by SPE. The SPE is performed mostly as in the previous approach with sorbents based on HLB [88,90,94] and mixed-mode cartridges containing strong or weak cationic exchangers [95]. Interestingly, a home-made cartridge that mixes several sorbents (Oasis HLB, Isolute ENV+, Strata-X-AW and Strata-X-CV) have also been proposed [91,99]. The aim was to broaden as much as possible the spectrum of compounds that can be retained to be able to identify as many compounds as possible. It is reported that this cartridge was very advantageous as it also eliminated many interferences in a matrix (wastewater) that is very complex.

Regarding the instrumentation used, most studies focus on the quadrupole time-of-flight (QqTOF) [88,90,94,100], which has a lower resolution than the Orbitrap [101]. However, there is no reason for this, except perhaps that the QqTOF instruments have been on the market longer. It is to be expected that the number of applications with orbitrap will soon increase.

Another important aspect is how to acquire mass spectral information. There is a division between those studies using data independent acquisition (DIA) that acquires simultaneous mass spectra with different collision energies: (i) a low energy mode (LE) where low collision energy was selected for detection of protonated

Table 2
Summary of the bottom-up platforms to selected biomarkers of human exposure and consumption.

Substances	Extraction	Determination	Approach	Ref.
Markers of cancer	On-line SPE of 800 mL using solid disk sorbents in an automated system (SPDESK® 4790). Sample treated with EDTA	UHPLC-QqTOF-MS/MS ESI+ 50–800 Da (DIA: MS^E) Acquity BEH C18 column (150 mm x 2,1 mm i.d. 1,7 μm) and H_2O–MeOH 0.1% FA	Suspected list based on biomarkers of prostate and breast cancer, chemotherapy drugs associated with them, pharmaceuticals/ therapies such mitotic inhibitors, anti-metabolites, hormones and immunotherapeutic agents.	[100]
Exposure to Bisphenol A (bisphenol sulphate)	50 mL adjusted to pH 7–8, SPE with Oasis HLB and MeOH. FEV: 0.5 μL	UHPLC-QqTOF-MS/MS ESI ± 50–800 Da (DIA: MS^E) BEH C18 column (50 × 2.1 mm, 1.7 μm) and H_2O–MeOH 2.5% NH_4F	Post-acquisition data mining of analyzed wastewater samples	[88]
Markers of multi chemical exposure	50 mL adjusted to pH 7–8, SPE with Oasis HLB and MeOH. FEV: 0.5 μL	UHPLC-QqTOF-MS/MS ESI +/− (DIA: broad band bbCID) BEH C18 column (50 × 2.1 mm, 1.7 μm) and H_2O–MeOH 2.5% NH_4F	Retrospective identification and quantification of bisphenol sulphate, triclosan sulphate, 3-Bisphenol A	[90]
NPSs and their TPs	100 mL adjusted to pH 6.5 SPE homemade (Oasis HLB, Isolute ENV+, Strata-X-AW and Strata-X-CV) and MeOH/ethyl acetate with NH_4OH. FEV: 0.5 μL	LC-QqTOF-MS/MS ESI +/− (DDA: isolation *m/z* values) Acclaim RSLC C18 column (2.1 × 100 mm, 2.2 μm) and H_2O –MeOH with NH_4CO_2+0.1% FA (+) and $NH_4C_2H_3O_2$ (−)	Suspected database of TPs was built using two in silico prediction tools, the Eawag-Biocatalysis/ Biodegradation Database Pathway Prediction System and the MetabolitePredict software, and a list of reported metabolites and TPs from the literature. In silico HPLC retention times estimation is used for identification	[91]
Illicit drugs and other biomarkers specially amphetamine and its TPs	100 mL adjusted to pH 7 HLB SPE. FEV: 0.5 μL	UHPLC-Q Orbitrap-MS/MS ESI 50–1300 Da (DDA: most intense *m/z*) XBridge BEH C18 XP column (150 mm × 2.1 mm, 2.5 μm) and H_2O –MeOH 0.1% FA	Suspected screening against a homemade list of compounds Non-target screening searching major peaks in libraries available through internet.	[95]
NPSs	100 mL adjusted to pH 6.5 SPE homemade (Oasis HLB, Isolute ENV+, Strata-X-AW and Strata-X-CV) and MeOH/ethyl acetate with NH_4OH. FEV: 0.5 mL	LC-QqTOF-MS/MS ESI + DIA in source CID (bbCID) Acclaim RSLC 120C18 column (2.1 × 100 mm, 2.2 μm) and H_2O –MeOH with NH_4CO_2+0.1% FA	Wide-scope target and suspect screening methodologies against suspect list of 451 NPSs and their metabolites	[93]
NPSs and conventional drugs	250 mL SPE Strata X and MeOH/DCM. FEV: 1 mL	UHPLC-QqTOF-MS/MS ESI +50–100 Da (DDA: most intense *m/z*) Kinetex™ XB-C18100A (50 × 2.1 mm, 1.7 μm) and H_2O–MeOH 0.1% FA	Suspected and non-target workflows In-silico software to identify TPs	[94]
Cathinones and TPs	Direct injection of 2 μL after centrifugation and further filtration to 0.45 μm filter	UHPLC-QqTOF-MS/MS ESI (±) (DIA: MS/MS^E) Phenomenex Biphenyl (100 mm × 2.1 mm, 2.6 μm) and H_2O –MeOH 0.04% FA	Suspect lists comprising the name and molecular formulae from previously identified metabolites Non-target screening using the 'COmponent Detection Algorithm' and 'COMPARE LCMS' algorithm from the ACD/MS Workbook suite were used for a non-target data analysis strategy.	[92]

