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Simultaneous ultra-high-pressure liquid chromatography–tandem mass spectrometry determination of amphetamine and amphetamine-like stimulants, cocaine and its metabolites, and a cannabis metabolite in surface water and urban wastewater☆

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ABSTRACT

An ultra-high-pressure liquid chromatography–tandem mass spectrometry (UHPLC–MS/MS) method has been developed for the simultaneous quantification and confirmation of 11 basic/acidic illicit drugs and relevant metabolites in surface and urban wastewater at ng/L levels. The sample pre-treatment consisted of a solid-phase extraction using Oasis MCX cartridges. Analyte deuterated compounds were used as surrogate internal standards (except for norbenzoylecgonine and norcocaine) to compensate for possible errors resulting from matrix effects and those associated to the sample preparation procedure. After SPE enrichment, the selected drugs were separated within 6 min under UHPLC optimized conditions. To efficiently combine UHPLC with MS/MS, a fast-acquisition triple quadrupole mass analyzer (TQD from Waters) in positive-ion mode (ESI+) was used. The excellent selectivity and sensitivity of the TQD analyzer in selected reaction monitoring mode allowed quantification and reliable identification at the LOQ levels. Satisfactory recoveries (70–120%) and precision (RSD < 20%) were obtained for most compounds in different types of water samples, spiked at two concentration levels [limit of quantification (LOQ) and 10LOQ]. Thus, surface water was spiked at 30 ng/L and 300 ng/L (amphetamine and amphetamine-like stimulants), 10 ng/L and 100 ng/L (cocaine and its metabolites), 300 ng/L and 3000 ng/L (tetrahydrocannabinol-COOH). Recovery experiments in effluent and influent wastewater were performed at spiking levels of three and fifteen times higher than the levels spiked in surface water, respectively. The validated method was applied to urban wastewater samples (influent and effluent). The acquisition of three selected reaction monitoring transitions per analyte allowed positive findings to be confirmed by accomplishment of ion ratios between the quantification transition and two additional specific confirmation transitions. In general, drug consumption increased in the weekends and during an important musical event. The highest concentration levels were 27.5 μg/L and 10.5 μg/L, which corresponded to 3,4-methylenedioxymethamphetamine (MDMA, or ecstasy) and to benzoylecgonine (a cocaine metabolite), respectively. The wastewater treatment plants showed good removal efficiency (>99%) for low levels of illicit drugs in water, but some difficulties were observed when high drug levels were present in wastewaters.

1. Introduction

The consumption of alcohol and illicit drugs during weekends and special events (e.g. concerts, festivals, local festivities) is significantly higher than on weekdays. Generally, drug consumption by individuals has been discovered by analyzing biological fluids (e.g., blood, serum, oral fluids, or urine) [1–4]. However, the estimation of drug consumption of an audience during these events is rather complicated and unreliable, due to the voluntary participation of the consumers in these specific analysis studies. An approach to obtain reliable data to monitor and estimate the consumption of illicit drugs of a population was proposed by Daughton in 2001 [5] and put into practise in 2005 by Zuccato et al. [6]. This approach, consisting of the determination of illicit drugs in urban wastewaters, got worldwide attention by the media and has been supported by various scientists [7–14], becoming a major issue nowadays. Information on accurate real-time data is of interest, both in environmental and social scientific studies. Results may also contribute

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to the evaluation of the removal efficiency of wastewater treatment plants (WWTPs) [11], to the understanding of the potential impact of these compounds on the aquatic ecosystem [15], and can help social scientists and authorities to combat drug abuse.

The analysis of illicit drugs in wastewater and surface water has been recently reviewed by Castiglioni et al. [16]. The authors pointed out that the principal difficulty deals with the low concentration levels of drugs in combination with the complexity of the matrix. Developed analytical methods are based on solid-phase extraction (SPE), for sample pre-treatment and pre-concentration, and the analytical technique of choice is liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS).

Drugs to be analyzed have been generally extracted using Oasis SPE polymer cartridges built of a balanced mixture of hydrophilic and lipophilic (HLB) monomers [8,9,11], or Oasis MCX, a strong cation-exchange mixed mode polymeric sorbent built upon HLB copolymers [6,7]. The different properties (acid, neutral or basic) of the illicit drugs make the selection of SPE cartridges of great importance. Besides, the optimization of SPE process is required to avoid analyte losses, but SPE pre-concentration, which is normally required to obtain the intended sensitivity, can also concentrate matrix interferences resulting in signal enhancement or suppression. In this way, the presence of matrix effects is found to be one of the main factors affecting the sensitivity of the established analytical method [17]. The addition of appropriate internal standards is one of the best approaches to compensate for matrix effects, especially when using analyte isotope labelled internal standard, as one expects that the internal standard is affected by matrix effects in the same way than the analyte [7,12,18–20]. When the internal standard is used as surrogate (i.e. added to the sample previously to sample treatment), it can also compensate for potential analytical errors associated to the sample manipulation.

In relation to LC–MS/MS methodology for the determination of illicit drugs, recent papers have reported methods using ultra-high-pressure liquid chromatography (UHPLC) coupled to MS/MS for ultra fast separations and reliable determination of these analytes [20–22]. Besides ultra fast separation, UHPLC allows better resolution, largely due to advancements in the particle size and bridging structure of the column packing. This advantage is important in terms of sample throughput and sensitivity improvement. Moreover, the use of MS/MS with triple quadrupole (QqQ) analyzers allows to minimize, or in some cases to eliminate, matrix interferences, improving selectivity and sensitivity due to the possibility of adequate precursor and product ion selection, leading to low chemical noise in the chromatograms. Thus, UHPLC–MS/MS QqQ in selected reaction monitoring (SRM) mode is considered nowadays as one of the most selective and sensitive techniques for the quantification and confirmation of organic contaminants in environmental samples and it is highly suitable for trace determination of drugs of abuse in water. However, the applications cited above do not take full advantage of UHPLC separations, because fast scanning triple quadrupole was not used. Therefore longer separations were needed.

The confirmation of the identity of compounds detected in samples, even at the limit of quantification (LOQ) level, is of great importance in order to avoid the reporting of false positives. Different approaches can be used for confirmation, most of them based on the collection of analyte mass information. One of the most frequently used confirmation criteria [23] is based on the collection of identification points (IPs), which are earned depending on the mass analyzer used. Regarding MS/MS analysis with low resolution instruments, as QqQ, a minimum of two SRM transitions should be monitored for a safe positive finding, together with the measurement of the ion ratio between both recorded transitions. However, the quality of the transitions should be taken into account, as acquiring non-specific transitions might lead to report false positives or even false negatives, because of the non-compliance of the expected ion ratios [18,24].

The aim of this work is to develop and validate a rapid and sensitive method for the simultaneous determination of amphetamine and amphetamine-like stimulants, cocaine and its metabolites, and a cannabis metabolite in surface water and in urban wastewater. The developed method, using Oasis MCX SPE cartridges for clean-up and pre-concentration, followed by UHPLC–MS/MS measurement, has been applied to the analysis of 24-h composite effluent and influent urban wastewaters samples from a WWTP of the Castellón province (Spain). Daily 24-h composite samples were collected along two complete weeks, one in June and another one in July 2008, during an important rock event. Special emphasis is placed on the reliable confirmation of illicit drugs detected in water, so that three SRM transitions have been acquired to simultaneously detect, quantify and confirm positive samples.

