**A fully automated approach for the analysis of 37
psychoactive substances in raw wastewater based on on-line
solid phase extraction-liquid chromatography-tandem mass
spectrometry**

Ester López-García¹, Nicola Mastroianni¹, Cristina Postigo^{1*}, Damià Barceló^{1,2}, Miren
López de Alda^{1*}

¹ Water and Soil Quality Research Group, Department of Environmental Chemistry,
Institute of Environmental Assessment and Water Research (IDAEA-CSIC), Jordi
Girona 18-26, 08034 Barcelona, Spain.

² Catalan Institute for Water Research, Girona, H2O Building, Scientific and
Technological Park of the University of Girona, Emili Grahit 101, 17003 Girona, Spain.

***Corresponding Authors:** Cristina Postigo, Miren López de Alda
Institute of Environmental Assessment and Water
Research (IDAEA-CSIC),
Department of Environmental Chemistry
C/ Jordi Girona 18-26, 08034 Barcelona, Spain.
cprgam@cid.csic.es, mllaqam@cid.csic.es
Tel: +34-934-006-100, Fax: +34-932-045-904

29

30 **ABSTRACT**

31 This work presents a multi-residue method for the simultaneous determination of 37
32 legal and illicit psychoactive substances in wastewater, including the most common
33 illicit drugs (cocaine-related compounds, amphetamine type stimulants, hallucinogens,
34 opiates/opioids, and cannabinoids), new psychoactive substances (two synthetic
35 cathinones, the synthetic opioid AH-7921 and the arylcyclohexylamine
36 methoxetamine), and legal but controlled psychoactive substances (stimulants,
37 benzodiazepines, antidepressants, sedatives, antipsychotics and hypnotics). To this end
38 a fully automated analytical approach based on solid phase extraction coupled in series
39 to liquid chromatography tandem mass spectrometry detection (on-line SPE-LC-
40 MS/MS) was used. The methodology developed was validated in terms of linearity,
41 recovery, repeatability, and sensitivity in wastewater. Method linearity was between 0.1
42 ng/L (or the analyte limit of quantification if higher) and 2000 ng/L (10250 ng/L in the
43 case of caffeine). Absolute recoveries were variable (between 5% and 132%),
44 depending on the analyte. However, the use of isotopically labeled compounds
45 corrected for analyte losses during the extraction process and matrix effects (relative
46 recoveries within the range of 80-120%). Repeatability of the method was satisfactory
47 for all analytes, with RSD values lower than 13% for most compounds. Limits of
48 detection and quantification in wastewater were below 7 and 23 ng/L, respectively, for
49 all analytes except lormetazepam (10 and 32 ng/L), caffeine (13 and 44 ng/L), and the
50 cannabinoids 11-nor-9-carboxy- Δ^9 -tetrahydrocannabinol (18 and 61 ng/L) and 11-
51 hydroxy- Δ^9 -tetrahydrocannabinol (69 and 228 ng/L). The method was applied to the
52 analysis of wastewater samples collected daily in Barcelona for one week. Twenty-five
53 of the 37 analytes were detected in the samples analyzed. Average concentrations
54 ranged from 7 ng/L in the case of zolpidem to 54 μ g/L in the case of caffeine.

55

56 **KEYWORDS:** drugs of abuse, psychotropic pharmaceuticals, new psychoactive
57 substances, on-line solid phase extraction, liquid chromatography-tandem mass
58 spectrometry, sewage water.

59

1. Introduction

According to the last World Drug Report of the United Nations Office on Drugs and Crime, 255 million people between 15 and 64 years used illicit drugs at least once in 2015. Although this indicates a clear increase in the number of illicit drug users since 2006, illicit drug use has remained stable in the last five years worldwide. Such trend has also been observed in Spain, with cannabis being the most consumed drug (9.5% of last year prevalence of use among the 15-64 age band in 2015), followed by cocaine (2%), ecstasy (0.6%), hallucinogens (0.6%), and amphetamines (0.5 %) [1]. In recent years new psychoactive substances (NPS) or “legal highs” have appeared in the market. These substances produce similar effects than traditional illicit drugs; however, they are not controlled by the 1961 United Nations Single Convention on Narcotic Drugs or the 1971 United Nations Convention on Psychotropic Substances [2]. In Spain, the consumption of this type of substances is still not very widespread with a last year prevalence of use among people aging 15-64 years of 0.9% [1]. Last year prevalence of use of hypnotosedatives including tranquilizers, sedatives and somniferous has continuously increased in Spain since 2005 (ca. 10 points in 10 years) [1]. Although these substances are legally prescribed to relieve pain or to treat depression, seizure disorders, anxiety and/or insomnia, among others [1], their abusive use without medical supervision can lead to health issues or even dependence. In this respect, non-prescribed use of these substances has doubled in the last 10 years (last year prevalence among people aging 15-64 of 2.3%) [1].

Given the widespread use of illicit and legal psychoactive drugs, these substances in their original form or their metabolites have been introduced into the water cycle, and their presence has been reported in wastewater, surface water, groundwater, and even drinking water [3-5]. As a result, they have been recognized as a new class of emerging organic contaminants of concern in the environment. The determination of psychoactive drugs in water matrices is justified to evaluate their environmental occurrence, required for proper risk assessment, and to back-calculate their use by a specific population, which is possible from their analysis in untreated wastewater [6].

Measurement of these substances, medium to highly polar in most cases, in aqueous environmental matrices relies on the use of sensitive and selective analytical techniques, such as liquid chromatography (LC) coupled to mass spectrometry (MS) detection.

While targeted LC-MS(/MS) methods have been commonly employed for their quantitative analysis [5, 7-10] non-targeted approaches using sensitive high-resolution mass spectrometers have been used for the identification of NPS [11-13].

Despite the high sensitivity of MS instruments, the low concentrations at which these substances are present in the aquatic environment call for their preconcentration prior to LC-MS analysis. This has been commonly achieved by means of solid phase extraction [14]. Fully automated methodologies based on configurations that allow coupling the SPE step to the LC-MS system allow reducing analytical lab-effort, cost, and time [15] as compared to offline SPE based methods. Furthermore, minimum sample manipulation in on-line approaches contributes to obtaining accurate, precise and reliable results. Large volume injection in modern and sensitive MS instruments has been reported to replace well the SPE preconcentration step in the analysis of clean water samples [9]. However, on-line SPE based methodologies are still appointed as the best approaches for the automated analysis of highly complex matrices such as untreated wastewater.

In this context, this work presents a multi-residue analytical method based on on-line SPE-LC-MS/MS for the simultaneous determination of 37 selected psychoactive substances in raw wastewater. The list of target psychoactive substances included the most common illicit drugs such as cocaine and related metabolites, amphetamine-type stimulants (ATS), hallucinogens, opiates, and cannabinoids; NPS, in particular, two synthetic cathinones, one synthetic opioid and one arylcyclohexylamine; and the most consumed psychoactive pharmaceuticals in Spain, and in general worldwide, such as benzodiazepine-type anxiolytics, antidepressants, sedatives, antipsychotics and hypnotics. Despite the fact that there are already several fully automated methodologies published in the peer-reviewed literature for the analysis of illicit drugs in wastewater, prescribed psychoactive drugs and NPS are usually not included. In this regard, to the authors' knowledge this is the first time that α -hydroxy-alprazolam (OH-ALPZ), α -hydroxy-midazolam (OH-MIDZ), midazolam (MIDZ), temazepam (TEMZ), zolpidem (ZOPD), 3,4-methylenedioxypyrovalerone (MDPV), 3,4-dichloro-N-[[1-(dimethylamino)cyclohexyl]methyl]benzamide (AH-7921), mephedrone (MEPH), and methoxetamine (MXE) are analyzed in raw wastewater using a methodology based on on-line SPE. The method developed was applied to the analysis of raw wastewater of the city of Barcelona, to obtain a first general picture on the occurrence of legal and

illicit psychoactive substances previously not investigated in the area, e.g., NPS and most of the psychoactive pharmaceuticals included in the developed methodology.

