Talanta 85 (2011) 157–166

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Development of a solvent-free method for the simultaneous identification/quantification of drugs of abuse and their metabolites in environmental water by LC–MS/MS

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ARTICLE INFO

Article history:
Received 18 January 2011
Received in revised form 11 March 2011
Accepted 20 March 2011
Available online 19 April 2011

Keywords:
Direct injection
Illicit drugs
Metabolites
Environmental water
LC–QqLIT-MS/MS
Green analytical chemistry

ABSTRACT

This work details a rapid analytical method using direct sample injection for the simultaneous identification/quantification of 22 drugs of abuse, including some of their major metabolites, in environmental samples. This has been developed using a hybrid triple quadrupole-linear ion trap-mass spectrometer (QqLIT). With the increasing sensitivity of today's tandem mass spectrometers, direct injection analysis of water samples has become an attractive alternative to traditional analytical protocols, which often include a preliminary pre-concentration step. What's more, this kind of analysis is in accordance with many of the main objectives of so-called green analytical chemistry, or environmentally friendly practice. The analytical performance of the LC–MS/MS method was evaluated in three different water matrices (surface water, influent and effluent wastewater). Data acquisition was carried out in selected reaction monitoring (SRM) mode under time-scheduled conditions, monitoring two SRM transitions for simultaneous identification/quantification of all target compounds in the samples. Additionally, an experiment was performed using the information-dependent acquisition (IDA) scan to carry out the identification of those analytes for which the second transition was present at a low intensity. Finally, the two methodologies developed were applied to real samples for evaluation.

1. Introduction

The abuse of illicit drugs has become a serious global problem in our contemporary society. The United Nations Office of Drugs and Crime (UNODC, 2010) has shown a shift towards the use of new drugs and towards new markets. In spite of stabilization in the cocaine and heroin markets, problems relating to the misuse of prescription drugs are growing in many parts of the world. Currently, the number of people using amphetamine-type stimulants globally even exceeds the number of opiate and cocaine users [1]. On the other hand, due to current social interest, both in the general public and with policy makers, in connection to knowledge about trends and the prevalence of drug consumption, rapid and reliable analytical strategies are being developed by the scientific community to detect these analytes and their active metabolites in the aquatic environment in order to start prevention campaigns or to take targeted action. In Italy in 2005, for the first time, Zuccato et al. [2] applied a methodology based on cocaine residue measurements excreted in the consumers' urine into both surface water and wastewater – to serve as quantitative indicators of inter-community drug consumption. Since then, various research groups have applied this methodology for the analysis of drugs of abuse in urban sewage samples collected from different European countries such as Italy [3], Spain [4,5], Ireland [6] and Belgium [7].

At present, sample preparation based on solid-phase extraction (SPE) followed by analysis with liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS), with triple quadrupole (QqQ) analyzers, has been the most reported analytical method for quantifying low concentrations of illicit, as well as legal, drugs in environmental samples [2–7]. The high sensitivity, versatility and selectivity of LC–MS/MS make it a very suitable technique for the analysis of organic compounds present at trace levels in the aquatic environment. However, in spite of the advantages of SPE for sample concentration and clean up, some of its limitations have been commented on in previous publications [8,9], such as low and variable analyte recovery, matrix effects and the time and/or cost involved. In order to tackle this problem, Gonzalez-Mariño et al. [9] used molecularly imprinted polymers (MIPs) sorbents, as a further alternative, for the determination of amphetamines in wastewater. On the other hand, Chiaia and Banta-Green [10] used another