2. Experimental

2.1. Chemicals and materials

Illicit drugs and metabolites studied were the following: amphetamine, methamphetamine, 3,4-methylenedioxyamphetamine (MDA), 3,4-methylenedioxymethamphetamine (MDMA, or ecstasy), 3,4-methylenedioxyethylamphetamine (MDEA), cocaine, cocaethylene, benzoylecgonine, norbenzoylecgonine, norcocaine, and 11-nor-9-carboxy-Δ^9-tetrahydrocannabinol (THC-COOH), a relevant metabolite of Δ^9-tetrahydrocannabinol (cannabis). These compounds were obtained from Sigma–Aldrich (Madrid, Spain), Cerilliant (Round Rock, TX, USA) and the National Measurement Institute (Pymble, Australia) as solutions in methanol, acetonitrile or as salt. Some features of the studied compounds such as chemical structure, mono-isotopic molecular weight and CAS number are shown in Fig. 1. Standard stock solutions of each compound were prepared at 100 mg/L in methanol or acetonitrile. Intermediate solutions (10 mg/L) were prepared by diluting the stock solution ten times with methanol. Infusion solutions of individual standards were prepared at a concentration of 1.5 mg/L in methanol:water (50:50, v/v) just before infusion experiments. Mixed working solutions containing all analytes were prepared from intermediate solutions at different concentrations by appropriate dilution with water, and were used for preparation of the aqueous calibration standards and for spiking samples in the validation study.

Deuterated compounds were all purchased from Cerilliant as solutions in methanol or acetonitrile at a concentration of 100 mg/L and were used as surrogate internal standards for quantification: [$^2H_6$]amphetamine (amphetamine-d_6), [$^2H_5$]methamphetamine (methamphetamine-d_5), [$^2H_5$]3,4-methylenedioxyamphetamine (MDA-d_5), [$^2H_5$]3,4-methylenedioxymethamphetamine (MDMA-d_5), [$^2H_5$]3,4-methylenedioxyethylamphetamine (MDEA-d_5), [$^2H_3$]benzoylecgonine (benzoylecgonine-d_3), [$^2H_3$]11-nor-9-carboxy-Δ^9-tetrahydrocannabinol (THC-COOH-d_3), [$^2H_3$]cocaine (cocaine-d_3) and [$^2H_8$]cocaethylene (cocaethylene-d_8). A mixed surrogate standard working solution at 100 μg/L (except: THC-COOH-d_3 at 1 mg/L) was prepared in water.

All standard solutions were stored in amber glass bottles at −20 °C.

HPLC-grade methanol, ammonia solution (25%) and formic acid (98–100%) were acquired from Scharlau (Barcelona, Spain). HPLC-grade water was obtained by purifying demineralised water in a Milli-Q plus system from Millipore (Bedford, MA, USA).

SPE cartridges, mixed reversed-phase/cation-exchange cartridges (Oasis-MCX; 6 mL, 150 mg) and SPE cartridges, built of a hydrophilic and a lipophilic monomer(Oasis-HLB; 6 mL, 200 mg) were purchased from Waters (Milford, MA, USA).

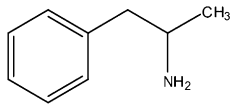
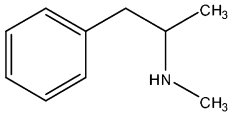
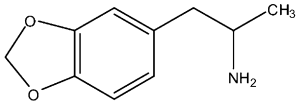
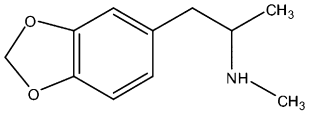
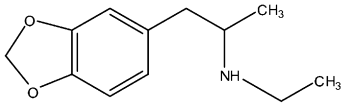
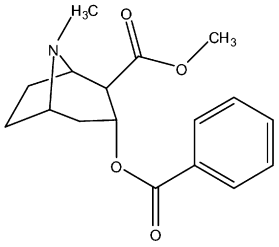
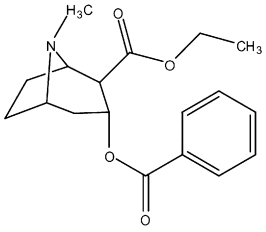
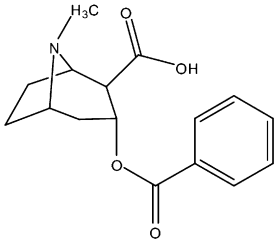
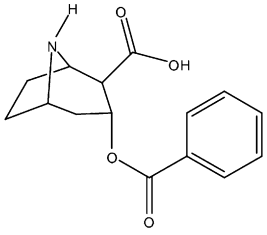
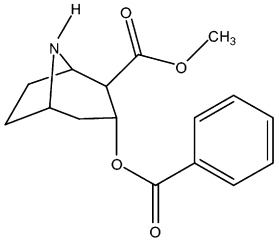
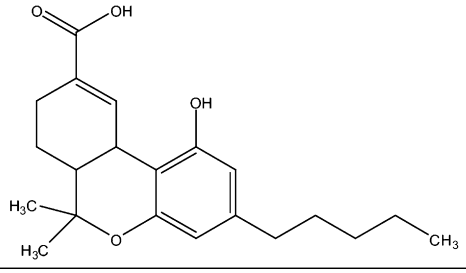
Chemical structure	Compound Mono-isotopic Molecular weight CAS number	Chemical structure	Compound Mono-isotopic Molecular weight CAS number
	Amphetamine 135.1 [300-62-9]		Methamphetamine 149.1 [537-46-2]
	MDA 179.1 [4764-17-4]		MDMA 193.1 [42542-10-9]
	MDEA 207.1 [82801-81-8]		Cocaine 303.1 [50-36-2]
	Cocaethylene 317.4 [529-38-4]		Benzoylecgonine 289.1 [519-09-5]
	Nor-benzoylecgonine 275.2 [60426-41-7]		Norcocaine 289.1 [61585-22-6]
			11-nor-9-carboxy-Δ^9-tetrahydrocannabinol 344.5 [56354-06-4]

Fig. 1. Chemical structure, mono-isotopic molecular weight, and CAS number of the selected illicit drugs.

2.2. Samples

A number of 28 wastewater samples were collected in polyethylene high density bottles and stored in the dark at −20 °C. The 24-h composite samples were taken from influent and effluent from a WWTP of the province of Castellón (Eastern Spain), during the third week of June 2008 (average week) and the third week of July 2008 (during an important music event). This WWTP is designed to treat wastewaters (urban or mixed urban and industrial) of a small population of approximately 32 000 inhabitants, with a mean flow rate of 8250 m^3/day. In this study, no surface water samples were taken. However, surface water was collected for validation for future studies, and it was taken in February 2008 from the Mijares river (Villarreal, Castellón).

2.3. Ultra-high-pressure liquid chromatography

A triple quadrupole mass spectrometer was interfaced to a Waters Acquity UPLC system (Waters). Chromatographic separation was carried out using an Acquity UPLC BEH C_{18} column (50 mm × 2.1 mm I.D., particle size 1.7 μm) (Waters). An optimized gradient was used at a constant flow rate of 0.3 mL/min using methanol (solvent A) and 5 mM ammonium acetate 0.1% formic acid (solvent B). The gradient elution was: 0 min, 10% A; 0–3 min linear

from 10 to 90% A; 3–3.5 min, 90% A; 3.5–3.6 min linear from 90 to 10% A, return to initial conditions; 3.6–6 min 10% A, equilibration of the column.

2.4. Mass spectrometry

A TQD triple quadrupole mass spectrometer with an orthogonal electrospray ionization source Z-spray (Waters) was used. Cone gas as well as desolvation gas was dry nitrogen generated from pressurized air in a N_2 LC–MS (Claind, Teknokroma, Barcelona, Spain) nitrogen generator. The cone gas and the desolvation gas flows were optimized at approximately 60 L/h and 1100 L/h, respectively. For operation in the MS/MS mode, collision gas was Argon 99.995% (Praxair, Madrid, Spain) with a pressure of 2×10^{-3} mbar in the T-wave collision cell. Other parameters optimized were: capillary voltage, 3.5 kV in positive ionization mode; lens voltage 0.3 V; source temperature, 120 °C and desolvation temperature, 500 °C. Dwell times of 0.01 s/transition were selected.

All data were acquired and processed using MassLynx v 4.1 software.

