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Fully Automated Determination in the Low Nanogram per Liter Level of Different Classes of Drugs of Abuse in Sewage Water by On-Line Solid-Phase Extraction-Liquid Chromatography−Electrospray-Tandem Mass Spectrometry

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The present work describes the first fully automated method, based on on-line solid-phase extraction (SPE)-liquid chromatography-electrospray-tandem mass spectrometry, developed for the determination of drugs of abuse (17 compounds and metabolites belonging to the classes of amphetaminics, cannabinoids, cocainics, opiates, and lysergics) in sewage waters. On-line SPE is performed by passing 5 mL of the water sample through a PLRP-s cartridge for analytes measured in positive ionization mode (all but cannabinoids) and through an Oasis HLB cartridge for analytes measured in negative ionization mode (cannabinoids). For unequivocal identification and confirmation two selected reaction monitoring transitions are registered per compound, thus achieving the four identification points requested by the European Union for banned substances. Quantitation is performed by the internal standard method, indispensable to correct for matrix effects. The main advantages of the method developed are high sensitivity (limits of determination between 0.69 and 5.97 ng/L), selectivity and reliability of results, minimum sample manipulation, full automation, and fairly high throughput (analysis time per sample is 2 × 35 min). As a part of the validation procedure, the method developed has been applied to the analysis of various influent and effluent samples from four Spanish sewage treatment plants.

In the last 5−6 years, various authors have proposed to analyze the content of drugs of abuse in river and sewage water as a method, alternative to the traditional ones, to estimate drug abuse by the population. Traditional methods used for this purpose are based on population surveys and social, medical, and crime statistics (e.g., number of arrests and detentions). These methods provide inaccurate, not real-time data by difficult, lengthy, expensive, and usually invasive means.[1] In contrast, the analysis of water provides real-time data, which allows the immediate adoption of appropriate measures by the responsible authorities, and is less expensive and anonymous (avoiding potential privacy conflicts).

This strategy was first proposed by Daughton in 2001.[1] Four years later, Zuccato et al. put it into practice for the first time to estimate cocaine abuse in the north of Italy.[2] Since then, various authors have supported this idea and have developed different analytical methods for the determination of various drugs of abuse in sewage and surface waters.[3−7] All the methods developed so far are based on off-line solid-phase extraction (SPE) followed by liquid chromatography−tandem mass spectrometry (LC−MS/MS) analysis and are rather straightforward. However, hyphenation makes it possible to develop fully automated methods, which are characterized by advantageous features (as compared to classical approaches), such as cost and time savings and improved analytical performance. In this context, the main objectives of this work were (1) to develop a fully automated method based on on-line SPE-LC−MS/MS for the multianalyte determination of the most relevant drugs of abuse and their metabolites in water, and (2) to apply this method to the analysis of various real sewage water samples in order to obtain a first, general picture about their environmental occurrence and patterns of consumption in a few selected locations.

A total of 19 compounds, belonging to 5 different classes, were initially investigated: 3 cocainics (cocaine (CO), its metabolite benzoylecgonine (BE), and the transesterification product cocaethylene (CE) formed when cocaine is taken together with

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ethanol), 5 amphetamine-like compounds (ALC) (amphetamine (AM), methamphetamine (MA), 3,4-methylenedioxymethamphetamine (MDMA or ecstasy), (*R*,*R*)(−)-pseudoephedrine (PS-EPH), and (1*S*,2*R*)(+)-ephedrine hydrochloride (EPH-HCl), the last two measured together as total ephedrine (EPH)), 5 opiates (heroine (HER), morphine (MOR), the hydrolyzed product of heroine 6-acetylmorphine (6ACM), and the conjugates morphine-3β-D-glucuronide (M3G) and morphine-6β-D-glucuronide (M6G)), 3 cannabinoids (Δ^9-tetrahydrocannabinol (THC) and its metabolites 11-nor-9-carboxy-THC (nor-THC) and 11-hydroxy-THC (OH−THC)), and lysergic acid diethylamide (LSD) and its metabolites nor-LSD and nor-iso LSD (nor-LSD), and 2-oxo-3-hydroxy-LSD (O-H-LSD).

Out of this selected list of compounds, 11 have been analyzed before in water and 8 are investigated for the first time. These are as follows: (*R*,*R*)(−)-pseudoephedrine and (1*S*,2*R*)(+)-ephedrine hydrochloride, heroin, morphine-6-glucuronide, the LSD metabolites nor-LSD and O-H-LSD, and THC and its metabolite OH-THC.

In addition, this new analytical proposal requires smaller sample volumes, only 5 mL, compared to conventional off-line solid-phase extraction methods that require between 100 and 500 mL of water.[3−7]

EXPERIMENTAL SECTION

Chemicals. High-purity (>97%) standard solutions of the target compounds were obtained from Cerilliant (Round Rock, TX) as solutions in methanol or acetonitrile. 6ACM, MOR, HER, BE, CO, CE, LSD, MA, MDMA, PS-EPH, EPH-HCl, and THC were provided at a concentration of 1 mg/mL; AM, M3G, M6G, nor-THC, OH-THC, O-H-LSD, and nor-LSD were supplied at a concentration of 100 μg/mL. Their molecular structure, CAS number, and molecular weight are shown in Figure 1.

Several deuterated compounds, also purchased from Cerilliant (Austin, TX) as solutions in methanol or acetonitrile at a concentration of 1 or 0.1 mg/mL, were used as surrogate standards (SS) for quantitation: benzoylecgonine-d_8 (BE-d_8), cocaine-d_3 (CO-d_3), cocaethylene-d_3 (CE-d_3), LSD-d_3, amphetamine-d_5 (AM-d_5), methamphetamine-d_{14} (MA-d_{14}), MDMA-d_5, (1*S*,2*R*)-ephedrine-d_3-hydrochloride (EPH-d_3), heroin-d_9 (HER-d_9), morphine-d_3 (MOR-d_3), morphine-3β-D-glucuronide-d_3 (M3G-d_3), and Δ^9-THC-d_3 (THC-d_3).

Individual stock solutions were prepared by diluting each analyte solution with methanol to a concentration of 5 μg/mL. Working standard mixtures were then prepared at different concentrations by appropriate dilution of the individual stock solutions in methanol (concentration of internal standards 20 ng/mL).

Stock and working standard solutions were stored at −20 °C in the dark. The standard mixtures were used as spiking solutions for preparation of the aqueous calibration standards and in the recovery studies.

HPLC-grade methanol, acetonitrile and water, and formic acid (98−100%) were acquired from Merck (Darmstadt, Germany). Ammonium formate ($CH_2O_2{\cdot}NH_3$) and ammonium acetate ($NH_4C_2H_3O_2$) were purchased from Sigma-Aldrich Chemie (Steinheim, Germany).

Equipment. Preconcentration of the samples was performed using an automated on-line SPE sample processor Prospekt-2 (Spark Holland, Emmen, The Netherlands) configured for high sample volumes. The system consists of an automated cartridge exchange (ACE) module, which holds two trays for up to 96 cartridges each one, and a high-pressure dispenser module for handling of solvents and samples by way of a 2-mL high-pressure syringe. The ACE unit is equipped with two clamps and two high-pressure valves, a configuration that permits the elution of a cartridge in one clamp while the following sample in a sequence is being loaded in another cartridge in the other clamp. The Prospekt-2 is controlled by means of SparkLink version T2.20-01 (Spark Holland).

Three different 10 mm × 2 mm i.d. disposable trace enrichment cartridges were evaluated for their efficiency in the on-line SPE of the target drugs of abuse from water: the polymeric cartridge Oasis HLB (macroporous polymer of divinylbenzene and *N*-vinylpyrrolidone, 30-μm particle size) from Waters (Barcelona, Spain), the polymeric phase PLRP-s (cross-linked styrene−divinylbenzene polymer, 15−25-μm particle size) from Spark Holland, and the silica-based cartridge Hysphere C18 EC (end-capped octadecyl phase, 8-μm particle size,) also from Spark Holland.

