Evaluating substance use via wastewater analysis : an overview of analytical workflows

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Evaluating substance use via wastewater analysis: an overview of analytical workflows

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Abstract

From an epidemiological perspective, evaluating substance use (e.g. illicit drugs, alcohol, and tobacco) at the population level is a crucial aspect for the assessment of public health. Yet, currently used survey-based methods are subject to various limitations. Recently, wastewater analysis, known also as wastewater-based epidemiology (WBE), has become an established approach for retrieving additional epidemiological information. The added value of WBE to monitor illicit drug use in Europe has been recognised and adopted by the European Monitoring Centre for Drugs and Drug Addiction since 2011. WBE relies on the analysis of human metabolites (biomarkers) in urban wastewater to monitor and back-estimate substance (ab)use in the studied populations. Biomarkers of interest are typically identified and quantified with target analytical strategies using liquid chromatography coupled to tandem mass spectrometry. However, high resolution and accurate mass spectrometry is also employed in WBE as the most suitable technique to perform suspect and non-target screening for emerging substances, such as new psychoactive substances and their metabolites, in wastewater and public urinals. This article presents an overview of the recent analytical framework and workflows for target and suspect analyses using low and high resolution mass spectrometry and discusses the latest advances in WBE. Finally, future perspectives and developments are shortly discussed.

Consumption of illicit drugs is a global health issue which affects millions worldwide, with figures suggesting that as many as 29.5 million people are affected by drug-related disorders (1). There is thus an urgent need to implement fast, objective and evidence-based monitoring tools to better measure the extent of the phenomenon and assess the effectiveness of related drug policies (2). Commonly, illicit drug monitoring makes use of various indicators (e.g., general population surveys, treatment demands, police statistics and medical records) to estimate the prevalence of drug use. Yet these indicators suffer from various limitations which prevent obtaining a complete picture of the situation. In particular, they do not allow obtaining an estimate of the actual amounts of illicit drugs which are being consumed, nor do they allow to easily estimate the contribution of heavy drug users. In an attempt to overcome these limitations, an approach referred to as "wastewater-based epidemiology" (WBE) has been developed and implemented in the last decade (3). The WBE approach relies on the measurement of human metabolites of illicit drugs in wastewater samples collected at the influent of wastewater treatment plants (WWTP). Analogously to urine testing, types and levels of metabolites found in wastewater reflect the type and amount of illicit drugs consumed by the sampled community. Since its first application, WBE has seen an astonishing development, both in terms of the extent of locations monitored (4) and the analytical technologies implemented (5).

Conventional illicit drugs, such as cocaine, amphetamine-type stimulants (ATS, such as amphetamine, methamphetamine, 3,4-methylenedioxymethamphetamine (MDMA)), heroin and cannabis, and their urinary metabolites have been monitored in multiple locations across the world. Beyond conventional drugs, novel psychoactive substances (NPS), such as synthetic cathinones and phenethylamines, have also been the target of various studies (6,7). NPS represent an ever growing group of substances designed to mimic the effect of conventional drugs such as MDMA or cannabis (8). However, because their structure differs from that of controlled substances, they can be sold legally (mainly online) until they are detected and added to the list of scheduled chemicals. Currently, almost 700 NPS are being monitored, yet their number has been increasing in recent years (9) and measuring the prevalence of use of these compounds is extremely difficult. This is mainly because new substances are being continuously introduced and users, upon which rely many of the monitoring systems (e.g., household surveys), rarely know which substance they have been actually using. Two additional groups of substances have also been the target of WBE studies, namely pharmaceuticals with potential for abuse (e.g., benzodiazepines) and opiates (e.g., morphine, methadone, oxycodone, fentanyl) (10–12).

