INVESTIGATION OF DRUGS OF ABUSE IN WASTEWATER

ESTIMATION OF DRUG CONSUMPTION AND POTENTIAL ENVIRONMENTAL IMPACT

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ABSTRACT

Drugs of abuse (DOAs) have come to be in the spotlight of the society in recent years. Consumption of these substances can be estimated from the analysis of urban wastewater, as drugs consumed by human beings are finally excreted, mainly in urine, and end up in the wastewater. In addition, when these waters are treated in wastewater treatment plants (WWTPs) there is not a complete removal of compounds such as pharmaceuticals and drugs of abuse; therefore, treated wastewater may have an important impact on aquatic environment. Both issues, the estimation of consumption of DOAs as well as the potential impact on effluent wastewater on the environment, are considered in this study.

The study is focused on the determination of several drugs usually consumed and some major metabolites: amphetamine, 3,4-methylenedioxymethamphetamine (MDMA or ecstasy), methamphetamine, 11-nor-9-carboxy-Δ9-tetrahydrocannabinol, cocaine, benzoylecgonine and ketamine. Modern and sensitive analytical methodology based on the ultra-high performance liquid chromatography – tandem mass spectrometry was applied. Solid-phase extraction (SPE) was used for clean-up and to pre-concentrate the samples. To correct for matrix effects, isotope-labelled internal standards, available for all compounds tested, were added to the samples. Moreover, for influent wastewater samples, a four-fold dilution was done before SPE with the aim to facilitate the loading of the samples through the cartridge. The volume of samples injected into the LC-MS/MS system was 3μL. The method applied was previously validated by researchers of the group to ensure the reliability of data reported.

The experimental procedure was applied to 14 influent and 14 effluent wastewater samples daily collected from WWTPs of Castellón and Madrid over a whole week.

At the same time, removal efficiency of these WWTPs was calculated to estimate the percentage of compound that was not eliminated in the WWTP and consequently was discharged to the aquatic environment generating a potential negative impact. The toxicity of the drugs studied was also considered in the study.

1. INTRODUCTION

Nowadays, the estimation of drugs of abuse (DOAs) consumption has become an issue of great concern for governments as it has been demonstrated to be directly related to (inter)national trafficking with the subsequent negative socioeconomic effects (European Monitoring Centre for Drugs and Drug Addiction and Europol 2016). The traffic of these substances is usually a hidden activity. Apparently, it has not effect on general population directly but if there is not control over it, it would generate serious problems over the years as economic crisis or health problems.

According to the European Monitoring Centre of Drugs and Drug Addiction (EMCDDA) in its Drug Report 2017 (EMCDDA 2017), more than 93 million of people have tried some DOA during their lives, being more common in males than in females. Moreover, young adults, among 15 and 34 years old, are the group of population found to be the main consumers of DOA. The results show in this report show different trends in DOA consumption depending on the area of Europe. Whereas drugs such as cannabis or cocaine are the most consumed in south-Europe, other drugs such methamphetamine or 3,4-methylenedioxymethamphetamine (MDMA or ecstasy) are mainly consumed in northern countries (See **Figure 1**). In addition to the "classic", conventional DOAs, a new concern has appears in recent years. Newly designed drugs, known as new psychoactive substances (NPS), are appearing increasingly in the market to avoid the strict regulation on DOA consumption and trafficking (EMCDDA 2016). Most of NPS are created in clandestine laboratories and could generate really harmful effects on human health without neglecting the environmentally repercussion (EMCDDA 2015). In the case of Spain, the general drug use remains stable over the recent years. The consumption of some recreational drugs, such as cocaine and MDMA, is higher during the weekend while others such as cannabis remain constant over the week.

Traditionally, drug consumption used to be estimated through population surveys. However, these studies are not always reliable due to low response rate and response biases (Lai et al. 2016). Thus, a more robust strategy for the determination of DOA consumption is required. Nowadays, wastewater based epidemiology (WBE) provides more reliable data through the qualitative and quantitative analysis of DOA present in wastewater (WW) (Rousis et al. 2017).

Figure 1. Most frequently seized stimulant drug in Europe, 2015 or more recent data (EMCDDA)

The general procedure for WBE is the following: firstly, a 24h-composite sample is taken at the entrance of a wastewater treatment plant (WWTP); next, a sample extraction to pre-concentrate the analytes as well as the addition of isotopically labelled internal standards (ILIS) to correct the matrix effect is made; then, the samples are analysed by means of ultra-high pressure liquid chromatography coupled to mass spectrometry (UHPLC-MS/MS); finally, the consumption (mg/day/1000 habitants) is calculated by knowing some specific information about the WWTP (See **Figure 2**) (Bijlsma et al. 2014).
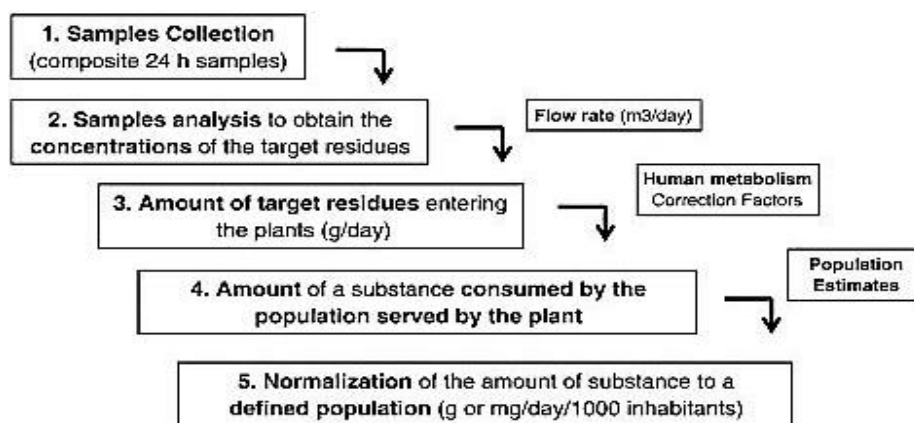
Figure 2. Wastewater based epidemiology workflow (Modified from Castiglioni et al. 2014)

It should be noted that in some cases both the drug and main human metabolites are analysed. This is due to the necessity of a more stable/abundant compound to target and to avoid faking the results because of dumping. The drug is transformed by the human metabolism in different metabolites depending on the kind of drug. The polar substances pass directly to the urine while no polar substances are converted into more polar ones by human metabolism and, then, pass to the urine too. Thus, the analysis of wastewater can be seen as a general test to estimate drugs consumption through the analysis of thousands of anonymous individual that excrete drugs and metabolites through urine into the toilet.

WBE has also been applied for the analysis of alcohol or tobacco but, up till now, the most important application is to study the consumption of illicit drugs in a delimited community (Bijlsma et al. 2016). Recently, this strategy has been also tested for its application to NPS (Bade et al. 2016). Furthermore, WBE has the advantage of allowing researchers to monitor DOAs consumption in real time and observe changes within weeks and seasons of the year. On the other hand, the methodology presents some limitations such as the stability of the compounds in WW, the variation in flow in WWTP, as well as census data to back-calculate DOA consumption rate (Castiglioni et al. 2013; EMCDDA 2016). For this reason, WBE data should be complemented with surveys or similar.

Nowadays it is possible find several studies around the world which apply this methodology. Bijlsma et al. (2016) estimated the illicit drug use in the main cities of Colombia. In the same way, Castiglioni et al. (2016) studied this process and were able to compare the drug use in several cities in Europe. USA (Subedi and Kannan 2014), China (Du et al. 2017) or Australia (Lai et al. 2016) have done different works and advances in recent years. Special mention must be also made of the first WBE approach on the Africa continent where DOA data is limited, published this year (Archer et al. 2018). WBE is a young methodology that is improving day by day. Thus, due to the good results obtained in these and other studies, the analysis of WW for establishing consumption of drugs of abuse has been proven to be valuable way to determine the intake of these kinds of substances.

1.1. Removal efficiency of WWTP and environmental impact

Water is one of the most important components in our planet. Life known today needs water to exist. Since the vast majority of living creatures have water in their organisms, it is essential to survive. However, humans use water resources for several activities, which unfortunately generate contaminant residues. Water is considered contaminated if there are some substances in it or exists a particular condition that do not allow water be used to other purposes (Owa 2014). Over time, humans have developed different kind of systems to achieve cleaning the sewage wastewater before to drop it again to the nature, normally to rivers or seas. Nowadays, the most important and common system is to treat WW in wastewater treatment plants (WWTPs). Influent wastewater (IWW) enters into the WWTP where it suffers different treatments based on chemical, physical and biological processes. In this way, most of pollutants are eliminated, so water can return to the most natural state being discharged. However, despite the treatments applied in most of WWTPs, effluents (EWW) usually still contain certain amounts of undesirable substances such as pesticides, pharmaceuticals, personal care products or DOA, which are thrown to nature (Hernández et al. 2011; Westlund and Yargeau 2017).