LC−MS/MS analyses were carried out in a system consisting of an Agilent HP 1100 pump (Agilent Technologies, Palo Alto, CA) equipped with an autosampler and connected in series with a 4000QTRAP hybrid triple quadrupole-linear ion trap mass spectrometer equipped with a Turbo Ion Spray source (Applied Biosystems-Sciex, Foster City, CA). The autosampler indicated above was used only in the optimization procedure to assess the absolute method recovery by comparing the peak areas obtained in the on-line analysis of spiked water samples with those obtained from the injection of standards mixtures of the analytes in methanol at equivalent concentrations.

For chromatographic separation, two different analytical columns were evaluated: a reversed-phase Purospher Star RP-18 end-capped column (125 mm × 2.0 mm, particle size 5 μm) from Merck (Darmstadt, Germany) and a Sunfire C18 (2.1 × 100 mm, 3.5 μm) from Waters (Milford, MA).

On-Line Trace Enrichment. On-line SPE preconcentration of all samples (previously filtered), aqueous standard solutions, and blanks was performed by loading 5 mL of the corresponding solutions at 1 mL/min through an Oasis HLB and a PLPR-s cartridge previously conditioned with 1 mL of acetonitrile and 1 mL of water (flow rate 1 mL/min). Oasis HLB is used for cannabinoids (THC and its metabolites OH-THC and nor-THC), which are analyzed in the negative ionization (NI) mode, and PLRP-s for all other compounds, which are analyzed in the positive ionization (PI) mode. After sample loading and prior to elution, the cartridges are washed with 1 mL of HPLC water at a flow rate of 1 mL/min to complete transfer of the sample and remove interferences such as inorganic salts.

Upon completion of each SPE protocol, which takes place in the left clamp of the Prospekt-2, the cartridge is moved to the right clamp where the trapped analytes are eluted to the LC column with the chromatographic mobile phase. Meanwhile, a new cartridge is placed in the left clamp, where preconcentration of the next sample in a sequence is simultaneously performed. This kind of configuration allows short cycle times, which in our approach are 35 min (the duration of the chromatographic run time). All steps of the sample preconcentration protocol are pro-

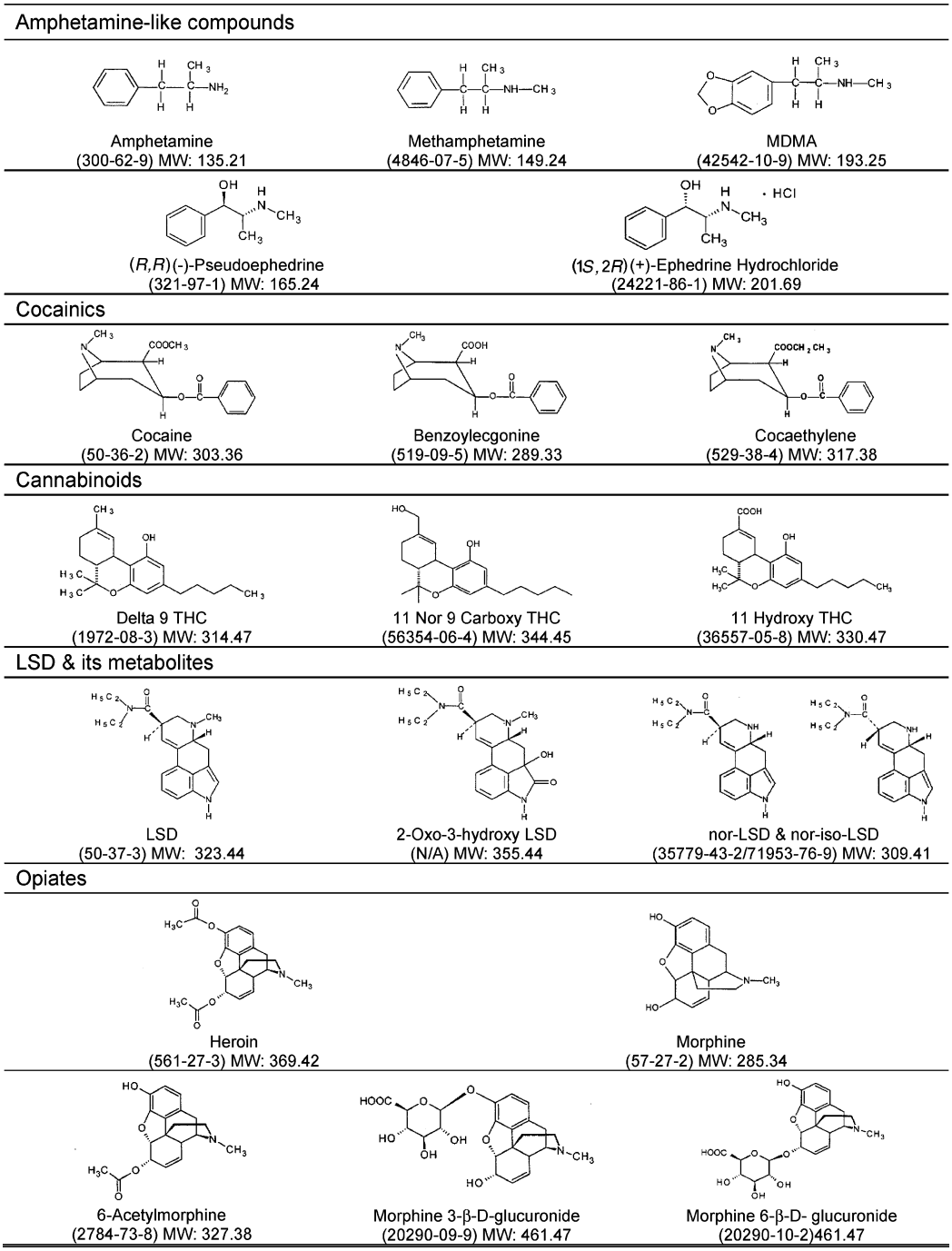

Figure 1. Molecular structure, CAS number, and molecular weight of the target analytes.

grammed on and automatically controlled by the Prospekt-2, which acts as an autosampler coupled to the LC−MS/MS instrument.

LC−ESI-(QqLIT) MS/MS Analysis. Chromatographic separation was performed with a Purospher Star RP-18 end-capped column (125 mm × 2.0 mm, particle size 5 μm) preceded by a guard column (4 × 4 mm, 5 μm) of the same packing material, both from Merck. Elution of the trapped analytes to the LC system was performed with a chromatographic mobile phase consisting of gradient acetonitrile/water (flow rate 0.3 mL/min). The proportion of the organic solvent was programmed to increase from 10 to 50% in the first 5 min and then to 100% in the following 13 min; afterward the column was cleaned with 100% acetonitrile for 5 min and finally readjusted to the initial conditions by programming the amount of organic solvent to 10% in 2 min. These conditions were held for 10 min to allow re-equilibration of the column before the next injection. The total time of chromatographic analysis is 35 min.

LC−ESI-MS/MS analysis of cannabinoids (extracted with Oasis HLB cartridges) is performed in the NI mode, and the analysis of the remaining compounds (extracted with PLRP-s cartridges) is performed in the PI mode. For quantitative analysis, and in order to get enough identification points to achieve analyte confirmation, data acquisition is performed in selected reaction monitoring (SRM) mode, recording the transitions between the precursor ion and the two most abundant product ions for each target analyte.