Wastewater is however a complex matrix containing thousands of compounds deriving from human metabolism, personal care products, household appliances, industrial processes and environmental runoff (e.g., agriculture). Furthermore, whilst some analytes can be measured in the high ng/L or even µg/L range (i.e., benzoylecgonine and methamphetamine), and can thus be analysed by direct injection, others are present in much lower concentrations and require laborious sample preparation strategies (e.g., opiates, NPSs). This requires furthermore the use of highly sensitive and selective methods. Moreover, because of a large number of target compounds, methods are needed to allow the robust, accurate and simultaneous analysis of multiple compounds. The present paper aims at providing an

overview of sample preparation and instrumental techniques used in the field of WBE to process and analyse wastewater samples (Figure 1).

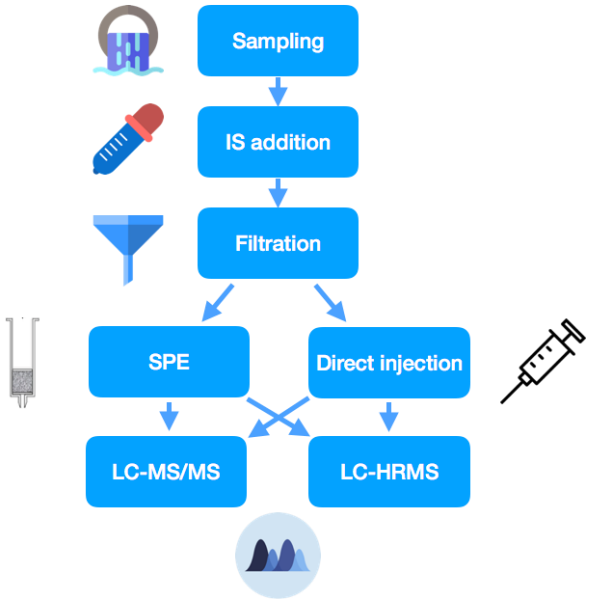

Figure 1: Scheme of the procedures used to analyse metabolites of illicit drugs in WBE.

Sample preparation

Sampling

Wastewater sampling plays a crucial role in WBE as it directly influences the representativeness of the collected samples and, consequently, the validity of the obtained estimates of illicit drug use (13). Sampling is generally carried out using automated samplers installed at the influent of a WWTP. These can be programmed to collect wastewater at fixed intervals (i.e., time-proportional sampling) or as a function of the flow or volume of wastewater (i.e., flow- or volume-proportional sampling, respectively) (14). Incorrect sampling approaches can have a significant influence on the total uncertainty of WBE and, even when working in ideal conditions (i.e., flow-proportional sampling (14)), errors in the final estimate ranging from 5% to 10% can be expected (15).

Sample preparation

After collection, samples are generally immediately frozen to ensure the stability of the analytes during storing and transport. Once received in the laboratory, samples are thawed, spiked with mass labelled internal standards and then filtered (e.g., glass microfiber filters with 1.6 µm pore size) (16) and/or centrifuged (17), as wastewater contains large amounts of suspended matter (18). The latter is generally not analysed in WBE due to logistic reasons (i.e., long sample preparation procedures involving filtration, drying, homogenisation and multiple cycles of pressurized liquid extraction (19)) and because sorption is expected to be limited due to the polarity of the target analytes. Yet, for more lipophilic compounds, adsorption onto particulate matter has been shown to be substantial. For instance, in the case of methadone and its major metabolite 2-ethylidene-1,5-dimethyl-3,3-diphenyl-pyrrolidine

(EDDP), 11-20% and 21-50% of the total concentration found in wastewater is adsorbed onto suspended matter, respectively (20).

Following filtration of wastewater samples, two approaches are most commonly implemented, namely direct injection or offline solid-phase extraction (SPE). In the first case, no further processing of the samples is applied and these are directly analysed using liquid chromatography coupled to tandem (LC-MS/MS) or high-resolution mass spectrometry (LC-HRMS) (21). Large-volume injection, with injected sample volumes of 1800 µL, has also been reported (22).