2. Material and methods

2.1. Reagent and materials

High purity (> 97 %) standards of the target compounds and isotopically labeled analogs were purchased from Cerilliant (Round Rock, TX, USA) as solutions in methanol (MeOH) or acetonitrile (ACN) at a concentration of 0.1 or 1 mg/mL. The illicit drugs investigated included cocaine (COC), its major metabolite benzoylecgonine (BE) and cocaethylene (CE), a metabolite formed when cocaine and ethanol are simultaneously consumed; the ATS amphetamine (AM), methamphetamine (MA) and 3,4-methylenedioxymethamphetamine (MDMA or ecstasy); the hallucinogenic substances ketamine (KET) and lysergic acid diethylamide (LSD); the opioids/opiates morphine (MOR), heroin (HER) and its metabolite 6-acetylmorphine (6ACM), methadone (METH) and its metabolite 2-ethylidene-1,5-dimethyl-3, 3-diphenylpyrrolidine (EDDP); and the cannabinoids 11-nor-9-carboxy- Δ^9 -tetrahydrocannabinol (THC-COOH) and 11-hydroxy- Δ^9 THC (OH-THC), which are the main THC metabolites. The NPS included were the synthetic cathinones MEPH and MDPV, the synthetic opioid AH-7921, and the synthetic arylcyclohexylamine MXE, whereas the list of prescribed psychoactive substances covered the benzodiazepine-type anxiolytics alprazolam (ALPZ), its metabolite OH-ALPZ, diazepam (DIAZ), lorazepam (LORZ), lormetazepam (LRMZ), MIDZ, its metabolite OH-MIDZ, oxazepam (OXZ) and TEMZ; the stimulants caffeine (CAF) and ephedrine (EPH); the antidepressants citalopram (CTLP), fluoxetine (FLX), sertraline (STR), and venlafaxine (VFX); the sedative hydroxyzine (HXZ), the antipsychotic chlorpromazine (CPMZ), and the hypnotic ZOPD. Table 1 in shows the physico-chemical properties including pKa and log K_{ow} of the target analytes.

Working standard mixture solutions were prepared in MeOH at different concentrations in the range of 0.1 and 2000 ng/mL (10250 ng/mL in the case of CAF) by appropriate dilution of individual stock solutions. All of them contained the isotopically labeled (surrogate) standard (SS) mixture at a concentration of 5050 ng/mL in the case of CAF-d₃, 50 ng/mL in the case of BE-d₃, COC-d₃, CTLP-d₆, EDDP-d₃, EPH-d₃, LORZ-d₄,

LRMZ-¹³Cd₃, METH-d₃, MOR-d₃, OH-THC-d₃, OXZ-d₅, STR-d₃, THC-COOH-d₃ and VFX-d₆, or 20 ng/mL in the case of the remaining surrogate compounds. A separate solution containing only the SS at the aforementioned concentrations was also prepared for sample analysis. Working standard mixtures were used for calibration purposes and as fortification solutions in the validation studies. All standard solutions were stored in the dark at – 20 ° C until use.

All solvents used were from J.T. Baker (Serviquimia, Barcelona) and were HPLC grade. Formic acid (> 98 %) and ammonium formate (> 99%), used as mobile phase modifiers, were purchased from Merck and Fluka Analytical (Sigma Aldrich), respectively.

2.2.On-line SPE-LC/MS-MS method

The developed automated methodology is based on a previously published method for the determination of 18 illicit drugs and metabolites and alprazolam [16] in wastewater.

Main modifications included were:

1. the expansion of the list of target compounds with 21 new target substances, i.e., AH-7921, caffeine, citalopram, chlorpromazine, diazepam, fluoxetine, hydroxyzine, ketamine, lorazepam, lormetazepam, MDPV, mephedrone, midazolam, methoxetamine, α -hydroxy-alprazolam, α -hydroxy-midazolam, oxazepam, sertraline, temazepam, venlafaxine and zolpidem, and their corresponding isotopically labeled compounds for isotope dilution quantification,
2. the centrifugation of the samples instead of their filtration in order to reduce the adsorption of the less polar compounds (e.g., cannabinoids) onto the filters,
3. the use of an acidic mobile phase (5 mM ammonium formate/formic acid, pH 3.8) to reduce chromatographic peak tailing and improve compound ionization,
4. the extraction of all target analytes onto the wettable polymeric sorbent PLRPs (cross-linked styrene-divinylbenzene polymer, 10x2 mm, 15-25 μ m particle size) (Spark Holland, Emmen, The Netherlands),

5. a cartridge-washing step with a 5% MeOH aqueous solution to reduce the extent of matrix interferences, and
6. the MS analysis of cannabinoids (i.e., THC-COOH and OH-THC) in the positive electrospray ionization mode.

On-line SPE was performed using an automated sample processor SymbiosisTM Pico that is composed of an AliasTM autosampler equipped with a 5 mL sample loop, an HPLC binary pump, an automated cartridge exchange (ACE) module, and a high-pressure dispenser (HPD) module (Spark Holland). For extraction, five-mL of a ten-fold diluted wastewater sample containing the SS mixture was loaded onto previously conditioned (3 mL of ACN followed by 2 mL of HPLC-grade water at a flow rate of 5 mL/min) PLRPs cartridges at a flow rate of 1 mL/min. Upon sample loading, the cartridge was washed with 1 mL of HPLC-grade water containing 5% of MeOH at a flow rate of 5 mL/min. Finally, elution of the analytes from the cartridge to the LC system was performed with the chromatographic mobile phase (water containing 5 mM of formic acid/ammonium formate buffer and ACN at a constant flow rate of 0.3 mL/min). Chromatographic elution and separation was performed with a Purospher Star RP-18 end-capped column (125 mm × 2.0 mm, particle size 5 µm) preceded by a guard column of the same packing material, both from Merck (Darmstadt, Germany) and the following organic gradient: from 5% to 40% in 12 min, then to 70% in 6 more minutes, to 80% in the next minute and to 100% in the following 9 minutes. Pure organic conditions were held for 5 min to clean the column. Return to initial conditions was done in 4 min, and they were maintained for 10 min for column re-equilibration before the next injection. Since SPE of a sample in a sequence is performed at the same time that the LC-MS/MS analysis of the previously extracted one, total SPE-LC-MS/MS analysis time is defined by the longest process, which in this case corresponds to the 47 min needed for the sample chromatographic separation and column cleaning and re-equilibration.

MS analysis was performed with a 4000 QTRAP hybrid triple quadrupole-linear ion trap (QqLIT) mass spectrometer equipped with a Turbo Ion Spray Source (AB-Sciex, Framingham, MA, USA) set in the positive ionization mode (ESI+). Data acquisition was performed in the selected reaction monitoring (SRM) mode recording two SRM transitions per compound and one for the corresponding SS.

218 **Table 1.** Physical-chemical properties of target compounds: molecular structure, CAS number, molecular weight, octanol water partition
 219 coefficient (log Kow) and acid dissociation constant (pKa).

Class of compounds	Target analyte	Molecular structure	CAS number	Molecular weight	Log Kow ^a	pKa
Cocaine compounds	Cocaine (COC)		50-36-2	303.36	2.30	8.61
	Benzoyllecgonine (BE)		519-09-5	289.33	-1.32*	Strongest Acidic: 3.15** Strongest Basic: 9.54**
	Cocaethylene (CE)		529-38-4	317.39	2.66*	Strongest Basic: 8.77**
Amphetamine-type stimulants	Amphetamine (AM)		300-62-9	135.21	1.76	10.1
	Methamphetamine (MA)		4846-07-5	149.24	2.07	9.87
	3,4-Methylenedioxy methamphetamine (MDMA)		42542-10-9	193.25	2.15	Strongest Basic: 10.14**

Hallucinogens	Ketamine (KET)		1867-66-9	237.73	2.18	7.5
	Lysergic acid diethylamide (LSD)		50-37-3	323.44	2.44	7.8
Opioids/opiates	Morphine (MOR)		57-27-2	285.34	0.89	8.21
	Heroin (HER)		561-27-3	369.42	1.58	7.95
	6-Monoacetylmorphine (6 ACM)		2784-73-8	327.38	0.40*	Strongest Acidic: 10.19 ** Strongest Basic: 9.08 **

	Methadone (METH)		76-99-3	309.45	3.93	8.94
	2-Ethyl-1,5-dimethyl-3,3-diphenylpyrrolinium (EDDP)		66729-78-0	277.41	4.94*	Strongest Basic: 9.64**
Cannabinoids	11-Hydroxy- Δ^9 THC (OH-THC)		36557-05-8	330.47	5.33*	Strongest Acidic: 9.34** Strongest Basic: -2.7**
	11-Nor-9-carboxy- Δ^9 THC (THC-COOH)		56354-06-4	344.45	6.36*	Strongest Acidic: 4.21** Strongest Basic: -4.9**
New Psychoactive substances (NPS)	3,4-Dichloro-N-[[1-(dimethylamino)cyclohexyl)methyl]benzamide (AH-7921)		55154-30-8	329.27	4.39*	

