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Determination of seven drugs of abuse and their metabolites in surface and waste water using solid-phase extraction coupled to liquid chromatography-high resolution mass spectrometry

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Drugs of abuse in water by liquid chromatography high resolution mass spectrometry

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Abbreviations used in the text:

ACN – acetonitrile; BE – benzoylecgonine; COC – cocaine; COD – codeine; DIC – dihydrocodeine; EDDP – 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidone; HRMS – high resolution mass spectrometry; LOD – limit of detection ; LOQ – limit of quantitation; MCX – mixed-mode strong cation exchange; MDPV – methylenedioxy-pyrovalerone; ME – matrix effect; MeOH – methanol; MEP – 4-methylephedrine; MET – methadone; MDL – method detection limit; MMC – mephedrone; MOR – morphine; MQL – method quantitation limit; PE – process efficiency; PTFE – polytetrafluoroethylene (Teflon); QqQ – triple quadrupole mass analyzer; %RSD – relative standard deviation in percentage; R_{SPE} – recovery of the SPE method; SRM – selected reaction monitoring; WWTP – waste water treatment plant

Keywords: cathinones; drugs of abuse; environmental water samples; high resolution mass spectrometry; mixed-mode solid-phase extraction

ABSTRACT

A method based on liquid chromatography-(electrospray)high resolution mass spectrometry (Exactive Orbitrap) combined with solid-phase extraction using strong cationic exchange mixed-mode sorbent has been developed for the determination of seven drugs of abuse, including two synthetic cathinones, as well as some of their metabolites in environmental water samples. The method provides low detection limits and a high confirmation power thanks to the diagnostic and two fragment ions monitored for each compound in high resolution mass spectrometry, providing six identification points for each analyte. The clean-up step based on methanol in the extraction step adequately decreased the matrix effect, mainly for river and effluent water, and provided suitable process efficiency. Method detection and quantitation limits for environmental waters were at low ng/L. The method was applied to analyze the samples of influent and effluent waste water, as well as surface water. Codeine, methadone, and its metabolite were determined in all samples of waste water and the metabolite of cocaine, benzoylecgonine, was found at the highest concentration.

1. INTRODUCTION

In recent years, the interest in determination of emerging organic pollutants has considerably increased for several reasons. Some of them have a significant biological activity that could compromise the functioning of living beings in the environment: pharmaceutical drugs [1-5], drugs of abuse [3,5-11], residual compounds from the personal care products [4] and others. Drugs of abuse are of particular concern, since some of them have been found to be poorly eliminated by waste water-treatment plants [9], resulting in the presence of original compounds and their metabolites in surface [12,13] and even tap water [13]. In surface waters, drugs of abuse have been shown to cause detrimental effects to water animals at environmentally relevant concentrations [14]. Further water treatment such as chlorination may cause the transformation of original compounds into new ones with a different toxicity, including more toxic compounds [15]. Presently, new waste water-treatment procedures are being developed and tested for the elimination of these substances from waste water [4,16-17]. With the data obtained from waste water analysis, an assessment of drug consumption in the population can also be made, named »waste water epidemiology« [10,11].

Amongst the drugs of abuse found in waste water, the most frequently encountered are opiates and opioids, cannabinoids, cocainics and amphetamine-based substances and their metabolites [3,5-11], but there is also an emergence of novel psychoactive substances. Among them, synthetic cathinones are of particular

concern due to their increased consumption [18-23], with mephedrone (MMC) and methylenedioxy-pyrovalerone (MDPV) being the ones most consumed.

Concentrations of drugs of abuse and their metabolites in waste water are usually at the ng/L level, requiring very sensitive analytical methods. Liquid chromatography (LC) coupled to tandem mass spectrometry (MS/MS) with a triple quadrupole (QqQ) analyzer has to date been the most frequently employed technique for this purpose [3,5,7,11,24], offering good selectivity and low detection limits due to the background noise elimination in the selected reaction monitoring (SRM) mode. In recent years, however, high resolution mass spectrometer instruments (HRMS) based on time-of-flight (ToF) or Orbitrap mass analyzers have become more affordable. The latter has been shown to offer similar or lower detection limits compared to QqQ instruments in SRM mode, while an enhanced resolution allows for simultaneous confirmation of analytes from their exact mass [24,25]. Hybrid instruments such as Q-Orbitrap are also a good alternative but at higher cost. With HRMS instruments, retrospective analyses can be achieved which might contribute to detect non-targeted analytes in the sample besides the quantification of the targeted ones [10]. There are several reports on the determination of most frequently used drugs of abuse (opiates and opioids, cocainics) in waste water, mainly using QqQ [3,5,7,11], while also including studies using HRMS by hybrid Q-Orbitrap [10,24]. In contrast, synthetic cathinones have to date been analyzed mainly in biological matrices and there are only some studies [18-24] where a few cathinones were determined in waste water samples, in most cases together with other illicit drugs. Most of these studies use LC-MS/MS with QqQ analyzer [19-23] and only one study [24] evaluates the use of Q-Exactive whose performance was compared to QqQ analyzer for one cathinone, MMC. Other

alternative methods based on GC-MS [26] or GC-vacuum ultraviolet detector (VUV) [27], as well as supercritical chromatography coupled to MS [28], have already been reported in the literature.