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doi:10.1016/j.talanta.2011.03.051

methodology, combined on large-volume injection and a stainless steel sample loop extension (RPLC/MS/MS) for the analysis of illicit and legal drugs in wastewater, as an alternative to conventional SPE. This technique involves the direct injection of sample volumes (ranging from 100 μL to 5000 μL) rather than conventionally injected volumes (10–20 μL). Therefore, it is possible to increase the sensitivity of the analytical procedure, minimizing cost and sample preparation steps associated with other separation procedures, which are considered complex and time consuming. In another study, Choi et al. [11] demonstrated that the sample amount introduced into the ion source of the chromatographic system has a significant effect on the extent of matrix signal suppression. Choi concluded that lower injection volumes likely reduce the loading of matrix components onto the column, minimizing the matrix effects associated with the column overloading effects. The appearance of new generation highly sensitive analytical systems onto the market has allowed for a significant reduction in the sample volume injected into the system (between 5 μL and 20 μL) without significant evidence of sensitivity loss. Such is the case with the hybrid triple quadrupole-linear ion trap-mass spectrometer (QqLIT), the high sensitivity of which even permits sample dilution in order to reduce the amount of matrix load into the system, and, thereby, dramatically decreases the signal-enhancement/suppression problems generated by the matrix effect, so common in these kinds of matrices. As has been mentioned before, direct sample injection has many advantages, such as reducing sample handling and, as a consequence, improving analysis reproducibility and minimizing potential sample contamination. In addition, this methodology decreases the amounts of solvents, waste and cost associated with other extraction techniques such as liquid–liquid or solid-phase extraction. For all of these reasons, direct sample injection can be considered an environmentally friendly practice, also sometimes termed "green analytical chemistry", the main concepts of which have been gaining interest over recent years [12,13].

On the other hand, Commission Decision 2002/657/EC [14], relating to the performance of analytical methods, emphasises the need of obtaining at least three identification points (IPs) for correct confirmation of the presence of target compounds when using LC–MS/MS. However, the main problem is that, sometimes, the three IPs are not obtained from those analytes that only show a single selected reaction monitoring (SRM) transition, or that the second transition has an intensity difference of up to 10 times regarding the first transition. To solve these problems, the information-dependent acquisition (IDA) scan allows the combining of several survey scans acquired in different operation modes, and is thus able to obtain a great deal of structural information in a single run. In this work mode, the identification is additionally aided by the use of a MS/MS spectral library. This strategy has been described for the rapid screening of 301 drugs in blood and urine samples, combining sequential SRM and MS/MS experiments [15]. Martínez Bueno et al. [16] also tested this kind of methodology but in the analysis of 14 organic pollutants in wastewater.

An innovative aspect of the present study has been the use of direct sample injection for the analysis of 22 drugs of abuse, along with some of their respective metabolites, in wastewater and surface water, without prior treatment of the sample. Until now, the direct analysis of these compounds in environmental water, without sample pre-concentration, has not been widely used, due in part to the typically low sensitivity associated to trace levels, all of which are present in the aquatic medium. Another innovative aspect of the present work has been to carry out an extra analysis based on the use of an IDA scan. This combines SRM as the survey scan along with two analyses in full scan, using enhanced product ion (EPI) mode as the dependent scans, all within the same chromatographic run, allowing the confirmation of some of the target drugs of abuse in various aquatic matrices. The two analytical methods were applied to real samples, both in sewage and river water, in order for them to be evaluated. Additionally, in this study, the removal percentages of each drug of abuse were estimated, as well as their metabolites, during their passage through a municipal sewage treatment plant (STP), with secondary treatment. The STP is located in the southeast of Spain. Finally, the daily variations of these compounds in wastewater influent were evaluated over the week.

