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Screening and Selective Quantification of Illicit Drugs in Wastewater by Mixed-Mode Solid-Phase Extraction and Quadrupole-Time-of-Flight Liquid Chromatography–Mass Spectrometry

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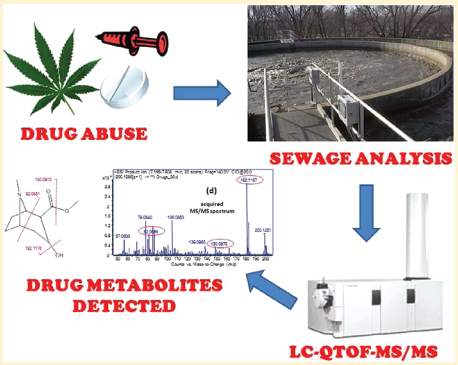
Supporting Information

ABSTRACT: For the first time, a mixed-mode solid-phase extraction with fractionation of basic analytes from neutral and acidic species during cartridge elution and liquid chromatography–quadrupole-time-of-flight mass spectrometry (LC–QTOF-MS) was combined for the quantitative determination of 24 illicit drugs and metabolites in urban sewage samples. The effects of several sample preparation and instrumental parameters in the sensitivity and selectivity of the quantitative method are thoroughly discussed. Under final working conditions, recoveries above 63% and 82% were attained for all species in raw and treated sewage, respectively; whereas, the limits of quantification of the method, defined for a signal-to-noise of 10 (S/N = 10), ranged from 2 to 50 ng L^{-1}. Sequential elution of mixed-mode cartridges allowed a significant reduction of matrix effects observed during electrospray ionization of basic drugs versus those measured for hydrophilic balance reversed-phase sorbents and the same mixed-mode polymer without fractionated elution. Analysis of raw wastewater samples confirmed the ubiquity of cocaine (COC), benzoylecgonine (BE), and 11-nor-9-carboxy-Δ^9-tetrahydrocannabinol (THCCOOH) in this matrix. The capability of the above methodology to identify new illicit drugs and/or metabolites in sewage samples is also discussed. With this aim, a two step strategy is proposed. First, high-resolution MS chromatograms, acquired throughout each chromatographic run, are automatically searched against an in-house built database, a reduced list of candidate drugs is generated, and the corresponding extracted ion chromatograms are obtained. In a further LC run, the tandem mass spectrometry (MS/MS) spectra of unknown peaks are acquired using different collision energies and compared with those existing in public libraries, or interpreted, to assign the unknown peak to one of the previously selected candidates.



Abuse of illicit drugs has become a problem of global concern. According to the "World Drug Report 2011" of the United Nations Office of Drugs and Crime (UNODC), between 149 and 272 million people consumed any illicit substance at least once in the past year and between 15 and 39 million were considered addicted.[1] Because of excretion after consumption and occasional direct disposals into sewage systems, illicit drugs and their metabolites are continuously discharged into wastewaters.[2−12] Since their removal during sewage treatments is usually incomplete, they are released into surface waters[2,9,10,13−15] and they have even reached drinking water sources.[13,16−18] Moreover, analysis of raw wastewater can be used to monitor the consumption of drugs in a specific location. This approach was applied for the first time in 2005 by Zuccato et al.,[19] and since then, other research groups have used it to estimate drug abuse in different countries.[10,11,13,17,20−24]

Most procedures developed for the analysis of illicit drugs residues in water samples comprise a sample concentration step followed by liquid chromatography–tandem mass spectrometry (LC–MS/MS) determination, normally on triple quadrupole (QqQ) instruments.[2,4,9,15,18,24−27] In regards to sample preparation, solid-phase extraction (SPE) is the preferred technique. Analytes are concentrated using either the hydrophilic reversed-phase type[3,7,11,28,29] or mixed-mode (reversed-phase plus cation-exchange) materials[2,4,9,24,25,27] and then recovered using an organic solvent or mixture of solvents compatible with further LC separation. The selectivity of the above approaches is rather limited since the washing step considers only aqueous solutions for the removal of inorganic salts. As a consequence, significant signal suppression effects have been reported during electrospray ionization (ESI), particularly for wastewater samples with high loads of organic compounds.[4,30] Although deuterated analogues are available to compensate for those matrix effects, they certainly result in increased limits of detection (LODs) and quantification (LOQs). Recently, we have shown that an improved SPE protocol can provide cleaner extracts and lower LODs for amphetamine type drugs.[31]

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Table 1. Experimental Parameters Used for the Quantification of the Target Analytes and Instrumental Performance Data[a]

compound	IS	precursor (m/z)	product(m/z)	CE (V)	mass error[b] (mDa)	mass error[b] (ppm)	R^2[c]	RSD[b] (%)	LOQ (pg)
AMP	AMP-d_6	136.1121	91.0542	8	0.2	2.2	0.9997	5.6	50
MAMP	MAMP-d_5	150.1277	91.0542	10	0.3	3.0	0.9995	7.7	50
MDA	MDA-d_5	180.1019	163.0754	12	1.0	6.2	0.9947	17.8	50
MDMA	MDMA-d_5	194.1176	163.0754	12	0.7	4.5	0.9993	8.1	30
MDEA	MDEA-d_5	208.1332	163.0754	12	0.7	4.1	0.9998	4.9	20
COC	COC-d_3	304.1543	182.1176	20	0.8	4.3	0.9981	5.0	20
BE	BE-d_3	290.1387	168.1019	20	0.7	4.2	0.9995	6.3	20
COE	COC-d_3	318.1700	196.1322	20	0.4	2.1	0.9982	5.5	30
SCO	COC-d_3	304.1543	138.0913	20	0.5	3.4	0.9986	6.9	20
LSD	LSD-d_3	324.2070	223.1230	25	0.7	3.1	0.9997	5.5	20
O-H-LSD	LSD-d_3	356.1968	237.1022	25	0.6	2.7	0.9985	6.4	30
BZP	BZP-d_7	177.1386	91.0542	25	0.2	1.9	0.9996	8.8	50
mCPP	BZP-d_7	197.0845	154.0418	20	0.9	6.1	0.9998	8.0	50
PCP	PCP-d_5	244.2060	86.0964	10	0.3	3.0	0.9997	7.1	20
FEN	FEN-d_5	337.2274	188.1434	25	0.8	4.1	0.9988	5.2	20
MOR	MOR-d_3	286.1438	201.0910	35	0.4	1.3	0.9981	3.6	10
6-AM	MOR-d_3	328.1543	165.0699	40	0.1	0.4	0.9976	5.2	10
COD	COD-d_3	300.1594	165.0699	40	1.2	4.0	0.9939	3.9	10
HER	MOR-d_3	370.1649	165.0699	40	0.3	0.7	0.9942	4.6	10
MET	MET-d_3	310.2165	265.1587	15	0.4	1.6	0.9993	2.2	10
EDDP	KET-d_4	278.1903	234.1277	30	0.5	2.2	0.9957	6.4	20
KET	KET-d_4	238.0993	125.0153	20	0.6	4.9	0.9993	9.5	50
THC	THC-d_3	313.2173	245.1547	35	1.8	7.5	0.9974	19.7	100
THCCOOH	THCCOOH-d_3	343.1915	299.2017	22	2.0	6.8	0.9988	19.2	100