DOAs present high biological activity, psychoactive properties and probably worse effects which are still an issue of study, when entering the aquatic environment (Boix 2014). Every contaminant that arrived to the nature after passing WWTP can be constitute a serious problem that must be studied and solved, if necessary. Unfortunately, when EWW reaches the sea or a river, it may affect aquatic organisms living there or others animals who drink from there. In the same way, if this supposed "clean" water is used to irrigation, the plants and crops may absorb some of the harmful substances. So, the main problem appears when the contaminated water arrives to animals, crops and plants which will be a food source for humans in the near future. Moreover, an occasionally direct contact with this treated water might not generate big problems, but a continued treatment or contact with it might become a problem in long-term exposure. Anyway, the possible presence of any substance which is not usual to be present in the environment may result an important issue that deserves to be studied in detail.

For all above mentioned reasons, removal efficiency (RE) of WWTP is a key aspect that must be considered when dealing with environmental pollution. To calculate RE, **Eq. 1** (Bijlsma et al. 2012) is applied:

$$\text{Removal Efficiency (\%)} = \left(1 - \frac{\text{Mass load}_{EWW}}{\text{Mass load}_{IWW}}\right) \cdot 100$$

<div align="right">**Eq. 1**</div>

The mass load of EWW from day (*x+1*) is compared with the mass load of IWW from day (*x*) considering that the time of residence of water in the WWTP is approximately 24 hours. High percentage of removal efficiency indicates that the concentration of contaminants in EWW is rather low respect to their concentration in IWW as a consequence of the efficiency of the WWTP.

Moreover, the interest on knowing which pollutants end up into the aquatic environment as well as their potential impact on the ecosystem motivated the development of some tools to evaluate environmental risk. Environmental Risk Assessment (ERA) is one of these tools used to assess the potential environmental impact of chemicals and human activities (Smit, Holthaus, and Tamis 2005) with the aim of identifying possible risks and looking for a solution.

For the evaluation of the sensitivity of environment towards a given pollutant in an established way, the most common approach is to compare predicted environmental concentrations (PECs) with measured environmental concentrations (MECs). Moreover, PECs and MECs can be compared with aquatic predicted no effect concentrations (PNECs). When these ratios (PEC/PNEC and MEC/PNEC) exceed 1 there are more probability that undesirable effects on living organisms occur (Smit et al. 2005).

Unfortunately, the estimation of these ratios is sometimes costly and high-demanding. For this reason, other approaches, such as computational tools, are often used. The Threshold of Toxicological Concern (TTC) approach provides a system to estimate if a compound presents risk to provoke health problems (Laufersweiler et al. 2012). According to Schnabel 2009, the concept of TTC is based on the assumption that similar chemicals have similar behaviour in the same medium. The computational systems are usually open source software for chemoinformatics data management,

which allows storage a large number of chemical structures and their toxicological data. Although they are normally used to study hazard when the compound is orally administered to the human body, it can be obtained to analyse hazard in aquatic medium. A technique used to classify chemical compounds according their hazard is based on Cramer's rules which separate compounds in three classes. These rules were published in 1978 and were validated with 82 compounds with no observed effect levels (NOEL) data and other carcinogens compounds (Curios-IT 2009).

In Class I substances with simple chemical structures are included, the level of toxicological hazard is rather low. On the other side, Class III includes substances that not allow a strong initial presumption of safety or even suggest a certain level of toxicity or have reactive functional groups. Lastly, in Class II are covered compounds that are among characteristics of the other classes (Curios-IT 2009).

## 1.2. Analytical techniques used in this study

The analytical technique used in this work is ultra-high performance liquid chromatography coupled to tandem mass spectrometry (UHPLC-MS/MS) due to the excellent performance in sensitivity, selectivity and robustness at low concentration levels. However, the complexity of the matrix generates a sample with a large amount of undesirable substances that may need to be eliminated. Commonly, the sample must be cleaned-up and pre-concentrated because of the low analyte concentrations. The most widely sample treatment is Solid Phased Extraction (SPE) (Bijlsma et al. 2014).

1.2.1. Solid-phase extraction

SPE is a technique widely used to pre-concentrate and purify mixtures of different components in different kind of samples. Depending on the physic-chemical properties of the target compounds, this process is able to retain specific groups of compounds from a problem solution. The SPE process consists basically on four steps: conditioning, loading, washing and elution (Alkarawi 2016) (See **Figure 3**).

Firstly, the cartridges need to be conditioned using the proper solvent or a mix of solvents to saturate the surface and then, a buffer with the same composition than the

sample to activate the sorbent is used. Next, the sample is loaded and passed through the cartridge by gravity or under vacuum. Analytes are retained by the surface, although some interfering substances can be also retained. Using the correct solvents, the cartridge is washed to eliminate interfering compounds remaining and to purify the sample. When the cartridges are completely dried, the analytes are eluted with an appropriate solvent.
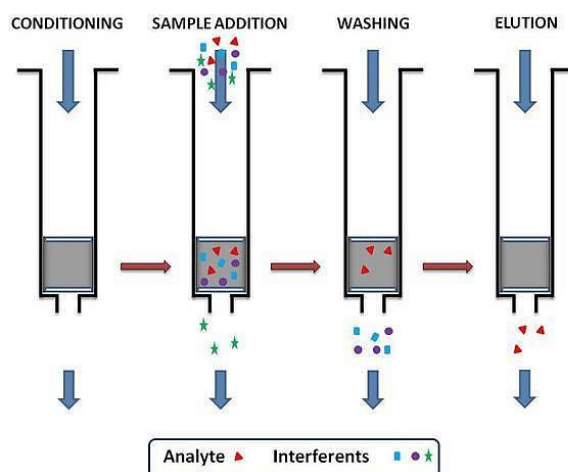

Figure 3. SPE's steps (Dheyaa Alkarawi, 2016).

Using this methodology and final analysis by LC-MS/MS, one of the main problems reported is matrix effect, which strongly affect the quantification of the analytes in complex matrix samples. To correct matrix samples, several ILIS were used in this work. ILIS were added to the samples before SPE. In this way, not only matrix effects are corrected but also any other error related to samples treatment (e.g. potential errors associated to losses in SPE process).

1.2.2. Chromatographic separation

Chromatography is an analytical technique used for the physical separation of a mixture of chemical substances into its individual components. This technique has been modified and improved over the years resulting, nowadays, in different kinds of chromatographic systems commercially available (Levinson 2001). Chromatography is a separation technique essential in many areas of application of modern analytical chemistry, as for example, doping control analysis, food safety, environmental analytical chemistry and metabolomics, among others.

In this study, reverse phase ultra-high performance liquid chromatography (UHPLC) is used. UHPLC in front of HPLC provides better resolution and sensitivity of analysis (Cooper et al. 2007). The sample is injected into the column with the mobile phase which is propelled by a pump. Depending on the polarity of analytes in comparison to mobile and stationary phases, these analytes are retained and eluted at different times. After being separated in the LC column, a detector is required to transform the different signals in understandable information i.e. the analyst can observe the signals as chromatographic peaks with different retention time and intensity (See **Figure 4**). Although many detectors are available to be coupled to a UHPLC system, the most suitable one for this type of analysis is a mass spectrometer. The coupling of chromatography and mass spectrometry is in fact a hyphenation of two powerful techniques. Thus, instead of using the term "detection", as used in conventional systems such as UV and fluorescence, is more appropriate to name this coupling as chromatography hyphenated to mass spectrometry (LC-MS).
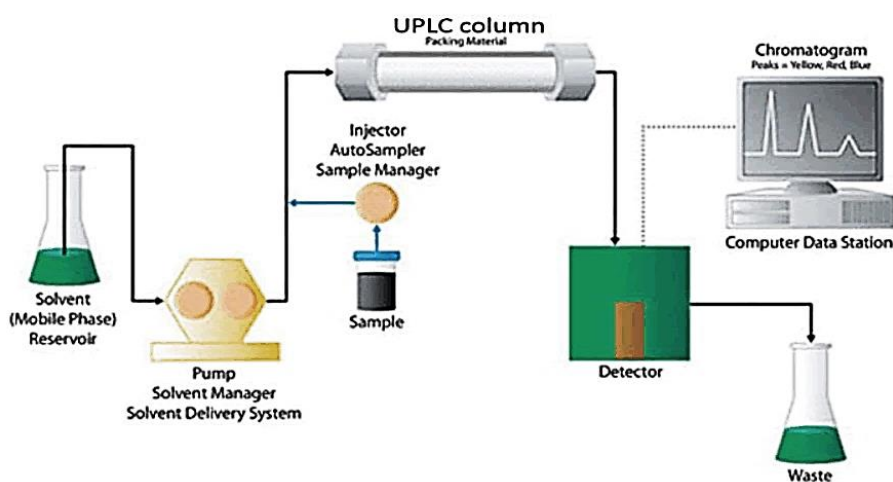

Figure 4. Working of UPLC (Chandraman, 2016).