2.5. Recommended procedure

Prior to solid-phase extraction all water samples were centrifuged for 5 min at 4500 rpm. 50 mL of surface water or effluent wastewater, or five times diluted influent wastewater, were spiked with a mixed surrogate labelled standard and the pH was adjusted to 2.0 with formic acid. The final concentration in sample for each surrogate labelled internal standard was 0.1 μg/L, except for THC-COOH (1 μg/L). SPE was performed using Oasis MCX cartridges that were conditioned by washing and rinsing with 6 mL of MeOH, 3 mL of Milli-Q water and 3 mL of acidified water (pH 2). Samples were percolated through the cartridges by gravity, and then cartridges were washed with 5 mL of 2% ammonia in water and vacuum dried for 15 min. Analytes were eluted using 8 mL of a 2% ammonia solution in MeOH.

The extracts were evaporated to dryness at 35 °C under a gentle stream of nitrogen [20] and reconstructed in 1 mL of 10% methanol aqueous solution. The sample extract (20 μL) was injected directly into the UHPLC–MS/MS system without filtration (Fig. 2).

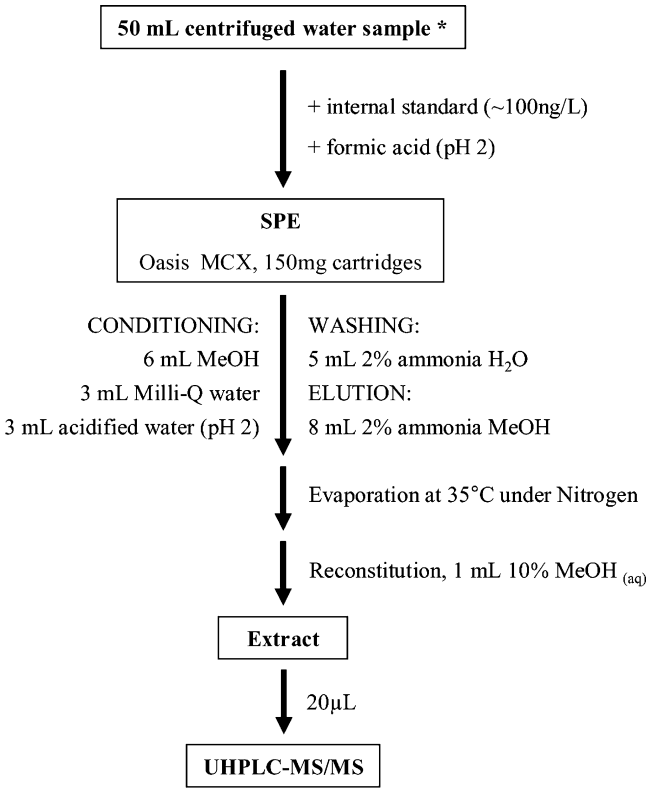


Fig. 2. Diagram of the recommended procedure.

2.6. Quantification and method validation

Acquisition was performed in SRM mode, with the protonated molecular ion of each compound chosen as precursor ion. The most abundant product ion of each target analyte was typically used for quantification and two additional product ions were used for confirmation. LC retention time was also compared with that of the reference standards (within ±2.5%) to help to confirm the compounds detected in samples. Each compound was quantified using its corresponding labelled analyte as surrogate internal standard, except norcocaine, which was quantified using a deuterated analogue (cocaine-d_3), and norbenzoylecgonine, for which no adequate internal standard was found.

The linearity of the method was studied by analyzing standard solutions in triplicate at six concentrations, in the range from 2 μg/L to 70 μg/L for amphetamine and amphetamine-like stimulants; from 0.5 μg/L to 25 μg/L for cocaine and its metabolites; and from 20 μg/L to 600 μg/L for THC-COOH, depending on the sensitivity reached for each analyte. Satisfactory linearity was assumed when the correlation coefficient (r) was >0.99, based on analyte/internal standard peak areas measurement.

The limit of quantification (LOQ) objective was taken as the lowest concentration level for which the method was fully validated using spiked samples with satisfactory recovery (between 70 and 120%) and precision (relative standard deviation (RSD) ≤20%). Confirmation by using three MS/MS transitions was also required at the LOQ level.

The limit of detection (LOD), defined as the lowest concentration that the analytical process can reliably differentiate from background levels, was estimated for a signal-to-noise of three from the quantitation SRM chromatograms of samples spiked at the lowest analyte concentration tested.

Accuracy (estimated by means of recovery experiments) and *precision* (expressed as repeatability in terms of RSD) were evaluated by analyzing water samples spiked at different concentration levels (LOQ and 10LOQ). In surface water, the LOQs objective were 30 ng/L for amphetamine and amphetamine-like stimulants, 10 ng/L for cocaine and its metabolites, and 300 ng/L for THC-COOH. In effluent and influent wastewater the LOQs were around three and fifteen times higher, respectively. All recovery experiments were performed in quintuplicate for each type of water sample tested.

3. Results and discussion

3.1. MS/MS optimization

Full-scan mass spectra and MS/MS compound-dependent parameters (e.g. cone voltage, collision energy) were optimized by direct infusion of individual standard solutions at 1.5 mg/L in methanol/water (50:50, v/v) and at a flow rate of 10 μL/min, using the build-in syringe pump directly connected to the interface. All analytes were measured by electrospray ionization (ESI) operating in positive ionization mode, including THC-COOH. Optimum MS source and analyzer conditions for SRM determination of each target and internal standard compound are listed in Table 1.

TQD mass spectrometer is a fast-acquisition triple quadrupole mass analyzer that allows decreasing dwell times and ionization

Table 1
UHPLC–MS/MS parameters established for the SRM acquisition mode (quantification and confirmation transitions). For labelled internal standards, only typically the quantification related transition was acquired.

Compounds	t_R (min)	Precursor ion (*m*/*z*) $[M+H]^+$	CV[a] (V)	CE[b] (eV)	Product ion[c] (m/z)	*Q*/*q* ratio (RSD %)
Amphetamine	1.47	136.2	25	20	91.1	
				10	119.1	0.56 (7)
				30	65.1	7.69 (9)
MDA	1.47	180.2	25	10	163.2	
				20	105.1	2.63 (7)
				20	133.1	3.00 (7)
MDEA	1.60	208.3	35	25	105.1	
				40	77.1	1.57 (7)
				25	135.1	1.82 (8)
MDMA	1.49	194.3	30	15	163.2	
				25	105.1	1.58 (4)
				40	77.1	2.83 (8)
Methamphetamine	1.50	150.3	35	20	91.1	
				10	119.1	2.65 (7)
				35	65.1	11.99 (9)
Cocaine	1.84	304.1	30	20	182.2	
				30	82.0	1.90 (9)
				50	77.0	2.93 (7)
Cocaethylene	2.05	318.3	45	20	196.2	
				30	82.0	9.74 (9)
				25	150.2	1.64 (8)
Benzoylecgonine	1.68	290.1	40	20	168.2	
				30	82.0	1.60 (8)
				30	105.0	0.81 (8)
Norbenzoylecgonine	1.76	276.2	45	15	154.1	
				20	136.1	1.61 (10)
				45	77.0	4.67 (14)
Norcocaine	1.94	290.1	30	15	136.1	
				25	168.2	0.85 (10)
				35	68.0	2.14 (10)
THC-COOH	3.68	345.3	40	15	193.2	
				25	299.3	0.46 (6)
				20	327.3	0.33 (10)
Amphetamine-d_6	1.45	142.2	25	20	93.1	
MDA-d_5	1.45	185.2	25	10	168.2	
MDEA-d_5	1.58	213.3	35	25	135.2	
MDMA-d_5	1.48	199.3	30	15	165.3	
Methamphetamine-d_5	1.49	155.3	35	20	92.3	
Cocaine-d_3	1.83	307.1	30	20	185.3	
Cocaethylene-d_8	2.05	326.3	45	20	204.3	
Benzoylecgonine-d_3	1.69	293.1	40	20	171.2	
THC-COOH-d_3	3.67	348.3	40	15	330.3	

[a] CV, cone voltage.
[b] CE, collision energy.
[c] Top, product ion used for quantification; below, the two product ions used for confirmation.

mode switching time, without apparent sensitivity losses. This gave us the possibility of acquiring up to three SRM transitions per compound at 10 ms dwell time. The most sensitive SRM transition was typically selected for quantification whereas two additional transitions were acquired to render a highly reliable confirmative method. Non-specific transitions, e.g. loss of water, were avoided as possible in order to minimize the risk of false positives [24]. Confirmation of positive findings was carried out by calculating the peak area ratios between the quantification (*Q*) and confirmation (*q*1 and *q*2) transitions and comparing them with mean *Q*/*q* value obtained from the calibration standards. The sample was considered positive when the experimental ion ratio fell within the tolerance range, in the line of the European Union Guidelines [23]. Retention time had also to fit with that of the reference standard (within ±2.5%) to be reported as positive.