[a]Underlined compounds were quantified in single MS mode, acquiring their MS/MS for confirmation. THC and THCCOOH were analyzed in ESI−, all remaining compounds in ESI+. [b]Mean of eight replicates of the same standard (20 ng mL^{-1}) acquired at 2 GHz during a 24 h period. [c]Calibration range LOQ−1000 ng mL^{-1} (IS 200 ng mL^{-1}).

Regarding the determination step, LC−MS/MS methods developed with QqQ instruments usually render an unmatched sensitivity. However, for some analytes with low m/z values for their precursor ions, as amphetamine class drugs, it is not possible to obtain two intense transitions, which are required for their proper identification in the selected reaction monitoring (SRM) mode.[30,31] Similarly, the possibility of interferences from coeluting isobaric compounds can alter SRM transition ratios required for proper identification,[32] and in some cases a "too rich" MS/MS fragmentation pattern is obtained (e.g., opiate drugs and metabolites[30]), causing a significant loss of sensitivity.

The replacement of QqQ systems by high-resolution/accurate-mass analyzers such as hybrid quadrupole-time-of-flight (QTOF) mass spectrometers can overcome many of those problems and allows the unambiguous identification of a given species from its accurate mass measurements and isotope patterns matching.[33−36] In addition, when working in the MS mode as a single TOF, these systems offer the possibility to screen for a theoretically unlimited number of compounds after the LC−MS run (post-target analysis), without the need for reference standards.[34,37,38] This may become very useful for drugs of abuse to detect the consumption of new substances, which continuously appear in the market. Although the quantitative possibilities of LC−QTOF-MS/MS have already been shown for some groups of contaminants in environmental and food samples,[38−41] in the field of illicit drugs analysis only its screening capabilities based on unspecific pseudo-MS/MS have been evaluated.[42]

Hence, the goal of this study was to develop and to validate a new method for the determination of 24 analytes, corresponding to a wide range of illicit drugs and some of their major urinary metabolites, in wastewater samples, placing special emphasis on its selectivity. Target drugs were selected based on the levels reported in wastewater[43] and recent abuse trends according to the UNODC[1] and the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA).[44] The method is comprised of a selective SPE step using a mixed-mode (Oasis MCX) sorbent, which allows the separation of neutral and acidic compounds from basic species during the elution step, reducing matrix effects. The quantitative and screening capabilities of the LC−QTOF-MS/MS system are also discussed. The screening potential was evaluated by performing post-target analysis over the chromatograms of the real samples, using an empirical formulas database of 130 drugs (Supporting Information, Table S1).

EXPERIMENTAL SECTION

Standards, Solvents, and Sorbents. (±)-Amphetamine (AMP), (±)-methamphetamine (MAMP), (±)-3,4-methylenedioxyamphetamine (MDA), (±)-3,4-methylenedioxymethamphetamine (MDMA), (±)-3,4-methylenedioxyethylamphetamine (MDEA), cocaine (COC), cocaethylene (COE), benzoylecgonine (BE), lysergic acid diethylamide (LSD), 2-oxo-3-hydroxy-LSD (O-H-LSD), benzylpiperazine (BZP), 1-(3-chlorophenyl)piperazine (mCPP), 1-(1-phenylcyclohexyl)-piperidine (PCP), fentanyl (FEN), morphine (MOR), 6-acetylmorphine (6-AM), codeine (COD), heroine (HER), (±)-methadone (MET), (±)-2-ethyl-1,5-dimethyl-3,3-diphenylpyrrolinium (EDDP), ketamine (KET), (−)-scopolamine (SCO), (−)-Δ^9-tetrahydrocannabinol (THC), and (−)-11-nor-9-carboxy-Δ^9-tetrahydrocannabinol (THCCOOH) were pur-

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chased from Cerilliant (Round Rock, TX) as 1 or 0.1 mg mL^{-1} solutions in acetonitrile (ACN) or methanol (MeOH). Scopolamine (SCO) was supplied as pure substance by Sigma-Aldrich (Madrid, Spain). Deuterated compounds were also purchased from Cerilliant (0.1 mg mL^{-1} in ACN or MeOH) and used as surrogated internal standards (ISs) for the quantification of their analogue native analytes. For those species whose deuterated analogue was not available, a structural or retention time related IS was used instead (Table 1). Mixed standard solutions (containing all the analytes or all the ISs) were prepared in MeOH at 2 mg L^{-1} and stored in the dark at −20 °C.

LC-grade ACN and MeOH, aqueous ammonia (NH_3) solution (25%), hydrochloric acid (37%), and acetic acid were supplied by Merck (Darmstadt, Germany). Ultrapure water was obtained by purifying demineralized water in a Milli-Q system (Millipore, Bedford, MA).

SPE cartridges containing either 200 mg of the Oasis HLB reversed-phase sorbent or 150 mg of the mixed-mode (reversed-phase and cation-exchanger) Oasis MCX material were purchased from Waters (Milford, MA).