Due to the low concentrations of emerging contaminants in the environmental samples, analytes should be extracted and preconcentrated from waste water samples prior to analysis, with solid-phase extraction (SPE) as the most used extraction technique. Depending on the polarity and acidic or basic properties of the analytes, the most frequently used solid phases are polymeric sorbents either with polar moieties or cationic or anionic exchange moieties that combined turn out to mixed-mode sorbents. While the mechanism of analyte retention on the former sorbents is mainly reversed-phase interactions, the retention on the latter is also due to ionic interactions, which may involve better selectivity. The inclusion of an effective washing step during sample treatment improves selectivity and can also minimize the matrix effect (ME) that emerges when using electrospray ionization (ESI) in LC-MS. Several studies [7,18,21-23] used Oasis MCX (mixed cationic/RP sorbent) cartridges to extract therapeutic drugs, drugs of abuse and metabolites, including some novel psychoactive substances. Andrés-Costa et al. [9] used Strata-X (RP) cartridges to extract 8 drugs of abuse and metabolites, while Borova et al. [5] found Strata-XC (mixed cationic/RP sorbent) a better choice than Strata-X for drugs of abuse and pharmaceutical drugs. Heuett et al. [10] used an on-line SPE system equipped with HyperSep Retain PEP cartridges to extract 18 drugs of abuse. Besides commercially available sorbents, in-house polymer-based sorbents with various functional groups have been successfully synthesized and evaluated for the extraction of these and similar compounds [3,29-31]. Although sorbent choice is one of the most important

129 factors governing the efficiency of SPE, the experimental conditions are important
130 parameters as well [7,8].
131
132 The aim of the present study was to develop an SPE combined with LC-HRMS
133 (Exactive Orbitrap) analytical method for the determination of some drugs of abuse
134 and their metabolites in surface and waste water, in order to further exploit the
135 benefits offered by HRMS in terms of detection limits and confirmation power on the
136 selected group of drugs of abuse. These included were: morphine, codeine,
137 dihydrocodeine, cocaine, methadone, mephedrone and MDPV, and their metabolites
138 benzoylecgonine, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidone (EDDP) and 4-
139 methylephedrine.
140

141 2. EXPERIMENTAL

142

143 *2.1 Materials and standards*

144 Standards of drugs of abuse and their metabolites (Table 1 [3,10,32,33]) were
145 obtained either as solids or as solutions in ampullae: methylenedioxy-pyrovalerone
146 HCl (MDPV), mephedrone HCl (MMC) and its metabolite 4-methylephedrine (MEP),
147 cocaine metabolite benzoylecgonine (BE), morphine (MOR) purchased from LGC
148 (Luckenwalde, Germany); dihydrocodeine (DIC), methadone metabolite 2-ethylidene-
149 1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP) from Cerilliant (Round Rock, TX, USA);
150 and codeine (COD), cocaine (COC) and methadone (MET) from Sigma-Aldrich (St.
151 Louis, MO, USA). Solvents methanol (MeOH) and acetonitrile (ACN) were of HPLC
152 gradient grade purity, obtained from J.T.Baker (Deventer, Netherlands). Ammonia
153 solution was 28.0-30.0 % from Sigma-Aldrich, formic acid from J.T.Baker. Ultra-pure

water was obtained from a water purification system (Purelab Ultra, Veolia water, Sant Cugat del Vallès, Spain). For SPE, Oasis MCX (500 mg) extraction cartridges from Waters Corp. (Milford, MA, USA) were used.

Stock standard solutions of analytes were prepared by dissolving the weighed solid standard in MeOH at the concentration of 1000 mg/L. For purchased standards available as solutions in glass ampullae, the content of the ampulla was diluted with MeOH to obtain solution at the concentration of 100 mg/L. These solutions were kept in the freezer at –20 °C and were stable for several months. They were further diluted with mobile phase to obtain working solutions for injection into liquid chromatograph or with water for extraction optimization.

2.2 Samples

Samples of influent and effluent waste water were collected in waste water treatment plant (WWTP) from Tarragona (Spain) and Reus (Spain). Surface water was obtained from Ebro River (Spain). All samples were stored in the freezer at –20 °C until analyzed. Before extraction, waste water samples were consecutively filtered through 1.20 μm glass fiber filter (Fisher Scientific, Pittsburgh, PA, USA), and 0.45 μm Nylon filter (Magna, Thomas Scientific, Swedesboro, NJ, USA).

2.3 Solid-phase extraction

A SPE Vacuum Manifold from Supelco (Bellefonte, PA, USA) was used for solid-phase extractions. Extracts were evaporated in MiVac-Duo concentrator (GeneVac Inc., Ipswich, UK).

Extraction cartridge was conditioned with 5 mL of MeOH and rinsed with 5 mL of ultrapure water acidified to pH 3. Sample (100 mL or 250 mL) adjusted to pH 3 was

passed under vacuum through the cartridge at approximate speed 10 mL/min using the vacuum manifold. Cartridge was dried for 2 min by applying vacuum. The cartridge was then rinsed with 5 mL of MeOH. 15 mL of 5 % ammonia in MeOH were used for the elution of the retained compounds. The eluate was evaporated to the aqueous residue of approximately 0.5-1 mL in the vacuum concentrator at 40 ^{o}C. The residue was transferred into a 2 mL measuring flask, diluted to mark with 5 % MeOH in ultrapure water and filtered through a 0.45 μm PTFE syringe filter before injection into LC-HRMS.

2.4 Liquid chromatography-high resolution mass spectrometry conditions

For LC-HRMS experiments, liquid chromatograph Thermo Scientific Accela with autosampler and quaternary pump coupled to Thermo Scientific Exactive Orbitrap mass spectrometer equipped with heated electrospray ion source (HESI II) (Thermo Scientific, Waltham, MA, USA) was used.