To achieve higher sensitivity, resolution at the first quadrupole (Q1) is fixed low and resolution at the third quadrupole (Q3) is set to unit. Settings for source-dependent parameters, common to both polarity modes, are as follows: curtain gas (CUR), 30 V; source temperature, 700 °C; nitrogen collision gas (CAD) high, and ion source gases 1 and 2 as default, 50 V each. Conversely, the ion spray voltage in the NI and in the PI modes is set to −4500 and 5500 V, respectively. In both cases, the pause between SRM transitions is 5 ms.

Instrument control and data acquisition and evaluation are performed with Analyst 1.4.2 software (Applied Biosystems).

Sample Collection and Treatment. One set of samples was collected from the major STP of Barcelona (El Prat STP). This plant is located at the mouth of the Llobregat River, which ends up in the Mediterranean Sea. It serves more than 2 million equivalent inhabitants from the southwestern metropolitan area of Barcelona and other towns located at the west side of the main city. At this plant, influent and effluent 24-h composite samples were collected every day during the first week of July 2007.

A second set of influent and effluent 24-h composite samples was collected on the 26th of July 2007 from three STPs of various selected tourist cities of the Autonomous Community of Valencia: Valencia, Benicasim, and Gandía.

All samples were taken in amber glass bottles, vacuum filtered through 1-μm glass fiber filters, followed by 0.45-μm nylon membrane filters (Whatman International Ltd., Maidstone, England), and stored in the dark at −20 °C.

RESULTS AND DISCUSSION

Sample Preparation and Preservation. In this fully automated methodology, sample handling is limited to the filtration step, performed to eliminate the particulate matter present in the samples, and the addition of the internal standard mixture. Due to the low sample volume requirements (5 mL), samples, even from extensive monitoring programs, can be stored in the freezer at −20 °C. This means of preservation, which halts any biological activity and avoids the risk of contamination or alteration of the sample nature due to the addition of preserving agents, gives the analyst a wide margin of time to carry out the extraction and analysis (months vs typically days or weeks in methods using simple cooling, addition of preservation agents, and storage of the samples in the cartridges or extracts after extraction).

LC Column and Mobile-Phase Composition Optimization. In the optimization of the best LC conditions, two different columns and three different mobile-phase compositions with varying flow rates (0.2, 0.3, and 0.4 mL/min) were tested.

Chromatographic separation is not a crucial issue when using MS/MS for detection because the probability of finding two compounds with the same retention time and the same SRM transitions is fairly low. However, in LC−MS/MS, an efficient LC separation is still important to avoid or minimize matrix effects as it is also important the selection of the mobile-phase composition to enhance the detector response.

Of the two columns tested, the Purospher STAR RP-18e was selected for separation because the MS/MS signals obtained for the target analytes MOR, BE, HER, 6ACM, O-H-LSD, and AM and the peak shape of ephedrine were better with this column than with the Sunfire C18, and the run time and analytes separation were similar in both.

For SPE elution and LC separation, the first mobile phase tested was gradient acetonitrile/water without the addition of modifiers. However, under these conditions, amphetamine-like compounds showed considerable peak tailing. In an attempt to solve this problem, two other mobile-phase compositions, selected from the literature, were tested: (1) a binary mobile phase consisting of (A) acetonitrile containing 0.1% formic acid and (B) HPLC water containing 30 mM formic acid, adjusted to pH 3.5 with ammonium formate;[6] (2) a binary mobile phase consisting of (A) methanol containing 0.1% formic acid and (B) HPLC water with 0.1 mM NH_4AC and 0.05% formic acid.[8]

Figure 2 shows the chromatograms obtained for some selected target analytes with all three mobile phases tested. As can be seen, the acidified acetonitrile/water mobile phase selected by Huerta-Fontela et al.[6] did not reduce the peak tailing observed for ALC and lowered the response obtained for many analytes like MOR, O−H-LSD, 6ACM, LSD, HER, and AM. Conversely, the acidified methanol/water mobile phase proposed by Applied Biosystems[8] reduced peak tailing slightly, but the detector response for some compounds was again lower (as compared to plain acetonitrile/water) and the MOR peak was markedly disturbed.

Based on the above observations, the mobile phase finally selected for analysis was gradient acetonitrile/water (without additives) with an optimized flow rate of 0.3 mL/min.

Optimization of MS/MS Conditions. Optimization of the different parameters influencing the MS signal was performed by on-column off-line injection (5 μL) of standard solutions of the individual target analytes and of mixtures of all of them. Selection of parent ions, ionization mode, and optimum ionization conditions was performed in full-scan mode at different values of declustering potential (DP). Out of the 19 drugs investigated, 16 showed higher response in PI mode and 3 (the cannabinoids) in NI mode. THC and O-H-THC can actually be measured in both ionization modes, but NI provides more intense MS signals.

Further identification of the most abundant fragment ions and selection of the optimum gas collision energies (CE) for each analyte were carried out in the product ion scan mode. Table 1 shows the most relevant (in terms of sensitivity) SRM conditions selected for each target and surrogate compound.

As shown in this table, the two ephedrine forms (PS-EPH and EPH-HCl) have equal retention time and fragmentation patterns thus not being possible their differentiation in the analysis; they were therefore quantified together as total ephedrine (EPH). Due to their similar fragmentation pattern, M3G and M6G also share the same SRM transitions; however, in this case, LC separation allows distinguishing between them. Both compounds show two chromatographic peaks (corresponding probably to two epimeric forms):[9] one peak at retention time (RT) 1.17 min, which is common to both compounds, and another peak at RTs 1.84 and 3.49 min for M3G and M6G, respectively, that can be used for their identification.

Due to the high sensitivity provided by the 4000Qtrap instrument, there is no need to create different time windows to improve detection sensitivity; i.e., all transitions are scanned during the whole analysis run time. Representative SRM chromatograms obtained from the analysis of an aqueous standard mixture of the

(8) Application Note 114AP46-01, Applied Biosystems/MDS SCIEX.

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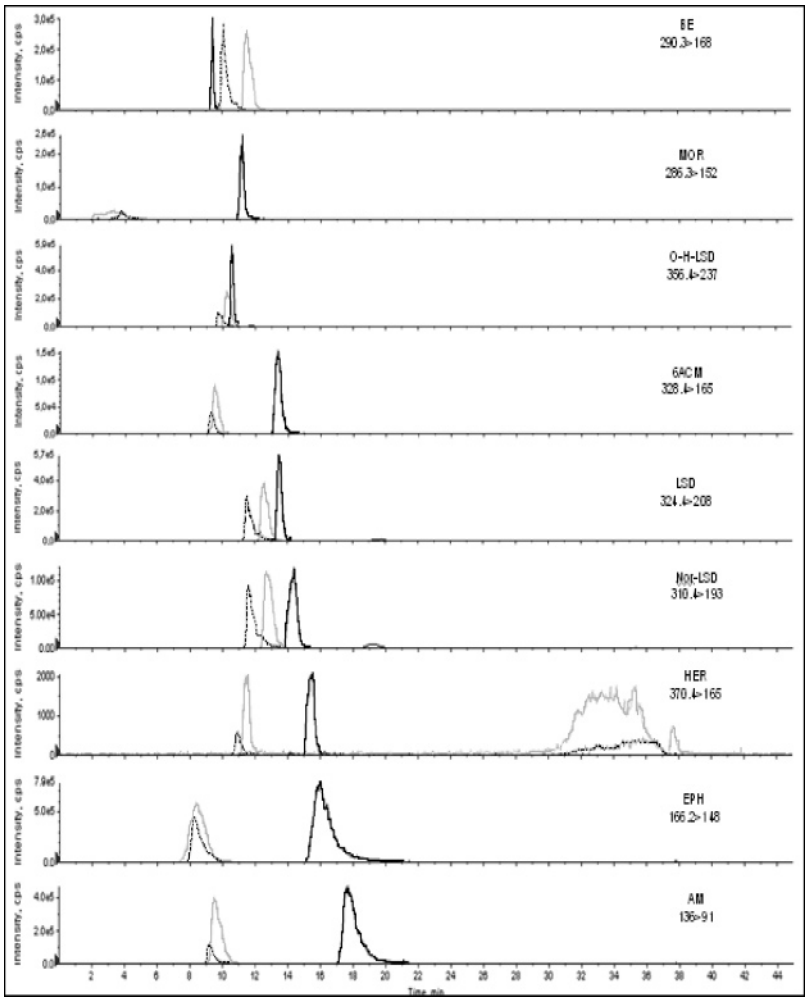

Figure 2. LC−MS/MS analysis of BE, MOR, O-H-LSD, 6ACM, LSD, nor-LSD, HER, EPH, and AM in a standard mixture (1000 ng/mL). Mobile phase: acetonitrile/water (black line), acidified acetonitrile/water[6] (dashed line), and acidified methanol/water[8] (gray line).

analytes at a concentration of 50 ng/L applying the optimum method conditions are illustrated in Figure 3.