2. Experimental

2.1. Chemicals and reagents

The analytes studied comprise a group of 22 drugs of abuse belonging to two different categories, in respect to their effects on the nervous system: (i) tranquilising drugs: morphine, acetylmorphine, ethylmorphine, methadone, 2-ethyllidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP), heroine and codeine, and (ii) stimulant drugs: nicotine, cotinine, caffeine, paraxanthine, cocaine, benzoylecgonine, ephedrine, phenylephrine, ketamine, amphetamine, methamphetamine (METH), ethylamphetamine (EAMP), 3,4-methylenedioxyamphetamine (MDA), 3,4-methylenedioxymethamphetamine (MDMA) and 3,4-methylenedioxyethamphetamine (MDEA). Two labelled standards, nicotine-d_3 and $^{13}C_3$-Caffeine isotope were used as surrogate standards. All the chemicals included in this study were purchased from Sigma–Aldrich (Steinheim, Germany) at analytical grade (purity >90%). Individual stock standard solutions were prepared by diluting each analyte solution in methanol for tuning study. A mixed working solution of these compounds at 2 mg/L in methanol was used for the preparation of calibrations. Calibration standards were then prepared at different concentrations by appropriate dilution of the mixed working solution using acetonitrile: water (10:90, v/v). All the standard and working solutions were stored at −20 °C and calibration standards in water were freshly prepared for each new series of samples. Table 1 summarizes all of the target drugs of abuse evaluated in this study along with some of their respective metabolites.

Methanol (MeOH) and HPLC-grade acetonitrile (AcN) were supplied by Merck (Darmstadt, Germany). Water used for LC–MS analysis was generated from a Direct-Q™ 5 Ultrapure Water System from Millipore (Bedford, MA, USA) with a specific resistance of 18.2 MΩ cm. Formic acid (98% purity) was purchased from Fluka (Buchs, Germany).

2.2. Sample collection and treatment

Wastewater samples used in this study were collected from a municipal sewage treatment plant (STP) located in the southeast

Table 1
Stimulant and tranquilising drugs of abuse and some of their respective metabolites evaluated in this study.

Family	Compounds	Metabolites
Stimulant Drugs	Nicotine	Cotinine
	Caffeine	Paraxanthine
	Cocaine	Benzoilecgonine
	Amphetamine (speed)	Methamphetamine
		Ethylamphetamine
		MDA
		MDEA
		MDMA (Ecstasy)
	Ephedrine	Phenylephrine
	Ketamine	–
Tranquilising drugs	Morphine	Acetylmorphine
		Ethylmorphine
	Methadone	EDDP
	Heroin	–
	Codeine	–

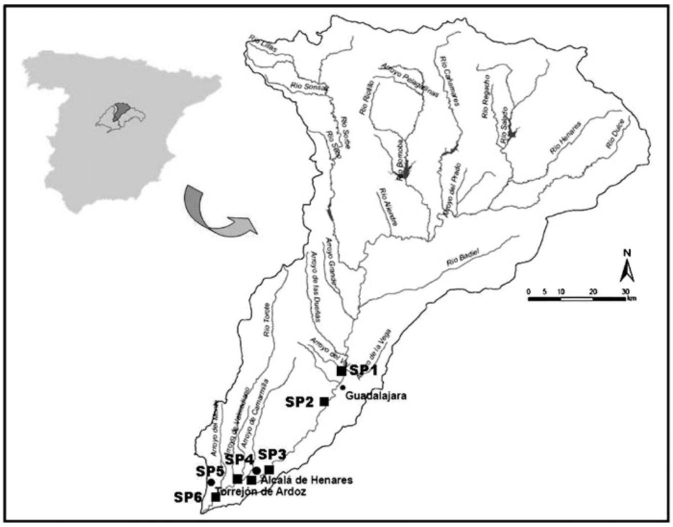

Fig. 1. Location of the sampling points (SP1-6).

of Spain (Almería). The plant is connected to a sewage system servicing a municipal area with ~78,000 inhabitants. It is strategically situated in a very productive agricultural area and very close to a hospital which discharges into the urban network. This plant applies a pre-treatment for solid removal, a primary treatment to eliminate suspended material, an activated sludge biological treatment and a final clarification. Analysis of the corresponding daily composite samples of raw influent and treated effluent were performed over seven consecutive days and over two consecutive months (May–June 2010). Integrated samples were representative of a single day's work in the STP and were taken at one-hourly intervals. Sampling was carried out by an automatic device (0.5 L/3 h).