Different columns, mobile phases, gradient profiles and temperatures were tested for the separation of the analytes. The optimal conditions were: LC column Ascentis Express C_{18} (100 x 4.6 mm, 2.7 μm) with fused-core particles from Supelco. Mobile phase was 0.1 % formic acid in ultrapure water (A) and ACN (B) and the following gradient was applied: 0-1.5 min 5 % B, 1.5-3.5 min to 15 % B, 3.5-8.5 min to 25 % B, 8.5-11 min to 55 % B, 11-13 min to 100 % B, 13-14 min 100 % B, 14-15 min to 5 % B, post-time 3 min. Mobile phase flow-rate was 0.5 mL/min, column temperature, 35 ^{o}C and injection volume 25 μL.

Heated electrospray ion source (HESI-II) conditions in positive mode ionization and fragmentation conditions for the analyzed compounds were selected to obtain the optimal signal for the diagnostic and two fragment ions (confirmation ions). Group 1

(MOR, COD, DIC): sheath gas 25 a.u., auxiliary gas 6 a.u., heater temperature 400 ^{o}C, capillary temperature 300 ^{o}C, spray voltage 2.00 kV, capillary voltage 45 V, tube lens voltage 110 V, skimmer voltage 25 V; collision energy (HCD) 60 eV. Group 2 (COC, BE, MET, EDDP, MDPV, MMC, MEP): sheath gas 35 a.u., auxiliary gas 10 a.u., heater temperature 400 ^{o}C, capillary temperature 300 ^{o}C, spray voltage 4.20 kV, capillary voltage 15 V, tube lens voltage 85 V, skimmer voltage 22 V; collision energy (HCD) 30 eV. Two time windows (both in positive ionization mode) were settled for acquiring the data. In each window two scan events were used: one at full scan (injection time 250 ms, resolution 50,000 FWHM) and one fragmentation in HCD cell in "all ion fragmentation" mode (injection time 50 ms, resolution 10,000 FWHM). Analytes were identified based on the accurate mass of diagnostic and two fragment ions with less than ± 5 ppm error and on the basis of retention time.

2.5 Method validation

Instrumental quality parameters, including linear range of the method, determination coefficients, repeatability of retention times and repeatability of signal (peak area of diagnostic ion), detection limits (LOD) and quantification limits (LOQ) were determined. Working standard solutions of all analytes in the concentration range 0.1–200 μg/L and blank solvent (5 % MeOH in ultrapure water) were injected in at least 4 replicates. LODs were determined as the concentration giving an ion signal of at least 10^3, and LOQs as the lowest point of the calibration curve.

For the validation of SPE/LC-HRMS method for surface and waste water, the same parameters as above were evaluated by extracting and analyzing at least 3 replicates of spiked samples. Besides that, matrix effect (ME), SPE recovery (R_{SPE}) and process efficiency (PE) were also evaluated. ME was calculated by comparing the

peak areas for each compound spiked to blank matrix extract (spiking after extraction), with peak areas for analytes in standard solution. Interfering coeluting compounds from the sample matrix either suppress ionization of the analytes in HESI source (negative ME) or enhance it (positive ME). R_{SPE} was calculated by comparing peak areas of analytes in the spiked sample extract (spiking before extraction) and analyte peak areas in blank matrix extract spiked with analytes (spiking after extraction). PE was calculated from peak areas for analytes in the spiked sample extract (spiking before extraction) compared to peak areas for analytes in standard solution and therefore includes ME and R_{SPE}.

3. RESULTS AND DISCUSSION

3.1 Optimization of liquid chromatography-high resolution mass spectrometry conditions

The aim of LC optimization was to achieve a good separation of the analytes in the shortest time possible. Two columns were tested: Ascentis Express C_{18} 100 x 4.6 mm and Ascentis Express RP-Amide 100 x 2.1 mm, both with 2.7 μm fused-core particles. The tested mobile phase consisted of ACN and either ultra-pure water or 0.1 % formic acid in ultra-pure water. Besides the column and composition of the mobile phase, gradient and column temperature (25–35 ^{o}C) were also optimized. The best separation in a reasonably short time was achieved with Ascentis Express C_{18} column, using the gradient detailed in Section 2.5, mobile phase flow-rate of 0.5 mL/min and column temperature set at 35 ^{o}C. Some analyte pairs were not completely baseline separated even at these optimized conditions: MMC and MEP, as well as MDPV and COC. They showed some peak overlap, which was still

acceptable due to different *m/z* of their diagnostic and fragment ions (Table 1). Moreover, the poor retention of MOR on any of the tested columns was also quite problematic, and it was not possible to achieve any longer retention time even at the initial mobile phase composition of 3 % ACN. Due to the low content of ACN in the initial LC conditions, the content of organic solvent in the injected standard solutions or sample extracts had to be kept below 10 %, otherwise the peak for MOR was distorted.