SPE Optimization. The most important parameters affecting the extraction efficiency of a SPE procedure are the cartridge, the sample volume, and the sample loading flow rate.

In the optimization of the present SPE process, three different cartridges were evaluated: Oasis HLB, PLRPs, and Hysphere C18 EC. The extraction efficiency of each of these cartridges was estimated from the recovery percentage obtained for each target compound when loading 5 mL of HPLC water spiked with the analytes at 1000 ng/L (triplicate analysis). Based on these recoveries (see Figure 4) and on the preferential ionization (PI or NI mode) of the target analytes, Oasis HLB was selected for extraction of the cannabinoids measured in NI mode (actually, either Oasis HLB or PLRP-s could be used for this class of compounds since both show similar extraction efficiencies) and PLRP-s for extraction of all other compounds, measured in PI mode. Recoveries in the C18 cartridge were very low or zero for most compounds. M3G and M6G were not efficiently extracted by any of the tested cartridges and were thus not given further consideration as target analytes.

The sample volume (5 mL) was selected at the beginning of the optimization process as a compromise between sensitivity and matrix effects. Small sample volumes may compromise the method sensitivity, but large sample volumes may also affect very negatively the method sensitivity due to ionization suppression effects.

The sample loading flow rate may also affect the efficiency of the SPE process (the time of contact between the sample analytes and the sorbent surface, and thus the extraction efficiency, decreases with increasing flow rates), but in this particular case, optimization of this parameter was not necessary because even at the selected very low value of 1 mL/min, the time needed for the whole extraction procedure (10 min) is lower than that needed for the chromatographic analysis (35 min) performed simultaneously.

Method Performance. The performance of the method was evaluated through estimation of the linearity, sensitivity, repeatability, recovery, and matrix effects of the method.

Quantification, based on peak areas, was performed by the internal standard (IS) method. For each analyte, the corresponding, or the most similar in terms of structure, deuterated compound was used as internal standard. Seven to ten point calibration curves were constructed, using least-squares linear regression analysis, from application of the overall method to 5-mL aliquots of LC-grade water spiked with the analytes at concentrations ranging from 0.1 ng/L (or the limit of quantification if higher) to 1000 ng/L (5000 ng/L for BE and cocaine). The calibration

Table 1. Optimized SRM Conditions Used for On-Line SPE-LC−MS/MS Analysis of Drugs of Abuse in Sewage Water

target compounds	abbrev	retention time (min)	SRM transitions (m/z) precursor ion → product ion	DP[a] (V)	CE[b] (V)	SRM ratio (SRM1/SRM2)
Compounds Analyzed in Positive Ionization Mode						
(1*S*,2*R*)-(+)-ephedrine hydrochloride	EPH	9.06 ± 0.16	166.2 → 148.0	40	20	3.81 ± 0.47
			→ 133.0	30	30	
(*R*,*R*)-pseudoephedrine	EPH	9.06 ± 0.16	166.2 → 148.0	40	20	3.81 ± 0.47
			→ 133.0	30	30	
(1*S*,2*R*) (+)-ephedrine-d_3 hydrochloride	*EPH−d*$_3$	9.02 ± 0.18	169.3 → 151.0	40	20	–
amphetamine	AM	10.03 ± 0.17	136.2 → 91.0	30	20	1.68 ± 0.25
			→ 119.0	30	15	
amphetamine-d_5	*AM−d*$_5$	9.93 ± 0.19	141.2 → 96.0	30	20	–
MDMA	MDMA	10.84 ± 0.21	194.3 → 163.0	50	20	2.41 ± 0.13
			→ 105.0	50	35	
MDMA-d_5	*MDMA−d*$_5$	10.83 ± 0.19	199.2 → 135.0	40	35	–
methamphetamine	MA	10.82 ± 0.21	150.2 → 91.0	50	30	3.29 ± 0.42
			→ 119.0	50	20	
methamphetamine-d_{14}	*MA−d*$_{14}$	10.77 ± 0.21	164.2 → 98.0	50	30	–
benzoylecgonine	BE	7.00 ± 0.02	290.3 → 168.0	80	35	2.29 ± 0.15
			→ 77.0	70	100	
benzoylecgonine-d_8	*BE−d*$_8$	6.94 ± 0.02	298.2 → 171.0	80	30	–
cocaine	CO	13.73 ± 0.19	304.4 → 182.0	70	30	3.53 ± 0.43
			→ 77.0	70	90	
cocaine-d_3	*CO−d*$_3$	13.71 ± 0.22	307.4 → 185.0	70	25	–
cocaethylene	CE	14.52 ± 0.10	318.4 → 196.0	70	30	4.71 ± 0.66
			→ 77.0	70	95	
cocaethylene-d_3	*CE−d*$_3$	14.51 ± 0.16	321.4 → 199.0	70	30	–
2-oxo-3−hydroxy LSD	O−H−LSD	8.31 ± 0.02	356.4 → 237.0	50	35	2.10 ± 0.28
			→ 222.0	60	40	
nor−LSD, nor−iso-LSD	Nor−LSD	10.63 ± 0.10	310.4 → 193.0	60	40	0.37 ± 0.02
			→ 209.0	60	70	
LSD	LSD	10.52 ± 0.11	324.4 → 208.0	70	40	0.82 ± 0.05
			→ 223.0	60	40	
LSD-d_3	*LSD−d*$_3$	10.54 ± 0.13	327.4 → 226.0	60	35	–
morphine 6-β-D-glucuronide	M6G	–	462.5 → 286.0	80	45	–
			→ 201.0	80	65	
morphine 3-β-D-glucuronide	M3G	–	462.5 → 286.0	80	45	–
			→ 201.0	80	65	
morphine *3-β -D*-glucuronide-d_3	*MOR−d*$_3$	–	465.2 → 289.0	80	50	–
morphine	MOR	7.94 ± 0.06	286.3 → 152.0	90	75	1.54 ± 0.04
			→ 128.0	90	95	
morphine-d_3	*MOR−d*$_3$	7.83 ± 0.09	289.3 → 152.0	90	75	–
6−acetylmorphine	6ACM	9.50 ± 0.10	328.4 → 165.0	90	80	1.42 ± 0.10
			→ 152.0	90	75	
heroin	HER	11.04 ± 0.15	370.4 → 268.0	70	50	2.50 ± 0.06
			→ 165.0	70	70	
heroin-d_9	*HER−d*$_9$	10.98 ± 0.15	379.4 → 272.0	70	45	–
Compounds Analyzed in Negative Ionization Mode						
11-nor-9-carboxy-THC	Nor−THC	12.08 ± 0.04	343.5 → 299.5	−100	−35	5.45 ± 0.45
			→ 191.2	−100	−35	
11-hydroxy-THC	OH−THC	15.49 ± 0.03	329.5 → 311.2	−70	−25	7.69 ± 0.59
			→ 268.0	−70	−35	
Δ9-THC	THC	19.54 ± 0.05	313.5 → 245.1	−70	−40	1.13 ± 0.07
			→ 191.0	−70	−40	
Δ9-THC-d_3	*THC−d*$_3$	19.50 ± 0.02	318.4 → 196.0	−70	−40	–

[a] Declustering potential. [b] Collision energy.

curves obtained for both SRM1 and SRM2 were always linear with correlation coefficients (r^2) higher than 0.99 for all compounds (see Table 2).