Samples. Several wastewater samples were collected in the course of the study in February 2011 from a sewage treatment plant (STP) serving an urban population of ∼130 000 inhabitants of the northwest of Spain. Grab samples of treated and raw wastewater were taken in different week days and extracted (SPE) within 6 h after sampling in order to avoid analyte hydrolysis.[4,5] Composite samples of raw wastewater were collected in the course of a week by an automatic device working in a time-proportional mode (every 10 min during 24 h). Again, the combined sample was concentrated within 6 h after sampling.

Sample Preparation. Prior to extraction, samples (200 and 500 mL for raw and treated wastewater, respectively) were vacuum filtered, first through glass fiber prefilters and subsequently through 0.45 μm nitrocellulose filters (Millipore, Bedford, MA). The filtrate was adjusted to the desired pH, spiked with isotopically labeled standards (100 ng each), and subjected to the SPE process.

Under final working conditions, samples were adjusted to pH 4.5 and passed through Oasis MCX cartridges (∼10 mL min^{-1}) previously conditioned with 2 mL of a MeOH/NH_4OH (95:5) solution and 2 mL of pH 4.5 ultrapure water. Immediately after loading, SPE cartridges were washed with 10 mL of ultrapure water (adjusted to pH 4.5) and dried by a continuous nitrogen stream for 30 min. Finally, analytes were eluted in two separated fractions: cannabinoids (together with neutral/acidic matrix components) were first eluted by 2 mL of MeOH, and the remaining (basic) compounds were recovered straight afterward with 4 mL of MeOH/NH_4OH (95:5). Both fractions were concentrated down separately to ∼0.5 mL with a gentle stream of nitrogen (99.999%) in a Turbovap II concentrator (Zymark, Hopkinton, MA), adjusted to a final volume of 1 mL with MeOH and injected (10 μL) into the LC−MS system.

Liquid Chromatography−Quadrupole-Time-of-Flight-Mass Spectrometry. Analyses were performed using an Agilent 1200 series HPLC comprising a membrane degasser, a binary high-pressure gradient pump, a thermostatted LC column compartment, and an autosampler. Separations were carried out on a Nucleosil 100-3 C18 HD column (Macherey-Nagel GmbH & Co. KG, Düren, Germany) of 125 mm × 2 mm (length × i.d.) and 3 μm of particle size, thermostatted at 40 °C. The dual eluent system consisted of (A) 5 mM of ammonium acetate (NH_4OAc) in ultrapure water adjusted to pH 8.5 with NH_3 and (B) 5 mM of NH_4OAc in MeOH made to an apparent pH of 4.5 (by adding the equivalent amount of acetic acid to have such a pH in an aqueous solution). The flow rate was set at 0.2 mL min^{-1}, and the gradient program was as follows: 0 min (2% B), 0.2 min (50% B), 25 min (100% B), 29 min (100% B), 30 min (2% B), and 40 min (2% B).

The LC was coupled to an accurate-mass QTOF MS (Agilent 6520) equipped with a dual-ESI ion source. Nitrogen, used as the nebulizing and drying gases, was provided by a nitrogen generator (Erre Due srl, Livorno, Italy). Nitrogen of 99.9995% purity, for collision induced dissociation, was purchased from Carburos Metálicos (A Coruña, Spain). The capillary voltage of the ESI was set at 4 kV either in the positive or negative mode. The latter mode was used for the determination of cannabinoids, whereas remaining analytes were ionized in the positive mode. The temperature of the ESI chamber was set at 275 °C, the drying gas flow was set at 9 L min^{-1}, and the nebulizing gas pressure was set at 45 psig. The fragmentor voltage was maintained at 140 V for all compounds, and the pressure of nitrogen in the collision cell was adjusted at 18 mTorr.

Except the opioids, the analytes were quantified in the MS/MS mode from the MS/MS base peak extracted ion chromatogram using an accurate mass window of ±20 ppm. Opioids (COD, HER, MOR, and 6-AM) were quantified in the MS mode, extracting the $[M + H]^+$ ion chromatogram with a ±10 ppm mass window and acquiring also their MS/MS spectra just for confirmation purposes. This decision did not involve any extra analysis, since the QTOF system switches intermittently to single MS during an MS/MS run to allow the continuous calibration of the mass axis. With that aim, one of the ESI nebulizers was continuously infused with a reference solution according to the manufacturer specifications (5 psig), for which in negative mode the reference masses selected were 112.985587 and 980.016375 m/z, and in positive mode 121.050873 and 922.009798 m/z. MS spectra were recorded at 2 spectra per second and MS/MS spectra at 6 spectra per second in the positive mode and at 2 spectra per second in the negative mode. Spectral data were acquired at 2 GHz (extended dynamic range mode) when used for quantification measurements and at 4 GHz (high resolution mode) for screening purposes. Instrument control, data acquisition, and evaluation were performed with the Mass Hunter software (Agilent Technologies). The most relevant MS/MS parameters are summarized in Table 1.

Matrix Effects Evaluation. Matrix effects during ESI were evaluated spiking an aliquot of the final SPE extracts with 200 ng of all analytes and considering, in addition, nonspiked aliquots from each sample. Hence, the response of the spiked extracts (R_2) after nonspiked sample signal (R_B) subtraction was compared to the response factor of a standard prepared in MeOH (R_1) with the same concentration. Matrix effect percentages (% ME) were calculated as % ME = 100 × (R_2 − R_B)/R_1.[31,45,46]

Recoveries and Real Samples Analysis. Recoveries (% R) of the whole procedure were evaluated with spiked aliquots of different water samples: ultrapure water, treated wastewater, and raw wastewater. Deuterated ISs were added (100 ng) as surrogates in all cases to compensate matrix effects and losses during sample preparation. Differences between the corrected responses (analyte peak area divided by the signal of the IS) for spiked and nonspiked fractions of each sample were compared