In the optimization of ionization and fragmentation conditions, the following parameters in the ion source were optimized for each compound separately: sheath gas, auxiliary gas, heater temperature, capillary temperature, spray voltage, capillary voltage, tube lens voltage and skimmer voltage. Optimal ionization conditions for the early eluting opiates (MOR, DIC and COD) were significantly different than ionization conditions for the remaining analytes eluting at the higher percentage of ACN in the mobile phase. These conditions are given in Section 2.4 as conditions for Group 1 (MOR, DIC, COD) and Group 2 (COC, BE, MET, EDDP, MMC, MEP, MDPV), respectively. Fragmentation conditions in the Exactive Orbitrap mass analyzer were optimized for each diagnostic ion separately to achieve the highest abundance of two fragment ions for each analyte (Table 1). Collision energies were slightly different for each diagnostic ion, but were selected as 60 eV for Group 1 and as 30 eV for Group 2 in the final conditions for practical purposes. The most abundant fragment ions for the target analytes were the same as were already identified in low-resolution MS [7] as well as in HRMS [10,24,34]. Using a diagnostic and two fragment ions from HR mass spectra with exact mass (within ± 5 ppm) provides 6 identification points according to the criteria of the European Directive 2002/657/EC [35].

Under the optimized LC-HRMS conditions, instrumental validation parameters were established. A diagnostic ion of each analyte was used for the quantification. Instrumental linear ranges for all analytes were from LOQ up to 25,000 ng/L, for some analytes up to 50,000 ng/L, R^2 were above 0.9937 and %RSD of the peak area (n = 4) were 2.7–6.6 % except for MET (9.1 %). LODs, corresponding to the concentration at which ion signals were at least 10^3, ranged from 30 to 220 ng/L except for MET (320 ng/L) and MOR (360 ng/L); while LOQs were 1100 ng/L for MET, 1200 ng/L for MOR and from 100 to 700 ng/L for the remaining analytes. Instrumental detection limits are equal or better than those published for QqQ instruments in SRM mode, e.g. 0.05–1.50 μg/L [3]. The reason for the higher LOD and LOQ for MET and MOR was the ionization instability, and poor ionization, respectively.

3.2 Optimization of solid-phase extraction procedure

To achieve the best recoveries of the analytes and at the same time eliminate interfering substances from the complex samples of waste water, extraction conditions have to be carefully optimized. Besides the type of sorbent, sorbent mass, sample volume and pH, and eluting solvent have to be selected. Moreover, the washing step included prior to the elution has to be optimized to remove interfering substances from the cartridge. The first step of optimization was the choice of the type of sorbent and sorbent mass. Due to the slightly basic nature of the analytes (see Table 1) and on the basis of previous experience [7], we selected the Oasis MCX sorbent. Mixed-mode cation exchange polymeric sorbents are considered a better option for basic drugs than reversed phase or hydrophilic-lipophilic sorbents

[5,7]. Cartridges with 500 mg sorbent bed were selected to provide higher retention capacity, allowing the processing of large volumes of the sample, as is usual in environmental samples. Recoveries were initially evaluated using 100 mL of ultra-pure water. Two different sample pH were tested: pH adjusted to 3 and pH of ultra-pure water, which was 6.0-6.5 with no further adjustment. Average recoveries from ultra-pure water (no washing step applied) at pH 3 were from 87 % to 101 % for all analytes except for MMC (79 %) and EDDP (64 %). At pH 6.0-6.5, the recoveries were from 86 % to 100 % except for MMC (81 %), EDDP (68 %) and COC (81 %). All the analytes have a basic nitrogen moiety in their structure and weakly basic properties (Table 1); therefore they are protonated both at pH 3 and at pH 6, which explains similar recoveries. Based on the satisfactory recoveries, sample volume was increased to 250 mL and similar recoveries were achieved.

The usual eluent in combination with mixed-mode cation-exchange sorbent is an organic solvent with the addition of a weak base, e.g. ammonia, which enables the analytes to be deprotonated and desorbed from cation-exchange functional groups on the sorbent. The solvent of choice is most frequently MeOH [5,7], while less polar solvents have also been used in combination with ammonia for the elution of synthetic cathinones [18,34]. The concentration of ammonia used in most experiments, i.e. 2 % [5,7,34], and elution volume up to 10 mL [5,7,34] were in our case not sufficient to give acceptable recoveries, probably due to high sorbent capacity. Consequently, we optimized both the percentage of ammonia and elution volume. Good recoveries listed above were obtained only after elution with 15 mL of 5 % ammonia in MeOH. In our experiments, MeOH was proven to give acceptable recoveries for synthetic cathinones as well.

The greatest emphasis in the SPE optimization has been given to the washing step in order to remove matrix compounds which affect ionization of analytes in the electrospray ionization source. Acidified ultra-pure water, mixture of water and MeOH (1:1), and different volumes of MeOH (2-10 ml) were tested. The aim was to use the washing solvent with the highest eluting strength without significantly affecting the recoveries of the analytes. Washing step experiments were performed after loading 250 mL of sample at pH 3 or at pH 6 to check for possible influence of sample pH on the retention of matrix compounds and retention of analytes under the washing conditions. The results of these tests for selected analytes at sample pH 3 are shown in Figure 1. One representative analyte of each compound group is shown and other analytes from the same group showed very similar trends. At sample pH 6, a similar profile was obtained. Finally, 5 mL of MeOH was selected for the washing step and the selection was re-checked during the experiments with surface and waste water samples by evaluating the ME. Based on the overall optimization results, we decided to adjust the pH of all samples to 3 prior to extraction.

The final sample preparation step was the eluate evaporation. Evaporation to dryness, which is commonly applied in most SPE procedures, was found to decrease the amount of MMC and MEP by up to 40 % in the final extract. Synthetic cathinones are more prone to this behavior because of their higher volatility, which also enables their GC-MS analysis without derivatization [26,27]. Therefore, eluate had to be evaporated to the low volume of 0.5-1.0 mL, diluted to the final volume of 2.0 mL in a volumetric flask and analyzed on the same day because of the instability of some compounds in the final solvent. Thus, the overall recoveries from ultra-pure water under optimized SPE conditions including a washing step with 5 mL of MeOH were

between 79 and 91 %, somewhat lower for MET and EDDP. The latter has shown the lowest recoveries of all analytes under every tested condition.