The sensitivity is one of the method parameters usually enhanced in on-line systems. This is because the whole sample, instead of an aliquot of the final extract as in off-line protocols, is transferred to the chromatographic system. Thus, to achieve the present method sensitivity with a typical off-line procedure (where, for instance, the sample extract is reduced to 500 μL, from which 20 μL is injected in the LC−MS/MS system) the volume of sample to be extracted would have to be 125 mL (vs 5 mL on-line).

The limits of detection (LODs) and quantification (LOQs) of the present method were experimentally estimated from the on-line analysis of both influent STP water and spiked HPLC water as the concentration of analyte giving a signal-to-noise ratio of 3 and 8, respectively. The LODs and LOQs in HPLC water correspond to the average of the LODs estimated for the lowest concentration (0.1 ng/L) included in the calibration curve. Because the LODs and LOQs in sewage waters vary considerably from sample to sample, these limits have been calculated as the average of the LODs and LOQs, respectively, estimated for each of the sewage waters analyzed from the El Prat STP. In the case

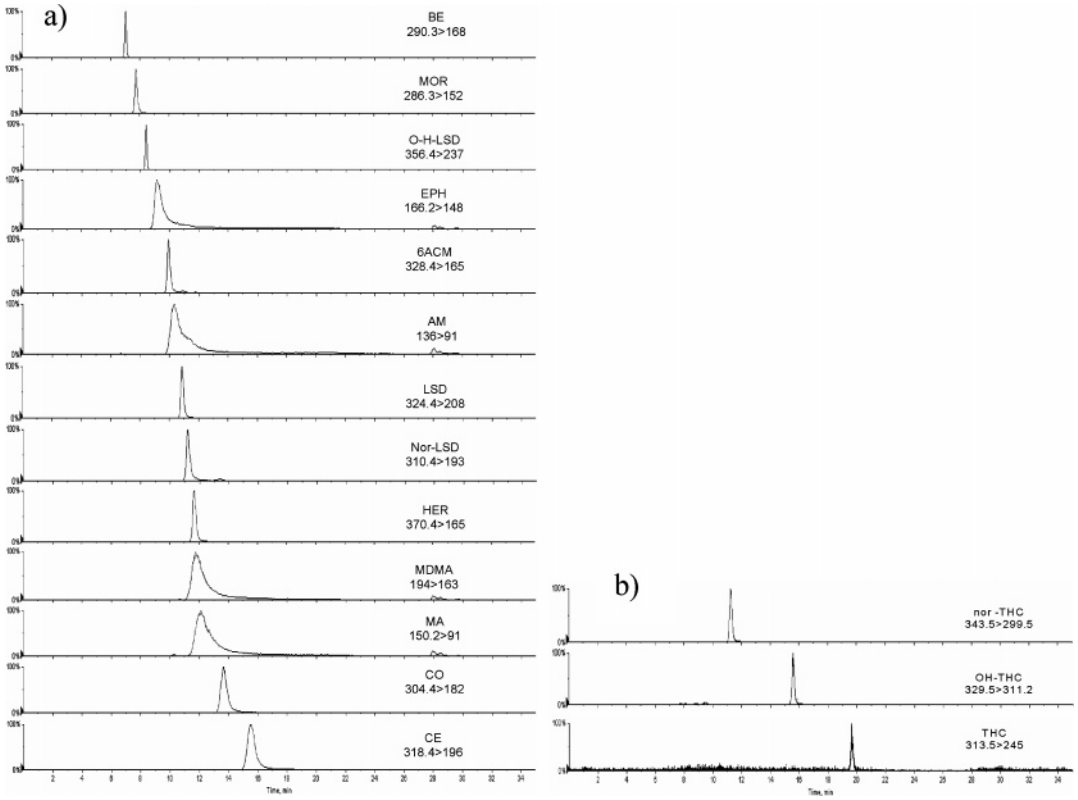

Figure 3. SRM chromatograms corresponding to the on-line analysis of an aqueous standard solution at a concentration of 50 ng/L: (a) analytes determined in PI mode; (b) analytes determined in NI mode.

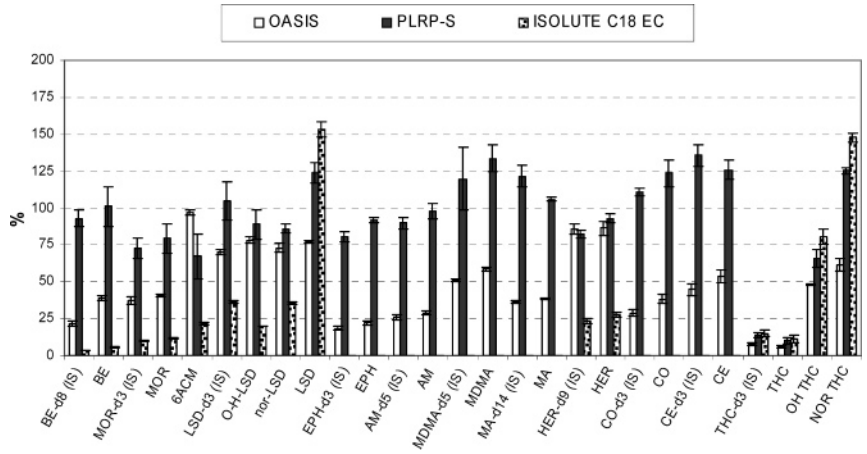

Figure 4. Comparison of the absolute recovery percentages and corresponding standard deviations obtained for the various target analytes in the replicate ($n = 3$) on-line SPE-LC−MS/MS analysis of spiked (1000 ng/L) HPLC water with Oasis HLB, PLRP-s, and HySphere C18 EC cartridges.

of THC and HER (undetected in all samples from the El Prat STP), the LOD and LOQ in sewage water were calculated from the on-line analysis of a randomly selected influent sewage water sample spiked with the standards at a concentration of 50 ng/L.

Table 2 shows the method LODs and limits of determination (minimum concentration of a compound that can be quantified (>LOQ, SRM1) and confirmed (>LOD, SRM2)) calculated for both influent sewage and HPLC water. Limits of determination in HPLC water were in the picogram per liter range for all compounds except THC (3.06 ng/L). This comparatively higher LOD for THC was not surprising, since this compound is the most hydrophobic of the various investigated and its ionization would be more efficient with other interfaces, different from electrospray, such as photoionization.[10] In sewage water, the sensitivity of the method was strongly affected. LODs and LOQs obtained in this matrix were found to be up to 75 times higher (depending on the compound) than in HPLC water. On average, the limits of determination in sewage water were 25 times higher than in HPLC water. The highest limit of determination in sewage water was obtained for MOR (5.97 ng/L). This marked difference is the result of pronounced matrix ionization suppression effects rather than, simply, higher background noise due to increased matrix complexity combined with insufficient detector selectivity.

(10) Cailleux, A.; Diquet, B.; Duretz, B.; Soares-Granja, J. Poster 042, Applied Biosystems/MDS SCIEX.