However, since some compounds, such as synthetic opiates (e.g., fentanyl and substitutes) and NPS, are present in particularly low concentrations, a pre-concentration step is necessary. SPE has been carried out mainly offline, using sample volumes ranging from 10 mL to 500 mL, although successful analysis of illicit drug metabolites in wastewater using online SPE and sample volumes of only 5 mL have been reported (23). Various types of sorbents have been contemplated, as shown in Table 1, yet, because most illicit drugs are weak bases, ion-exchange and, in particular, mixed reversed-phase cation-exchange sorbents have been most commonly used (24). Reversed-phase sorbents have also been contemplated, in particular because these provide improved recovery for 11-nor-9-carboxy-delta-9-tetrahydrocannabinol (THC-COOH), one of the metabolites of the active ingredient in cannabis, tetrahydrocannabinol (THC) (Table 1). Furthermore, because these sorbents are universal (25), they are generally preferred for suspect and non-targeted screening approaches (6). The use of molecularly imprinted polymers (MIPs) has also been reported, yet applications were limited to ATS (26). The authors nevertheless reported excellent results in terms of recovery, matrix effects, accuracy and precision.

SPE and direct injection have been commonly used in combination with LC-MS/MS, since the main objective is to obtain optimum conditions in terms of recovery and matrix effect, thus providing the highest sensitivity for a selected number of compounds. In the specific case of (targeted or unknown) screening approaches, however, sample preparation plays a crucial role. Particularly because the use of more selective pre-concentration protocols based on cation-exchange SPE sorbents will limit the universality of the screening approaches, whilst some analytes could potentially not be detected via direct injection because of their low concentrations. In this situation, researchers have often opted for using universal reversed-phase sorbents (6), which allow the recovery of a broader range of chemicals.

Table 1: Example of SPE sorbents used to extract illicit drugs from wastewater samples. Recovery data taken from (16).

Considered SPE sorbents	Oasis MCX	Oasis HLB	PLRPs	SupelMIP Amphetamine	Strata XC	UCT x RDAH
Description	Mixed-mode cation exchange	Hydrophilic-lipophilic balance	Cross-linked styrene-divinyl-benzene copolymer	Molecularly imprinted polymer	Mixed-mode strong cation mixed mode	Mixed-mode Ion Exchange
Compounds	**Recoveries [%]**					
Cocaine	91 - 102	86 - 121	59	-	56 - 100	-
Benzoylecgonine	87 - 107	40 - 100	8	-	53 - 85	57
Amphetamine	10 - 110	70 - 96	15	90	63 - 63	-
Methamphetamine	65 - 99	80 - 96	20	75	80	83
MDMA	86 - 102	84 - 125	27	98	52 - 60	88
Morphine	75 - 107	29 - 83	14	-	4 - 65	-
6-monoacetylmorphine	90 - 139	85 - 90	21	-	100	-
Methadone	97 - 112	44 - 100	-	-	55	-
EDDP	38 - 88	68 - 68	-	-	59	-
THC-COOH	51 - 68	67 - 96	-	-	-	-

Chromatographic approaches

Liquid chromatography hyphenated to mass spectrometry has by far been the most common instrumental technique used to detect and quantify illicit drug residues in wastewater. High performance liquid chromatograph (HPLC) or ultra-high performance chromatography (UHPLC) equipped with reverse-phase (RP) chromatographic columns have been generally used. Water, ammonium formate or acetate (1- 50 mM) acidified with formic or acetic acid (0.05-0.1%) have commonly been used as aqueous phases, whilst methanol or acetonitrile as organic phases (24). Some applications using hydrophilic interaction liquid chromatography (HILIC) have also been reported (27,28), which provided better retention for more polar compounds, such as ecgonine methyl ester (a metabolite of cocaine), poorly retained on RP columns. Enantiomeric analysis has also been reported in the field of WBE (29). In particular, authors contemplated the possibility of using chiral columns, operated under isocratic conditions and using different modifiers (e.g., 10% propanol and 1 mM ammonium acetate), to separate and quantify the enantiomeric fractions of various classes of illicit drugs and pharmaceuticals (30–32). These studies allowed to discern consumption from the direct disposal of unused drugs (in particular for MDMA, which is preferentially excreted as *R*-enantiomer whilst it is synthesised as racemate (32)), obtain clues about their potency and synthesis route (31).