	Mephedrone (MEPH)		1189805-46-6	177.25	2.39*	Strongest Acidic: 18.66 ** Strongest Basic: 8.05**
	Methoxetamine (MXE)		1239943-76-0	247.34	3.05*	Strongest Acidic: 13.61** Strongest Basic: 9.28**
	3,4-Methylenedioxy pyrovalerone (MDPV)		687603-66-3	194.19	3.97*	8.4
Benzodiazepines	Alprazolam (ALPZ)		28981-97-7	308.77	2.12	Strongest Acidic: 18.3** Strongest Basic: 5.08**

	α -Hydroxy-alprazolam (OH-ALPZ)		37115-43-8	324.77	2.41*	Strongest Acidic: 13.72** Strongest Basic: 4.97**
	Diazepam (DIAZ)		439-14-5	284.74	2.82	3.4
	Lorazepam (LORZ)		846-49-1	321.16	2.39	13
	Lormetazepam (LRMZ)		848-75-9	335.18	2.23*	Strongest Acidic: 10.68** Strongest Basic: -2.2**

	Midazolam (MIDZ)		59467-70-8	326.76	4.33*	5.5
	α -Hydroxy-midazolam (OH-MIDZ)		59468-90-5	342.76	2.87*	Strongest Acidic: 13.95** Strongest Basic: 4.99**
	Oxazepam (OXZ)		604-75-1	286.05	2.24	1.55
	Temazepam (TEMZ)		846-50-4	300.74	2.19	Strongest Acidic: 10.68** Strongest Basic: -1.4**

Antidepressants	Citalopram (CTLP)		59729-33-8	324.40	3.74*	Strongest Basic: 9.78**
	Fluoxetine (FLX)		54910-89-3	309.33	4.05	Strongest Basic: 9.8**
	Sertraline (STR)		79617-96-2	306.23	5.29*	Strongest Basic: 9.85**
	Venlafaxine (VFX)		93413-69-5	277.41	3.28*	10.09
Stimulants	Caffeine (CAF)		58-08-2	275.35	-0.07	10.4

	Ephedrine (EPH)		321-97-1	165.24	0.89	9.65
Sedative	Hydroxyzine (HXZ)		68-88-2	374.91	2.36*	2.47
Hypnotic	Zolpidem (ZOPD)		82626-48-0	307.40	3.85*	6.2
Antipsychotic	Chlorpromazine (CPMZ)		50-53-3	318.86	5.41	9.3

220 ^a Data extracted from EPI Suite TM. Available at: <https://www.epa.gov/tsca-screening-tools/epi-suitetm-estimation-program-interface> . Experimental data
221 and/or estimations (*) provided by KOWWIN v1.68 software.

222 ** Data extracted from <https://www.drugbank.ca/>. Estimations (**) provided by ChemAxon.

2.3.Method performance

The performance of the methodology was evaluated in terms of linearity, recovery, repeatability, sensitivity, and matrix effects.

Linearity of the method was evaluated within the concentration range 0.1-2000 ng/L (equivalent to 1 ng/L and 20 µg/L in wastewater), for all analytes except for caffeine, whose linearity was evaluated between 0.1 and 10250 ng/L (equivalent to 1 and 102.5 µg/L) due to the high concentrations expected in wastewater. For this, ten-point calibration curves were prepared in HPLC-grade water by appropriate dilution of the working standard solutions and analyzed with the optimized methodology. Calibration curves were constructed using the relation between the area of the analyte and the area of its deuterated analog through the isotope dilution approach, and weighted least-squared linear regression using $1/x^2$ as weighting factor was used to build the calibration curves in order to minimize the influence of higher concentrations in the model.

Analyte recovery was evaluated in raw wastewater. For this, five replicate analysis of fortified raw wastewater was performed at three different levels (50 ng/L, 500 ng/L and 2500 ng/L for all drugs except for caffeine that was evaluated at 10050 ng/L, 50500 ng/L and 102500 ng/L). Absolute recoveries (AR) of the target and surrogate compounds were calculated by comparing the peak areas obtained after on-line SPE-LC-MS/MS analysis of fortified wastewater samples (after subtraction of the background signal (n= 3) in the wastewater sample used in the recovery study, if present) with those obtained after LC-MS/MS analysis of solutions with equivalent amounts of analytes. Relative recoveries (RR) were also evaluated. These figures corresponded to the amount of analyte recovered in relation to the recovery of its corresponding SS.

The repeatability of the method was also examined at the aforementioned three different levels as the relative standard deviation (RSD) of the analysis of five replicates of fortified raw wastewater samples.

The sensitivity of the method was appraised through the limits of detection (LOD) and quantification (LOQ) observed for each target analyte. These limits were experimentally estimated from the analysis of raw wastewater samples as the concentration giving a

signal to noise ratio of 3 and 10, respectively. Due to the matrix heterogeneity of the wastewater samples, average LODs and LOQs are provided. Method LOD and LOQ of those compounds that were not detected in the analyzed wastewater samples were extracted from the MS signals observed after analysis of wastewater samples fortified at the lowest level.

In order to investigate matrix effects (ME), analyte peak areas obtained after on-line SPE-LC-MS/MS analysis of a wastewater sample fortified with the standard mixture at 1000 ng/L (after subtracting the amount of the analyte in the wastewater sample, if present) were compared with those obtained after on-line SPE-LC-MS/MS analysis of HPLC-grade water fortified at equal concentration (100 ng/L after wastewater dilution), according to Eq. 1:

$$\% \text{ ME: } ((A_{\text{ww}-100\text{ng/L}} - A_{\text{ww-blank}})/A_{\text{H}_2\text{O}-100\text{ng/L}})*100)-100 \quad (\text{Eq. 1})$$

In the absence of ME, similar peak areas should be obtained in fortified wastewater and HPLC-grade water. Negative values of ME should result when the analyte signal observed in wastewater is lower than that in the HPLC-grade water and hence, they indicate ionization suppression of the signal during MS analysis. On the contrary, positive values of ME that should result when the analyte signal in wastewater is higher than that in HPLC-grade water point out to signal enhancement due to matrix components.

2.4. Sample collection and sample preparation

24-h composite wastewater samples were collected on consecutive days during one week in March 2015 at the inlet of a WWTP located in Barcelona (Northeast of Spain). Sample collection was carried out by means of an automatic ISCO 6712C compact portable sampler (Instrumentación Analítica, El Prat de Llobregat, Barcelona, Spain) programmed to sample 50 mL every 10 min. One-liter of the homogenized sample was transferred to an amber polyethylene terephthalate (PET) flask and transported in cool conditions to the laboratory. Upon arrival, the sample was 10-fold diluted with HPLC-grade water, fortified with the SS mixture and centrifuged at 3500 rpm for 10 minutes. Then the supernatant was transferred to a PET bottle and stored at -20 °C in the dark until analysis.

3. Results

3.1. Method optimization

MS conditions for the psychoactive drugs included in the methodology were individually optimized for each compound after infusion of an acidified standard solution (0.5 – 1 µg/mL) with the aid of Analyst 4.2 software and manually confirmed. Optimum declustering potential was selected in full scan mode, and the two most abundant and selective SRM transitions, and optimum collision energies that maximize their intensity were selected after product ion scan experiments. THC-COOH and OH-THC were the only molecules that ionized better with negative than with positive electrospray. However, the method sensitivity for THC-COOH and OH-THC was compromised in view of reducing analysis time and cost.

Despite the fast acquisition rate of the MS analyzer, the registration of the target SRM transitions (two per target analyte and one per SS) was allocated in three different time-scheduled acquisition windows to maximize method sensitivity and ensure method reproducibility.

Chromatographic separation and peak signal were evaluated using different buffer (ammonium formate/formic acid) concentrations in the aqueous mobile phase (5 mM, 10 mM, and 20 mM). For that, a solution (MeOH:HPLC water, 1:1, v/v) containing the analytes at a concentration of 125 ng/mL was injected in the LC-MS/MS system. For most compounds, less signal suppression was observed at the lowest buffer concentration (1.3-2 times lower signal at 10 mM and 2-5 times lower signal at 20 mM as compared to 5 mM); therefore 5 mM ammonium formate/formic acid was selected as optimum LC modifier. Total Ion Chromatograms (TIC) obtained using the different mobile phases tested are shown in Figure 1.