1.2.3. Mass spectrometry

Mass spectrometry (MS) is a technique based on the generation of ions which are then separated and quantitatively detected. Ions with different mass/charge (m/z) ratios present different trajectories when an electrical and/or magnetic field is applied (Galen and Feiters 2016).

As the mass spectrometer can only observed charged ions in gas phase and the elution of the liquid chromatography is in liquid phase, the coupling of both instruments need to be considered since a change in the physic state of the elute is needed. Usually the chosen option is electrospray source of ionisation (ESI) which is able to eliminate the solvent at the same time that is ionising the analytes without producing in-source fragmentation.

When the sample exits from the LC column, a voltage is applied generating positive charged particles. The high temperature evaporates the solvent till the particle cannot deal with the charge and the repulsion break the particle obtaining positive ions which enter in the MS (Banerjee and Mazumdar 2012) (See **Figure 5**).
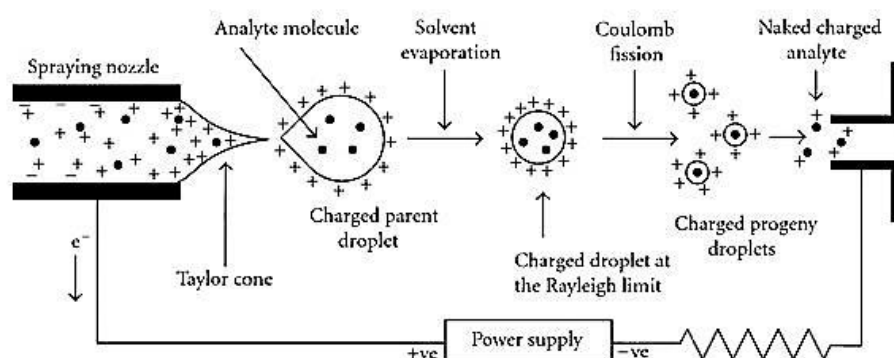
Figure 5. Working of ESI (**Banerjee and Mazumdar 2012**)

Once the components are in gas form, is possible to talk about MS. There are many mass analysers available for mass spectrometric analysis, but all of them work under the same premises. The mass analyser mostly used in quantitative analysis of organic micro-pollutants is triple quadrupole due to its high selectivity and sensitivity, as well as robustness and wide dynamic linear range (Schreiber 2010).
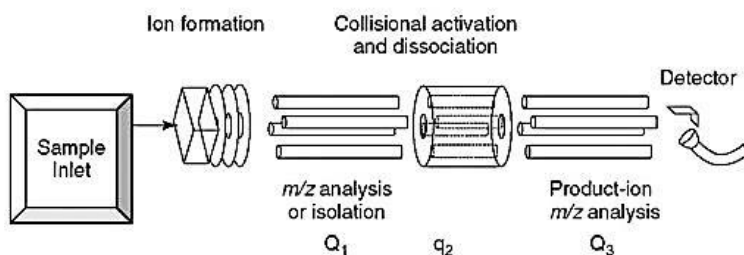
Figure 6. Working of triple quadrupole (Wageningen, University & Research).

Triple quadrupole (See **Figure 6**) is based on monitoring transitions between molecular ions (or precursor ions) and fragment ions (or product ions). For this purpose, only certain *m/*z ratios are allowed to pass through the first quadrupole (Q_1). Then, in the collision cell (second quadrupole q_2), a collision energy is applied for the breakdown of ions coming out from Q_1 into fragments. Finally, third quadrupole (Q_3) is setup for the monitoring of certain specified fragment ions. In that way, after the selection of molecular ions in Q_1, those are fragmented in the collision cell, and finally, only certain ions are traced in Q_3, resulting in the monitoring of transitions between molecular and fragment ions. (Heeren 2006).

2. MATERIAL AND METHODS

2.1. Reagents and chemicals

The standards of DOA and metabolites were purchased from Cercilliant (Round Rock, TX, USA) and the National Measurements Institute (Pymble, Australia) as solutions in methanol (MeOH), acetonitrile (ACN) or salt. ILIS used for quantification were: amphetamine-d_6, methamphetamine-d_5, MDMA-d_5, cocaine-d_3, benzoylecgonine-d_3 (BE-d_3), THC-COOH-d_3 and ketamine-d_3. All of them were acquired from Cercilliant in forms of solution in MeOH or ACN. Solvents for liquid chromatography are HPLC-grade MeOH, HPLC-grade ACN, ammonium acetate, formic acid (>98%) and primary secondary amine (PSA, 40-60 μm) which were obtained from Scharlau (Barcelona, Spain). HPLC-grade water was obtained purifying demineralised water using a Milli-Q plus system from Millipore (Bedford, MA, USA).

Standard stock solutions of each compound were prepared at 100 mg·L^{-1} in MeOH and ACN. Stock solution is diluted ten times with MeOH to obtain intermediate solutions. Infusion solutions of individual standards were prepared at a concentration of 1.5 mg·L^{-1} in MeOH:H_2O (50:50 v/v) Intermediate solutions suffers an appropriate dilution with Milli-Q water to obtain mixed working solutions containing all the analytes. They were used for preparation of the calibration standard, internal quality controls and fro spiking samples in the validation work too.

Individual stock solutions of ILIS were prepared in ACN or MeOH at a concentration of 10 mg·L^{-1}. Using water, a mixed standard working solution at 100 μg·L^{-1} was prepared and was used as surrogate internal standard.

All standard solutions were stored in amber glass bottles at -20ºC.

SPE cartridges used were Oasis HLB 3 cm^3 (60 mg) and Oasis MCX 6 cm^3 (150 mg) from Waters (Milford, MA, USA).

2.2. Instrumentation

The instrument used to carry out chromatography is a Waters UHPLC system (Milford, MA, USA), interfaced to a triple quadrupole MS (Xevo, TQS, Waters Micromass, Manchester, UK) equipped with T-Wave devices and ESI operated in positive-ion mode. Chromatography column was Acquit UPLC BEH C18, 1.7 μm, 50 mm x 2.1 mm (i.d.) (Waters) at a flow rate of 0.3 mL·min^{-1}. The column's temperature is kept at 40 ℃ while the sample manager was at 5 ℃. The mobile phase consisted of 5 mM ammonium acetate and 0.01% formic acid as solvent A and MeOH as solvent B. The percentages of these solvent changed as follows: 0 min, 90:10 (A:B v/v); 3 min, 10:90; 3.5 min, 10:90; 3.6 min, 90:10; 6 min, 90:10, equilibration of the column. The cone gas and desolvation gas flows were 250 and 1200 L·h^{-1}, respectively, of dry nitrogen. In MS/MS mode, argon 99.995% (Praxair, Madrid, Spain) was the collision gas. Other parameters optimized were: capillary voltage, 3.0 kV; source temperature, 150 ℃ and desolvation temperature, 650 ℃.

MassLynx v 4.1 software (Waters, Manchester, UK) was used to acquire and process all data.

2.3. Experimental

2.3.1. Sample collection

The sample analysed consisted on sample 24 h composite samples. The sampling took place in the WWTP along a whole week, specifically in WWTPs of Castellón and Madrid. 100 mL of IWW and EWW were taken every15 minutes and, at the final of the day, these aliquots were mixed in one composite sample. Finally, seven composite samples were available. The samplings started at 8 a.m. (Wednesday 13th December for Madrid and Tuesday 6th April for Castellón) and finished the next week (Wednesday 20th December for Madrid and Tuesday 13th April for Castellón) at the same hour. Thus, the weekends' samples were in the middle and it was possible to evaluate whether the consumption of DOAs differs in these days. So, in total, 28 samples were collected – 14 IWW samples and 14 EWW samples. All of them were collected in polyethylene high density bottles and directly transported to the laboratory (maximum 24 hours). Next, they were fortified with a mixed surrogate ILIS, filtered and immediately at -20 ℃ until analysis.