Mass spectrometry

Tandem mass spectrometry

Tandem mass spectrometry has by far been the most common mass analyser implemented in WBE studies, in particular because of its higher sensitivity compared to high resolution approaches. In particular, triple quadrupole (QqQ) and quadrupole-linear ion trap (QLIT) mass spectrometers have been used to analyse illicit drug residues in wastewater. These were generally operated in multiple reaction monitoring (MRM, Figure 1), providing both sensitivity and selectivity through the monitoring of at least two specific transitions. Electrospray (ESI), operated both in positive and negative modes, was used for ionisation of illicit drugs in WBE applications; other techniques such as atmospheric pressure chemical ionisation (APCI) have not been contemplated. LC-MS/MS has been commonly used to monitor consumption of conventional drugs (e.g., cocaine, ATS, heroin, cannabis), but also for more recent NPS such as synthetic cathinones and phenethylamines (7,33,34). Examples of common limits of quantitation are reported in Table 2.

Table 2: Range of method quantitation limits [ng L^{-1}] reported for conventional illicit drugs (16) and NPS (7,33,34).

	Target compound	Reported method quantitation limit (ranges) [ng L^{-1}]
Conventional illicit drugs	Cocaine	0.2 - 20
	Benzoylecgonine	0.2 - 20
	Amphetamine	1 - 20
	Methamphetamine	0.4 - 20
	MDMA	0.5 - 20
	Morphine	4 - 20
	6-acetylmorphine	3 - 20
	Methadone	0.3 - 50
	EDDP	0.7 - 20
	THC-COOH	2 - 100
Novel psychoactive substances	Methoxetamine	0 - 0
	Butylone	0.2 - 2
	Ethylone	0.6 - 2
	Methylone	0.05 - 2
	Methiopropamine	2
	PMMA	2
	PMA	2

High-resolution mass spectrometry

The implementation of LC-MS/MS is however limited by the availability of certified reference standards which, in the particular case of NPS, are rarely available. In fact, the ever growing number of

NPS detected makes it impossible for manufacturers and laboratories to have reference standards of the newest compounds available.

High-resolution mass spectrometry is thus an extremely appealing approach to tentatively screen for these compounds in wastewater and thus detect and highlight their potential consumption. In fact, the possibility of acquiring accurate mass full spectra opens the way for conducting screening studies to detect illicit drugs, in particular NPS, their metabolites and transformation products as well as to elucidate unknowns (5). Furthermore, the possibility of performing retrospective analyses allows determining if specific chemicals were present in previously analysed samples, opening the way to conduct longitudinal studies. Specific workflows based on mass filtering and peak detection algorithms, molecular formula determination, isotope pattern matching, database comparison, retention time prediction and MS/MS fragmentation pattern matching have been developed and implemented for suspect and non-target screening approaches in the field of WBE (35,36) (Figure 1). In particular Quadrupole-TOF (QTOF-MS) and linear ion trap Orbitrap (LIT-Orbitrap) mass spectrometers have been implemented in WBE studies (5). The main advantage of these instruments lies in the possibility of conducting MS/MS experiments for data on product-ion spectra. The obtained data is highly useful during the tentative identification process, in particular for structure elucidation. Whilst MS/MS experiments can be carried out in data-dependent (DDA) and data-independent acquisition (DIA), the former was generally preferred in WBE studies (6,37,38), as it reduces the load of data needing to be processed. Yet, it also increases the risk of missing relevant compounds which fall beneath the selected thresholds. However, regardless of the selected acquisition mode, methods need to undergo qualitative validation and, in particular, need to be tested on their ability to detect target compound at the established concentrations.