The effect of using different amounts of methanol (0%, 2.5%, and 5%) in the aqueous solution used to wash the on-line SPE cartridge on analyte breakthrough and matrix removal was also evaluated. For this, HPLC-grade water and raw wastewater (diluted 1 to 10) fortified at a working concentration of 250 ng/L were extracted in triplicate using the different washing conditions. Analysis of HPLC-grade water showed that no analyte was washed out from the cartridge using the washing solution containing the highest percentage of organic solvent tested (5%). Analyte signals observed after analysis of

fortified HPLC-grade water and raw wastewater were similar for all analytes but for fluoxetine, OH-THC and THC-COOH whose signal was increased about 2 times when the cartridge was washed with HPLC-grade water containing 5% MeOH as compared to pure HPLC-grade water. Since cannabinoids were the compounds providing the lowest instrumental method sensitivity, HPLC water containing 5% of MeOH was selected as washing solvent as it improved its sensitivity due to matrix interferences removal. Figure 2 shows the extracted ion chromatograms obtained for OH-THC, fluoxetine, and THC-COOH after on-line SPE-LC-MS/MS analysis of a fortified wastewater sample using the different evaluated washing solutions.

The chromatographic retention time, the SRM transitions and the optimum ionization conditions for the analysis of each target analyte and its SS are provided in Table 2. Extracted ion chromatograms of the target analytes obtained after on-line SPE-LC-MS/MS analysis of a wastewater sample fortified with the standard mixture at 2500 ng/L (102500 ng/L in the case of caffeine) are shown in Figure 3.

3.2. Method performance

Table 3 and Table 4 summarize the method performance in wastewater and HPLC-grade water, respectively, in terms of linearity, recovery, repeatability, and sensitivity.

The linearity of the method (in HPLC-grade water) expanded between 0.1 ng/L or the analyte limit of quantification (see Table 4) if higher and 2000 ng/L for most of the compounds (10250 ng/L in the case of caffeine). Shorter linearity ranges (see Table 4) were obtained for AH-7921, α -hydroxy-midazolam, and lorazepam. All calibration curves, constructed with a minimum of six data points, presented a coefficient of determination (r^2) higher than 0.99.

Method recovery in HPLC-grade water was evaluated at two concentration levels (50 ng/L and 250 ng/L) (see Table 4). Absolute recoveries ranged between 9 and 113%, and relative recoveries were between 82 and 120%. Analyte recoveries in raw wastewater (accounting for both extraction and matrix effects) were evaluated at three different levels: 50 ng/L, 500 ng/L, and 2500 ng/L (10050, 50500 and 102500 ng/L for caffeine). Method performance in this respect could not be assessed at the lowest tested level for

cocaine, benzoylecgonine, ephedrine (not even at 500 ng/L), lorazepam, lormetazepam, oxazepam, temazepam, citalopram, venlafaxine and caffeine, due to the background levels of these substances in the wastewater used in the recovery study; and for THC-COOH and OH-THC, because of their poor method sensitivity.

Analyte absolute recoveries, in good agreement at the different levels tested, were, in general, lower than those observed in HPLC-grade water: between 30 and 60% for 55% of the analytes, between 10 and 30% for 13% of the analytes, and below 10% for 5% of the analytes. Average absolute recoveries higher than 120% were occasionally obtained for lorazepam, lormetazepam, and oxazepam. However, analyte relative recoveries, always between 80 and 120%, and in good agreement with those obtained in HPLC-grade water, proved that the use of isotopically labeled compounds allows compensating for matrix effects as well as for analyte losses during the extraction process and the suitability of HPLC-grade water based calibration solutions to quantify levels in wastewater samples.

The overall method repeatability calculated as the relative standard deviation (RSD) of n=5 replicate analysis of fortified wastewater samples was satisfactory with RSD values always below 20%.

Regarding method sensitivity, average LODs and LOQs in raw wastewater were between 0.2 and 77 ng/L and from 0.6 to 257 ng/L, respectively. Compounds presenting the lowest sensitivity were OH-THC, THC-COOH, lormetazepam, and caffeine with LOQs ranging between 32 and 228 ng/L. Overall, most of the target analytes could be detected in wastewater with the described methodology at 5 ng/L (80% of the target compounds) and quantified at concentrations below 16 ng/L (78% of the compounds). This high sensitivity, considering the low sample volume used (5 mL), is possible thanks to the complete transfer of the sample through the on-line SPE-LS-MS/MS instrument.

(a) 5 mM

(b) 10 mM

(c) 20 mM

Figure 1. Total ion chromatogram (TIC) obtained after LC-MS/MS analysis of a standard solution containing 125 ng/mL of the target analytes using different buffer (ammonium formate/formic acid) concentrations in the aqueous solutions of the mobile phase: (a) 5 mM; (b) 10 mM; (c) 20 mM

Figure 2. Extracted ion chromatograms of (a) FLX, (b) OH-THC, and (c) THC-COOH obtained after on-line SPE-LC-MS/MS analysis of wastewater fortified at a concentration of 2500 ng/L using different cartridge-washing solutions.

389 **Figure 3.** Extracted ion chromatograms (XIC) of the target analytes after on-line SPE-
390 LC-MS/MS analysis of a wastewater sample fortified with the standard compounds at a
391 concentration of 2500 ng/L (102500 in the case of caffeine)

392 **Table 2.** LC-ESI-MS/MS conditions used to determine the target analytes and
393 corresponding isotopically labeled analogs.

Analyte	Retention time (min)	Declustering Potencial (V)	SRM transition	Colision Energy (eV)	SRM ratio (SRM1/SRM2)
0 - 12 min					
Morphine (MOR)	3.49 ± 1.65	90	286 > 152 286 > 128	75 95	1.8 ± 0.2
<i>MOR-d₃</i>	3.49 ± 1.25	90	289 > 152	75	
Ephedrine (EPH)	5.04 ± 0.83	40	166 > 148 166 > 133	20 30	5.2 ± 0.7
<i>EPH-d₃</i>	5.03 ± 0.79	40	169 > 151	20	
Amphetamine (AM)	5.86 ± 0.71	30	136 > 119 136 > 91	15 20	0.8 ± 0.1
<i>AM-d₅</i>	5.78 ± 0.74	30	141 > 96	20	
Caffeine (CAF)	5.92 ± 0.62	60	195 > 138 195 > 110	30 40	5.6 ± 0.2
<i>CAF-d₃</i>	5.92 ± 0.59	60	198 > 140	35	
6-Acetylmorphine (ACM)	6.28 ± 0.73	90	328 > 165 328 > 152	75 80	1.5 ± 0.2
<i>6ACM-d₆</i>	6.25 ± 0.77	90	334 > 165	75	
Methamphetamine (MA)	6.46 ± 0.92	50	150 > 91 150 > 119	20 30	3.2 ± 0.4
<i>MA-d₁₄</i>	6.46 ± 0.92	50	164 > 98	30	
3,4-Methylenedioxy- methamphetamine (MDMA)	6.77 ± 0.78	50	194 > 163 194 > 105	20 35	2.7 ± 0.3
<i>MDMA-d₅</i>	6.75 ± 0.73	40	199 > 135	35	
Benzoylecgonine (BE)	7.14 ± 0.49	80	290 > 168 290 > 77	35 100	1.8 ± 0.3
<i>BE-d₈</i>	7.08 ± 0.51	80	298 > 171	30	
Mephedrone (MEPH)	7.20 ± 0.63	40	178 > 160 178 > 145	20 30	1.7 ± 0.2
<i>MEPH-d₃</i>	7.19 ± 0.62	40	181 > 163	20	
Ketamine (KET)	7.48 ± 0.50	40	238 > 125 238 > 179	40 40	2.3 ± 0.1
<i>KET-d₄</i>	7.45 ± 0.54	40	242 > 129	40	
Methoxetamine (MXE) ¹	8.36 ± 0.46	50	248 > 203 248 > 121	20 40	1.3 ± 0.1
Heroin (HER)	9.08 ± 0.49	70	370 > 165 370 > 268	70 50	4.5 ± 0.5
<i>HER-d₉</i>	9.03 ± 0.46	70	379 > 272	45	
3,4-Methylenedioxy- pyrovalerone (MDPV)	9.50 ± 0.48	70	276 > 175 276 > 135	30 40	1.3 ± 0.1
<i>MDPV-d₈</i>	9.47 ± 0.54	70	284 > 185	41	

394 **Table 2 (cont.).** LC-ESI-MS/MS conditions used to determine the target analytes and
395 corresponding isotopically labeled analogs.