bbCID: broadband collision induced dissociation; FEV: final extract volume; DCM: dichloromethane; DDA:_ data dependent acquisition; DIA: data independent acquisition ESI: electrospray ionization; FA: formic acid; MeOH: methanol; MS^E: all in one mass spectra; MS/MS^E: all in one tandem mass spectrometry; NPSs: new psychoactive substances; SPE: solid phase extraction; TPs: transformation products; UHPLC-Q-Orbitrap-MS/MS: ultra-high performance quadrupole orbitrap tandem mass spectrometry; UHPLC-QqTOF-MS/MS: ultra high performance liquid chromatography coupled to a quadrupole time-of-flight tandem mass spectrometry.

or deprotonated molecular ions, and (ii) a high energy (HE) mode with a high collision energy for detection of fragment ions [88,90,93,100]. Instruments are versatile in DIA and can use collision energy ramps (instead of an only value) or acquire more than one HE scans segments in MS mode alternating the collision energies [92]. The main drawback of this mode is that it does not provide a true MS/MS of the precursor ion but the fragmentation takes place in the skimmer that selects the ions. DIA main advantage is that it provides a spectrum in which all ions were fragmented. To get a MS/MS spectrum of all ions present in the sample, recently sequential window acquisition of all theoretical fragment-ion spectra (SWATH) or $MSMS^{ALL}$ has been developed. In this mode, the instrument systematically acquire fragment data from precursor ion ranges chosen to cover the mass range of interest. The second scan mode was a Data Dependent Acquisition (DDA), which provided a full scan spectrum (MS), in which the X (between 5 and 20) most abundant precursor ions for each instrumental cycle were isolated and fragmented resulting in their HRMS/MS spectra [93]. In the case of WBE, DIA methods work better than DDA based on ion intensity because biomarkers in wastewater are not always major constituents.

Two types of methods have been used to identify biomarkers: suspect screening, and non-target analysis. Using suspect screening, Fernando-Climent et al. [100] searched cancer markers using a suspect list based on two cancer types, prostate and breast considered as the most frequent in Norway. Furthermore, the chemotherapy drugs associated with the treatment of these cancers, together with a large group of pharmaceuticals/therapies such mitotic inhibitors, anti-metabolites, hormones and immunotherapeutic agents were added to this list of suspected compounds. The high number of potential compounds as well as the low concentrations forced the use of a series of filters, inclusion/exclusion criteria and databases, which makes the analysis long, tedious and complicated compared to the top-down system. Fig. 3 illustrates the complexity the different filters and database that are selected pre-analysis and during the processing of the data to achieve proper results using bottom-up approaches and hep to understand why this approach has been so little used in comparison to the top-down. Among many other substances, several antineoplastic hormones, were identified (such as medroxyprogesterone, see Fig. 4). Similar suspect screening has been reported for NPSs and their metabolites [91–95]. Interestingly, Kinyua et al. [91] built a suspect database of potential TPs using two in silico prediction tools, the Eawag-Biocatalysis/Biodegradation Database Pathway Prediction System and the Metabolite Predict software, and a list of reported metabolites and TPs from the literature. Samples were screened using not only filters but also an in-house retention time prediction model. In the same way, Andrés-Costa et al. [94] used a metabolite finder software that works after the selection of the parent compounds, looking for a list of possible phase I (debenzylation, deethylation, nitroreduction, demethylation, etc.) and II (hydroxylation, methylation, different conjugations, etc.) reactions.