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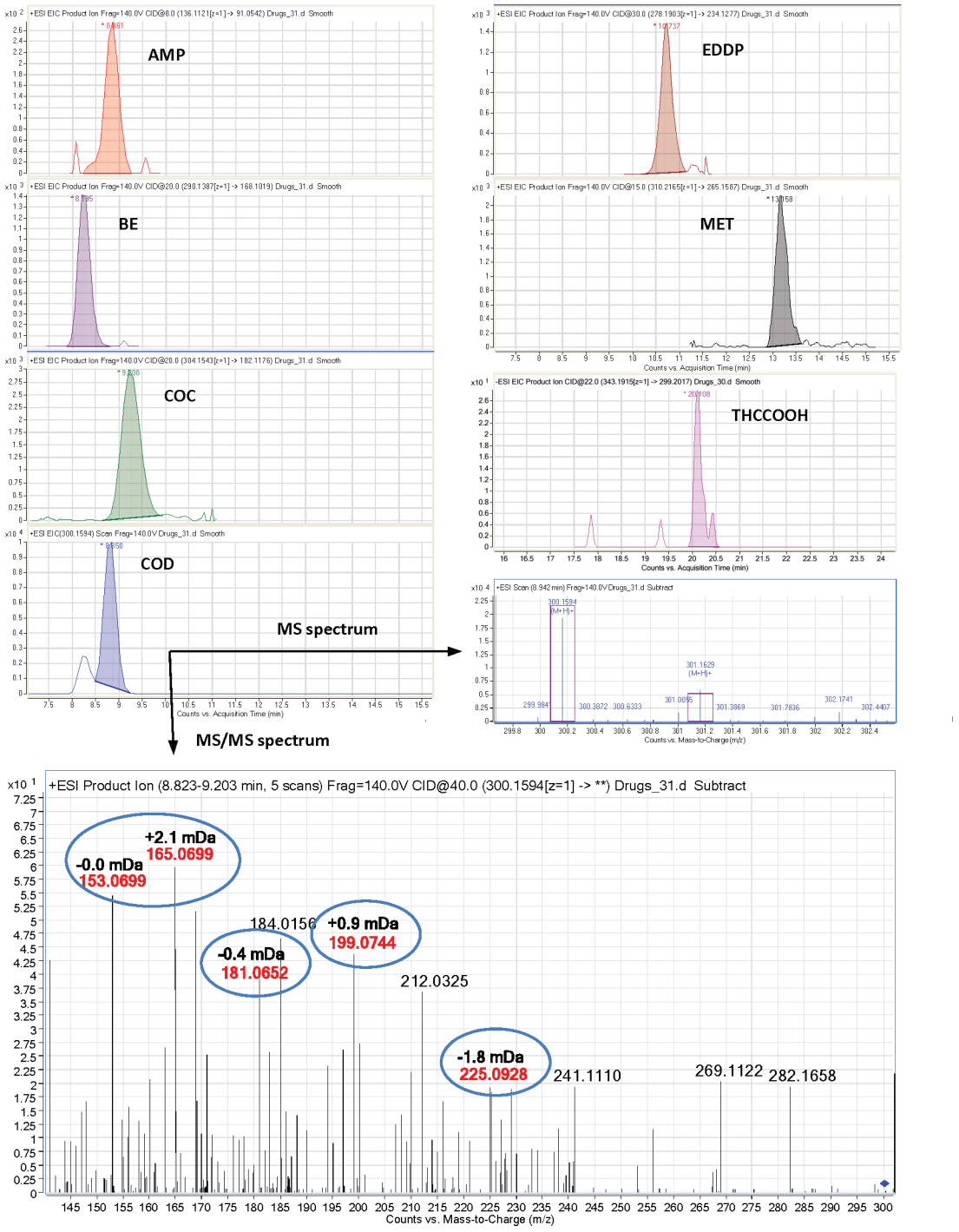

Figure 1. Chromatogram of a wastewater influent sample showing the compounds detected. For codeine, measured in single-MS mode, the accurate-mass MS and MS/MS spectra are shown. The expected and experimental isotopic patterns are presented in the MS spectrum. Confirmation product ions with mass deviation from the expected values are highlighted in the MS/MS spectrum.

with calibration curves obtained for standards in MeOH containing the ISs.

RESULTS AND DISCUSSION

Liquid Chromatography–Mass Spectrometry. First of all, the two different ionization modes were tested. Cannabinoids could be determined in both modes but showed higher responses in ESI−, agreeing with previous findings,[4,47] whereas remaining analytes, with a basic character, could only be determined in ESI+.

The LC−QTOF system used in this study does not allow one to switch the polarity of the ESI source in a single time segment when operating in the MS/MS mode. Thus, chromatographic conditions were adjusted to obtain a good separation between the two cannabinoids and the rest of the basic analytes, in order to group them in two different temporal segments. To this end, the organic phase was acidified to an apparent pH of 4.5, whereas the aqueous phase buffer was made to a pH of 8.5. In this way, basic compounds could be effectively retained in the C18 column at low organic content

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and, at the same time, the organic content gradient was accompanied by a pH gradient, increasing the retention of THCCOOH (pK_a 4.21) and decreasing the retention of MET (the basic drug displaying the highest retention time) so that they could be separated into two well-defined segments. The method is comprised of a first segment (until 17 min) using ESI+ and a second one operating the source in ESI− for the sensitive determination of THC and THCCOOH. As an example, a chromatogram of a 50 ng mL^{-1} standard is presented in the Supporting Information (Figure S1). In both segments, MS and MS/MS spectra were alternatively recorded using the m/z values compiled in Table 1. According to the 2002/657/EC Directive,[48] one single high-resolution MS/MS transition is enough to fulfill the identification points guideline. Yet, in the case of the four opioid compounds (MOR, 6-AM, COD, and HER), their MS/MS collision-induced dissociation leads to a multitude of fragments,[30] which compromises the sensitivity of MS/MS quantification. Actually, Boleda et. al.[3] decided to use a pseudo-MS/MS transition on a QqQ instrument in order to gain sensitivity in detection, but confirmation still relied on the low yield MS/MS products. Alternatively in this work, opioids were quantified from their single MS $[M + H]^+$ narrow-mass extracted ion chromatograms, recording MS/MS spectra for confirmation purposes. Besides MS/MS and due to the high resolution and mass accuracy of the QTOF system, the $[M + H + 1]^+$ ion can also be used as a sensitive confirmation ion (for opioids its intensity is ∼20% of the $[M + H]^+$) in order to comply with the 2002/657/EC Directive[48] identification points guideline. An example is shown in Figure 1, where the chromatogram of a sample is presented. In the case of COD, the identity of the chromatographic peak can be confirmed by the single MS $[M + H + 1]^+$ ion and characteristic MS/MS product ions in spite of the presence of other background ions and spectrum complexity.