Analyte	Retention time (min)	Declustering Potencial (V)	SRM transition	Colision Energy (eV)	SRM ratio (SRM1/SRM2)
0 - 12 min					
Cocaine (COC)	9.61 ± 0.51	70	304 > 182 304 > 77	30 90	4.5 ± 0.5
COC- <i>d</i> ₃	9.61 ± 0.50	70	307 > 185	25	
Zolpidem (ZOLP)	9.92 ± 0.39	70	308 > 235 308 > 263	46 36	2.4 ± 0.4
ZOLP- <i>d</i> ₇	9.85 ± 0.38	86	315 > 242	50	
Lysergic acid diethylamide (LSD)	10.14 ± 0.49	70	324 > 223 324 > 208	40 40	1.6 ± 0.3
LSD- <i>d</i> ₃	10.14 ± 0.49	60	327 > 226	35	
Venlafaxine (VFX)	10.23 ± 0.44	54	278 > 58 278 > 260	47 17	1.4 ± 0.1
VFX- <i>d</i> ₆	10.23 ± 0.44	56	284 > 64	45	
Cocaethylene (CE)	10.94 ± 0.45	70	318 > 196 318 > 77	95 35	3.1 ± 0.4
CE- <i>d</i> ₃	10.94 ± 0.45	70	321 > 199	30	
12 - 18.30 min					
Citalopram (CTLP)	12.65 ± 0.40	66	325 > 109 325 > 262	37 28	2.0 ± 0.2
CTLP- <i>d</i> ₆	12.64 ± 0.40	72	331 > 109	37	
3,4-Dichloro-N-[[1-(dimethylamino)-cyclohexyl]methyl]benzamide (AH-7921)	12.29±0.55	56	329 > 284 329 > 173	20 45	1.9 ± 0.2
AH-7921- <i>d</i> ₅	12.26±0.53	55	332 > 287	25	
Midazolam (MIDZ)	12.94 ± 0.39	102	326 > 291 326 > 249	38 52	4.3 ± 0.3
MIDZ- <i>d</i> ₄	12.90 ± 0.24	107	330 > 295	38	
2-Ethylidene-1,5-dimethyl-3, 3-di-phenylpyrrolidine (EDDP)	13.36 ± 0.49	70	278 > 234 278 > 249	45 35	2.0 ± 0.1
EDDP- <i>d</i> ₃	13.35 ± 0.42	70	281 > 234	45	
α-Hydroxy-alprazolam (OH-ALPZ)	13.39 ± 0.54	92	325 > 297 325 > 216	35 55	2.9 ± 0.4
OH-ALPZ- <i>d</i> ₅	13.33 ± 0.36	102	330 > 302	37	
α-Hydroxy-midazolam (OH-MIDZ)	13.63 ± 0.34	92	342 > 324 342 > 203	31 37	2.4 ± 0.2
OH-MIDZ- <i>d</i> ₄	13.60 ± 0.20	80	346 > 328	33	

396 **Table 2 (cont).** LC-ESI-MS/MS conditions used to determine the target analytes and
397 corresponding isotopically labeled analogs.

Analyte	Retention time (min)	Declustering Potencial (V)	SRM transition	Colision Energy (eV)	SRM ratio (SRM1/SRM2)
12-18.30 min					
Hydroxyzine (HXZ)	13.98 ± 0.51	34	375 > 201 375 > 166	57 29	2.8 ± 0.4
<i>HXZ-d₈</i>	13.97 ± 0.54	47	383 > 201	25	
Oxazepam (OXA)	14.00 ± 0.18	49	287 > 241 287 > 269	31 23	1.1 ± 0.1
<i>OXA-d₅</i>	13.92 ± 0.30	56	292 > 246	36	
Alprazolam (ALPZ)	14.42 ± 0.29	70	309 > 205 309 > 281	60 35	2.2 ± 0.4
<i>ALPZ-d₅</i>	14.38 ± 0.30	60	314 > 286	40	
Lorazepam (LORZ)	14.43 ± 0.32	60	321 > 275 321 > 303	20 30	1.6 ± 0.1
<i>LORZ-d₄</i>	14.41 ± 0.17	50	325 > 279	30	
Methadone (METH)	14.55 ± 0.35	45	310 > 265 310 > 105	20 40	2.8 ± 0.3
<i>METH-d₃</i>	14.54 ± 0.38	60	313 > 268	20	
Fluoxetine (FLX)	14.86 ± 0.33	44	310 > 44 310 > 148	47 13	4.6 ± 0.4
<i>FLX-d₆</i>	14.85 ± 0.34	46	316 > 44	46	
Sertraline (STR)	15.02 ± 0.41	41	306 > 275 306 > 159	19 39	1.6 ± 0.2
<i>STR-d₃</i>	15.02 ± 0.44	46	309 > 159	43	
Chlorpromazine (CPMZ)	15.10 ± 0.49	49	319 > 86 319 > 58	29 59	1.3 ± 0.2
<i>CPMZ-d₃</i>	15.09 ± 0.46	52	322 > 89	28	
Temazepam (TEMZ)	15.50 ± 0.11	73	301 > 255 301 > 283	33 20	2.1 ± 0.3
<i>TEMZ-d₅</i>	15.41 ± 0.19	56	306 > 260	32	
Lormetazepam (LRMZ)	15.95 ± 0.32	77	335 > 289 335 > 177	34 57	8.4 ± 0.7
<i>LRMZ-¹³CD₃</i>	15.92 ± 0.27	56	339 > 293	31	
Diazepam (DIAZ)	17.00 ± 0.10	75	285 > 193 285 > 222	40 40	1.4 ± 0.1
<i>DIAZ-d₅</i>	16.91 ± 0.21	75	290 > 154	40	
18.30 - 47 min					
11-hydroxy- Δ^9 - tetrahydrocannabinol (OH-THC)	20.43 ± 0.23	65	331 > 313 331 > 193	20 30	9.1 ± 0.3
OH-THC-d ₃	20.52 ± 0.18	65	334 > 316	15	

Table 2 (cont). LC-ESI-MS/MS conditions used to determine the target analytes and corresponding isotopically labeled analogs.

Analyte	Retention time (min)	Declustering Potencial (V)	SRM transition	Colision Energy (eV)	SRM ratio (SRM1/SRM2)
12-18.30 min					
11-nor-9-carboxy- Δ^9 -tetrahydrocannabinol (THC-COOH)	20.56 $\pm$ 0.24	50	345 > 299	20	0.43 $\pm$ 0.09
			345 > 327	29	
THC-COOH-d ₃	20.52 $\pm$ 0.18	55	348 > 330	25	

¹ Ket-d4 used as surrogate standard

401 **Table 3.** Method performance in terms of analyte (absolute and relative) recoveries, repeatability (RSD) and sensitivity (limits of detection and
402 quantification) in wastewater