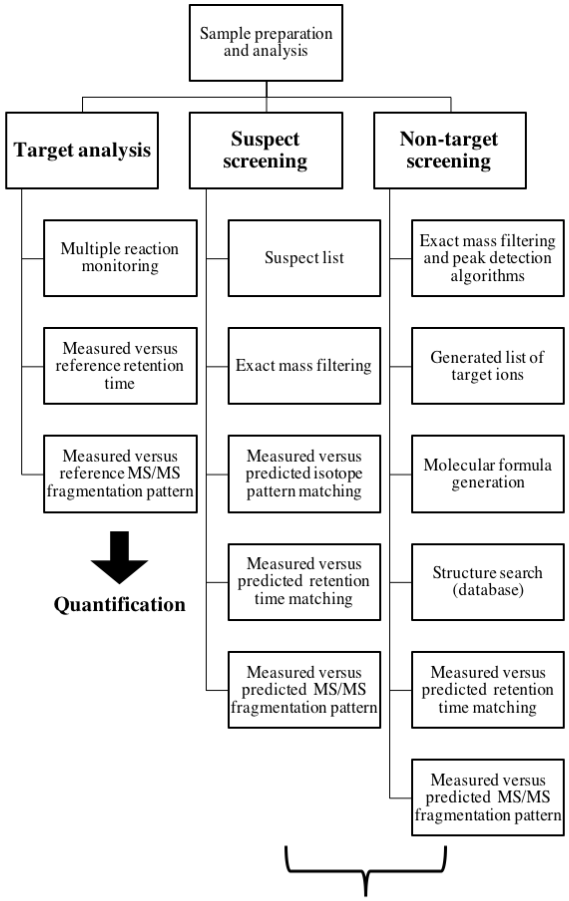

Figure 2: Scheme of workflows commonly implemented in target analysis, suspect and non-target screening approaches for WBE applications.

Screening for new psychoactive substances in wastewater

Detecting and identifying trace level compounds in a complex matrix such as wastewater has been shown to be a nontrivial task. This is particularly important for NPS, whose lifetime on the market is relatively short (few months to 1-2 years from introduction into the market until they are detected and banned). Individual substances will thus have a limited number of users and concentrations in wastewater will consequently be extremely low, further complicating the task of analysts trying to detect, identify and inform public health officials about the consumption of specific NPS. Recently, researchers active in the field of WBE have contemplated an alternative matrix to gain information about consumption of NPS, namely pooled urine samples collected from urinals (disposed in public spaces or at festivals). The main advantage of this matrix, compared to wastewater, is that target compounds are expected to be present in substantially higher concentrations. In one study, the authors optimized both sample preparation protocols and instrumental conditions for various groups of substances (i.e., general and basic drugs, as well as synthetic cannabinoids screening) (39). The analysis was carried out using an LIT-Orbitrap system and, in addition to full scan MS, MS/MS and MS^n experiments, allowed to tentatively identify 13 different NPS in samples collected over the course of 6 months. Amongst these were methiopropamine, 1,4-trifluoromethylphenylpiperazine, 4-methyl-

buphedrone, methcathinone, ethyl-methcathinone, and 1,4-methoxyphenylpiperazine. Following a similar strategy, researchers also implemented a suspect screening approach, involving an in-house data base containing approximately 2000 entries, to identity NPS in wastewater samples collected during a major public event (6). The authors reported the (tentative) identification of 8 NPS, ranging from identification level 1 to 3 on the scale proposed by Schymanski et al. (40). LC-HRMS has also been used to elucidate transformation products of NPS in wastewater. In particular, the use of a hybrid quadrupole-Orbitrap system to identify microbial transformation products of methylenedioxy-pyrovalerone (MDPV) has been reported (41). Through the interpretation of HR-MS^2 spectra the authors were able to tentatively identify 12 transformation products as well as their biotransformation pathways (i.e., demethylation followed by *O*-methylation, hydroxylation followed by oxidation or methylation, *N*-demethylation and hydroxylation).