Given that the Agilent 6520 QTOF system uses an analog-to-digital-converter (ADC) that can be operated either at 4 GHz (highest mass resolving power; FWHM resolution, ∼9500 at m/z 113 and ∼22 000 at m/z 980) or 2 GHz (resolution ∼half of 4 GHz but expanded linear range), both ADC acquisition modes were compared in terms of mass accuracy in both single MS and MS/MS modes. In the single MS mode, at 4 GHz, mass errors increased with the concentration of the target species, reaching the 50 ppm threshold at 500 ng L^{-1}, whereas at 2 GHz mass errors stayed below 5 ppm even at concentrations near the LOD (Figure S2 in the Supporting Information). In MS/MS operation, though the effect was less significant, still less mass accuracy was provided by the 4 GHz mode. Hence, particularly taking into account that the four opioids included in this research were quantified in single MS, the ADC was operated at 2 GHz when performing quantitative measurements. As compiled in Table 1, in this way the mass error was not higher than 4 ppm for the analytes determined in the single MS mode and lower than 8 ppm in MS/MS. Therefore, extracted ion chromatograms used for quantification were taken with a mass tolerance of ±10 ppm in MS and ±20 ppm in MS/MS (in the worst case, equivalent to ±3.7 and ±6 mDa, respectively) leading to a very low noise baseline.

The LC−MS(/MS) method produced a good linearity in the LOQ−1000 ng mL^{-1} range and relative standard deviation (RSD) values not higher than 20%, even at levels close to the LOQ (Table 1). Also, the instrumental LOQs of the QTOF instrument were in the 10−100 pg range, which are higher than those reported on UPLC−QqQ-MS/MS instruments (0.05−4 pg)[3,29] but on the same order of magnitude of those achieved with a standard LC−QqQ-MS/MS system (12−530 pg).[4]

Solid-Phase Extraction. As mentioned in the introduction, the Oasis MCX sorbent was selected for the preconcentration of the analytes on the basis of its demonstrated retention efficiency[2,9,25] and its capability to provide more selective extractions than other materials for basic compounds.[31]

Initially, the effect of the sample pH on the retention of the analytes was investigated with 200 mL aliquots of spiked ultrapure water (2 ng mL^{-1}) adjusted to different pHs in the range from 2.5 to 10 units. After loading the sample, cartridges were rinsed with 10 mL of ultrapure water adjusted to the corresponding pH and eluted with 10 mL of MeOH/NH_4OH (95:5). Most of the basic analytes, e.g., BE and COD, showed recoveries around 90% within the range of the investigated pH values (Figure S3 in the Supporting Information). This trend indicates that even the neutral forms of these species, existing at basic pHs, are efficiently retained in the mixed-mode SPE cartridge through reversed-phase interactions. However, some few compounds (BZP, PCP, KET, and MET) showed lower recoveries at pH 10, requiring also the ionic interactions between their positively charged forms and the sulfonic moiety of the sorbent to be quantitatively extracted from the sample. In the case of THCCOOH, recoveries increased, surprisingly, with sample pH. This compound exists only as neutral (pH 2.5) or negatively charged species (rest of tested pHs) interacting with the MCX sorbent just through the reversed-phase mechanism. Consequently, recoveries are not expected to improve with the increase of the pH. However, the trend observed for this compound (Figure S3 in the Supporting Information) is likely the consequence of sorption losses for its neutral form (log K_{ow} ≈ 6.2) in the walls of sample vessels and connections between the sample and the SPE cartridge at low pHs. On the other hand, at higher pHs, THCCOOH exists as a negatively charged, more polar species (log K_{ow} ≈ 2.9 at pH 7),[49] less prone to sorption processes. On the basis of the above results, samples were adjusted at pH 4.5 in order to favor the dual-retention mechanism of basic drugs, which represent 22 of the 24 analytes involved in this research.

Subsequently, breakthrough studies were performed and it was found that 150 mg MCX cartridges can concentrate up to 500 mL of raw wastewater without significant losses for any of the investigated analytes (data not shown). Working sample volumes were finally set at 500 mL in the case of treated wastewater but reduced to 200 mL for raw samples in order to prevent the bed of sorbent from clogging. In further experiments, the sequential elution of MCX cartridges was optimized. It was found that about 95% of the two cannabinoids were recovered with only two fractions (2 × 1 mL) of MeOH, which did not contain any trace of the basic analytes. On the other hand, the successive elution with 4 × 1 mL of MeOH/NH_4OH represented around 98% of the basic drugs and metabolites (data not shown). Thus, in the optimized method, MCX cartridges were eluted first with 2 mL of MeOH and finally with 4 mL of MeOH/NH_4OH (95:5). Both extracts were collected separately, concentrated, made with MeOH to a final volume of 1 mL, and analyzed in two different LC−MS injections.

The above optimized SPE scheme (protocol A) was compared in terms of selectivity (as % ME, see Matrix Effects Evaluation) with two other different SPE methods, representing the approaches more frequently used in the literature.[43] In one

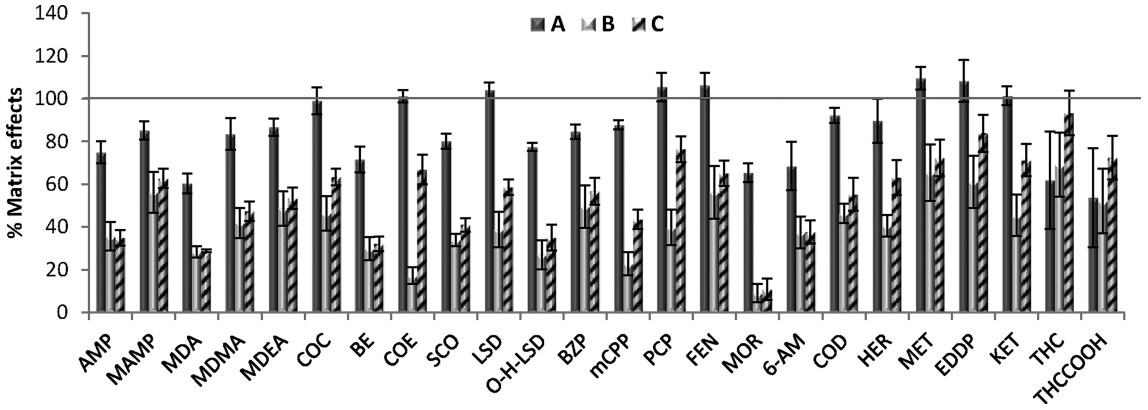

Figure 2. Matrix effects (% ME) in effluent wastewater depending on the SPE protocol: (A) Oasis MCX with fractionated elution (this work); (B) Oasis MCX, single elution; and (C) Oasis HLB.