As a consequence of the higher flow rate of the mobile phase, an elevated desolvation temperature was necessary. By increasing the desolvation temperature and the desolvation gas flow rate, more reproducible data were acquired, which finally lead to a more robust method. In order to improve desolvation efficiency, analyte ionization and reproducibility, the following parameters were optimized and set up as follows: source temperature, 120 °C; desolvation temperature, 500 °C; cone gas flow rate, 60 L/h; desolvation gas flow rate, 1100 L/h.

Determination of THC-COOH has been reported both in negative-ion mode [atmospheric pressure chemical ionization (APCI) or ESI] [7,12,25], and in positive-ion mode [21]. In principle, more abundant ionization would be obtained in negative mode, due to the expected higher trend towards the ionization of the acidic group. However, in our work THC-COOH presented more abundant ionization in ESI positive-ion mode, which would indicate better ionization towards basic groups. In addition to the higher sensitivity, ionization in positive-ion mode was also of our interest in order to get a simultaneous determination method for all analytes

selected using the same LC separation. Special effort was done to optimize sample preparation and LC separation to incorporate THC-COOH in our method, but we still observed some difficulties related to the confirmatory capability and sensitivity for this analyte. According to Postigo et al. [12] a single relevant indicator of cannabinoid consumption cannot be pointed out yet. Nevertheless, we expected to have a rough estimation of the consumption of cannabis by determining THC-COOH, which has been used as an indicator of cannabis usage by other authors as well [7,21].

3.2. UHPLC optimization

Chromatographic separation might not be 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 important to avoid or minimize matrix effects. In addition, the selection of the mobile phase composition can be relevant to enhance the detector response [17].

In this paper, different mobile phase compositions – variations of buffer (ammonium acetate and ammonium formate), pH (addition of formic acid) and organic solvents (acetonitrile, methanol) – were tested. The effects of pH and mobile phase ionic strength in the peak shapes, resolution and efficiencies were evaluated by varying the buffer concentration. An optimum mobile phase consisting of ammonium acetate (5 mM) with 0.1% formic acid (pH 2) and methanol was selected. This mobile phase showed satisfactory results for the drugs selected, and also for THC-COOH, the compound with lower sensitivity. Applying the UHPLC recommended gradient shown in Section 2.3, the selected drugs were separated within 4 min.

As it can be seen in Table 1 and Fig. 3, benzoylecgonine and norcocaine presented a common transition (290.1 → 168.2). In this particular case, chromatographic separation becomes an important issue and co-elution needs to be avoided. In our case, this transition was used for quantification of benzoylecgonine being necessary a complete resolution with norcocaine to obtain reliable data. From Fig. 3, the baseline peak-to-peak resolution (R) for benzoylecgonine and norcocaine was calculated to be 1.8. A value which illustrates complete resolution of both peaks [26].

3.3. Sample preparation and matrix effects

During the optimization of the SPE process, two different cartridges were evaluated: Oasis HLB and Oasis MCX. Their extraction efficiencies were estimated from the recovery percentage obtained for each target compound when loading a small sample volume (50 mL) of Milli-Q water at a low sample loading flow rate (gravity) (triplicate analysis). 50 mL Milli-Q water sample was spiked with a mixed standard solution at individual concentrations of 2 μg/L. All selected analytes showed satisfactory absolute recoveries (80–120%) for both cartridges. Oasis MCX were selected for further optimization with surface water, because the mixed mode material allows improved selectivity towards basic analytes due to pH and polarity changes during loading, washing and elution steps. The MCX cartridges were washed with aqueous basic solution (2% ammonium in water) instead of organic (methanol), to remove interferences such as inorganic salts, which resulted in lower matrix effect. We observed that the washing had no negative effect on the recoveries or on the sensitivity of the target analytes. Finally, analytes were eluted from the cartridges using a 2% ammonium solution in MeOH.

Filtration of the extract is of importance in order to maintain maximum life-time of the sub-2-μm analytical column. Consequently we tested analyte losses due to the filtering of extracts before injecting into the UHPLC–MS/MS system. In this work, 0.2 μm filters (13 mm) of different materials (polypropylene and PTFE) were tested, but the results were not satisfactory, showing recoveries lower than 70% for cocaine, cocaethylene, norcocaine and THC-COOH. At the end, we decided not to filter the final extracts before injection into the UHPLC system, until an adequate material is found.

Absolute recoveries, to estimate the extraction efficiency of the MCX cartridges, are shown in Fig. 4A. In the case of surface water, recoveries were slightly lower (65–94%) compared to Milli-Q water and for three compounds (amphetamine, methamphetamine and THC-COOH) values below 70% were obtained. We assumed that absolute recoveries of selected analytes in effluent and influent wastewaters would possibly be worse, as these sample matrices are more complex and problematic from an analytical point of view. Therefore, the internal standards were added to the samples as sur-

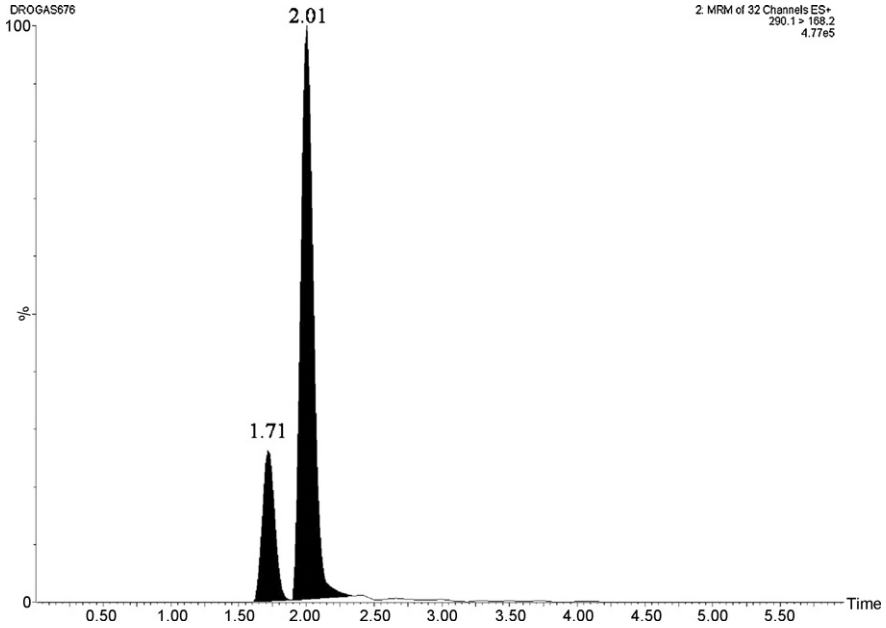


Fig. 3. UHPLC–MS/MS chromatogram (SRM transition 290.1 → 168.2) from a mixed reference standard of benzoylecgonine (1.6 μg/L, t_R = 1.71 min) and norcocaine (1.8 μg/L, t_R = 2.01 min).

rogates, just before the SPE process, to compensate for potential losses of compounds along sample treatment.