Perspectives

Whilst most applications of WBE focused on obtaining information about the consumption of illicit drugs, the potential information contained in wastewater is not limited to these substances. Recent studies have explored other applications of WBE which encompass various aspects related to lifestyle. For instance, the analysis of nicotine and its metabolites (42,43) as well as other tobacco alkaloids (i.e., anatabine and anabasine) (44) in wastewater to monitor the use of tobacco has been reported by various groups. Sample preparation protocols similar to illicit drugs, namely SPE using mixed-mode cation-exchange sorbents (43,44) or direct injection (42), were developed and analyses were carried out using LC-MS/MS systems. A comprehensive method was recently developed to analyse a broad range of tobacco-related compounds, including nicotine metabolites, alkaloids, toxicants and carcinogens (i.e., *N*-nitrosoanabasine, *N*-nitrosoanatabine, 4-(methyl-nitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and *N*-nitrosonornicotine (NNN)) and their metabolites (4-(methyl-nitrosamino)-1-(3-pyridyl)-1-butanol (NNAL)), for epidemiological purposes (45). The authors optimized the extraction protocol after testing various types of sorbents including mixed mode reversed-phase cation and anion exchangers, hydrophilic-lipophilic balanced sorbents and MIPs. Best results were obtained with mixed mode cation exchange cartridges.

Consumption of alcohol has also recently been monitored through wastewater analysis. This is achieved through the analysis of phase-II metabolites of ethanol, namely ethyl sulfate (EtS) and ethyl glucuronide (EtG). Analyses were performed by direct injection however, because of the low retention of EtS and EtG on reversed-phase columns, various ion-paring agents have been used. In one case, authors added dihexylammonium acetate (7 mM) to the mobile phases (i.e., water and methanol) (46), whilst in another study 50 mM tetrabutylamonium bromide were added directly to the sample (47). In one study, however, authors reported the use of a reversed-phase column with 0.1% acetic acid in water and acetonitrile as mobile phases, without any particular sample preparation (48).

Pharmaceuticals with a potential for recreational use or abuse (e.g., oxycodone, fentanyl, ketamine and benzodiazepines) have also been the target of various WBE studies (49–51). Protocols using reversed-

phase SPE cartridges and LC-MS/MS or LC-HRMS were developed and levels ranging from < LOQ (i.e., diazepam (49)) to 2.4 µg/L (i.e., tramadol (50)) have been measured, highlighting the widespread use of some substances. The analysis of phosphodiesterase type V inhibitors (i.e., sildenafil, vardenafil, and tadalafil) in wastewater and sludge has also been reported (52). Authors developed a method based on SPE (i.e., reversed-phase hydrophilic-lipophilic balance) and pressurized liquid extraction (for sludge samples) followed by LC-MS/MS analysis. Recoveries ranged from 45% to 103%, whilst measured levels were between 10-50 ng/L and 3-10 ng/g in wastewater and sludge, respectively. Following the results of this study, another group used measured levels of sildenafil and its metabolites in wastewater to assess the extent of illicit consumption of erectile dysfunction medications in the Netherlands (53).

Another field in which WBE has recently been implemented is the monitoring of human health, in particular through the measurement of (endogenous) biomarkers of effect or disease and biomarkers of exposure (54,55). Regarding the first group of biomarkers, the development of a method to measure oxidative stress biomarker 8-iso-prostaglandin F2alpha (8-iso-$PGF_{2\alpha}$) was recently reported (56). Instead of conventional sample preparation using SPE, the authors used an immunoaffinity clean-up step. Whilst recoveries were lower compared to conventional SPE sorbents (i.e., anion exchange and reversed-phase sorbents), almost no matrix effects were. Analyses were carried by LC-QTOF-MS and method quantitation limits of 0.3 ng/L were reported.

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