3.3 Evaluation of the method for surface and waste water samples

The final method comprised the optimized SPE extraction procedure and the LC-HRMS determination. Its performance was evaluated on the samples of surface water, as well as samples of influent and effluent waste water.

When 250 mL of the surface water were analyzed, ME was in the range –3 % to 41 % at the concentration level evaluated (100 ng/L) with the majority of the compounds showing an ionization enhancement, as can be seen from Table 2. We also performed the evaluation of ME, R_{SPE} and PE at a lower concentration level of 20 ng/L and ME was similar for most of the compounds. R_{SPE} were at both levels very similar to those observed for the ultra-pure water, with the exception of BE and EDDP, which showed lower recoveries. PE was good, even within the range 100 ± 20 % for all compounds except MET, due to the complete SPE extraction and ion enhancement. Slightly lower PEs were observed for BE and EDDP at both levels because of lower recoveries, as previously mentioned.

Due to the low levels of the target analytes present in surface water, matrix matched calibration was performed by using 250 mL of surface water spiked at different levels of analytes; determination coefficients, shown in Table 2, were higher than 0.9933. Repeatability, expressed as %RSDs (*n*=3, Table 2) are generally quite good even without the use of isotope-labeled internal standards. Less satisfactory %RSDs was

obtained for EDDP because the recovery of this compound on the Oasis MCX cartridges was the lowest.

Method detection limits (MDL) were in the range 0.1 to 2 ng/L. The obtained MDLs and MQLs (lower limit of linear range) are in good agreement with the results reported in the literature using QqQ instruments [3,5,7,9,12,13,23]. The proposed method is thus fully comparable to those already published for similar analytes. However, it should be emphasized that the literature results were in all cases obtained by using isotope-labeled internal standards, while in the case of the proposed method, comparable performance was obtained without any internal standards.

For influent and effluent waste water, ME, R_{SPE} and PE were evaluated for samples from the waste water treatment plant (WWTP) located in Tarragona. Due to higher complexity of the matrix and rather high concentration of target analytes in the samples, only 100 mL of the samples were processed and the sample was spiked at high levels (100 ng/L in effluent water and 500 ng/L in influent water). Some of the analytes were already present in the blank samples and in these cases, signals obtained for original samples were subtracted from the signals obtained for the spiked samples (pre- or post-extraction). Table 3 shows the results. As can be seen, all compounds (except MEP) suffer from ion suppression in effluent waste water, but process efficiencies were acceptable for all analytes except DIC and MMC. As expected, effluent waste water has less matrix effect than influent waste water, but still more than surface water. In the influent waste water, a significant suppression of the ionization, up to –71 %, was observed, resulting in generally low process

efficiencies. For BE and COC, it was not possible to calculate some of these parameters because of high concentration in the blank samples. In order to diminish ionization suppression and enhance process efficiency for the influent waste water sample, we tested the feasibility of diluting the extract 1:5, with the results shown in Table 3. Compared to the undiluted extract, the matrix effects decreased. Further dilution of the extract (1:10) did not bring any improvement in these parameters.

Due to the high content of the analytes in these kinds of samples, matrix-matched calibration was not possible. Therefore, these samples were quantified by an external calibration method and by applying the process efficiency for the respective sample.

3.4 Application to the real samples

The method was applied to determine the target compounds in samples of Ebro River and influent and effluent waste water from two waste water treatment plants (WWTPs) in the southeastern part of Spain (Tarragona and Reus). The compounds were identified on the basis of their retention time, their diagnostic and two fragment ions given in Table 1. The advantage of using a high-resolution mass analyzer was clearly shown by the possibility of high specificity for identification of the compounds by comparing the *m/z* for all three selected ions with those of the standard compounds.

Some of the drugs of abuse were detected in surface water samples from Ebro River: COD, BE, EDDP and MET. In all cases, their concentration was between MDL and MQL. In a previous study [7], analyzing samples from the same river, BE was already found in the range 19-35 ng/L. In the report from the nearby geographical region of

428 València, Spain, compounds COD, MOR, COC, BE and MET were found in fresh
429 water lagoon surface waters at concentrations up to 11 ng/L [12]. On the other hand,
430 Mendoza et al. [13] found concentrations up to 823 ng/L of BE and up to 150 ng/L of
431 COC, MOR, MET and EDDP in two rivers from the Madrid region. These results
432 show that the most frequently abused drugs can be present in a broad concentration
433 range in surface waters depending on the region and especially the density of
434 population.
435
436 The results for waste water are shown in Table 4. As can be seen, most drugs of
437 abuse were determined while cathinones were not present in any sample. The mass
438 errors for diagnostic ions were below ±5 ppm which is considered an accurate
439 determination. An exception was for some fragment ions for the compounds present
440 at low concentrations. Also, the ratio of fragment ions was in most cases within the
441 range established for direct injection of analytes, with the exception of compounds at
442 low concentrations. The European Directive 2002/657/EC [35] concerning the
443 performance of analytical methods sets the need for 4 identification points for
444 compound confirmation. Diagnostic and two fragment ions from HR full mass spectra
445 give 6 identification points [35], which fully satisfies these criteria. An example of an
446 extracted ion chromatogram for the sample of influent waste water is given in Fig. 2.
447
448 All analyzed samples contained COD, MET and its metabolite EDDP (Table 4). COD
449 is a prescription drug that is often abused, while MET is often used to treat heroin
450 addiction, therefore their presence in all samples is not surprising. They were found
451 in a similar concentration range in the studies by Pedrouzo et al. [7] and Gilart et al.
452 [3], both conducted in the same WWTPs as the present study. In the study on waste