Matrix effects can lead to a suppression or enhancement of the analyte response due to co-eluting matrix constituents. Several approaches are typically applied to deal with matrix effects in quantitative analysis: improvement of the sample pre-treatment and/or the chromatographic separation, matrix-matched standards calibration, sample dilution or the use of stable-isotopically labelled internal standards, the latest being widely accepted to be the most satisfactory approach [17,18]. Preliminary experiments on surface water were performed by spiking SPE extracts in order to avoid the influence of potential losses in the SPE process. Therefore, SPE blank surface water extracts were spiked with the selected drugs (native and labelled) and matrix effects were evaluated for each compound calculating the absolute (without internal standard) and relative (with internal standard) responses in comparison to those of reference standards in solvent. Matrix ionization suppression was observed for all compounds, except for norbenzoylecgonine, which showed ionization enhancement (~200%) (Fig. 4B). As the deuterated analyte was not available for this compound, benzoylecgonine-d_3 was tested as analogue IS to compensate for norbenzoylecgonine matrix effects, but the results were not satisfactory in none of the samples tested. For the rest of compounds, the use of labelled IS corrected the matrix effects, as expected.

An estimation of matrix effects in wastewater samples could be made throughout the signal variations observed in the deuterated IS, which were added to all type of samples as well as to standards in solvent. As expected, matrix effects seemed to be more important in wastewater (mainly in the influent) and notable ionization suppression was observed for all analytes, norbenzoylecgonine included.

In our work, to compensate for potential errors associated to both sample preparation and matrix effects, each compound was quantified using its corresponding deuterated standard added as surrogate internal standard. In the case of norcocaine and norbenzoylecgonine, their labelled analogues were not commercially

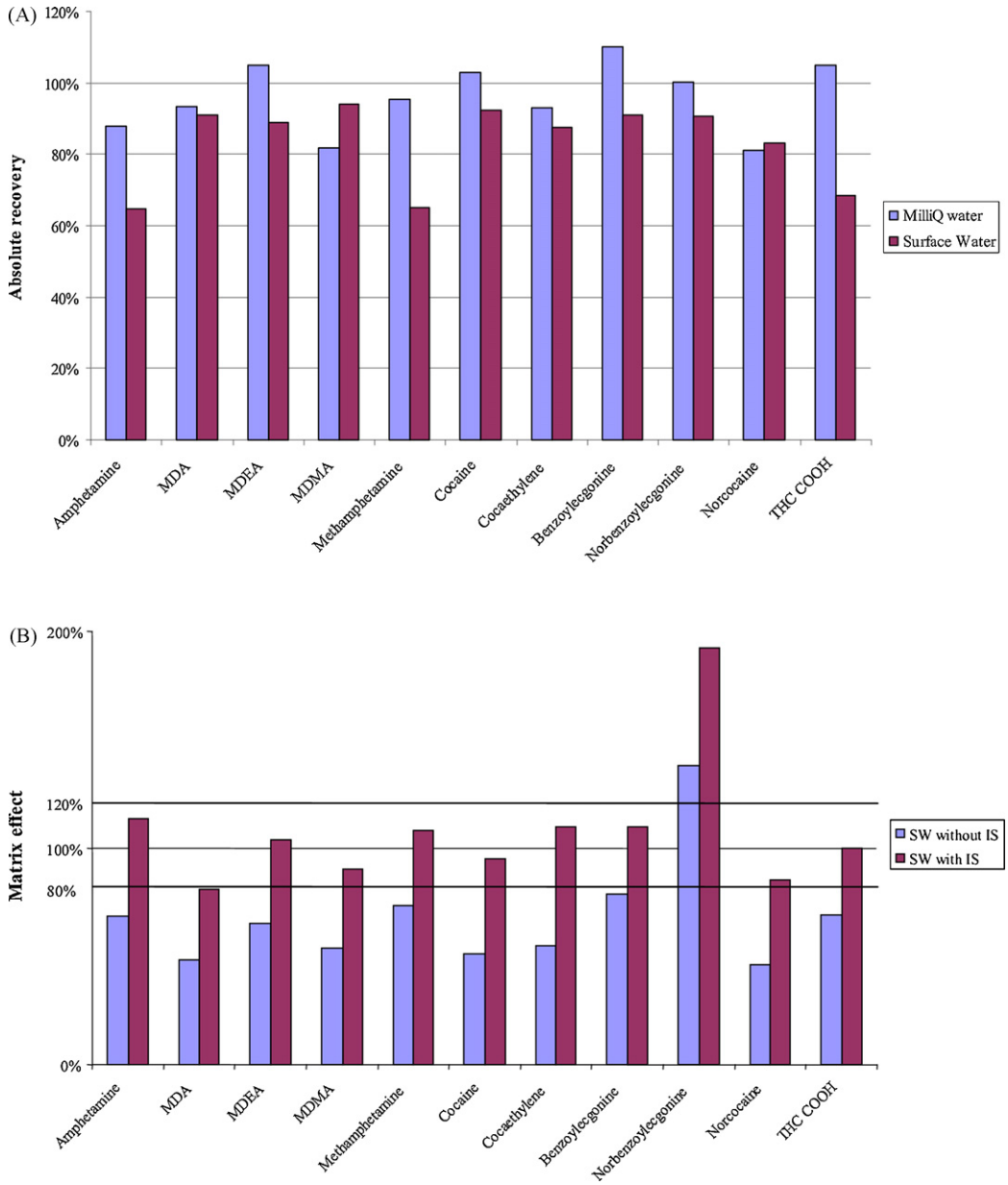


Fig. 4. (A) Absolute recoveries of all selected analytes in Milli-Q and surface water using MCX cartridges. (B) Matrix effects in surface water for all selected analytes with and without correction of internal standards.

Table 2
Method validation in surface water ($n = 5$).

Compound	Recovery (RSD)		LOD (ng/L)	Q/q_1 ratio deviation (%)[a]	Q/q_2 ratio deviation (%)[a]
	LOQ[b]	10LOQ			
Amphetamine	102 (5)	95 (6)	2	4 (from −10 to +7)	6 (from +1 to +12)
MDA	106 (7)	96 (6)	17	1 (from −10 to +10)	8 (from −3 to +25)
MDMA	92 (13)	97 (9)	4	1 (from −8 to +6)	10 (from +3 to +17)
MDEA	94 (4)	96 (3)	0.5	3 (from −6 to +16)	4 (from −12 to +15)
Methamphetamine	90 (7)	98 (6)	0.6	6 (from −17 to +19)	6 (from −25 to +33)
Cocaine	90 (15)	76 (9)	0.8	6 (from −14 to +17)	0 (from −19 to +20)
Cocaethylene	70 (9)	82 (3)	0.3	11 (from +3 to +22)	4 (from −18 to +19)
Benzoylecgonine	103 (12)	84 (8)	0.05	5 (from −4 to +13)	3 (from −12 to +14)
Norbenzoylecgonine	92 (6)	107 (5)	2	5 (from −13 to +6)	4 (from −3 to +15)
Norcocaine	73 (11)	61 (7)	2	7 (from −20 to +0)	4 (from −13 to +17)
THC-COOH	91 (14)	120 (11)	30	3 (from −19 to +11)	2 (from −14 to +12)

[a] Average deviation and range (in %) of the experimental Q/q ratios obtained from sample extracts spiked at the lowest level validated ($n = 5$) in relation to those calculated from reference standard solutions in solvent (see Table 1).
[b] LOQ objective was 0.03 μg/L for amphetamine and amphetamine-like stimulants; 0.01 μg/L for cocaine and its metabolites; 0.3 μg/L for THC-COOH.

Table 3
Method validation in effluent wastewater ($n = 5$).