		Absolute recovery (n=5)			Relative recovery (RSD) (n=5)			LOD (ng/L)	LOQ (ng/L)
		50 ng/L	500 ng/L	2500 ng/L	50 ng/L	500 ng/L	2500 ng/L		
Cocainics	COC	n.c.	30	40	n.c.	96 (3)	97 (3)	0.2	0.8
	BE	n.c.	n.c.	70	n.c.	n.c.	98 (4)	0.5	1.5
	CE	31	33	40	108 (8)	107 (6)	105 (2)	0.5	1.4
Amphetamine-type stimulants	AM	21	50	66	106 (15)	111 (5)	112 (3)	4.0	13
	MA	37	38	44	119 (6)	110 (6)	109 (4)	2.6	8.5
	MDMA	30	36	43	98 (12)	102 (9)	98 (7)	1.0	3.4
Hallucinogens	KET	35	39	46	101 (8)	96 (5)	98 (6)	0.5	1.5
	LSD	28	32	38	96 (11)	85 (5)	99 (5)	0.8	2.8
Opioids/opiates	MOR	26	26	32	114 (20)	98 (12)	99 (8)	5.1	17
	HER	50	56	78	112 (20)	113 (7)	106 (16)	4.7	16
	6ACM	42	42	54	113 (20)	97 (9)	92 (9)	2.7	9.0
	METH	24	20	24	97 (18)	99 (7)	99 (10)	1.9	6.3
	EDDP	25	27	33	115 (6)	101 (7)	100 (2)	0.2	0.6
Cannabinoids	THC-COOH	<LOQ	102	118	<LOQ	89 (9)	94 (9)	18	61
	OH-THC	<LOQ	14	27	<LOQ	80 (8)	84 (19)	69	228
New psychoactive substances	AH-7921	36	34	37	119 (7)	103 (6)	101 (3)	1.6	5.4
	MDPV	36	34	41	109 (12)	101 (6)	98 (5)	1.3	4.3
	MEPH	43	40	47	112 (6)	101 (4)	100 (5)	3.0	10
	MXE	37	38	48	113 (9)	97 (3)	105 (6)	0.8	2.6

405 **Table 3 (cont).** Method performance in terms of analyte (absolute and relative) recoveries, repeatability (RSD) and sensitivity (limits of
406 detection and quantification) in wastewater.

		Absolute recovery (RSD) (n=5)			Relative recovery (RSD) (n=5)			LOD (ng/L)	LOQ (ng/L)
		50 ng/L	500 ng/L	2500 ng/L	50 ng/L	500 ng/L	2500 ng/L		
Benzodiazepines	ALPZ	48	62	86	112 (18)	100 (12)	93 (10)	0.6	1.9
	OH-ALPZ	80	95	117	96 (20)	110 (10)	100 (15)	3.9	13
	DIAZ	51	53	74	107 (8)	111 (8)	97 (5)	1.8	5.9
	LORZ	n.c.	99	137	n.c.	90 (5)	90 (3)	6.8	23
	LRMZ	n.c.	102	142	n.c.	118 (5)	89 (3)	10	32
	MIDZ	34	36	39	106 (8)	100 (9)	99 (5)	0.8	2.7
	OH-MIDZ	58	59	67	116 (9)	101 (9)	106 (4)	1.7	5.7
	OXZ	n.c.	81	132	n.c.	93 (8)	92 (11)	6.6	22
	TEMZ	n.c.	80	118	n.c.	100 (8)	81 (11)	2.4	7.9
Antidepressants	CTLP	n.c.	27	30	n.c.	107 (11)	104 (10)	1.9	6.4
	FLX	8	6	5	116 (18)	99 (9)	101 (9)	2.0	6.7
	STR	10	10	12	120 (13)	110 (11)	104 (13)	3.3	11
	VFX	n.c.	38	45	n.c.	103 (4)	100 (3)	0.8	2.5
Stimulants	CAF*	n.c.	45	58	n.c.	111 (8)	106 (7)	13	44
	EPH	n.c.	n.c.	52	n.c.	n.c.	102 (4)	4.7	16
Sedative	HXZ	37	29	32	115 (3)	110 (7)	110 (4)	0.2	0.8
Hypnotic	ZOPD	34	37	43	109 (6)	109 (3)	111 (7)	0.2	0.7
Antipsychotic	CPMZ	10	7	6	119 (20)	96 (13)	98 (18)	2.9	9.5

407 <LOQ: Concentration tested below the analyte limit of quantification; n.c.: not calculated, high background concentrations in the sample. *

408 Recovery studies were performed with wastewater samples spiked at 10050, 50500 and 102500 ng/L

409

Table 4. Linearity, analyte (absolute and relative) recovery, repeatability and limit of detection and quantification of the method in HPLC-grade water

		Linearity		Absolute recovery (n=5)		Relative recovery (RSD) (n=5)		LOD (ng/L)	LOQ (ng/L)
		r ²	Range (ng/L)	50 ng/L	250 ng/L	50 ng/L	250 ng/L		
Cocainics	COC	0.9908	0.1-2000	38	42	93 (3)	98 (3)	< 0.1	0.1
	BE	0.9916	0.1-2000	62	65	92 (4)	96 (2)	0.1	0.5
	CE	0.9920	0.5-2000	40	40	116 (3)	105 (4)	0.1	0.5
Amphetamine-type stimulants	AM	0.9930	1-2000	74	70	108 (6)	107 (3)	0.5	1.0
	MA	0.9930	1-2000	53	55	107 (6)	115 (5)	0.5	1.0
	MDMA	0.9922	0.1-2000	45	49	105 (11)	101 (13)	< 0.1	0.1
Hallucinogens	KET	0.9908	0.5-2000	49	51	98 (5)	93 (5)	0.1	0.5
	LSD	0.9914	0.5-2000	28	34	92 (11)	103 (12)	0.1	0.5
Opioids/opiates	MOR	0.9954	5-2000	54	59	97 (3)	104 (4)	1.0	5.0
	HER	0.9930	1-2000	56	60	87 (6)	97 (14)	0.5	1.0
	6ACM	0.9908	1-2000	57	64	101 (5)	93 (6)	0.5	1.0
	METH	0.9908	0.5-2000	33	33	100 (2)	105 (3)	0.1	0.5
	EDDP	0.9908	0.5-2000	30	33	97 (5)	100 (5)	0.1	0.5
Cannabinoids	THC-COOH	0.9902	10-2000	100	67	101 (8)	107 (8)	7.0	10
	OH-THC	0.9974	10-2000	34	12	94 (13)	82 (9)	7.0	10
New psychoactive substances	AH-7921	0.9912	0.1-100	35	36	98 (5)	98 (5)	< 0.1	0.1
	MDPV	0.9966	1-2000	38	39	104 (6)	94 (7)	0.1	1.0
	MEPH	0.9908	0.5-2000	55	54	101 (4)	99 (5)	0.1	0.5
	MXE	0.9902	0.5-2000	44	51	89 (3)	99 (8)	0.1	0.5
	ALPZ	0.9928	0.5-2000	70	68	103 (4)	102 (4)	0.1	0.5
Benzo-diazepines	OH-ALPZ	0.9970	5-2000	92	87	98 (9)	106 (6)	2.0	5.0
	DIAZ	0.9914	1-2000	60	62	99 (3)	103 (8)	0.5	1.0
	LORZ	0.9932	5-500	113	96	92 (5)	88 (3)	1.0	5.0
	LRMZ	0.9934	1-2000	98	92	96 (5)	91 (2)	0.5	1.0
	MIDZ	0.9936	1-2000	38	38	101 (6)	98 (7)	0.5	1.0
	OH-MIDZ	0.9942	1-1000	68	67	103 (7)	105 (3)	0.5	1.0
	OXZ	0.9930	1-2000	110	104	103 (3)	98 (4)	0.5	1.0
	TEMZ	0.9960	0.5-2000	87	83	95 (5)	93 (3)	0.1	0.5
	CTLP	0.9934	0.5-2000	32	35	101 (4)	102 (4)	0.1	0.5
Anti-depressants	FLX	0.9970	1-2000	12	13	90 (3)	90 (12)	0.5	1.0
	STR	0.9920	1-2000	14	18	109 (4)	109 (3)	0.5	1.0
	VFX	0.9928	0.1-2000	42	45	93 (5)	103 (3)	< 0.1	0.1
Stimulants	CAF*	0.9918	0.1-10250	78	77	120 (10)	119 (8)	< 0.1	0.1
	EPH	0.9908	1-2000	65	65	108 (4)	103 (1)	0.5	1.0
Sedative	HXZ	0.9900	0.5-2000	35	36	108 (8)	109 (4)	0.1	0.5
Hypnotic	ZOPD	0.9920	0.5-2000	36	38	106 (3)	113 (3)	0.1	0.5
Antipsychotic	CPMZ	0.9924	1-2000	9	12	98 (16)	105 (2)	0.5	1.0

* Recovery studies were performed with HPLC-grade water spiked at 1005, 5050 and 10250 ng/L

3.3. Matrix effects

The extent of matrix effects in the analysis of psychoactive substances in wastewater is shown in Figure 4. In general, low matrix effects, within $\pm 20\%$, were observed for most compounds. MS signal suppression greater than 20% was observed in the case of fluoxetine (-65%), sertraline (-54%), benzoylecgonine (-50%), methadone (-39%), morphine (-36%), ephedrine (-35%), caffeine (-30%), and amphetamine (-30%). This could explain the low absolute recoveries obtained for these compounds, particularly for sertraline and fluoxetine (AR < 10%). On the contrary, MS analysis of THC-COOH was affected by matrix components that enhanced its signal more than 20%. However, as previously mentioned, relative recoveries in the range of 80-120% for all compounds demonstrate how useful surrogate standards are at compensating matrix effects (in addition to extraction losses).