Table 2. Quality Control Parameters of the Analytical Method: Linear Correlation Coefficients (r^2), LOD, Limits of Determination (LDet), Repeatability (RSD), and Absolute (AR) and Relative Recoveries (RR) in HPLC Water and Influent Sewage Water

		HPLC water				sewage water				
	linearity r^{2a}	LOD[b] (ng/L)	LDet[c] (ng/L)	RSD[d] (%)	AR[e] (%)	LOD[b] (ng/L)	LDet[c] (ng/L)	RSD[d] (%)	AR[e] (%)	RR[f] (%)
EPH	0.9968	0.04	0.12	2.4	73	0.78	2.21	3.8	15	101
EPH-d_3(IS)					68				15	
AM	0.9990	0.07	0.20	2.3	85	0.34	0.92	12.4	15	94
AM−d_5(IS)					75				16	
MDMA	0.9994	0.05	0.14	8.2	121	1.10	2.93	9.1	27	121
MDMA-d_5(IS)					103				22	
MA	0.9979	0.03	0.08	10.4	97	0.28	0.75	2.7	20	114
MA-d_{14}(IS)					105				17	
BE	0.9941	0.01	0.02	8.2	98	0.67	5.24	2.5	8	115
BE-d_8(IS)					80				7	
CO	0.9974	0.01	0.04	8.7	85	0.18	2.40	11.7	59	173
CO-d_3(IS)					81				34	
CE	0.9945	0.01	0.04	4.2	120	0.07	0.69	6.5	52	105
CE-d_3(IS)					117				50	
O-H-LSD	0.9977	0.02	0.04	4.5	69	0.97	2.60	4.2	11	71
nor-LSD	0.9978	0.03	0.09	4.7	91	0.68	1.81	8.2	22	145
LSD	0.9975	0.01	0.02	8.2	112	0.27	0.89	3.9	17	107
LSD−d_3(IS)					96				15	
MOR	0.9974	0.04	0.10	3.4	69	1.51	5.97	2.2	14	77
MOR−d_3(IS)					60				18	
6ACM	0.9984	0.06	0.17	10	55	1.94	5.17	1.8	21	118
HER	0.9997	0.04	0.10	9.6	76	0.78	2.07	4.2	22	121
HER−d_9(IS)					67				18	
nor−THC	0.9949	0.05	0.12	1.4	93	0.43	1.13	7.0	13	266
OH−THC	0.9921	0.08	0.23	3.5	57	0.54	1.45	4.4	37	745
THC	0.9949	1.15	3.06	6.3	8	1.26	3.37	14.0	9	173
THC−d_3(IS)					8				5	

[a] Linearity. calibration range 0.1−1000 ng/L (0.1−5000 ng/L for BE and CO). [b] Limit of detection of the first SRM transition. [c] Limit of determination: minimum concentration that can be quantified (>LOQ, SRM1) and confirmed (>LOD, SRM2). [d] Relative standard deviation, spiking concentration: 50 ng/L ($n = 6$). [e] Calculated from the peak areas obtained in on-line analysis of spiked (50 and 1000 ng/L) water samples as percentages of the peak areas obtained from direct chromatographic injection (5 μL) of equivalent amounts of the standards in methanol (mean of the average results obtained at each concentration). [f] Relative to the associated deuterated surrogate standard.

Due to the similar detector response provided by the two SRM transitions selected per compound (SRM1/SRM2 ratio lower than 4 for all compounds but CE (4.75), OH-THC (7.69), and nor-THC (5.45)), the LODs and LOQs obtained for the second SRM transition (confirmation) are not too different from those obtained for the first SRM transition (quantitation). As a result, the method limits of determination remain fairly low. This is particularly important in the case of LSD that is the most potent psychoactive drug known and its metabolites because LSD doses (20−300 μg) are much lower than those of other drugs of abuse (in the mg range). These low levels make the detection of LSD and its metabolites much more difficult, as compared with other drugs, not only in the environment but also in biological fluids.[11]

Overall, the method limits of determination obtained in wastewater (between 0.69 and 5.97 ng/L) are in the same range of those reported by other authors, e.g., 0.48−8.7[4] and 0.2−2.1 ng/L.[6]

The overall method repeatability, calculated as the relative standard deviation (RSD) of the replicate ($n = 6$) analysis of HPLC grade and of sewage influent water spiked with a standard mixture of the analytes (50 ng/L), was satisfactory, with RSD values ranging between 1.4 and 10.4% in HPLC water and between 1.8 and 14.0% in sewage water (Table 2). The repeatability of results is one of the main advantages of automated on-line methods and is because manipulation of the samples and common intermediate steps of off-line methods are minimized or completely avoided.

Analyte Recoveries. Absolute recoveries in HPLC water and sewage water were calculated from the peak areas obtained for each analyte in the on-line analysis of spiked (50 and 1000 ng/L) water samples as percentages of the peak areas obtained from direct chromatographic injection (5 μL) of equivalent amounts of the standards in methanol. Relative recoveries in HPLC water and sewage water were determined from the absolute recoveries for each compound as percentages of the absolute recoveries of the associated surrogates.

Absolute and relative recoveries calculated at 50 ng/L were in general good agreement with those calculated at 1000 ng/L. As can be observed in Table 2, absolute recoveries in HPLC water were above 50% for all compounds but THC (the most apolar analyte, log $P = 7.60$) and the compounds already excluded from analysis, M3G and M6G.

On average, absolute recoveries in sewage water were ~10 times lower than in HPLC water (in most cases below 25%), which is attributed to matrix ionization suppression. These effects can be compensated with the use of appropriate surrogate standards (SS).

In both matrixes, the absolute recoveries of the target compounds and their associated deuterated surrogate standards

(11) Pizzolato, T. M.; Lopez de Alda, M. J.; Barceló, D. *Trends Anal. Chem.* **2007**, *26*, 609−624.

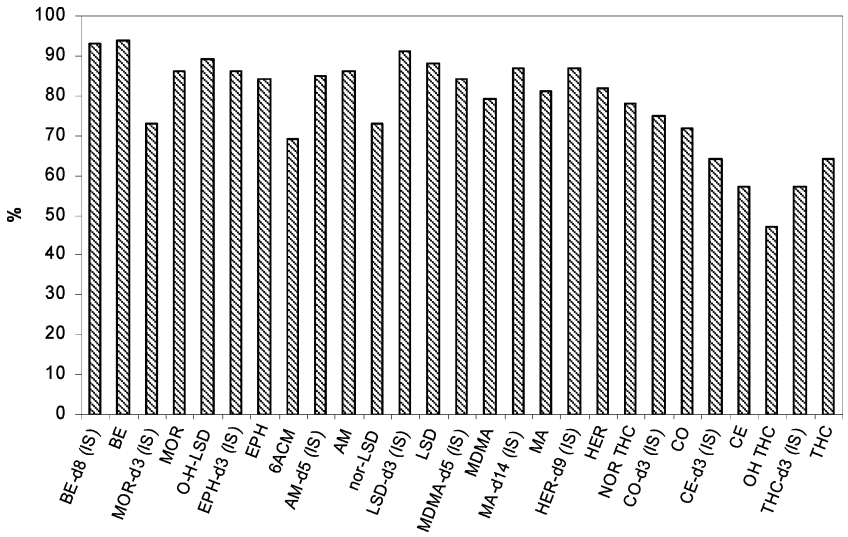

Figure 5. Matrix effects study. Bars show the percentage of signal reduction (ionization suppression) for each compound in influent sewage water compared to HPLC water.

were fairly similar (relative recoveries equal to 100 ± 25% for most compounds). This supports the theory that deuterated compounds behave in approximately (but not exactly) the same way as the corresponding nondeuterated compounds along the whole analytical procedure (including extraction, chromatographic separation, and response in the MS detector) and that their use as SS is therefore useful to correct for potential losses during sample manipulation and extraction as well as for matrix effects. What SS cannot correct is the loss of sensitivity due to ionization suppression.