Compound	Recovery (RSD)		LOD (ng/L)	Q/q_1 ratio deviation (%)[a]	Q/q_2 ratio deviation (%)[a]
	LOQ[b]	10LOQ			
Amphetamine	102 (5)	103 (9)	40	8 (from +5 to +17)	14 (from +2 to +26)
MDA	88 (5)	77 (9)	88	1 (from −9 to +10)	3 (from −6 to +10)
MDMA	84 (25)	100 (13)	9	6 (from +2 to +14)	4 (from −5 to +18)
MDEA	103 (3)	105 (2)	9	5 (from +1 to +13)	7 (from +1 to +23)
Methamphetamine	94 (17)	84 (16)	1	3 (from −25 to +24)	13 (from +7 to +27)
Cocaine	109 (4)	125 (1)	2	4 (from −8 to +16)	9 (from +4 to +14)
Cocaethylene	78 (11)	97 (8)	1	1 (from −22 to +20)	4 (from −16 to +14)
Benzoylecgonine	120 (7)	114 (14)	0.3	5 (from −16 to +8)	1 (from −6 to +10)
Norbenzoylecgonine	80 (9)	63 (3)	0.2	7 (from −20 to −1)	9 (from −27 to 0)
Norcocaine	76 (5)	70 (9)	2	3 (from −11 to +5)	9 (from −14 to +5)
THC-COOH	48 (8)	118 (11)	500	1 (from −17 to +17)	13 (from −20 to +1)

[a] Average deviation and range (in %) of the experimental Q/q ratios obtained from sample extracts spiked at the lowest level validated ($n = 5$) in relation to those calculated from reference standard solutions in solvent (see Table 1).
[b] LOQ objective was 0.1 μg/L for amphetamine and amphetamine-like stimulants; 0.03 μg/L for cocaine and its metabolites; 0.8 μg/L for THC-COOH.

available; so norcocaine was quantified using cocaine-d_3, for norbenzoylecgonine no labelled standards were found suitable and it was quantified without using a surrogate internal standard. Besides, influent wastewater needed to be at least five times diluted before the SPE process to handle with matrix effect. Otherwise, the ionization suppression was so high that it could not be properly compensated even using deuterated standards.

Before SPE, water samples were centrifuged for 5 min at 4500 rpm to avoid compound losses that could take place if filtration was used instead. After centrifugation, the surrogate/internal standards were added, assuring in this way the correct quantification of analytes in real water samples.

3.4. Method validation

Prior to its application, the overall analytical procedure was satisfactorily validated for surface water (Table 2), effluent wastewater (Table 3) and influent wastewater (Table 4), considering the following parameters: linearity, precision and accuracy, LOQs, LODs, and Q/q ratios used for confirmation. These tables show

Table 4
Method validation in influent wastewater ($n = 5$).

Compound	Recovery (RSD)		LOD (ng/L)	Q/q_1 ratio deviation (%)[a]	Q/q_2 ratio deviation (%)[a]
	LOQ[b]	10LOQ			
Amphetamine	113 (8)	108 (6)	54	3 (from −17 to +24)	6 (from −22 to +15)
MDA	90 (7)	81 (9)	91	5 (from −15 to +12)	6 (from −24 to +21)
MDMA	73 (5)	75 (8)	18	5 (from −15 to +13)	3 (from −8 to +10)
MDEA	75 (18)	71 (2)	40	2 (from −5 to +16)	4 (from −19 to +18)
Methamphetamine	116 (12)	119 (6)	7	3 (from −19 to +12)	2 (from −24 to +19)
Cocaine	87 (10)	112 (3)	3	0 (from −5 to +15)	0 (from −7 to +7)
Cocaethylene	85 (5)	95 (3)	2	4 (from −11 to +3)	6 (from −12 to +2)
Benzoylecgonine	[c]	[c]	[d]	1 (from −6 to +12)	4 (from −5 to +14)
Norbenzoylecgonine	50 (15)	57 (10)	1	1 (from −17 to +10)	11 (from +1 to +25)
Norcocaine	73 (8)	83 (5)	5	5 (from −2 to +12)	2 (from −7 to +15)
THC-COOH	72 (9)	120 (6)	2500	2 (from −8 to +11)	1 (from +1 to +23)

[a] Average deviation and range (in %) of the experimental Q/q ratios obtained from sample extracts spiked at the lowest level validated ($n = 5$) in relation to those calculated from reference standard solutions in solvent (see Table 1).
[b] LOQ objective was 0.5 μg/L for amphetamine and amphetamine-like stimulants; 0.15 μg/L for cocaine and its metabolites; 4.0 μg/L for THC-COOH.
[c] Not calculated due to the high concentration found in the "blank" sample.
[d] Not estimated, as validation at the LOQ level was unfeasible due to high levels found in the "blank" sample.

Table 5
Real-time concentrations of illicit drugs in influent and effluent wastewater of a WWTP placed in Castellón province, during weekdays (1) and weekends (2) of June and July, 2008.

Analyte	Influent wastewater (μg/L)				Effluent wastewater (μg/L)			
	June		July		June		July	
	Weekday	Weekend	Weekday	Weekend	Weekday	Weekend	Weekday	Weekend
Amphetamine	d*	d	–	1.40	–	–	–	0.21
	1 (<0.5)	2 (<0.5)	−1 (0.62)	3 (0.61–1.40)				2 (0.11–0.21)
MDA	–	–	d	1.69	–	–		0.68
			2 (<0.5–0.79)	3 (0.92–1.69)			−1 (0.41)	3 (0.50–0.68)
MDEA	–	–	–	d	–	–	–	
				2 (<0.5)				−1 (<0.1)
MDMA	–		3.26	27.5	d	d	0.71	21.2
		2 (<0.5)	4 (<0.5–15.9)	3 (18.3–27.5)	2 (<0.1)	3 (<0.1)	4 (0.20–6.23)	3 (14.9–21.2)
Methamphetamine	–	–	–	d	d	d	–	d
				2 (<0.5)	1 (<0.1)	1 (<0.1)		1 (<0.1)
Cocaine	0.82	0.50	0.55	0.56	d	d	d	0.54
	3 (0.41–0.82)	3 (0.50–0.70)	4 (0.37–1.24)	3 (0.47–0.72)	3 (<0.03)	3 (<0.03–0.04)	4 (<0.03–0.15)	3 (0.05–0.56)
Cocaethylene	d	d	d	0.15	–	–	d	0.08
	3 (<0.15)	3 (<0.15)	3 (<0.15–0.19)	3 (<0.15–0.15)			2 (<0.03)	3 (<0.03–0.08)
Benzoylecgonine	2.18	2.81	4.14	10.5	d	d	0.06	6.79
	4 (1.48–2.18)	3 (2.15–2.81)	4 (1.65–7.21)	3 (5.67–10.5)	3 (<0.03)	3 (<0.03)	4 (<0.03–1.45)	3 (0.76–6.79)
Norbenzoylecgonine	d	0.15	d	0.43	d	d	0.03	0.15
	4 (<0.15)	3 (<0.15–0.15)	2 (<015–0.31)	3 (0.20–0.43)	1 (<0.03)	3 (<0.03)	2 (0.03–0.11)	3 (0.12–0.17)
Norcocaine	–	–	–	–	d	d	d	d
					4 (<0.03)	3 (<0.03)	3 (<0.03)	3 (0.03)
THC-COOH	–	d	d	d	–	–	–	d
		1 (<4.0)	1 (<4.0)	2 (<4.0)				1 (<0.8)

d: detected at concentration level ≥LOD and ≤LOQ.
Representative 24-h composite influent and effluent wastewater samples of a weekday (collected on Thursday 19th of June 2008, and on Thursday 17th of July 2008).
Representative 24-h composite influent and effluent wastewater samples of a weekend (collected on Sunday 22nd of June 2008 and on Sunday 20th of July 2008).
* Top: individual concentration value found in the sample.
Bottom: number of days when target analytes were positively identified, and concentration range (in brackets). A week is considered from Tuesday to Friday (4 days), a weekend is considered from Saturday to Monday (3 days).