2.3.2. Analytical procedure

Firstly, samples were centrifuged to remove possible solids in WW. Next, 100 mL of four-fold diluted IWW (i.e. 25 mL IWW plus 75 mL approximately of HPLC-grade water) and 100 mL EWW samples were spiked with ILIS. The final concentration in sample for each ILIS was 20ng/L. SPE cartridges were conditioned washing and cleaning with 6 mL of MeOH and 6 mL of Milli-Q water. The samples were loaded through the cartridges by gravity at a flow rate around 3 mL/min. Then, the cartridges were vacuum dried for approximately 15 min and the analytes eluted using 5 mL of MeOH. These sample extracts were evaporated to dryness at 35 ºC under a gentle stream of nitrogen and then reconstituted in 1 mL MeOH:H_2O (10:90 v/v). Analyses were performed by injecting 3 µL of the final extract in the UHPLC-MS/MS system (See **Figure 7**).
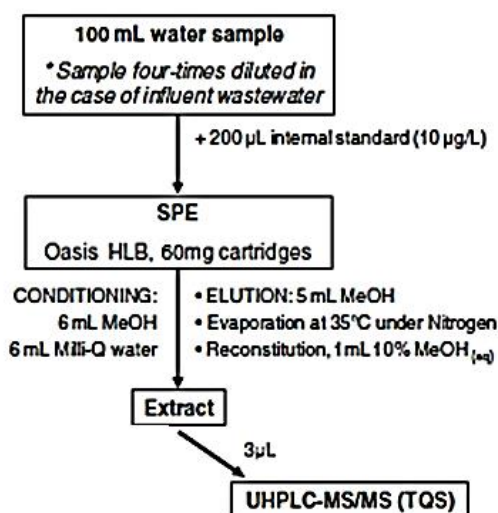
Figure 7. Graphical workflow of the analytical procedure (**Modified from Bijlsma et al. 2014**)

2.3.3. Selected drugs of abuse to be studied

In this work, 7 drugs and/or main metabolites were selected: amphetamine, methamphetamine, 3,4-methylenedioxymethamphetamine (MDMA or ecstasy), cocaine, benzoylecgonine (BE), 11-nor-9-carboxy-Δ_9-tetrahydrocannabinol (THC-COOH) and ketamine (See **Figure 8**). ILIS were available for all of them, and were used as surrogate for appropriate quantification: amphetamine-d_6, methamphetamine-d_5, MDMA-d_5, cocaine-d_3, BE-d_3, THC-COOH-d_3 and ketamine-d_4.

Amphetamine

Methamphetamine

MDMA

Cocaine

BE

THC-COOH

Ketamine
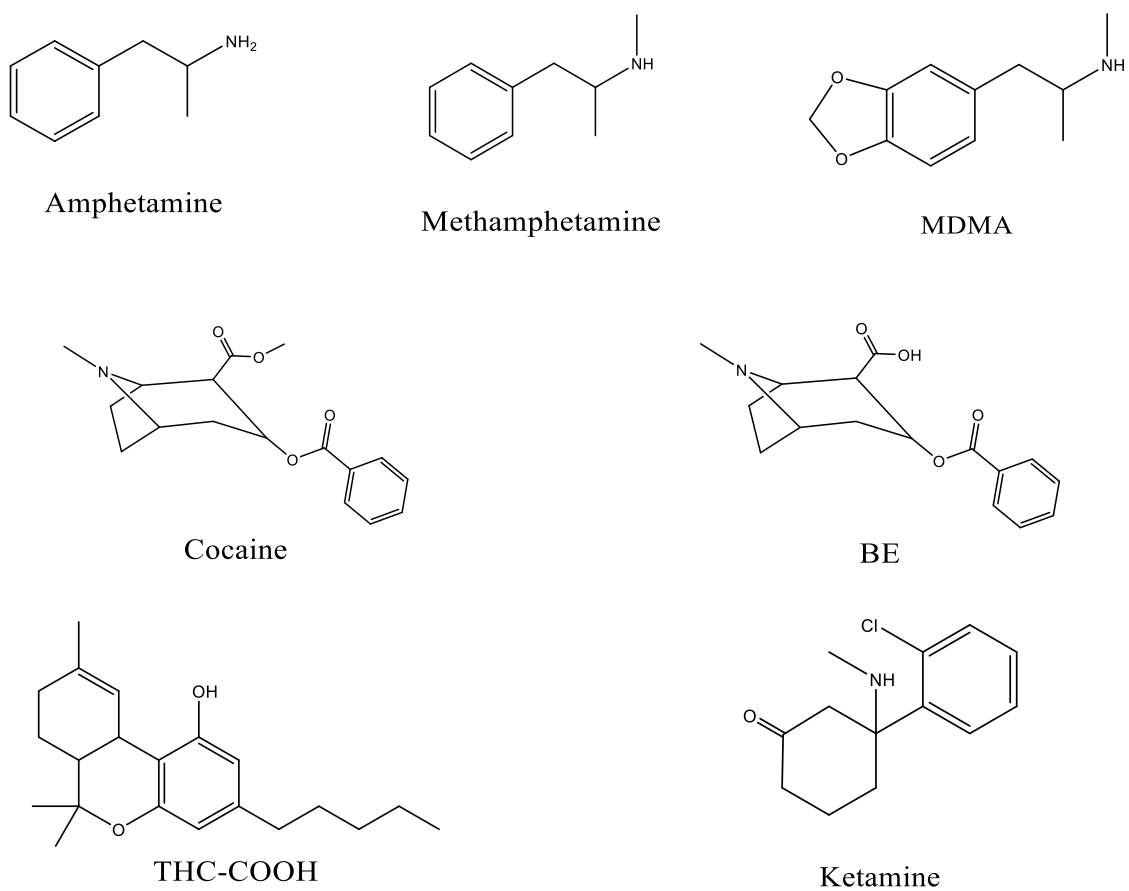
Figure 8. Chemical structures of DOAs studied

3. RESULTS AND DISCUSSION

In this study, data were obtained with ESI operating in positive mode, using the protonated molecule [M+H]$^+$ as precursor ion due to the good results obtained in previous works (Bijlsma et al. 2014). Three MS/MS transitions were acquired for each compound. The most sensitive and specific one (Q), was selected for quantification tasks whereas the two other transitions (q$_1$ and q$_2$), less sensitive or specific, were acquired with confirmation purposes. In **Table 1**, mass spectrometry parameters used in this study, as well as retention times for the different compounds and transitions selected are shown. Moreover, the average Q/q ratios (intensity ratio) together with the associated RSD from the calibration standards are also shown.

**Table 1. UHPLC-MS/MS parameters for the SRM acquisition mode (quantification (Q) and confirmation (q))**

Compound	Rt (min)	Q transition	CV (V)	CE (eV)	q$_1$ transition	CE (eV)	q$_2$ transition	CE (eV)	Q/q$_1$ (RSD %)	Q/q$_2$ (RSD %)
Amphetamine	1.80	136>119	20	10	136>91	20	119>91	10	1 (3)	2 (11)
Amphetamine-d$_6$	1.80	142>93	20	10	-	-	-	-	-	-
Methamphetamine	1.82	150>119	35	10	150>91	20	150>65	35	1 (0)	22 (0)
Methamphetamine-d$_5$	1.82	155>121	35	10	-	-	-	-	-	-
MDMA	1.81	194>163	30	15	194>105	25	194>77	40	2 (6)	7 (8)
MDMA-d$_5$	1.80	199>165	30	15	-	-	-	-	-	-
Cocaine	2.21	304>182	30	20	304>82	30	304>77	50	3 (1)	12 (2)
Cocaine-d$_3$	2.21	307>185	30	20	-	-	-	-	-	-
BE	1.99	290>168	40	20	290>105	30	290>82	30	3 (6)	5 (3)
BE-d$_3$	1.99	293>171	40	20	-	-	-	-	-	-
THC-COOH	3.96	345>193	40	25	345>299	20	345>327	15	1 (10)	0.3 (9)
THC-COOH-d$_3$	3.95	348>196	40	25	-	-	-	-	-	-
Ketamine	2.17	238>125	20	25	238>179	15	238>207	15	3 (3)	5 (6)
Ketamine-d$_4$	2.15	242>129	20	20	-	-	-	-	-	-

Rt (retention time), Q (quantification), q (confirmation), CV (cone voltage) and CE (collision energy)

In total, 28 SRM transitions, concretely three SRM transitions for quantification of each compound and only one transition for each individual ILIS, were acquired.

The method applied had been previously validated for the two types of urban wastewater, IWW and EWW, in terms of linearity limit of quantification (LOQ), instrumental limit of detection (LOD), precision and accuracy (Bijlsma et al. in 2014).