water samples from student dormitories in Florida, USA, Heuett et al. [10] detected COD in concentrations up to 981 ng/L, while MET and EDDP were generally absent. All three substances were also detected in several WWTP on the island Santorini, Greece [5]. Based on the pooled data from several studies, MET and its metabolite EDDP are most often found in waste waters in Australia, Spain and Belgium [36].

In our study, most samples also contained MOR, COC and its metabolite BE, the latter one in quite high concentration, up to few μg/L (Table 4). Earlier studies from the same WWTPs [3,7] found slightly lower concentrations of MOR, but comparable levels of COC and BE. By comparison, Heuett et al. [10] in Florida detected MOR and BE in more than half samples, but in lower concentrations than in our case, while COC was not detected in any sample. Borova et al. [5] determined the presence of COC and BE at elevated concentrations in waste water samples from all WWTPs on Santorini, Greece. COC and BE were also found in all samples analyzed in a big study conducted in the area of Bogota, Columbia [37]. In Slovakia, both compounds were found in lower levels (BE up to 200 ng/L) in waste waters from different cities [38]. However, pooled data from several waste water studies [36] show occurrence of COC and BE in waste waters and thus consumption at quite high levels in several European countries and in Australia [36]. By comparison, MOR was detected only in waste waters in Italy and Spain [36], and Florida, USA [10]. These data are hardly surprising, since COC is, besides cannabis, considered to be the most widely consumed drug of abuse worldwide [37,38]. Our results for the effluent waste waters (Table 4) demonstrate that both compounds are not completely degraded in the WWTP.

In the same geographical area as the present study, no previous studies were done on the presence of synthetic cathinones; therefore we were not able to compare our results. Nevertheless, in the study of Mwenesongole et al. [18], MMC was present in waste water from Cambridgeshire, UK, in an unusually high concentration (0.548 μg/mL). In contrast, van Nuijs et al. [19] in Belgium found that MMC and MDPV were below LOQ. Chen et al. [20] reported the detection of MMC and MDPV in Australian waste waters, although the concentrations are not given. In Italy, MMC was determined in waste water of only 2 cities out of 17 tested in concentrations up to 24 ng/L [21]. In Croatia, analyzed cathinones were detected only sporadically and below detection limit [23].

4. CONCLUSIONS

A method using liquid chromatography coupled to high resolution mass spectrometry (LC-HRMS) with Exactive Orbitrap mass analyzer was developed for the determination of some drugs of abuse in surface and waste water samples. The drugs of abuse included in this study were some commonly abused opiates, opioids and cocainics, as well as synthetic cathinones which belong to the group of novel psychoactive substances, along with some of their metabolites. The optimized SPE procedure using mixed-mode cation exchange Oasis MCX allowed for a significant removal of matrix components using a clean-up step with methanol. However, for the influent sample, a further dilution of the extract was necessary in order to obtain acceptable matrix effect. We have shown that an accurate analysis with low detection limits is possible even without the use of isotopically labeled internal standards in

case when they are not available or are too expensive. HRMS Exactive Orbitrap offers similar LODs and LOQs as the more frequently used QqQ MS instruments in selected ion monitoring mode, but gives and additional benefit of the high specificity for identification of detected compounds based on the accurate mass determination of diagnostic and two fragment ions, as well as a full mass spectrum for each compound. Studies on drugs of abuse in waste water employing HRMS Exactive Orbitrap are at present very scarce, therefore the proposed method is a novel contribution in this area.

In the samples of influent and effluent waste water, most of the analyzed compounds were present with the exception of synthetic cathinones. The presence of the studied drugs was unequivocally confirmed (Δm < 4 ppm) thanks to the Exactive Orbitrap analyzer. Results for effluent water from WWTP indicated that the analyzed compounds were insufficiently degraded during the waste water treatment process, which means their dissipation into the environment. Some compounds were thus detected also in surface water, although below the lower limit of quantitation.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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664

665 Figure Captions

666

667 **Fig 1**: Effect of cartridge washing on the recoveries for a representative analyte of

668 each family. For conditions see text. %RSD for *n*=3

669

670

671 **Fig 2**: Traces for diagnostic ions for the extract of Tarragona WWTP influent water

Table 1: Basic physico-chemical properties [3,10,32,33], retention time, accurate masses of the monitored diagnostic and fragment ions of the target compounds.