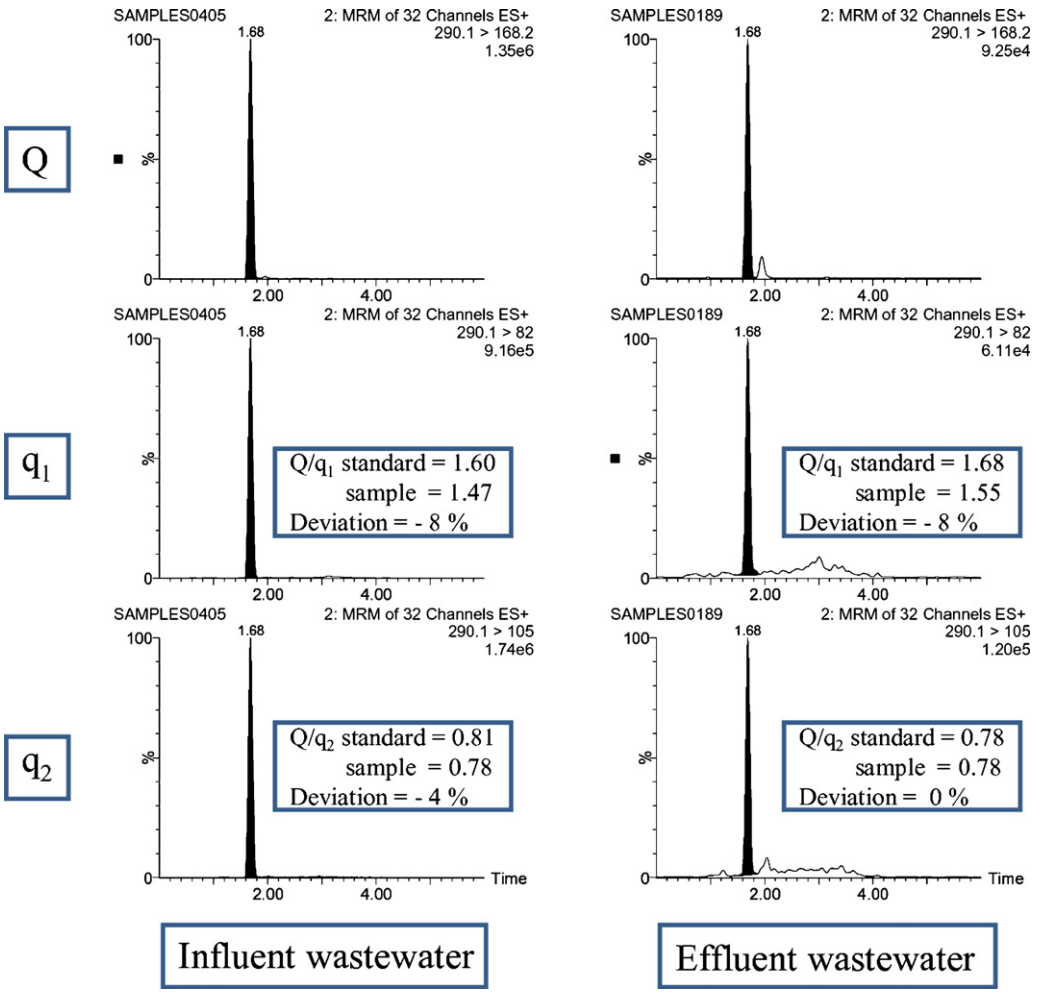


Fig. 5. UHPLC–MS/MS chromatograms corresponding to the positive finding of benzoylecgonine in influent (4140 ng/L) and effluent (60 ng/L) 24-h composite wastewater sample from a weekday of July 2008. (Q) quantification transition; (q_1) and (q_2) confirmation transition.

relative recovery as surrogate/IS were used in validation experiments.

Linearity was studied for all selected compounds. A six-point calibration curve, in the range from 2 μg/L to 70 μg/L for amphetamine and amphetamine-like stimulants; 0.5 μg/L to 25 μg/L for cocaine and its metabolites; 20 μg/L to 600 μg/L for THC-COOH, was generated by injecting mixed standard solutions with a fixed amount of mixed surrogate internal standard solution. Correlation coefficients obtained were, with few exceptions, greater than 0.99.

Precision and accuracy were evaluated by spiking "blank" water samples at two concentration levels (LOQ and 10LOQ), and analyzing them in quintuplicate. It was difficult to obtain genuine blank samples for wastewater, specially for influent. "Blank" samples were collected from the WWTP on Tuesday when lower concentration of drugs were expected in comparison to weekends. These "blank" samples were previously analyzed and positive findings were subtracted from the spiked samples. In surface water, the LOQs objective were fixed at 30 ng/L for amphetamine and amphetamine-like stimulants, at 10 ng/L for cocaine and its metabolites, and at 300 ng/L for THC-COOH. In effluent and influent wastewater the LOQs were around three and fifteen times higher, respectively. The LOQ objective was established depending on the sensitivity of the method for each analyte and on the type of water. Besides, all three SRM transitions could be acquired at these low levels for all analytes, making the reporting data highly confident as regards the identity of the compound detected. The method was found highly specific as no relevant interferences were observed in the blanks at the analytes retention times for each transition.

In general, recoveries (between 70 and 120%) and precision (RSD < 20%) were satisfactory for most compounds at both fortification levels. Table 3 shows data for effluent wastewater. As can be seen THC-COOH showed poor recovery (48%), but with satisfactory RSD (8%). At the 10LOQ level, cocaine showed a relatively high recovery (125%), but with good precision (1%). In influent wastewater (Table 4), benzoylecgonine precision and accuracy could not be calculated due to the high concentration found in the blank (around 2.5 μg/L). In both, effluent and influent wastewater, norbenzoylecgonine showed lower relative recoveries (in the range of 50–80%), but satisfactory RSD (<15%). The reason for the lower relative recoveries of norbenzoylecgonine was probably owing to the absence of an adequate internal standard.

As can be seen in Tables 2–4, the worst LODs were obtained for THC-COOH as a consequence of the poor sensitivity for this compound. With the exception of this metabolite, LODs in surface water varied between 0.05 ng/L and 17 ng/L, while in effluent wastewater increased up to the range 0.2–88 ng/L. In influent wastewater, LODs were in the range 1–91 ng/L. Regarding LOQs, we did not estimate them from a statistic point of view (e.g. from a signal-to-noise of 10). Instead, we have used a LOQ objective, which was established as the lowest level in sample for which the method was fully validated in terms of accuracy and precision. This criterion is normally applied in the field of pesticide residue analysis, accordingly to SANCO guidelines [27,28] and it leads to realistic LOQ values, nor-