Nonetheless, internal quality control tests (QCs) were also analysed in the course of the analytical procedure in order to assure the effectiveness, the robustness and the reliability of sample treatment and instrumental analysis. QCs consist of real-world wastewater samples with a known added concentration (spiked samples) – 2.5 ng (QC low) and 20 ng (QC high) - of all compounds. These samples are treated in the same way than real-world samples and were injected alternatively during the sequence of analysis.
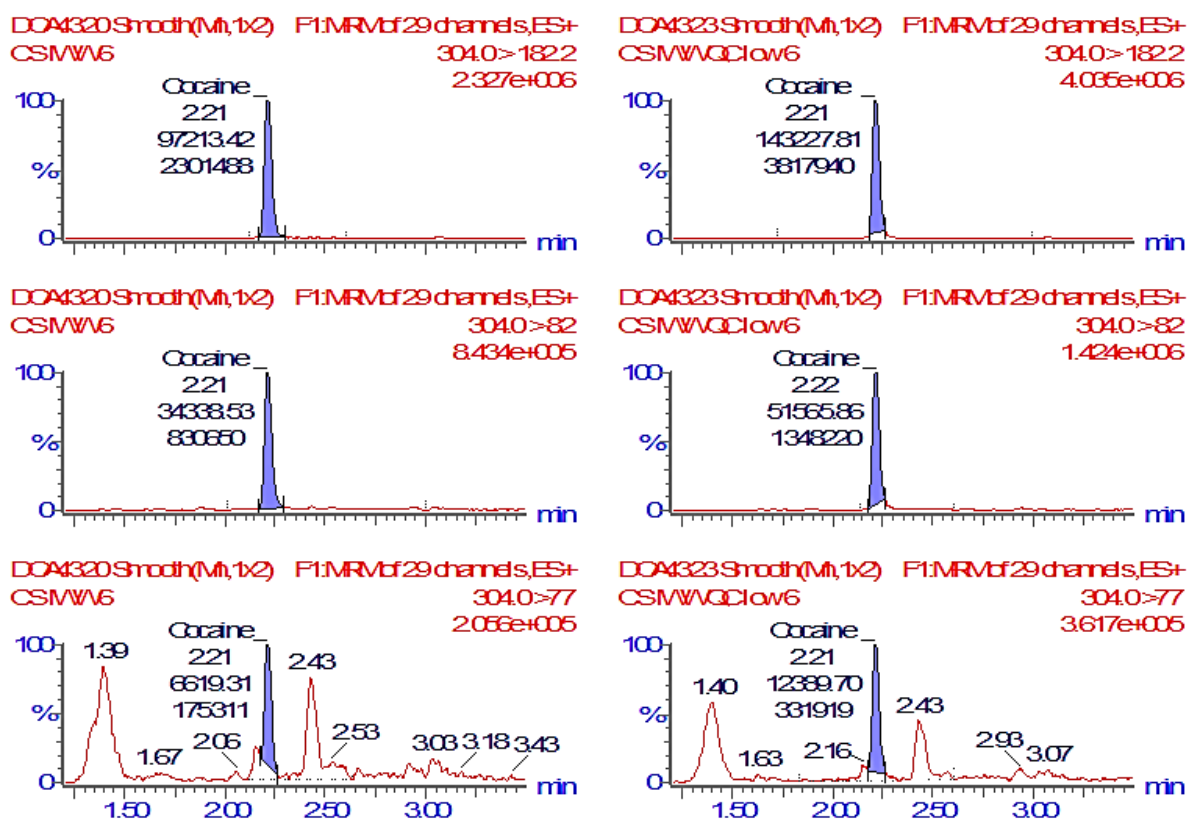
Figure 9. UHPLC-MS/MS chromatograms (three SRM transitions for cocaine) for a non-spiked IWW sample and spiked IWW sample collected in Castellon

Figure 9 shows the chromatograms for cocaine of the non-spiked IWW sample and the corresponding spiked sample collected in Castellón. This sample was chosen for fortification at the low concentration. By subtracting the concentration measured in the "blank" non-spiked samples to the QC's concentration is possible to obtain empirically the concentration spiked, and consequently calculate the total recovery of the methodology as a percentage.

Table 2. Recoveries (%) for each compound in QCs samples prepared at two concentration levels.

Compound	City	QC (µg/L)	Concentration spiked (µg/L)		Recovery (%)	
			IWW	EWW	IWW	EWW
Amphetamine	MAD	2.5	2.10	2.34	84	94
		20	13.90	24.30	70	122
	CAS	2.5	2.40	2.66	96	106
		20	23.00	13.97	115	70
MDMA	MAD	2.5	0.56	0.52	22	21
		20	4.08	3.39	20	17
	CAS	2.5	0.52	0.42	21	17
		20	4.82	3.98	24	20
Methamphetamine	MAD	2.5	0.44	0.56	18	22
		20	5.09	5.19	26	26
	CAS	2.5	0.58	0.53	23	21
		20	5.85	4.88	39	24
Cocaine	MAD	2.5	2.41	2.88	96	115
		20	18.97	22.62	95	113
	CAS	2.5	2.46	2.72	98	109
		20	28.75	24.12	144	121
BE	MAD	2.5	1.65	1.18	66	47
		20	2.40	14.2	12	71
	CAS	2.5	6.49	1.97	260	79
		20	29.57	15.84	148	79
THC-COOH	MAD	2.5	2.13	1.88	85	75
		20	17.75	18.86	89	94
	CAS	2.5	2.95	1.90	118	76
		20	24.20	21.37	121	107
Ketamine	MAD	2.5	1.05	1.02	42	41
		20	8.46	8.75	42	44
	CAS	2.5	0.93	1.01	37	40
		20	10.06	7.90	50	40

QCs recoveries are shown in **Table 2**. Recovery values between 60-140% are typically considered as acceptable when dealing with trace analysis of organic contaminants, as mentioned in some international guidelines (SANTE, 2015). As it can be seen in **Table 2** the results were satisfactory for most cases of amphetamine, cocaine,

BE and THC-COOH. Otherwise, for two of amphetamine-like stimulants (MDMA and methamphetamine) the recoveries were below 60% which revealed the difficulties for this type of analysis for these compounds in wastewaters, especially because of the huge differences from one wastewater sample to another. In any case, the values were in-sample consistent and a correction factor was applied for an accurate quantification. As an example, if the average QC recovery was 20%, extract concentrations were recalculated considering only 20% efficiency in the extraction. Therefore, concentrations were multiplied by a factor of 5 for obtaining more accurate and realistic values. The correction factor was calculated for each compound (MDMA, methamphetamine, BE and ketamine) in each matrix (IWW and EWW for Madrid, IWW and EWW for Castellón).

In addition to quantification issues, it is necessary to adopt strict criteria for a reliable identification of the compound detected in samples. These criteria are based on agreement in retention times and Q/q ratios between the compound detected and the calibration standards. Depending on the agreement of Q/q ratios in sample with those of the standard, the compound will be confirmed (and, therefore, quantitation permitted) or only detected. Normally, when both Q/q ratios (Q/q_1 or Q/q_2) are below ±30% deviation with respect to the average Q/q ratio of the calibration standards, the compound can be considered as fully identified (confirmed) and its quantification is possible. If at least one Q/q ratio is within this limit of acceptability, the compound can be considered as detected but not fully confirmed. The ideal situation is that the two Q/q ratios available are in agreement with the standards, a fact that, in our case, occurred in nearly all samples.

3.1. Consumption of DOAs

For the establishment of DOA consumption only IWW data was considered. A total of 28 samples were analysed. In each sequence of samples analysis, the calibration curve was injected twice, at the beginning and at the end of the sequence. Using calibration curves (See **Figure 10**), the concentration of drugs were calculated in ppt (ng/L). It must be noted that regression coefficient was higher than 0.99 for all calibration curves. Quantification was made using relative responses analyte versus ILIS in order to perform the appropriate correction of matrix effect and SPE recovery.