Compound	Abbrev.	Formula	CAS No.	Drug class	log*P*	pK_a	t_R[a] [min]	Diagnostic ion *m/z*	Fragm. ion *m/z*	Fragm. ion *m/z*
Morphine	MOR	$C_{17}H_{19}NO_3$	57-27-2	opiates	0.89	9.85	2.91	286.1438	152.0626	128.0626
Codeine	COD	$C_{18}H_{21}NO_3$	76-57-3	opiates	1.19	6.05	5.43	300.1594	165.0704	181.0653
Dihydrocodeine	DIC	$C_{18}H_{23}NO_3$	125-28-0	opiates	NA	8.4	5.30	302.1756	141.0704	165.0704
Cocaine	COC	$C_{17}H_{21}NO_4$	50-36-2	cocainics	2.30	8.6	8.73	304.1543	182.1181	82.0656
Benzoylecgonine	BE	$C_{16}H_{19}NO_4$	519-09-5	cocaine metabolite	−1.32	3.2; 9.5	7.69	290.1387	168.1024	82.0656
Methadone	MET	$C_{21}H_{27}NO$	76-99-3	opioids	3.93	9.1	12.07	310.2165	265.1592	223.1122
2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine	EDDP	$C_{20}H_{23}N$		methadone metabolite	4.94	7.7	11.74	278.1903	234.1282	186.1282
Mephedrone	MMC	$C_{11}H_{15}NO$	1189805-46-6	synthetic cathinone	2.39[b]	8.0[b]	6.57	178.1226	145.0885	119.0856
4-methylephedrine	MEP	$C_{11}H_{17}NO$	27465-53-8	mephedrone metabolite	NA	NA	6.48	180.1388	147.1041	131.0855
Methylenedioxy-pyrovalerone	MDPV	$C_{16}H_{21}NO_3$	687603-66-3	synthetic cathinone	3.97	7.3	8.60	276.1600	126.1278	135.0440

[a] %RSD (*n* = 10) of retention time 0.3–0.9 %, except MOR 2.8 %; [b] predicted property [32]; NA, not available

Table 2: Method validation parameters for the determination of analytes in surface water (250 mL). Parameters ME, R_{SPE}, PE and %RSD are given for spiking level 100 ng/L.

Compound	ME [%]	R_{SPE} [%]	PE [%]	Linear range [ng/L]	R^2	%RSD [%] (*n*=3)	MDL [ng/L]	MQL [ng/L]
MOR	23	93	115	4 – 100	0.9973	4	0.5	1.5
DIC	–3	93	91	4 – 160	0.9987	3	0.4	1.1
COD	8	93	101	4 – 160	0.9986	2	0.1	0.4
MEP	16	93	108	4 – 160	0.9977	4	2	6.8
MMC	14	73	86	4 – 160	0.9951	6	0.8	2.8
MDPV	26	90	115	4 – 160	0.9958	5	1	3.5
BE	17	56	68	4 – 160	0.9933	9	0.8	2.6
COC	41	82	116	4 – 100	0.9935	4	2	8.1
EDDP	37	49	70	4 – 100	0.9963	12	0.4	1.3
MET	34	117	154	4 –160	0.9971	5	0.7	2.3

Table 3: Matrix effect (ME), SPE recoveries (R_{SPE}) and process efficiency (PE) for the extraction of analytes from 100 mL of effluent (EWW) and influent (IWW) waste water at the concentration level of 100 ng/L and 500 ng/L, respectively. Column IWW 1:5 shows ME and PE after extract dilution with dilution solvent (5 % MeOH in ultra-pure water). ND - not determined.

	EWW			IWW			IWW 1:5	
	ME [%]	R_{SPE} [%]	PE [%]	ME [%]	R_{SPE} [%]	PE [%]	ME [%]	PE [%]
MOR	–18	173	98	–33	107	87	–30	91
DIC	–46	78	42	–71	84	28	–49	73
COD	–38	140	81	–57	129	55	–3	145
MEP	10	62	78	–46	107	67	–4	103
MMC	–44	88	56	–60	75	34	–19	63
MDPV	–10	86	104	–37	86	68	2	83
BE	–9	125	106	ND	ND	ND	ND	ND
COC	–40	119	92	–66	ND	128	–17	142
EDDP	–3	90	106	–37	54	65	16	96
MET	–11	70	84	–20	121	87	–5	86

%RSD (*n*=3) < 25%

Table 4: Average concentrations in ng/L and average mass accuracy as mass error (Δm) in ppm for the diagnostic ion of the identified compounds in the samples of influent (IWW) and effluent (EWW) waste water. %RSD and average mass acuracy for n =3.

	Tarragona IWW		Tarragona EWW		Reus IWW		Reus EWW	
	c^a [ng/L]	Δm [ppm]	c^b [ng/L]	Δm [ppm]	c^c [ng/L]	Δm [ppm]	c^d [ng/L]	Δm [ppm]
MOR	451	–0.8	178	–0.2	319	0.7	n.p.	n.p.
COD	343	–1.7	298	–2.4	665	–0.3	333	–1.0
BE	>2,500[e]	–0.9	97	–1.3	2,421	0.2	n.p.	n.p.
COC	253	–2.1	28	–1.9	n.p.	n.p.	n.p.	n.p.
EDDP	122	2.0	82	1.0	68	2.3	54	2.4
MET	169	2.9	32	1.9	86	2.8	58	3.4

[a] calculated for diluted (1:5) extract, %RSD 3-12 %; [b] %RSD 9-33 %; [c] calculated for diluted (1:5) extract, %RSD 4-12 %; [d] %RSD 7-34 %; [e] above the upper limit of quantitation for BE; n.p....not present (below MDL)