Figure 4. Matrix effects (%) observed for the on-line SPE-LC-MS/MS analysis of psychoactive substances in raw wastewater (fortified at 1000 ng/L).

3.4. Occurrence of psychoactive substances in wastewater

The validated methodology was applied to the analysis of raw wastewater samples collected at a WWTP located in Barcelona, Spain. Figure 5 shows the levels of psychoactive substances measured in the influent wastewater samples analyzed.

Figure 5. Concentration (ng/L) of psychoactive substances (mean $\pm$ SD) in raw wastewater (n=7).

Most of the investigated compounds were present in all wastewater samples analyzed. The exceptions were zolpidem, α -hydroxy-midazolam, and temazepam that were detected in 71, 86 and 86% of the analyzed samples, respectively, and 6-acetylmorphine, heroin, LSD, midazolam, α -hydroxy-alprazolam, OH-THC, chlorpromazine, AH-7921, methoxetamine, MDPV and mephedrone that were not detected in any sample. Alprazolam was detected in all samples; however, it could not be quantified because levels measured were below its LOQ (1.9 ng/L).

The most abundant compounds in the wastewater samples analyzed presented average concentrations in the $\mu\text{g/L}$ range and were the stimulants caffeine (53.8 $\mu\text{g/L}$) and ephedrine (2.3 $\mu\text{g/L}$), and indicators related to the most consumed illicit drugs in the area [1]: benzoylecgonine (2.2 $\mu\text{g/L}$), cocaine (1.3 $\mu\text{g/L}$), and THC-COOH (1.1 $\mu\text{g/L}$). The high levels of caffeine measured are in agreement with wastewater concentrations reported for this stimulant in other developed countries [14, 17]. This is attributed to the high consumption of beverages such as coffee, tea or soda drinks that contain this psychoactive substance, e.g., 1.8 cups of coffee or tea were consumed per capita per day in Spain in 2015-2016 [18]. The extended use of ephedrine could be attributed to the therapeutic use of this substance to treat asthma, bronchitis, and hypotension, in addition to its illegal use as an amphetamine type stimulant.

The antidepressants venlafaxine, with 838 ng/L, citalopram, with 442 ng/L and sertraline, with 381 ng/L, were the most abundant psychoactive pharmaceuticals. According to the Spanish Agency of Medicines and Medical Devices [19], consumption of antidepressants has continuously increased in Spain since 2000, being selective serotonin uptake inhibitors (SSRIs), i.e., paroxetine, fluoxetine, sertraline, citalopram and its S-stereoisomer escitalopram, and selective serotonin and norepinephrine reuptake inhibitors (SSNRI), i.e., venlafaxine and duloxetine, the most consumed drugs [19]. Such an increase in the use of antidepressants is mainly attributed to an increasing incidence of mood disorders among the population as well as to the prescription of these substances for additional therapeutic uses. Antidepressant levels measured in Barcelona were overall higher than those measured in raw wastewaters of Slovakian cities (256 ng/L in the case of venlafaxine and 102 ng/L in the case of citalopram) [20], in wastewaters of the city of New York (376 ng/L for venlafaxine, 62 ng/L for sertraline and 96 ng/L for citalopram on average) [21], in wastewaters of the Santorini island in Greece (11 ng/L for fluoxetine, 348 ng/L for venlafaxine, 290 ng/L for citalopram and 30 ng/L for sertraline on average) [8] and in wastewaters of other Spanish regions, e.g. Galicia (north Spain) (16 ng/L of fluoxetine, 401 ng/L of venlafaxine and 114 ng/L of citalopram on average) [22].

Illicit ATS amphetamine, methamphetamine and MDMA, and the opiate morphine and the synthetic opioid methadone and its metabolite EDDP presented similar concentrations in the wastewaters analyzed (between 145 and 255 ng/L). The presence of morphine in wastewater may originate from its therapeutic use but also from heroin

consumption. Despite the fact that neither heroin nor its exclusive metabolite 6-acetylmorphine were detected in the investigated samples, heroin use as a source of morphine cannot be discarded because heroin is highly metabolized after consumption and 6-acetylmorphine, excreted at a very low rate, is unstable in wastewater [23]. Levels of amphetamine and morphine measured in Barcelona were lower than those measured, for example, in England (830 ng/L for amphetamine and 481 ng/L for morphine); however, higher concentrations of methamphetamine and methadone were measured in Barcelona compared with England (2 ng/L and 87 ng/L on average) [7] while similar levels of EDDP were measured in both places.

As for benzodiazepines, average concentrations decreased in the order lorazepam (227 ng/L), oxazepam (163 ng/L), lormetazepam (99 ng/L), temazepam (93 ng/L) and alprazolam (<1.9 ng/L). The benzodiazepine metabolites analyzed were either below the method LOD (α -hydroxy-alprazolam) or measured at very low concentrations (24 ng/L for α -hydroxy-midazolam). Except for alprazolam, concentrations of benzodiazepines found in wastewaters of Barcelona were higher than in wastewaters analyzed in Greece (2 ng/L of diazepam, 21 ng/L of lorazepam, and 36 ng/L of oxazepam on average) [8], in Slovakia (84 ng/L of oxazepam on average) [20] and in the city of New York (4 ng/L of diazepam, 18 ng/L of lorazepam and 8 ng/L of oxazepam on average) [21].

Finally, the lowest average concentrations were observed for cocaethylene (42 ng/L), ketamine (20 ng/L), the sedative hydroxyzine (20 ng/L), diazepam (16 ng/L), and the hypnotic zolpidem (7 ng/L), which could be attributed to a low consumption of these substances in the investigated area and their likely high metabolization before excretion (ketamine and zolpidem are excreted after consumption as unchanged drugs in urine in 2.3% and < 1%, respectively) [24, 25]. This is in agreement with concentrations of these substances reported in wastewater in other investigated locations, e.g., levels of zolpidem measured in influent wastewater in the northwest of Spain ranged from 2.9 to 3.9 ng/L [26] and were below the method LOQ in another study performed in the northeast of Spain [27] while levels of ketamine in wastewater from Colombia were between <LOQ and 32 ng/L [28].

From the water management point of view, it may be worth noting that the results obtained correspond exclusively to influent raw waters, and that treated effluent

wastewaters usually contain much lower concentrations of substances not representing risk for the environment or human health.

4. Conclusions

A fully automated method has been developed and validated for the simultaneous analysis of 37 psychoactive substances in wastewater. The method, based on on-line SPE-LC-MS/MS, allows determining all target compounds in the low ng/L range in a fast and simple way. The use of isotopically labeled compounds compensates potential losses during the extraction process as well as matrix effects for most analytes.

Additional advantages include (i) the use of low sample volumes, which facilitates sample collection, transport/shipment, and storage, (ii) minimum manipulation of the sample, which allows to improve the reproducibility of the results, as well as reduce cross contamination processes, (iii) improvement of the sensitivity due to the introduction of the whole sample volume (instead of an aliquot of a sample extract) in the analytical system, and (iv) the provision of more reliable quantitative results because samples and calibration curves are processed in exactly the same way. In contrast, the methodology presents as disadvantages: (i) the SymbiosisTM Pico system cannot be used with ultra-high performance liquid chromatography columns due to the high back pressure generated in the system, (ii) only one SPE cartridge (instead of e.g. various cartridges in tandem) can be used at a time, and thus the extraction and sensitivity of specific analytes included in a multi-residue method can be compromised, and (iii) solvents used in the on-line SPE process (e.g. in the washing step) must be compatible with the LC mobile phase.

The application of the method to influent wastewater samples from a WWTP located in Barcelona revealed the ubiquitous presence of 68% of the investigated analytes in this matrix. The most abundant compounds were the stimulant caffeine, analytes related to the most illicit drugs consumed in the investigated area (THC-COOH, cocaine, and benzoylecgonine), and psychoactive pharmaceuticals such as ephedrine and the antidepressants venlafaxine, citalopram and sertraline. This methodology will be applied to the analysis of larger sets of samples to assess in more depth the presence of

these compounds in wastewaters and to explore the use of psychoactive pharmaceutical concentrations in wastewater as potential indicators of population's health.