Figure 11 shows the daily variance of concentrations (in ng/L) for DOAs quantified in IWW samples from Castellón. In this step, although consumption of DOA is not calculated yet, it is possible to have a rough idea about which drugs have more presence in the city studied and which days present the higher levels of drugs (i.e. higher consumption). No data are shown for Madrid due to confidential issues.
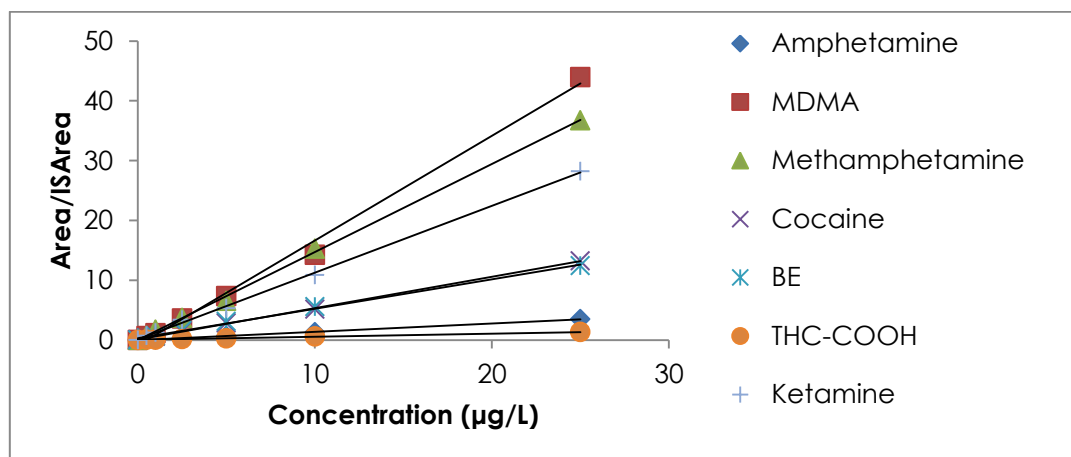
Figure 10. Calibration curves of DOA
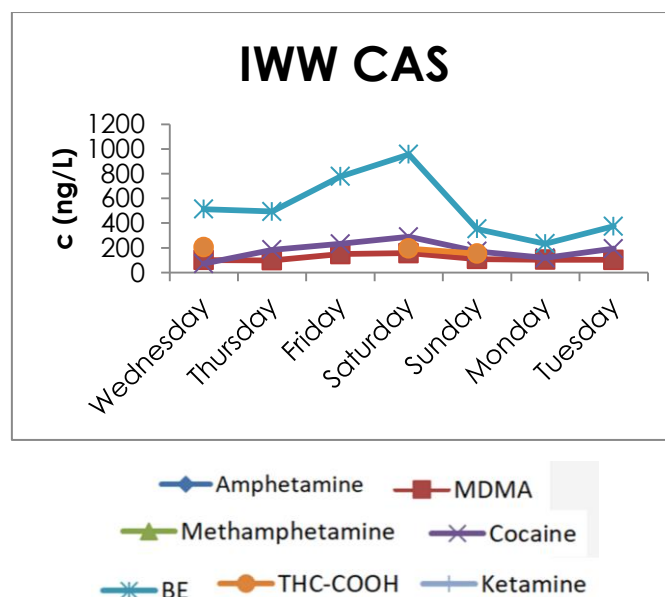
Figure 11. Daily variance of concentration of DOAs studied in IWW samples from Castellón during a week sampling.

In Castellón, the seven drugs studied were detected in IWW, although only four of them could be quantified because the levels of the remaining three drugs -amphetamine, methamphetamine and ketamine- were below the LOQ. BE was the most detected compound during the week, a fact that reveals a high consumption of cocaine. It was

observed during the weekend an increase of the concentration suggesting higher consumption during these days. On the contrary, consumption of the rest of drugs seems to remains constant during the week.

To estimate drug consumption **Eq.2** and then **Eq.3** (Foppe, Hammond-Weinberger, and Subedi 2018) were applied. It is important to note that data from the specific WWTP are required to perform the calculation. Specifically daily flow rate of raw wastewater entering the system and number of citizens served by that WWTP are required to this aim.

Mass load is obtained from **Eq.2** where C is the concentration of the certain drug in ppt (ng/L) and F is the daily flow rate in m^3/h of IWW in a period of 24h in the corresponding WWTP.

$$Mass\ load\ (mg/d) = C \cdot F \cdot 10^{-3} \cdot 24h \qquad\qquad \textbf{Eq. 2}$$

Using **Eq.3**, consumption of DOA per 1000 inhabitants is obtained. The population that WWTP covers in is 169,498 citizens.

$$\frac{Consumption}{1000\ inhabitants}\left(\frac{mg/d}{1000\ inhab.}\right) = Mass\ load\ (mg/d) \cdot \left(\frac{1000}{population}\right) \qquad \textbf{Eq. 3}$$

In **Table 3** the results for estimated consumption of the different drugs studied in this work in Castellón are shown.

Although amphetamine was detected in two IWW samples (11th and 12th of April), its quantification was not possible due to the values of Q/q ratio differed more than a 30% of Q/q ratio for standards. Quantification of THC-COOH was made in three IWW samples from Castellón corresponding to 6th, 9th and 10th of April. In the rest of samples, the compound was detected but it could not be fully confirmed as the Q/q ratios were out of the acceptable range of tolerance (±30%) in comparison to Q/q ratios for standards. Thus, although the compound detected was surely THC-COOH, there was not enough confidence in its identification, and additional experiments would be required to confirm its identity (e.g. additional injection modifying the chromatographic conditions). A similar situation was found in a few cases for amphetamine, methamphetamine and ketamine, which reveals the difficulties of the analysis in this

type of complex samples. The last two drugs were not found in the samples from Castellón, revealing very low consumption of these drugs.

Table 3. Results for drug consumption in Castellón during a week in April (2017).

	Drug Consumption Castellón (mg/d/1000 habitants)						
Day	Amphetamine	MDMA	Methamphetamine	Cocaine	BE	THC-COOH	Ketamine
(Thursday) 06/04/2017	N.D.	22	N.D.	15	109	44	N.D.
(Friday) 07/04/2017	N.D.	21	N.D.	39	106	-	N.D.
(Saturday) 08/04/2017	N.D.	32	N.D.	50	168	-	-
(Sunday) 09/04/2017	N.D.	31	-	57	189	38	N.D.
(Monday) 10/04/2017	N.D.	22	-	34	71	31	N.D.
(Tuesday) 11/04/2017	-	22	N.D.	25	50	-	N.D.
(Wednesday) 12/04/2017	-	22	N.D.	41	80	-	N.D.

Note: N.D.: Not Detected /- : the compound identity could not be confirmed due to the non-compliance of Q/q ratios

BE presented the highest mass loads, illustrating a notable consumption of cocaine in Castellón. During the weekend (Saturday and Sunday), the consumption of cocaine considerably increased, mainly because of its recreational utilization. In the case of MDMA, the consumption remained stable during the week with little variation among days. The study of cannabis took place through its major metabolite, THC-COOH. Observing the table, although the quantification was possible only for three days, results suggested that the cannabis consumption remained stable during the week.

3.2. Removal efficiency of Madrid and Castellón WWTP. Environmental impact.

The analysis of EWW samples allows to evaluate the potential removal of these DOAs in WWTP. In **Figure 12** it is possible to observe the daily variance of concentrations (in ng/L) for DOAs quantified in EWW samples from Castellón. **Figure 12** compared with **Figure 11**, suggest whether the removal of a certain compound took place or not. All drugs present concentration in EWW samples lower than in IWW, although there were variations between them. BE and cocaine presented rather lower

values of concentration. It can be seen on Monday April 10th, an important peak of cocaine probably due to low efficiency of the WWTP to eliminate the compound when the concentrations are higher i.e. during the weekend. Furthermore, despite of not having the most presence in IWW, the concentration of MDMA in EWW samples was higher than for the rest of drugs. This fact indicates a worse removal of the compound in the WWTP.
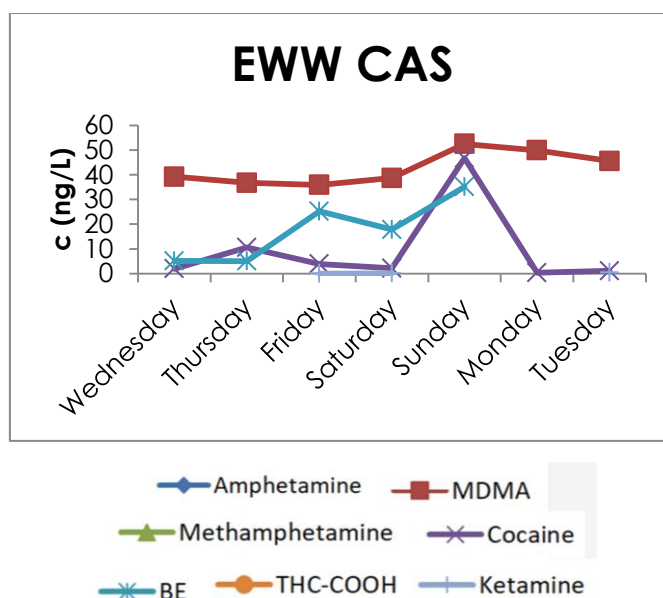
Figure 12. Daily variance of concentration of DOAs studied in EWW samples from Castellón during a week sampling.