In the absence of deuterated compounds for all target analytes, 6ACM was quantified using the structurally related compound MOR-d_3 as SS (relative recovery in sewage water 118%), LSD-d_3 was used to quantify the LSD metabolites O−H-LSD and nor-LSD (relative recoveries 71 and 145%, respectively) and THC-d_3 was used to quantify the THC metabolites OH-THC and nor-THC (relative recoveries 745 and 266%, respectively).

However, in this respect, it may be worth mentioning that an advantage of on-line SPE procedures is that correction for extraction recoveries is automatically performed because both the samples and aqueous standards are processed in exactly the same way through the whole analytical procedure.

Matrix Effects. Matrix effects were evaluated by comparing the peak areas obtained from the on-line analysis of a randomly selected influent sewage water sample spiked with the analytes at 1000 ng/L (after subtraction of the peak areas corresponding to the native analytes present in the sample) with those obtained from the on-line analysis of spiked (1000 ng/L) HPLC water. In the absence of matrix effects, the analytes' peak areas should be similar in both types of matrixes, whereas in the presence of matrix effects, the former are greater or lower than the latter depending on whether there is signal enhancement or suppression, respectively. In our case, all compounds were subject to matrix ionization suppression effects. These effects were quantified according with the following equation:

$$\text{signal suppression (\%)} = 100 - ((\text{Area}_{\text{sp sw}} - \text{Area}_{\text{sw}}) \times 100/\text{Area}_{\text{sp HPLC}})$$

where $\text{Area}_{\text{sp sw}}$ is the analyte peak area in the spiked sewage water sample, Area_{sw} is the analyte peak area in the nonspiked sewage water sample (if any), and $\text{Area}_{\text{sp HPLC}}$ is the analyte peak area in the spiked HPLC water sample. As shown in Figure 5, the percentage of signal reduction varied from 47% for OH-THC to 94% for BE. In general, matrix effects were shown to be considerably lower in effluent sewage samples (as compared to influent sewage) and to decrease with increasing chromatographic retention time; i.e., the first eluting (more polar) compounds experienced comparatively higher signal suppression probably due to the inefficient removal of matrix coeluting interferences, such as humic acids. Quantitation by the external standard method would have led in this case to inaccurate, lower than real, results. However, with the present methodology, the varying matrix effects and recoveries observed can be compensated through the use of the SS for quantitation, on the one hand, and the already mentioned automatic correction of extraction recoveries, on the other.

Confirmation Criteria. Identification of the target analytes is accomplished by comparing the retention time and the LC−MS/MS signals of the target compounds in the samples with those of standards analyzed under the same conditions. In order to avoid false positives, it is essential to monitor at least two different characteristic SRM transitions per compound and to consider the following confirmation criteria:[13,14]

(1) LC retention must be within ± 2% the retention time of the standard compound. In cases when this limit has been occasionally surpassed, the identification/confirmation has been deemed positive if the corresponding IS showed similar retention time deviation. This has been observed occasionally in the analysis of CE, cocaine, and ALC.

(2) The relative abundances of the two selected analyte SRM transitions in the sample must be within ±20−50% of the ions

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(13) Council of the European Communities, Commission Decision 93/256/EEC, *Official J. Eur. Commun.* 1993; L 118:1,
(14) Council of the European Communities, Commission Decision 2002/657/EC, *Official J. Eur. Commun.* 2002; L 221:8.

Table 3. Concentrations (ng/L) of Drugs of Abuse in Influent and Effluent Samples from Various STPs Located at the East Coast of Spain

	STP							
	EL PRAT (BARCELONA)[a]		VALENCIA		BENICASSIM		GANDÍA	
	INF (ng/L)	EFF (ng/L)	INF (ng/L)	EFF (ng/L)	INF (ng/L)	EFF (ng/L)	INF (ng/L)	EFF (ng/L)
EPH	591.9 ± 62.3	117.8 ± 17.3	394.0	266.0	444.0	138.0	360.0	162.6
AM	41.1 ± 9.1	**0.5 ± 0.1**	20.4	2.2	35.5	1.0	6.5	3.3
MDMA	133.6 ± 29.8	82.1 ± 22,2	113.0	38.2	245.0	376	47.4	30.3
MA	18.2 ± 5.8	6.30 ± 0.6	7.8	2.7	3.7	2.0	3.0	1.5
BE	4225.7 ± 1142.8	30.3 ± 17.6	1900.0	220.0	1450.0	49.9	1020.0	318.0
CO	860.9 ± 213.6	6.2 ± 3.7	651.0	33.2	540.0	83.6	316.0	105.0
CE	77.5 ± 33.2	1.7 ± 1.2	89.2	3.7	97.2	2.1	49.2	6.8
O−H−LSD	5.6 ± 12.1	0.7 ± 0.3	2.6	**0.8**	4.2	n.d.	n.d.	n.d.
nor−LSD	4.3 ± 1.8	0.6 ± 0.5	22.1	4.0	13.0	**1.5**	5.3	**1.3**
LSD	2.8 ± 1.2	0.3 ± 0,2	4.7	1.6	3.0	**0.6**	1.1	**0.2**
MOR	162.9 ± 20.0	21.8 ± 3.0	75.1	18.8	66.7	11.8	62.6	29.7
6ACM	12.8 ± 3.1	3.6 ± 0.5	10.5	**3.0**	8.8	**2.0**	5.9	**2.5**
HER	n.d.	n.d.	**2.3**	**1.2**	**2.4**	n.d.	n.d.	n.d.
nor−THC	4.3 ± 7.8	8.4 ± 3.8	13.8	3.9	32.5	19.0	16.8	10.8
OH−THC	8.4 ± 2.1	4.8 ± 1.9	37.2	23.0	77.6	14.1	24.2	8.0
THC	n.d.	n.d.	22.2	20.5	39.4	13.0	13.8	n.d.

[a] Average ± standard deviation of seven samples collected daily during one week. [b] nd, nondetected.

ratio produced by the standards.[14] The relative abundances of the SRM transitions monitored for each compound are provided in Table 1.

In principle, the analyte SRM transition providing the highest MS/MS signal, and therefore the best signal-to-noise ratio and LOD in HPLC water, was selected for quantitation. However, in the analysis of the sewage water samples investigated, it was observed that, for some compounds, namely, LSD and nor-LSD, the second most abundant transition was most appropriate for quantitation because it was more selective (less affected by matrix interferences) and provided better LODs than the most abundant one, which explains why for these compounds the SRM1/SRM2 ratio is lower than 1.

Drug of Abuse Levels in Real Samples. Table 3 lists the results obtained from the analysis of the various influent and effluent samples collected at the STPs located at the east coast of Spain, specified in the Experimental Section. As can be seen, some of the reported values are below the method limit of determination calculated for influent sewage samples. This is because each compound and sample is considered on a case-by-case basis and levels above the limit of determination for each compound and sample are thus considered valid. Nevertheless, to differentiate between the values above and below the method limit of determination, the latter are presented in the table in bold.

All target analytes were found to be present in the samples investigated; however, not all of them were found in every STP sampled. As an example, Figure 6 shows the analysis of an effluent sample collected in the STP of El Prat.

In the influent samples, the levels of the drugs of abuse classes monitored increased in the order, LSD and metabolites < cannabinoids < opiates < amphetamine-like compounds < cocainics.

The STP from Barcelona showed distinctly higher levels of cocainics and morphine and lower levels of cannabinoids as compared to the STPs of the Autonomous Community of Valencia.

CO and its metabolites BE and CE were found in 100% of the influent and effluent samples analyzed. BE was the most abundant compound within this group, with concentrations, on average, 4 and 32 times higher than those of CO and CE, which confirms it as a good indicator of cocaine consumption.