Acknowledgments

This work has been financially supported by the EU through the EU FP7 project SOLUTIONS (603437), and by the Government of Catalonia (Consolidated Research Groups: 2017 SGR 1404 - Water and Soil Quality Unit). It reflects only the author's views. The Community is not liable for any use that may be made of the information contained therein. Merck is acknowledged for the gift of LC columns. Nicola Mastroianni acknowledges the JAE Program (CSIC-European Social Funds) and Ester López-García acknowledges the Youth Employment Initiative and the European Social Fund for the "National System of Youth Guarantee" contract within the National Operational Programme on Youth Employment 2014–2020. Begoña Martínez López y Cristian Mesa García from Aguas de Barcelona are acknowledged for their assistance in the collection of wastewater samples.

References

- [1] OEDA, Observatorio Español de las Drogas y las Adicciones. Informe anual 2017. Alcohol, tabaco y drogas ilegales en España. Available at <https://goo.gl/jh46g9>. Accessed January 2018.
- [2] EMCDDA, European Monitoring Centre for Drugs and Drug Addiction. European Drug Report 2015. Trends and Developments. Available at <https://goo.gl/d2EJzx>. Accessed January 2018.
- [3] V. Calisto, V.I. Esteves, Psychiatric pharmaceuticals in the environment, *Chemosphere*, 77 (2009) 1257-1274.
- [4] R. Pal, M. Megharaj, K.P. Kirkbride, R. Naidu, Illicit drugs and the environment — A review, *Science of The Total Environment*, 463-464 (2013) 1079-1092.
- [5] E. López-García, C. Postigo, B. Zonja, D. Barceló, M. López de Alda, Analysis of Psychoactive Pharmaceuticals in Wastewater and Surface Water Using LC-MS, in: *Comprehensive Analytical Chemistry*, Elsevier, 2017.
- [6] S. Castiglioni, K.V. Thomas, B. Kasprzyk-Hordern, L. Vandam, P. Griffiths, Testing wastewater to detect illicit drugs: State of the art, potential and research needs, *Science of The Total Environment*, 487 (2014) 613-620.
- [7] D.R. Baker, B. Kasprzyk-Hordern, Multi-residue analysis of drugs of abuse in wastewater and surface water by solid-phase extraction and liquid chromatography–positive electrospray ionisation tandem mass spectrometry, *Journal of Chromatography A*, 1218 (2011) 1620-1631.
- [8] V.L. Borova, N.C. Maragou, P. Gago-Ferrero, C. Pistos, N.S. Thomaidis, Highly sensitive determination of 68 psychoactive pharmaceuticals, illicit drugs, and related human metabolites in wastewater by liquid chromatography-tandem mass spectrometry, *Analytical and Bioanalytical Chemistry*, 406 (2014) 4273-4285.
- [9] C. Boix, M. Ibáñez, J.V. Sancho, J. Rambla, J.L. Aranda, S. Ballester, F. Hernández, Fast determination of 40 drugs in water using large volume direct injection liquid chromatography–tandem mass spectrometry, *Talanta*, 131 (2015) 719-727.
- [10] K. Li, P. Du, Z. Xu, T. Gao, X. Li, Occurrence of illicit drugs in surface waters in China, *Environmental Pollution*, 213 (2016) 395-402.
- [11] M.J. Andrés-Costa, V. Andreu, Y. Picó, Analysis of psychoactive substances in water by information dependent acquisition on a hybrid quadrupole time-of-flight mass spectrometer, *Journal of Chromatography A*, 1461 (2016) 98-106.

- [12] J.A. Baz-Lomba, M.J. Reid, K.V. Thomas, Target and suspect screening of psychoactive substances in sewage-based samples by UHPLC-QTOF, *Analytica Chimica Acta*, 914 (2016) 81-90.
- [13] A. Causanilles, J. Kinyua, C. Ruttkies, A.L.N. van Nuijs, E. Emke, A. Covaci, P. de Voogt, Qualitative screening for new psychoactive substances in wastewater collected during a city festival using liquid chromatography coupled to high-resolution mass spectrometry, *Chemosphere*, 184 (2017) 1186-1193.
- [14] M.L. Hedgespeth, Y. Sapozhnikova, P. Pennington, A. Clum, A. Fairey, E. Wirth, Pharmaceuticals and personal care products (PPCPs) in treated wastewater discharges into Charleston Harbor, South Carolina, *Science of The Total Environment*, 437 (2012) 1-9.
- [15] A. Rubirola, M.R. Boleda, M.T. Galceran, Multiresidue analysis of 24 Water Framework Directive priority substances by on-line solid phase extraction-liquid chromatography tandem mass spectrometry in environmental waters, *Journal of Chromatography A*, 1493 (2017) 64-75.
- [16] C. Postigo, M.L. de Alda, D. Barceló, Evaluation of drugs of abuse use and trends in a prison through wastewater analysis, *Environment International*, 37 (2011) 49-55.
- [17] V. de Jesus Gaffney, V.V. Cardoso, E. Cardoso, A.P. Teixeira, J. Martins, M.J. Benoliel, C.M.M. Almeida, Occurrence and behavior of pharmaceutical compounds in a Portuguese wastewater treatment plant: Removal efficiency through conventional treatment processes, *Environmental Science and Pollution Research*, 24 (2017) 14717-14734.
- [18] ForumCafe, Consumo de café en España. Available at <https://goo.gl/XtWdWA>. Accessed January 2018.
- [19] AEMPS, Agencia Española de Medicamentos y Productos Sanitarios. Utilización de medicamentos antidepresivos en España durante el periodo 2000-2013. Informe de utilización de medicamentos U/AD/V1/14012015. Available at <https://goo.gl/jdHXt5>. Accessed January 2018.
- [20] T. Mackuľák, L. Birošová, M. Gál, I. Bodík, R. Grabic, J. Ryba, J. Škubák, Wastewater analysis: the mean of the monitoring of frequently prescribed pharmaceuticals in Slovakia, *Environmental monitoring and assessment*, 188 (2016) 18.
- [21] B. Subedi, K. Kannan, Occurrence and fate of select psychoactive pharmaceuticals and antihypertensives in two wastewater treatment plants in New York State, USA, *Science of the Total Environment*, 514 (2015) 273-280.

- [22] S. Esteban, Y. Valcárcel, M. Catalá, M.G. Castromil, Psychoactive pharmaceutical residues in the watersheds of Galicia (Spain), *Gaceta Sanitaria*, 26 (2012) 457-459.
- [23] E. Gracia-Lor, E. Zuccato, S. Castiglioni, Refining correction factors for back-calculation of illicit drug use, *Science of The Total Environment*, 573 (2016) 1648-1659.
- [24] D.R. Baker, L. Barron, B. Kasprzyk-Hordern, Illicit and pharmaceutical drug consumption estimated via wastewater analysis. Part A: Chemical analysis and drug use estimates, *Science of The Total Environment*, 487 (2014) 629-641.
- [25] D.M. Schwoppe, A. DePriest, D.L. Black, Y.H. Caplan, E.J. Cone, R. Heltsley, Determining Zolpidem Compliance: Urinary Metabolite Detection and Prevalence in Chronic Pain Patients, *Journal of Analytical Toxicology*, 38 (2014) 513-518.
- [26] I. Racamonde, R. Rodil, J.B. Quintana, E. Villaverde-de-Sáa, R. Cela, Determination of benzodiazepines, related pharmaceuticals and metabolites in water by solid-phase extraction and liquid-chromatography–tandem mass spectrometry, *Journal of Chromatography A*, 1352 (2014) 69-79.
- [27] M. Huerta-Fontela, M.T. Galceran, F. Ventura, Fast liquid chromatography–quadrupole-linear ion trap mass spectrometry for the analysis of pharmaceuticals and hormones in water resources, *Journal of Chromatography A*, 1217 (2010) 4212-4222.
- [28] L. Bijlsma, A.M. Botero-Coy, R.J. Rincón, G.A. Peñuela, F. Hernández, Estimation of illicit drug use in the main cities of Colombia by means of urban wastewater analysis, *Science of The Total Environment*, 565 (2016) 984-993.