Table 2. Overall Internal Standard Corrected Recoveries (n = 3) and LOQs of the Whole Method for the Different Matrixes Considered

	% R^a			LOQ (ng L^{-1})	
compound	ultrapure[b]	effluent[c]	influent[d]	effluent	influent
AMP	105.6 (10.1)	116.9 (12.9)	111.7 (7.0)	10	25
MAMP	107.1 (2.3)	106.6 (13.4)	91.5 (15.3)	10	25
MDA	109.7 (9.8)	116.6 (4.1)	114.2 (12.2)	10	25
MDMA	106.0 (5.4)	109.3 (2.6)	111.4 (9.8)	6	15
MDEA	105.7 (1.8)	105.3 (12.7)	115.4 (7.3)	4	10
COC	98.9 (7.5)	91.1 (6.5)	94.3 (4.2)	4	10
BE	105.8 (7.0)	122.7 (9.9)	121.8 (12.7)	4	10
COE	102.5 (3.1)	117.1 (9.9)	119.3 (3.3)	6	15
SCO	118.0 (5.8)	90.7 (8.7)	100.0 (6.1)	4	10
LSD	104.3 (3.5)	112.8 (6.5)	103.4 (3.9)	4	10
O-H-LSD	84.4 (11.9)	84.9 (6.1)	91.6 (7.4)	6	15
BZP	103.4 (4.5)	105.8 (16.8)	100.9 (8.6)	10	25
mCPP	108.5 (3.1)	104.6 (9.4)	80.4 (15.5)	10	25
PCP	106.5 (5.0)	106.3 (6.8)	111.6 (8.1)	4	10
FEN	103.8 (4.7)	109.2 (7.2)	109.9 (3.5)	4	10
MOR	99.1 (22.9)	128.2 (24.4)	130.8 (22.8)	2	5
6-AM	116.4 (11.3)	82.0 (17.9)	94.9 (11.7)	2	5
COD	105.2 (11.9)	128.9 (12.6)	94.3 (22.1)	2	5
HER	83.1 (20.6)	105.2 (31.2)	99.0 (29.7)	2	5
MET	101.9 (4.8)	117.2 (4.4)	108.4 (4.3)	2	5
EDDP	76.7 (21.6)	102.7 (11.7)	62.9 (7.7)	4	10
KET	109.2 (2.8)	119.1 (8.0)	115.8 (4.7)	10	25
THC	90.4 (27.0)	114.7 (7.9)	105.4 (20.5)	20	50
THCCOOH	116.9 (19.1)	123.8 (8.1)	107.4 (10.8)	20	50

[a]Expressed as "mean (RSD)". [b]SPE of 500 mL ultrapure water samples spiked with 100 ng L^{-1} of each analyte and 200 ng L^{-1} of each IS, n = 3 replicates. [c]SPE of 500 mL treated wastewater samples spiked with 200 ng L^{-1} of each analyte and 200 ng L^{-1} of each IS, n = 3 replicates. [d]SPE of 200 mL raw wastewater samples spiked with 500 ng L^{-1} of each analyte and 200 ng L^{-1} of each IS, n = 3 replicates.

case (protocol B), acidified samples (pH 4.5) were also concentrated using MCX cartridges, but the whole group of target drugs and metabolites was recovered in the same extract with 5 mL of MeOH/NH_4OH (95:5).[2,9,43] The third SPE scheme (protocol C) was based on the use of 200 mg Oasis HLB cartridges; in this case, samples were adjusted at pH 8.5, so basic analytes stayed in the neutral form[5] and elution was carried out with 5 mL of pure MeOH.

As it is displayed in Figure 2 for an effluent sample after a 500-fold preconcentration, protocol A % ME values were all above 60% for all basic drugs, whereas in protocols B and C they were as low as 10% in the case of MOR. For 200-fold preconcentrated influents (Figure S4 in the Supporting Information) differences in % ME were lower, but protocol A could still afford a ~30% more sensitive detection for basic compounds. These results are a consequence of the fractionated elution protocol A, where many interfering matrix constituents are removed in the first methanolic fraction. Hence, in the case of the two cannabinoid analytes, eluted in that fraction, % ME values are similar with any of the three protocols. Consequently, the SPE method optimized in this work can provide lower LODs/LOQs for all analytes with the exception of cannabinoids.

As shown in Table 2, estimated LOQs of the whole method varied from 2 to 20 ng L^{-1} in effluents and from 5 to 50 ng L^{-1} in influents, calculated as an S/N of 10. Recoveries (% R)

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Table 3. Mean Concentration (n = 3) Values for Analytes Occurring at Levels above Their LOQ in Different Real Samples[a]

	effluents (grab samples)[b]		influents (24 h composite samples)[b]						
C (ng L^{-1})	W	Th	Tu	W	F	Sa	Su	loads (g day^{-1})[c]	loads per 1000 inh (mg day^{-1})[c]
AMP	<LOD	14.2	26.4	<LOD	84.8	83.5	61.7	3.2	23.5
MDMA	<LOD	<LOD	<LOD	<LOD	<LOQ	<LOD	25.6		
COC	22.7	30.4	111.8	97.6	205.7	294.7	187.0	10.9	79.8
BE	170.8	207.3	257.7	173.0	504.6	707.7	591.9	27.1	198.8
COE	<LOD	<LOQ	<LOQ	<LOQ	<LOQ	24.4	<LOQ		
MOR	<LOD	<LOD	<LOD	<LOD	17.0	19.0	26.6	0.8	5.7
COD	105.5	<LOD	112.0	<LOD	<LOD	<LOD	<LOD		
MET	20.0	15.2	23.7	15.1	31.7	33.4	29.6	1.6	11.9
EDDP	33.9	21.0	54.6	22.3	35.5	43.1	46.7	2.5	18.0
THCCOOH	<LOD	32.2	98.3	55.7	228.0	147.0	101.0	7.6	56.0