There are some examples of non-target screening, Kinyua et al. [92] used the software of the instrument through 'COmponent Detection Algorithm' (CODA) and 'COMPARE LCMS' algorithm. CODA -a molecular feature detection algorithm- was useful for peak selection, which involved the removal of noise and background peaks, recovery of the mass spectra of pure compounds and separation of the co-eluting components within data sets. The COMPARE LCMS —a differential analysis algorithm— allowed for comparison of two or more data sets with extracted feature candidates and showed the difference between them. Instead, Boogaerts et al. [95] performed non-target screening on the most intense signals visually observed in the Orbitrap chromatogram. Then, the specific mases library searches were conducted in the open internet against mzCloud (HighChem Ltd., Slovakia), mzVault (Thermo Fisher Scientific Inc., USA) (with the mzVault May 2018 library), and Chemspider (Royal Society of Chemistry, USA) (with EAWAG biocatalysis/biodegradation, EPA DSSTox, EPA toxcast, Drugbank, ACToR, and FDA UNII — NLM databases), to give a more comprehensive list of compounds. These few examples show the great possibilities of HRMS within bottom-up methods. It is to be

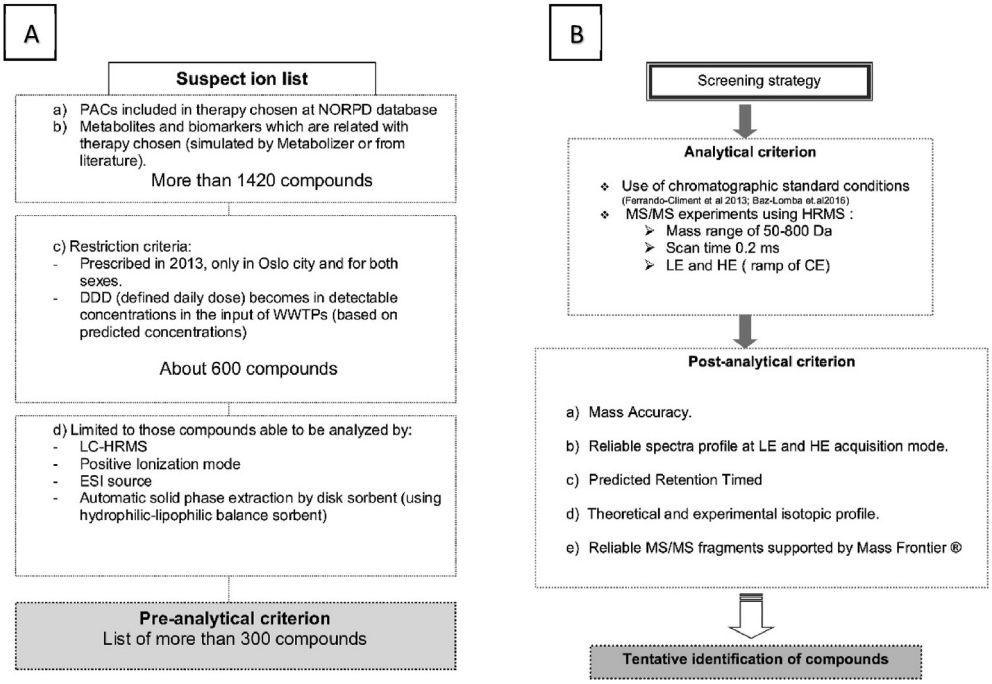

Fig. 3. Working flow in the bottom-up WBE (A) Diagram for the generation of a list of compounds suspected to be present in the input of WWTPs and (B) Strategy diagram of MS/MS screening for known-unknown compounds. Reproduced from Ref. [100] with permission of Elsevier.