Through the estimation of the removal efficiency in a WWTP (See **Tables 5 and 6**) it is possible to know if these treatment plants are efficient for the elimination of the drugs under study. High percentages of RE suggest that the compound studied is eliminated correctly. The ideal scenario corresponds to a RE around 100%. This would imply that the compound is present at a given concentration in IWW but it is not present (totally removed) in the EWW. Obviously, RE studies can only be considered for compounds present in IWW. The non-detection of a compound in EWW, if such compound is however present in IWW, would indicate an efficient removal in the WWTP.

Table 5. Removal efficiency for WWTP of Castellón.

	Day	MDMA	Cocaine	BE
		Removal efficiency (%)		
(WED)	06/04/2017	61.5	97.2	99.0
(THU)	07/04/2017	62.6	99.4	99.0
(FRI)	08/04/2017	76.0	98.3	96.8
(SAT)	09/04/2017	75.4	99.3	98.1
(SUN)	10/04/2017	51.6	72.6	90.0
(MON)	11/04/2017	52.0	99.7	-
(TUE)	12/04/2017	55.5	-	-

In the WWTP of Castellón, acceptable values of elimination (all above 50%) were obtained for MDMA. Despite these values, a rather important amount of drug exits the WWTP, arriving to the aquatic system and polluting it. In comparison to MDMA, higher RE were obtained for cocaine and BE, near the ideal situation (100%). It is important to note that on Sunday 10th, the removal efficiency for cocaine was lower, with RE around only 70%. During this day, the WWTP probably had problems to eliminate that compound, as it is also observed in **Figure 12**. Despite the good RE in most of cases, some compounds are still present in the EWW, and consequently can reach the aquatic environment through the discharges of EWW.

Table 6. Removal efficiency for WWTP of Madrid.

	Day	MDMA	Methamphetamine	Cocaine	BE	Ketamine
		Removal efficiency (%)				
(TUE)	13/12/2017	78.0	88.8	99.8	99.6	37.6
(WED)	14/12/2017	76.3	87.8	99.8	99.6	43.7
(THU)	15/12/2017	76.0	83.9	99.8	99.4	-53.1
(FRI)	16/12/2017	78.8	83.8	99.7	98.8	-45.5
(SAT)	17/12/2017	83.8	86.7	99.7	98.4	-121.2
(SUN)	18/12/2017	71.8	81.0	99.6	98.8	-20.1
(MON)	19/12/2017	67.0	86.9	99.7	99.6	-38.4

Values of removal efficiency of the Madrid's WWTP were similar for cocaine and BE in comparison with the WWTP of Castellón. Regarding MDMA, its elimination seems better in Madrid albeit the percentage of compound that arrives to the environment was still around 20-30%. In general, RE for most of the compounds under study was excellent, with the exception of ketamine. The high variability in the RE values for this compound may be due to the difficulties for its determination wastewater

because of the low analyte levels which can be hugely affected by matrix interference in the analysis.

Although the concentrations of DOAs were not much high in the EWW, a certain percentage of these compounds passed through the WWTP and consequently ended up in the aquatic environment. These compounds are normally not present in the environment from any natural source, and therefore, negative (eco)toxicological impact may occur. This impact needs to be studied to avoid unexpected consequences in our environment.

To this aim, the compounds found in EWW have been classified according Cramer's rules using the free software *Toxtree*. This tool allows predicting the toxicity and biodegradability of the compound studied. In **Table 7** the results obtained for each compound detected in EWW samples are shown.

Table 7. Toxic characteristics for each DOA detected in EWW samples

Compound	Toxic Hazard	Biodegradability
Methamphetamine	Class III	Unknown
MDMA	Class III	Persistent chemical
Cocaine	Class III	Persistent chemical
BE	Class III	Persistent chemical
THC-COOH	Class III	Persistent chemical
Ketamine	Class III	Persistent chemical

The software used follows a succession of rules which are interconnected among them. Depending on the structural characteristics of the certain compound, the computer programme classifies the substances according to their toxicological hazards. As it can be observed in **Table 7**, all DOAs studied are included into Class III of toxicological hazards i.e. considering the chemical structure of the compound, it is not possible to have a strong initial presumption of safety and even suggests a certain level of toxicity or to have reactive functional groups.

Methamphetamine and MDMA have similar structures. Likewise, cocaine and its metabolite, BE, barely present difference in their chemical structure. So, it is predictable that these compounds are included in the same class group respectively even though the software was unable to predict it

Respect of DOAs' biodegradability, they are mostly persistent in the medium. This would imply that their removal from the environment is not possible in a natural way. Methamphetamine biodegradability is unknown. Nevertheless, it is probably persistent in the medium because of its similarity with MDMA and methamphetamine (Schnabel, 2009).

Our data revealed that occurrence of DOAs in IWW and in EWW needs to be further studied. The removal efficiency of the WWTPs for these compounds needs to be also well established. The fact that DOAs studied in this work presented predictable high toxicity and persistence in the environment illustrated the relevance of this type of research, as it is necessary to prevent the negative (eco)toxicological impact of effluent wastewater into the aquatic environment.

4. CONCLUSIONS

The analytical approach used in this work, combining sample treatment by SPE and analysis by UHPLC-MS/MS, has been proved as an efficient and modern way to investigate the presence of drugs of abuse in wastewater samples. The use of quality control samples is compulsory to guarantee the reliability of data reported. From this point of view, QCs recovery data for amphetamine, cocaine, benzoylecgonine and cannabis were mostly satisfactory, but recoveries for amphetamine-like stimulants (MDMA and methamphetamine) and ketamine were below 70% in most of cases. The fact that these recoveries were rather consistent allowed us to apply a correction factor in the quantification.

The results obtained in this work illustrate that most of the DOAs studied were present in IWW. This allowed, using the approach known as "Wastewater Based Epidemiology", to estimate the drug's use in a certain community. The compound found at higher concentrations in IWW from Castellón was BE (the main metabolite of cocaine), a fact that indicates the high consumption of this drug. Cannabis seems also to be highly consumed in Castellón, as the concentration of its main metabolite, THC-COOH, revealed. Ecstasy (MDMA) was also found in IWW but at much lower concentrations.

Comparative analysis between IWW and EWW allowed estimating the removal efficiency of the WWTP for the compounds under study. This estimation was made for the WWTP of Castellón and a WWTP from Madrid. In general, RE (%) for both plants were high and the levels of the drugs in the treated wastewater were much lower than in the influent. Despite the mostly high RE, several of the compounds still arrived to the aquatic environment, which may have a negative impact on aquatic organisms and suppose an environmental risk that needs to be assessed.

Toxtree, the predictive tool applied in this work, estimates high levels of toxicity for the DOAs selected as well as high persistence (non-biodegradability) in the environment. Due to their relevant toxicity, the total removal of DOAs before being discharged into the aquatic medium would be the ideal situation.

Since this day arrived, the most reasonable solution is to promote awareness campaigns in a double way. On one hand, it is important to inform people about the

consequences of consumption of drugs of abuse and how they affect human health, trying to minimize the consumption (analysis of wastewater would be an excellent tool to measure the effectiveness of such campaigns). On the other hand, it is necessary to improve the removal efficiency of WWTPs, not only for these compounds but also for other emerging contaminants (e.g. pharmaceuticals and personal care products) in order to decrease water contamination and to reach a better health status of our environment.

REFERENCES

Alkarawi, Dheyaa. 2016. 'The Investigation of the Electrical Control of Hemimicelles and Admicelles on Gold for Analyte ...' (December 2016):1–67.

Archer, E., E. Castrignanò, B. Kasprzyk-Hordern, and G. M. Wolfaardt. 2018. 'Wastewater-Based Epidemiology and Enantiomeric Profiling for Drugs of Abuse in South African Wastewaters'. *Science of the Total Environment* 625:792–800. Retrieved (https://doi.org/10.1016/j.scitotenv.2017.12.269).

Bade, Richard et al. 2016. 'Ultra-High Performance Liquid Chromatography-Tandem Mass Spectrometry Determination of Synthetic Cathinones and Phenethylamines in Influent Wastewater'. *Submitted to Chemosphere* 1–10.

Banerjee, Shibdas and Shyamalava Mazumdar. 2012. 'Electrospray Ionization Mass Spectrometry: A Technique to Access the Information beyond the Molecular Weight of the Analyte'. *International Journal of Analytical Chemistry* 2012(January):1–40. Retrieved (http://www.hindawi.com/journals/ijac/2012/282574/).