[a]<LOQ, below limit of quantification; <LOD, below limit of detection; RSD < 30% in all cases. [b]Week day: Tu, Tuesday; W, Wednesday; Th, Thursday; F, Friday; Sa, Saturday; Su, Sunday. [c]Calculated from mean values for the influent samples, considering values lower than LOD and LOQ as equal to LOD/2 and LOQ/2, respectively, for statistical calculation. Loads not calculated for compounds that were below LOQ in more than two samples.

ranged from 76.7 to 118.0% in ultrapure water, from 82.0 to 128.9% in treated wastewater, and from 62.9 to 130.8% in raw wastewater. These recovery values and LOQs are in the range of those reported in the literature by SPE and LC−MS/MS.[43]

Application to the Quantification of Real Samples. The developed method was applied to determine the levels of the selected illicit drugs in two treated wastewater grab samples and in five 24 h composite influent samples, all of them collected from the same STP in different days during February 2011.

Mean concentration values for compounds occurring at levels above their LOQ are compiled in Table 3. As it is shown, the highest levels corresponded to BE (up to 708 ng L^{-1}), the main metabolite of COC, matching the findings reported by other authors[29,47,50] and highlighting the widespread consumption of this illicit drug. Both the parent drug and metabolite were quantified in all samples, with significantly higher concentrations in the composite influent samples collected during the weekend. The influent wastewater COC/BE ratio remained quite constant through the different days of the week (from 0.32 to 0.56) and was slightly higher than the expected excretion ratio of 0.22, although this value has a large uncertainty due to the lack of reliable metabolism studies in humans.[43] THCCOOH could also be determined in all influent samples, confirming the extended abuse of cannabis. On the other hand, MET and its main metabolite EDDP were also quantified in all raw and treated wastewater samples, but their concentrations stayed more constant through the different week days, probably as a result of the use of MET as a medical substitute of heroin in antiaddictive treatment. AMP and COD were also quantified at relatively high values (up to 84.8 and 112.0 ng L^{-1}, respectively) in some of the samples, whereas COE, MDMA, and MOR were measured at lower levels.

The concentrations from 24 h-composite influents were translated into mean loads and normalized per 1000 inhabitants-loads (Table 3). On the basis of the loads calculated for AMP, BE, and THCCOOH, the consumption per 1000 inhabitants of amphetamine, cocaine, and cannabis, respectively, was estimated.[43] It accounted for 76.4 mg day^{-1} 1000 inh^{-1} for amphetamine, 463 mg day^{-1} 1000 inh^{-1} for cocaine, and 8500 mg day^{-1} 1000 inh^{-1} for cannabis. Assuming an average dose of 30, 100, and 125 mg, respectively,[43] these data are equivalent to 2.5 doses day^{-1} 1000 inh^{-1} of amphetamine, 4.6 doses day^{-1} 1000 inh^{-1} of cocaine, and 68 doses day^{-1} 1000 inh^{-1} of cannabis. The above consumption is within the ranges published for these substances in Europe, with the exception of cannabis, whose maximum published consumption calculated through the sewage epidemiology approach until now had been 61 doses day^{-1} 1000 inh^{-1}.[43]

Screening of Other Drugs/Metabolites Using a Compound Database. As mentioned in the introduction, TOF systems provide high-resolution spectra that can be used, after the analysis, to search for not preselected analytes (post-target approach). In fact, such a possibility was tested by Hernández et al.[42] for screening drugs of potential abuse but using an unspecific pseudo MS/MS method, named as MS^E by the manufacturer. As no real MS/MS was recorded in that case, the reliability of the results depended on a very efficient chromatographic separation, such as UPLC used by the authors of that work.

In the present study, the post-target screening approach was also tested in order to find out other possible substances of abuse that may have appeared in the market recently or other metabolites that may be relevant under environmental conditions but would have been missed in the target selection by using pure MS and MS/MS data. To this end, a database containing more than 130 compounds was constructed, including the most popular illicit drugs of abuse and their metabolites[51,52] and also newly detected substances according to the last reports of UNODC[1] and EMCDDA.[44] The database (Table S1 in the Supporting Information) compiles the empirical formulas of the recorded species plus some additional data.

The screening protocol was based on the "Find by Formula" function of the Mass Hunter software provided by the manufacturer. This algorithm automatically searches for the ionized forms and potential adducts of the compounds included in the database (with a defined mass error tolerance of ±5 ppm) over the real samples, generating the accurate mass extracted chromatograms and comparing their peak spectra with the theoretical ones in terms of mass accuracy, isotopic match, and spacing between ions. These three parameters are combined into an overall score, where a value of 100 would represent a perfect match.[53] After a positive match, samples are reanalyzed in order to obtain their MS/MS product ion spectra,