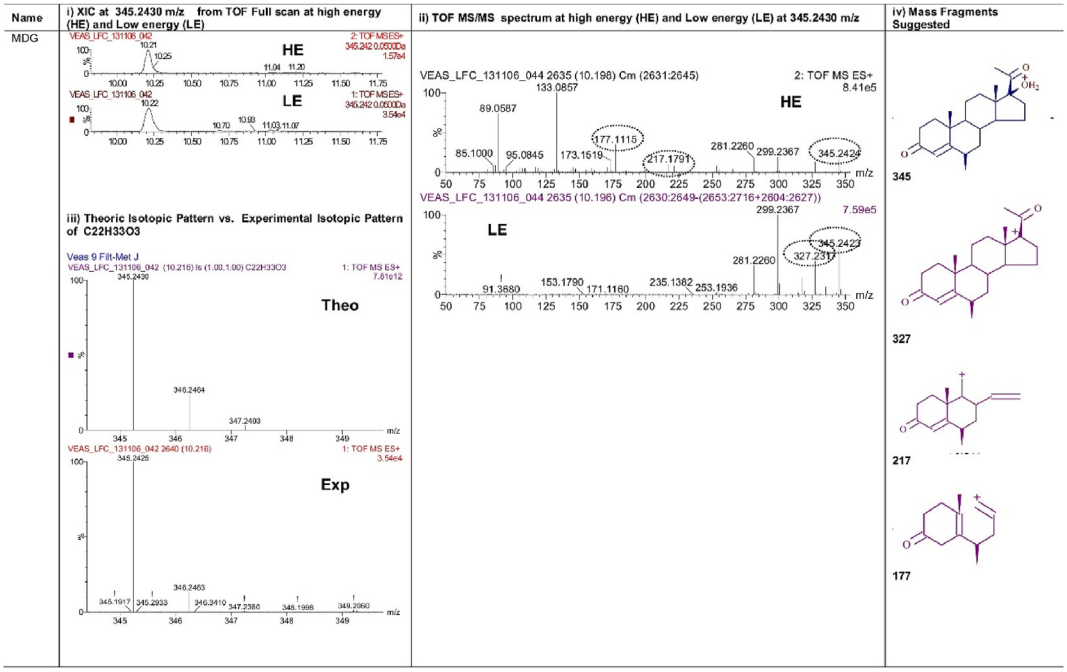

Fig. 4. Mass spectrometric criterion for Medroxyprogesterone identification: i) Peak mass detection by accurate mass, ii) spectra profile at LE and HE acquisition mode, iii) theoretical and experimental isotopic profile and iv) MS/MS fragments supported by Mass Frontier. Reproduced from Ref. [100] with permission of Elsevier.

hoped that in the future this type of methods will increase and that the possibilities of the technique in the identification of unknowns will be exploited.

Furthermore, in the non-target screening, wastewater fingerprinting involving the non-targeted chemical analysis of wastewater with subsequent multivariate data analysis based on the principle of the so-called metabolomics has also been applied [88]. Water samples were then, analyzed using a very generic chromatographic separations attaining detection of many potential compounds using HRMS. Then, after identification of as many compounds as possible in the sample retrospective data mining of their characteristic human metabolism markers is performed (most studied example is bisphenol A sulphate [88,90]). Finally, statistical analysis by multivariate methods is performed to classify the samples into specific groups according to different compounds present.

3. Conclusions and future trends

The top-down workflow is the most widely used within sewage epidemiology, based on identifying a priori metabolites and biomarkers that are determined in wastewater. In recent years, this system has evolved from focusing only on drugs of abuse to deploying a range of studies related to habits, lifestyle, consumption and health and disease of the population. This can provide a fingerprint of the characteristics of the city. From an analytical point of view, the top-down scheme uses a conventional target analysis based on SPE or direct injection of the sample into a LC-QqQ-MS/MS. This analysis, however simple, is not without complications, especially due to the complexity of the matrix and the poor stability of the analytes. For those analytes most studied, such as drugs of abuse, passive samplers and biosensors are becoming the next step that will facilitate the analysis of these substances.

Regarding the assessment of other indicators, such as those responsibles of health/disease, nutrition, etc., there is still a long way to go in this type of analysis to select uniquely human metabolites and to identify those linked to different types of diseases that are not related to drug treatment.

The bottom-up approach has been used very little in wastewater, although there is some work that highlights its incredible possibilities within the WBE. There are few but interesting papers that rely on broad searches for biomarkers directly in the wastewater itself as well as for degradation products and other metabolites. The future of WBE must take this route, especially to exploit the idea of city profiling. Bottom-up systems rely on HRMS, capable of detecting and identifying an infinite number of compounds, at least in theory.

In summary, the wide range of determinable biomarkers in wastewater identifies numerous behaviors (drug use or dietary habits, for example), exposures (to pesticides and industrial substances, for example) and health (pathogens or antibiotic resistance, for example) that profile one city versus another. Most biomarker studies are still academic and exploratory in nature. Soon, wastewater analysis will be able to provide a wealth of socially relevant information so that globally it can be provided with better services and a more sustainable environment.

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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