Figure 1

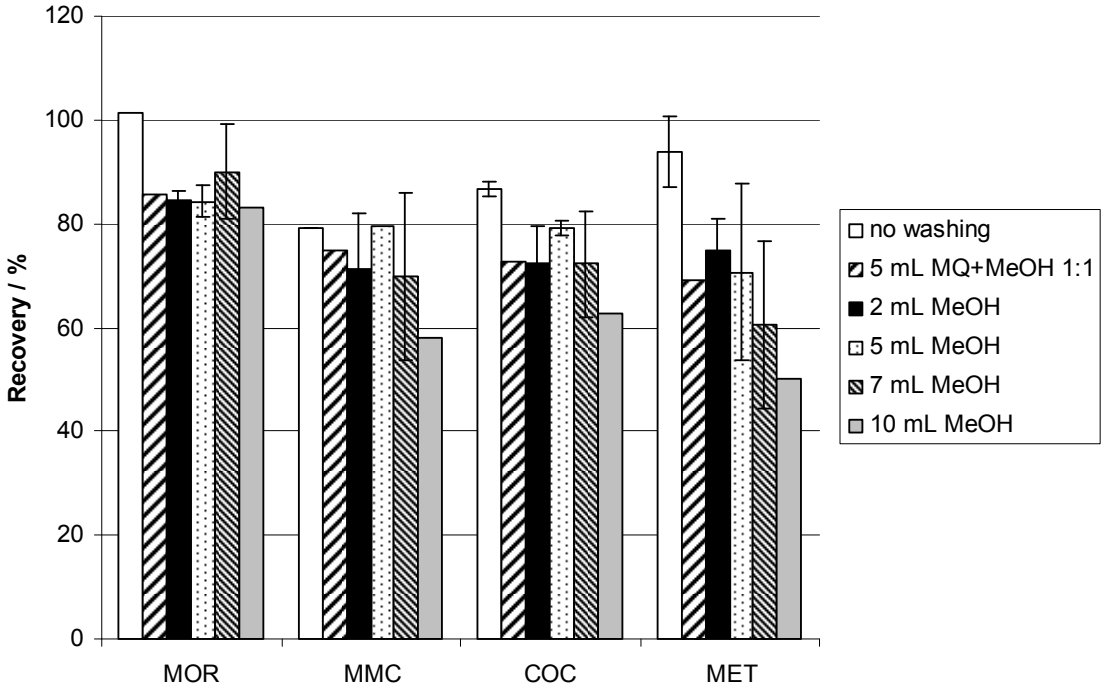
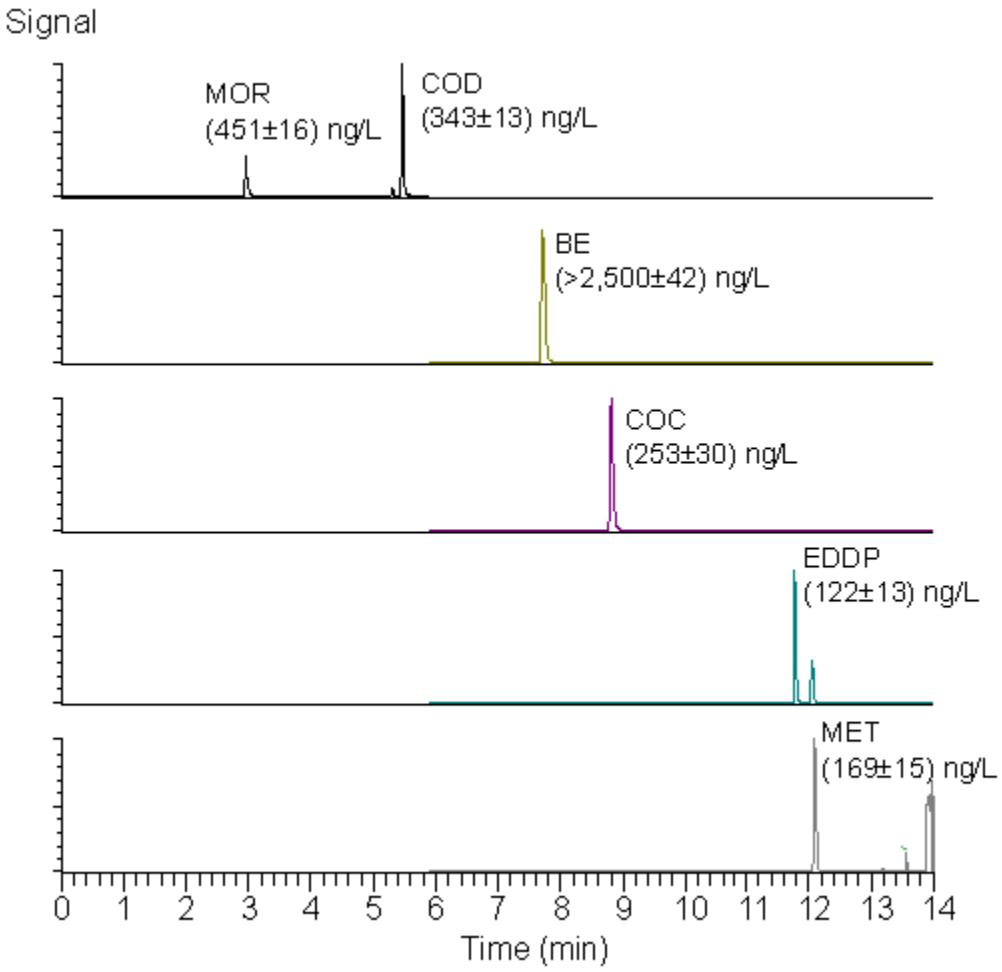


Figure 2


Signal
MOR
(451±16) ng/L
COD
(343±13) ng/L
BE
(>2,500±42) ng/L
COC
(253±30) ng/L
EDDP
(122±13) ng/L
MET
(169±15) ng/L
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
Time (min)