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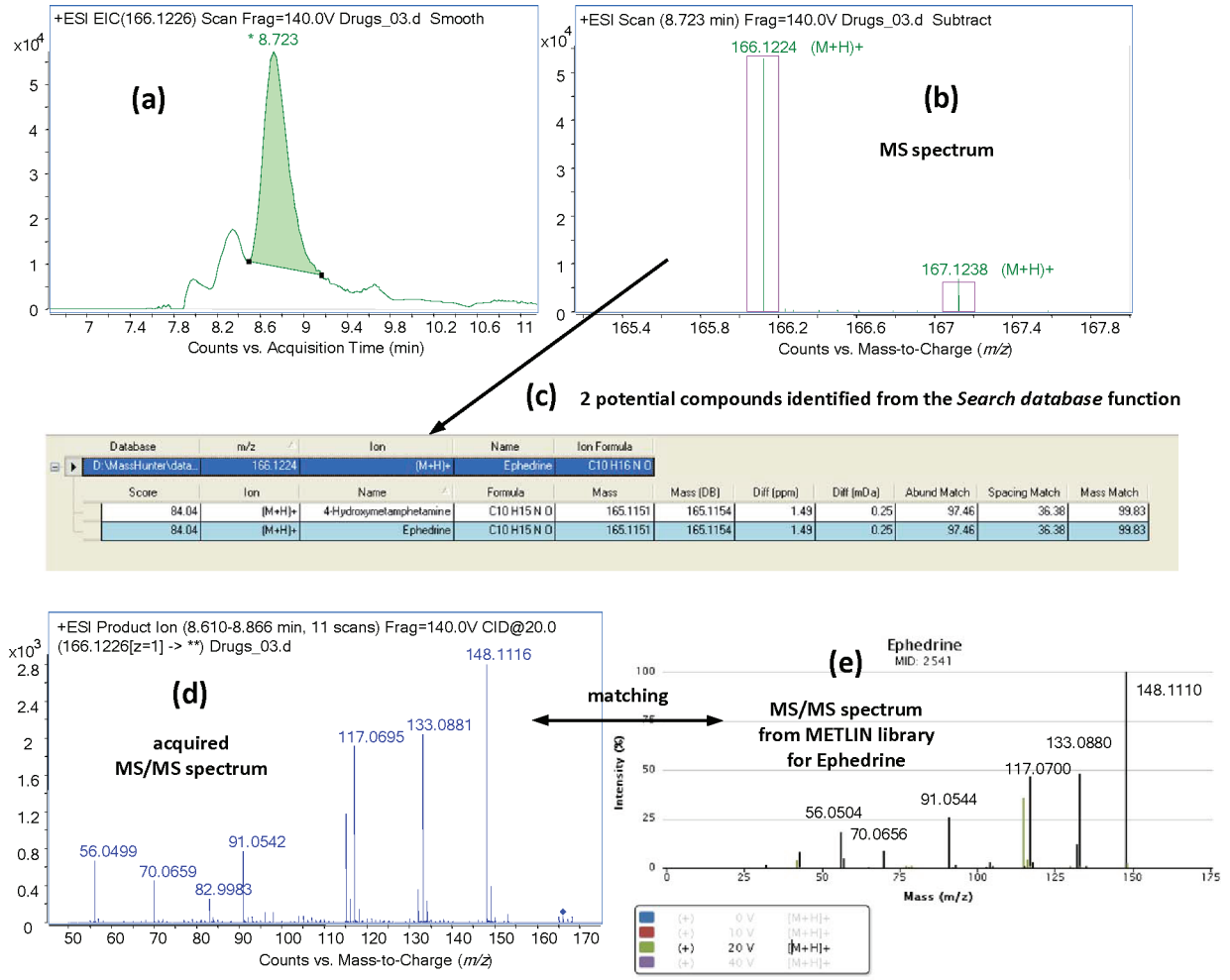

Figure 3. Identification workflow of ephedrine: (a) peak detected; (b) MS spectrum compared to database; (c) database match; (d) MS/MS spectrum acquired and contrasted with (e) METLIN library MS/MS spectrum for ephedrine.

which can provide relevant structural information necessary for structural confirmation.

Although the 4 GHz option is not recommended for quantitative operation due to detector saturation, leading to m/z shifts at high concentrations (as discussed in the first section of the Results and Discussion), this fact is compensated with the Mass Hunter Qualitative Analysis software for qualitative purposes, as saturated m/z peaks are automatically detected and their spectra automatically taken on the peak tails at a defined percentage below saturation, where mass accuracy is maintained. Therefore, as a first step, an influent wastewater extract was spiked with the 24 target compounds at two concentration levels (10 and 100 ng mL^{-1}, equivalent to 50 and 500 ng L^{-1} in the sample) and used as benchmark for the screening procedure at both 2 and 4 GHz. The results of this test showed that, at the highest spike level, 83% and 100% of the analytes were detected at 2 and 4 GHz, respectively, whereas at the lowest concentration only 50% and 62% of the compounds were positively identified (see Table S2 in the Supporting Information for details). These results highlight one of the main drawbacks of post-target screening: at low concentration levels, the chances to identify new drugs being consumed decreases. Moreover, the highest resolution provides greater possibilities of success in identifying post-target compounds than the 2 GHz mode, but then samples need to be reinjected.

In view of these results, influent samples were reinjected in the 4 GHz mode and the screening protocol applied. This methodology permitted the identification of ephedrine and ecgonine methyl ester in the influent samples, two substances already reported in wastewater.[28,47] As an example, Figure 3 shows the identification workflow for ephedrine. First, the extracted ion chromatogram was automatically generated by the software (Figure 3a) and its MS spectrum (Figure 3b) compared to the theoretical one. In this particular instance, there were two potential positive matches with the database (Figure 3c): 4-hydroxymetamphetamine and ephedrine, actually having the same empirical formula. Once candidates were detected, sample was reinjected and MS/MS spectra acquired at several collision energies. Then, in this case, the MS/MS spectra (Figure 3d) were compared to those available at the METLIN public library[54] (Figure 3e) so that the compound could be confirmed as ephedrine. In the case of ecgonine methyl ester (Figure S5 in the Supporting Information), no MS/MS spectra are available in the METLIN library, hence its structure was confirmed based on accurate product masses assignments and contrasted with the literature.[30]

On the other hand, an example of a compound initially identified as another potential drug or metabolites in the MS run and finally discarded on the basis of its MS/MS spectrum is

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presented in the Supporting Information (Figure S6). In this case, the MS/MS spectrum allowed the compound to be identified in the METLIN library as piperine, a natural alkaloid responsible for the pungency of pepper and other hot spices.

CONCLUSIONS

A new, selective SPE-LC–MS method for the simultaneous determination of 24 drugs of abuse and metabolites in wastewater samples was developed. Analytes were concentrated using mixed-mode Oasis MCX sorbents, improving the selectivity and LODs for basic drugs over other published SPE methodologies by adopting a fractionated elution strategy.

To the best of the authors' knowledge, a liquid chromatograph coupled to a hybrid QTOF mass spectrometer was employed for the first time for the quantification of drugs of abuse in waters. Although instrumental LOQs were, in some cases, higher than other values reported with QqQ systems, they were still low enough to allow the determination of several drugs and metabolites in real samples. Moreover, the high mass accuracy and resolution of the QTOF instrument permitted the single MS determination of opioids and better confirmation of low-mass amphetamine substances. Finally, the post-targeted capabilities of the QTOF system were used for the identification of originally nontargeted contaminants, such as ephedrine and ecgonine methyl ester.

ASSOCIATED CONTENT

Supporting Information

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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