Amphetamine-like compounds were found at comparatively lower concentrations than cocainics and showed fairly similar levels in both areas (Barcelona and the Autonomous Community of Valencia). Of the various compounds investigated, the most prevalent in influent sewage water was ephedrine (never studied before), followed by MDMA (ecstasy), amphetamine, and methamphetamine. However, ephedrine has different medical applications (treatment of acute hypertension, rinitis, sinusitis, depressive states, etc.) that can contribute to their presence in sewage waters. Huerta-Fontela et al.[6] investigated the occurrence of the MDMA metabolites, MDA (3,4-methylenedioxyamphetamine) and MDEA (methylenedioxyethamphetamine). However, MDA was not detected in any of the samples investigated and MDEA was found in only one sample and at lower concentrations than MDMA. Other potentially useful indicators of amphetamine-like compounds consumption, other than the parent compounds MDMA, AM, and MA, could be their conjugated metabolites (not yet investigated).

In general, the levels found in this study for both cocainics and amphetamine-like compounds are higher than those previously reported by other authors.[4−6]

Opiates showed distinctly higher concentrations in Barcelona than in the Autonomous Community of Valencia. Of the various compounds investigated, the most abundant was MOR, followed by 6ACM and HER. The presence of morphine is most likely due to its use as a potent analgesic in medicine, rather than to drug abuse. HER, which had not been investigated before in water, was found in only a few samples and at very low concentrations (1.2−2.4 ng/L).

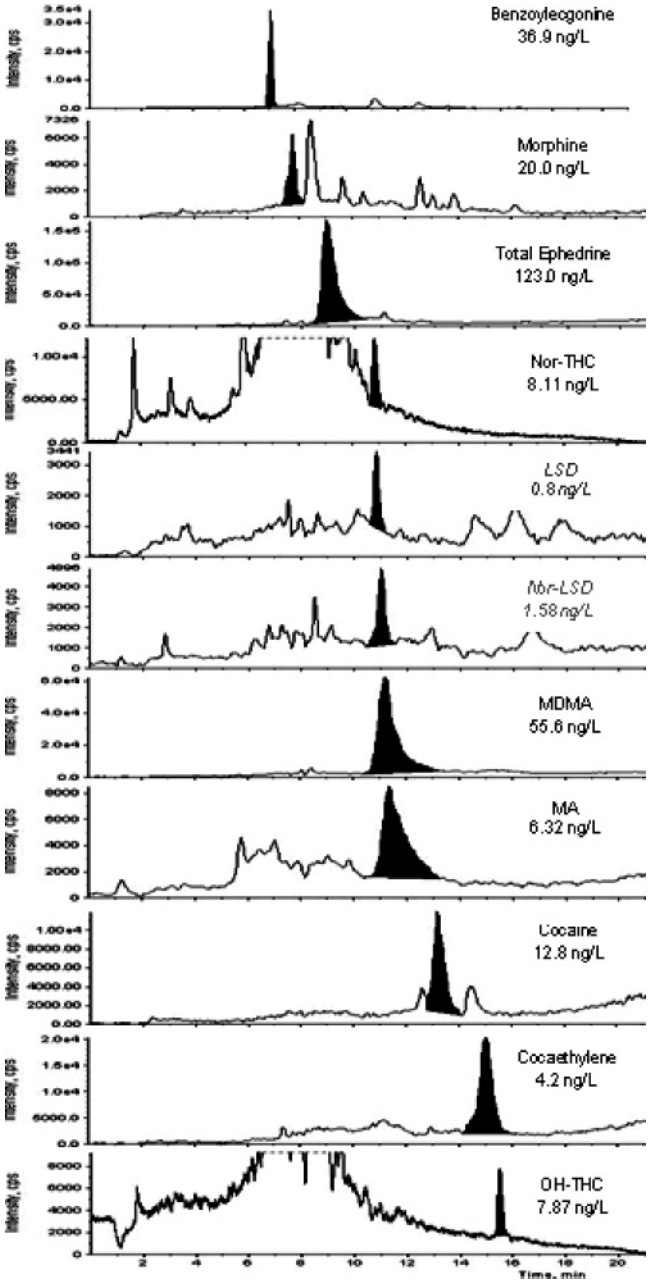

Figure 6. Analysis of an effluent sample collected at the El Prat STP. Only drugs positively identified and quantified are shown.

Cannabinoid concentrations were always below 100 ng/L and comparatively higher in the Valencian STPs than in the STP from Barcelona. The relative abundance of the parent compound THC and its metabolites nor-THC and OH-THC varied depending on the sample. On average the most abundant compound in the influent samples was OH-THC (37 ng/L), followed by nor-THC (17 ng/L) and THC (14 ng/L), and in the effluent samples THC (16.75 ng/L), but the differences are not very relevant; therefore, none of them can be pointed out as a single, clear indicator of cannabinoid consumption. Previous works had only analyzed nor-THC, finding higher concentrations (63−91 ng/L) in influent water[4] than in the present study (17 ng/L).

Taking into account that LSD is the most potent psychoactive drug known and that the LSD doses (20−300 μg) are much lower than those of other drugs of abuse (in the mg range), the low concentrations found for LSD and its metabolites (below 25 ng/L) were not surprising. Unlike LSD, its metabolites nor-LSD and O-H-LSD had not been investigated before in water; however, according to the results of the present study, nor-LSD (found at higher concentrations always than LSD) could be a better indicator of LSD consumption than LSD itself.

Average STP removal was between 95% for cocainics and 32% for cannabinoids. MDMA and nor-THC were occasionally found at higher concentrations in the effluent than in the corresponding influent. This finding, which has been reported also by Castiglioni et al.,[4] could be attributed to either desorption processes during wastewater treatment or, in the case of cannabinoids, deconjugation of the glucuronide forms in which they are partially excreted (11-nor-9-carboxy THC glucuronide (THC-COOH-glucuronide)).

CONCLUSIONS

In this work, the first fully automated method for the determination of different classes of drugs of abuse and their metabolites in sewage water ever described and the one covering the largest number of compounds (17 in total), some of them investigated for the first time, has been presented.

On-line methodologies allow considerably shortening of the overall analysis time and reducing the sample volume required, providing the same sensitivity levels achieved with off-line SPE. The use of small volumes, 5 mL in our method, facilitates sample preservation and storage in a freezer when immediate analysis is not possible. Sample pretreatment includes only filtration and addition of surrogate standards. Another advantage of on-line methods is that the intermediate evaporation steps typical of off-line procedures are avoided, with the corresponding time and cost savings. This is particularly important when amphetamine-like compounds are to be analyzed because these compounds may be lost during the evaporation step,[11,12] thus leading to inaccurate results (unless appropriate internal standards are used).

Despite the low sample volumes used, the method developed is sensitive enough to detect concentrations of drugs in the low nanogram per liter and even in the picogram per liter level in sewage waters. The use of two SRM transitions per compound (affording the required four identification points for controlled substances) and of deuterated surrogate standards for quantification and to correct potential matrix ionization suppression effects is essential to ensure obtaining reliable results.

Application of the method to influent and effluent water samples collected at various STPs has shown the presence of BE at microgram per liter levels, of CO, CE, EPH, MDMA, and MOR at high nanogram per liter levels, and of AM, MA, HER, 6ACM, and cannabinoids at low nanogram per liter levels. Overall, effluent samples show the lowest concentrations of the studied compounds, although in some cases, negative removals of specific compounds (nor-THC and MDMA) have been observed.

These data together with those coming from the application of the present methodology to other surveys are now being analyzed to deduct geographical and temporal drug consumption rates and patterns as well as STP removal efficiencies.

The main advantages of the proposed approach over official methods (surveys, etc.) are real-time information, accuracy, and cost-efficiency and, over previously published LC−MS/MS meth-

ods, automation, minimum sample manipulation, easy sample storage and preservation, and reliability of results.

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