Bijlsma, Lubertus, Eduardo Beltrán, Clara Boix, Juan V. Sancho, and Félix Hernández. 2014. 'Improvements in Analytical Methodology for the Determination of Frequently Consumed Illicit Drugs in Urban Wastewater'. *Analytical and Bioanalytical Chemistry* 406(17):4261–72.

Bijlsma, Lubertus, Ana M. Botero-Coy, Rolando J. Rincón, Gustavo A. Peñuela, and Félix Hernández. 2016. 'Estimation of Illicit Drug Use in the Main Cities of Colombia by Means of Urban Wastewater Analysis'. *Science of the Total Environment* 565:984–93. Retrieved (http://dx.doi.org/10.1016/j.scitotenv.2016.05.078).

Bijlsma, Lubertus, Erik Emke, Félix Hernández, and Pim De Voogt. 2012. 'Investigation of Drugs of Abuse and Relevant Metabolites in Dutch Sewage Water by Liquid Chromatography Coupled to High Resolution Mass Spectrometry'. *Chemosphere* 89(11):1399–1406. Retrieved (http://dx.doi.org/10.1016/j.chemosphere.2012.05.110).

Boix Sales, Clara. 2014. 'Investigación de Fármacos Y Drogas de Abuso En El Ámbito de La Salud Pública. Identificación de Productos de Transformación/metabolitos En El Medio Ambiente Acuático.' 474. Retrieved (http://www.tdx.cat/handle/10803/285328).

Castiglioni, Sara et al. 2013. 'Evaluation of Uncertainties Associated with the Determination of Community Drug Use through the Measurement of Sewage Drug Biomarkers'. *Environmental Science and Technology* 47(3):1452–60.

Cooper, K. A. et al. 2007. 'Rapid Reverse Phase-Ultra Performance Liquid Chromatography Analysis of the Major Cocoa Polyphenols and Inter-Relationship of Their Concentration in Chocolate'. *Journal of Agricultural and Food Chemistry* 55:2841–47.

Du, Peng et al. 2017. 'Estimating Heroin Abuse in Major Chinese Cities through Wastewater-Based Epidemiology'. *Science of the Total Environment* 605–606:158–65. Retrieved (http://dx.doi.org/10.1016/j.scitotenv.2017.05.262).

EMCDDA. 2017. *European Drug Report 2017: Trends and Developments*. Retrieved (http://www.emcdda.europa.eu/system/files/publications/4541/TDAT17001ENN.pdf%0A http://www.emcdda.europa.eu/system/files/publications/4541/TDAT17001ENN.pdf%0Ah ttp://www.emcdda.europa.eu/system/files/publications/4541/TDAT17001ENN.pdf%0Ahtt p://www.emcdda.eur).

EMCDDA. 2016. 'Wastewater Analysis and Drugs: A European Multi-City Study'. 1–5. Retrieved (http://www.emcdda.europa.eu/topics/pods/waste-water-analysis).

European Monitoring Centre for Drugs and Drug Addiction. 2015. 'New Psychoactive Substances in Europe'. *EU Early Warning System* (March):12.

European Monitoring Centre for Drugs and Drug Addiction and Europol. 2016. *EU Drug Markets Report. In-Depth Analysis*. Retrieved (http://www.emcdda.europa.eu/system/files/publications/2373/TD0216072ENN.PDF).

European Comission on health and food safety, 2017. *Guidance document on anlytical quality control and method validation procedures for pesticides residues and analysis in food and feed.*

Foppe, Katelyn S., Dena R. Hammond-Weinberger, and Bikram Subedi. 2018. 'Estimation of the Consumption of Illicit Drugs during Special Events in Two Communities in Western Kentucky, USA Using Sewage Epidemiology'. *Science of the Total Environment* 633:249–56. Retrieved (https://doi.org/10.1016/j.scitotenv.2018.03.175).

Galen, Peter M. Van and Martin C. Feiters. 2016. 'Mass Spectrometry Part of the Intrumental Analysis in (Bio) Molecular Chemitry'. (October):1–65.

Gros, Philippe. 2018. 'ANALYTICAL QUALITY CONTROL AND METHOD VALIDATION PROCEDURES FOR PESTICIDE RESIDUES ANALYSIS'.

Heeren, Ron M. A. 2006. 'Mass Spectrometry Fundamentals'. (April).

Hernández, Félix, Lubertus Bijlsma, Juan V. Sancho, Ramon Díaz, and María Ibáñez. 2011. 'Rapid Wide-Scope Screening of Drugs of Abuse, Prescription Drugs with Potential for Abuse and Their Metabolites in Influent and Effluent Urban Wastewater by Ultrahigh Pressure Liquid Chromatography-Quadrupole-Time-of-Flight-Mass Spectrometry'. *Analytica Chimica Acta* 684(1–2):96–106.

Lai, Foon Yin et al. 2016. 'Spatial Variations in the Consumption of Illicit Stimulant Drugs across Australia: A Nationwide Application of Wastewater-Based Epidemiology'. *Science of the Total Environment* 568(June):810–18. Retrieved (http://dx.doi.org/10.1016/j.scitotenv.2016.05.207).

Laufersweiler, Michael C. et al. 2012. 'Correlation of Chemical Structure with Reproductive and Developmental Toxicity as It Relates to the Use of the Threshold of Toxicological Concern'. *Regulatory Toxicology and Pharmacology* 62(1):160–82. Retrieved (http://dx.doi.org/10.1016/j.yrtph.2011.09.004).

Levinson, Ralph. 2001. '5. Chromatography'. *Modern Chemical Techniques* 116–59.

Manual, User. 2009. 'Cramer Rules with Extensions'. (April):1–7.

Owa, F. 2014. 'Water Pollution : Sources , Effects , Control and Management'. *International Letters of Natural Sciences* 3:1–6.

Rousis, Nikolaos I. et al. 2017. 'Wastewater-Based Epidemiology to Assess Pan-European Pesticide Exposure'. *Water Research* 121:270–79.

Schnabel, Jürgen. 2009. 'The Threshold of Toxicological Concern (TTC) and Its Application in the Safety Evaluation of Flavouring Substances'. *Toxicology Letters* 189(2009):S10. Retrieved (http://linkinghub.elsevier.com/retrieve/pii/S0378427409003208).

Schreiber, André. 2010. 'Advantages of Using Triple Quadrupole over Single Quadrupole Mass Spectrometry to Quantify and Identify the Presence of Pesticides in Water and Soil Samples'. *AB Sciex Applikáció* 6. Retrieved (http://sciex.com/Documents/brochures/msTripleQuad-Pesticides-Testing.pdf).

Smit, M. G. D., K. I. E. Holthaus, and J. E. Tamis. 2005. 'From PEC _ PNEC Ratio to Quantitative Risk Level Using Species Sensitivity Distribution'. (10).

Subedi, Bikram and Kurunthachalam Kannan. 2014. 'Mass Loading and Removal of Select Illicit Drugs in Two Wastewater Treatment Plants in New York State and Estimation of Illicit Drug Usage in Communities through Wastewater Analysis'. *Environmental Science and Technology* 48(12):6661–70.

Westlund, Paul and Viviane Yargeau. 2017. 'Investigation of the Presence and Endocrine Activities of Pesticides Found in Wastewater Effluent Using Yeast-Based Bioassays'. *Science of the Total Environment* 607–608:744–51. Retrieved (http://dx.doi.org/10.1016/j.scitotenv.2017.07.032).

http://toxtree.sourceforge.net/toxtree-plugins/toxtree-cramer/report/

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ABBREVIATIONS

BE	Benzoylecgonine
DOA	Drugs of abuse
ERA	Environmental Risk Assessment
ESI	Electrospray ionization
EWW	Effluent wastewater
ILIS	Isotopically labelled internal standards
IWW	Influent wastewater
MDMA	3,4-methylenedioxymethamphetamine
MEC	Measured environmental concentration
MS	Mass spectrometry
NOEL	No observed effect levels
NPS	New psychoactive substances
PEC	Predicted environmental concentration
PNEC	Predicted no effect concentration
QC	Quality control
RE	Removal Efficiency
SRM	Selected Reaction Monitoring
THC-COOH	11-nor-9-carboxy-Δ^9-tetrahydrocannabinol
TTC	Threshold of toxicological concern
UHPLC	Reverse phase ultra-performance liquid chromatography
UHPLC-MS/MS	Liquid chromatography coupled to tandem mass spectrometry
WW	Wastewater
WWTP	Wastewater treatment plants