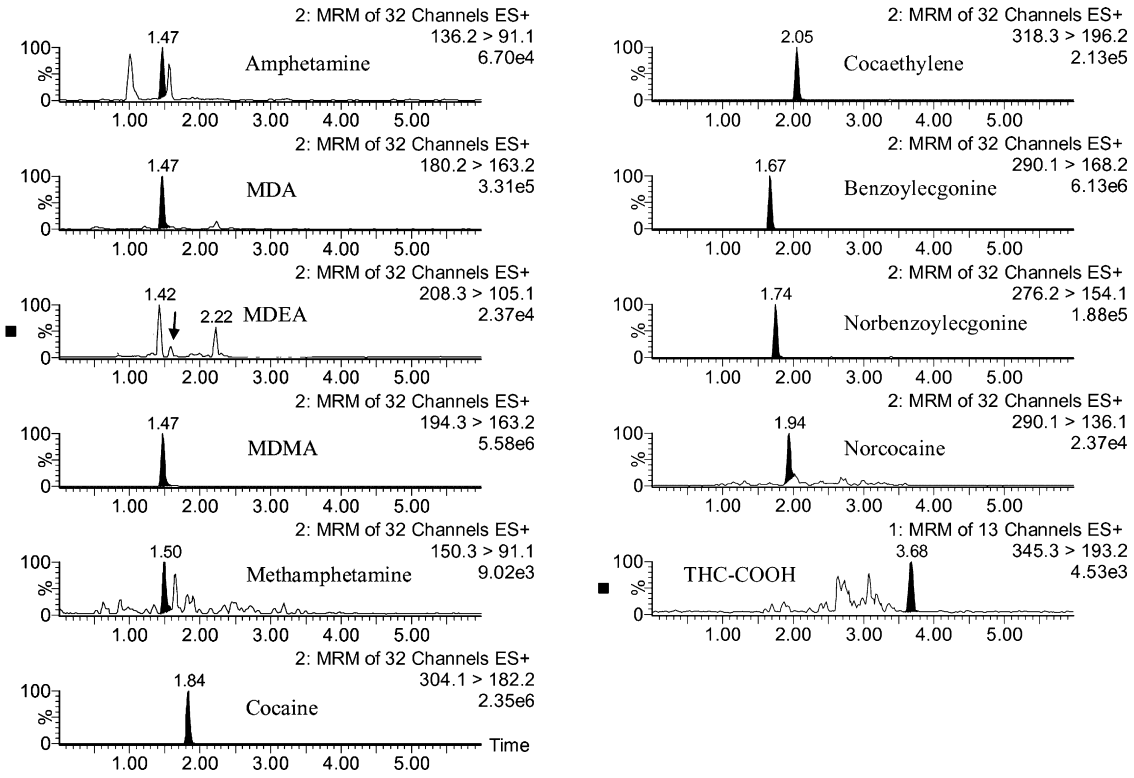


Fig. 6. UHPLC–MS/MS chromatograms (quantification transition) for an effluent wastewater sample collected on a weekend (20 July 2008). See Table 5 for concentration data.

mally higher than those estimated from statistical criteria. Besides, all compounds could be confirmed at the LOQ level by acquiring two additional transitions, in accordance with the two corresponding Q/q ratios.

Average intensity Q/q_1 and Q/q_2 ratios calculated from reference standards in solvent (see Table 1) were compared to those experimentally obtained from sample extracts spiked at the lowest level validated (i.e., the worst-case scenario). The aim was to test the robustness of these values and to check for potential matrix interferences that could affect the Q/q ratios and consequently, the confirmation process. As Tables 2–4 show, average Q/q_1 and Q/q_2 deviations were always below 20% (the maximum permitted tolerance varies between 20 and 50%, depending on the relative ion intensities (% of base peak)) [23].

3.5. Application to real samples

A number of 14 influent and 14 effluent 24-h composite wastewater samples were analyzed by the developed UHPLC–MS/MS method. 24-h composite water samples of Thursday and Sunday (of both June and July) have been selected as illustrative examples for a weekday and weekend, respectively, and real-time data obtained are summarized in Table 5.

In every sequence of analysis, water samples were injected in duplicate between two calibration curves. Two quality control samples (QCs), i.e. a blank water sample (previously analyzed) spiked at LOQ and 10LOQ levels, were also analyzed. QC recoveries were considered satisfactory when they were in the range of 70–120% for each analyte.

In general, concentrations of illicit drugs on weekdays were lower than during weekends. The consumption during the special musical event (July 2008) was considerably higher. THC-COOH showed a similar trend, although samples were not quantified, as all were below the LOQ objective of the method. In influent wastewater, cocaine and its main metabolite benzoylecgonine were the most abundant in the samples collected in June, with concentrations around 0.8 μg/L and 0.5 μg/L (cocaine) and 2.2 μg/L and 2.8 μg/L (benzoylecgonine), on weekdays and weekends, respectively. Benzoylecgonine was the most abundant cocaine metabolite in wastewater in all the samples analyzed and its concentration levels notably increased in July. This metabolite reached concentrations as high as 4.14 μg/L and 10.5 μg/L in influent wastewater on weekdays and weekends, respectively. It can be pointed out the impressive increase of amphetamine and amphetamine-like stimulants concentrations in July (especially MDMA with 3.26 μg/L and 27.5 μg/L in samples collected on Thursday and Sunday, respectively) during a special music event, which indicates that these drugs were mainly used for this special occasion. On the other hand, cocaine showed more constant concentration during the weeks analyzed, similar to the values found by Huerta-Fontela et al. [11], indicating a different pattern of use. Nevertheless, concentrations of benzoylecgonine, the main metabolite of cocaine, also increased significantly in July.

Confirmation of positive findings was carried out by calculating the peak area ratios between the quantification (Q) and confirmation (q_1 and q_2) transitions and comparing them with mean Q/q_1 and Q/q_2 values obtained from the calibration standards in the same sequence of samples. To consider a finding as positive, the experimental Q/q_i ratios should fit with those of reference standards with a maximum deviation ranging from 20 to 50% depending on the relative ion intensities. In our work, all findings were confirmed by the two Q/q_1 and Q/q_2 ratios when analyte concentration was above the LOQ of the method. Fig. 5 shows a positive finding of benzoylecgonine in influent and effluent wastewater. Samples, where analytes were detected at very low concentration levels (below the LOQ), were also considered as positive findings when all the three transitions acquired (with few exceptions) were observed and at least one Q/q ratio was within tolerance limits. Due to the higher chemical background in the chromatograms and to the low peak intensity as a consequence of the low analyte concentration, compliance of

Q/q ratio of both confirmatory transitions was not always possible.

The WWTP, designed to treat wastewaters (urban or mixed urban and industrial) of a small population, seemed to have good removal efficiency (>99%) for all compounds (see Table 5), but some difficulties were observed when high drug levels were present in wastewater samples (July). On weekdays, removal efficiency for MDMA and benzoylecgonine was around 80 and 95%, respectively. However, in samples collected on weekends, when the highest concentrations were reached, efficiency decreased to 25% (MDMA) and to 35% (benzoylecgonine). For other compounds detected, efficiencies were around 85% (amphetamine), 60% (MDA) and 65% (norbenzoylecgonine). Remarkable was the low removal efficiency observed for cocaine in the weekend sample of July (0.56 μg/L influent, 0.54 μg/L effluent), although this fact is in contradiction with the other samples analyzed.

Illustrative chromatograms (quantitative transition) are shown in Fig. 6 for a weekend effluent sample (Sunday), which contained almost all compounds investigated in this work. The excellent sensitivity of the method and its potential to detect low concentration levels of analytes are proved. Data concentrations in this sample are found in Table 5, where it can be seen that methamphetamine, norcocaine and THC-COOH are reported as detected (i.e. concentration below the LOQ). Having a look to the corresponding chromatograms, one realizes that satisfactory peaks were obtained for these analytes when acquiring the quantification transition. The reason for not performing quantification in these samples is because the method was not validated at these low levels by acquiring both the quantification and confirmation transitions. Therefore, although quantitative data could have been reported at lower levels, we assumed a conservative criterion and reported these analytes as detected (concentrations above the LOD of the method, but below the LOQ). The high number of analytes detected in this effluent weekend sample leads to the conclusion that the efficiency removal of the WWTP was poor along that day (20 July 2008), where high concentrations of illicit drugs were present in the influent sample. This fact might have possible consequence for aquatic ecosystems [15].

4. Conclusions

An analytical method based on UHPLC–MS/MS has been developed for the simultaneous quantification and confirmation of basic/acidic illicit drugs and relevant metabolites in surface and urban wastewater at the ng/L levels. Potential analytical errors associated to sample preparation and those resulting from matrix effects were compensated by using the analyte deuterated compound as surrogate internal standard. The overall analytical procedure, based on an off-line SPE step using Oasis MCX cartridges prior to the determination by UHPLC–MS/MS using a triple quadrupole analyzer, has been fully validated at the LOQ and 10LOQ level, obtaining satisfactory accuracy and precision. Confirmation of the analyte identity was guaranteed by acquiring 3 SRM transitions and the accomplishment of the ion ratios deviations for all analyte/matrix combinations, even at the LOQ level. The LOQ objective was established as a function of the sensitivity for each analyte, and in some cases was as low as 10 ng/L.

The application of this method to influent and effluent urban wastewater samples showed an increase of drug consumption during weekends and special events (e.g. festivals). The higher concentration levels reported corresponded to MDMA (ecstasy) and to the cocaine-metabolite benzoylecgonine. All positive findings were confirmed by accomplishment of ion ratios between the quantification transition (Q) and two specific additional confirmation transitions (q). The removal efficiency of the WWTP was satisfactory for low levels of illicit drugs in influent wastewater.

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