The river samples analyzed were collected from The Henares River, located in the centre of Spain (Madrid). This area is one of the most developed and densely populated of the country, with cities such as Madrid (>3,200,000), Alcalá de Henares (>203,000) or Guadalajara (>83,000), together with heavy industrial activity and several wastewater treatment plants (WWTPs) which lie along the river. Sampling points were selected to detect changes in the concentrations of the drugs so as to evaluate the effects of human influence and population density on river pollution. The Henares River sampling points 1, 2 and 3 were located between two small STPs (35 km, 24 km and 6 km before point 4, respectively), and which also had a very industrial surrounding area. Sampling point 4 receives the treated effluents from an important WWTP and it is a densely populated area. Sampling points 5 and 6 were situated after the discharge area from the sewage plant and downstream from a heavily populated area with large contributions from urban and industrial zones (20 m and 1.15 km after point 4, respectively). Grab samples (2 L) were collected from the 6 sites described above during a sampling campaign carried out in June 2010. The distribution and location of the sampling points can be found in Fig. 1.

All samples were collected using pre-rinsed amber glass bottles, and then they were sent in boxes packed with ice to the laboratory for subsequent analysis. Upon reception, samples were filtered through a 0.7 μm glass fibre filter (Teknokroma, Barcelona, Spain) and following this, samples were adjusted to pH 3 with 37% HCl and stored in the dark at −20 °C until analysis to prevent degradation of analytes during storage. Direct injection of samples at three different pHs (3, 6 and 8) were tested. Before analysis, all samples were spiked with the mixture of surrogate standards, nicotine-d_3 and $^{13}C_3$-Caffeine, in acetonitrile, and then all extracts were filtered using a 0.45-μm PTFE syringe filter (Millipore, USA) to remove suspended solids and particulate matter and to check the analysis.

2.3. LC–MS/MS analysis

A hybrid triple quadrupole/Linear Ion Trap mass spectrometer system (5500 QTRAP® LC/MS/MS, AB Sciex Instruments, Foster City, CA) was used to identify and quantify the target compounds. The triple quadrupole/linear ion trap (QqQLIT) is a hybrid system in which the final quadrupole can operate as a conventional mass filter or as a linear ion trap [16]. For the LC analysis, an Agilent 1200 HPLC system (Agilent Technologies, Wilmington, DE, USA) with a binary pump was used. The analytical column employed was a reversed-phase C8 of 150 mm × 4.6 mm and 5 μm particle size (Agilent ZorbaxEclipse XDB). Mobile phases A and B were acetonitrile and HPLC-grade water (containing 0.1% formic acid), respectively. The gradient program started with 10% of A, constant for 0.5 min, followed by a linear gradient up to 100% A in 10 min, and finishing with 100% A constant for 6 min. After this 16-min run time, 10 min of post-time followed using the initial 10% of A. The flow rate was set constant at 0.6 mL min^{-1} during the whole process, and the injection volume was 10 μL.

The HPLC system was connected to a QqQLIT-MS/MS with an electrospray interface (ESI) and was operated in positive ionization mode. The TurboIonSpray source settings were: IonSpray Voltage (IS), 5500 V; Source Temperature, 500 °C; Curtain Gas (CUR), 20 (arbitrary units); Collision Gas (CAD), Medium; Ion Source Gas (GS1 and GS2), 50 psi. Nitrogen was used as the nebulizer gas, curtain gas and collision gas.

2.3.1. Selected reaction monitoring (SRM) parameters

In order to obtain maximum sensitivity for identification and detection of all target compounds, two SRM transitions were optimized for each compound. All operation parameters, including declustering potential (DP), entrance potential (EP), collision energy (CE) and collision cell exit potential (CXP) were separately studied for each analyte by flow injection analysis (FIA) in the spectrometer. The best sensitivity in multiple reaction monitoring operation mode was achieved through the acquisition of selected reaction monitoring (SRM) transitions under time-scheduled conditions and with a time window of 90 s. The scheduled SRM enables optimized cycle time and maximized dwell times to be used during acquisition to provide higher multiplexing with good analytical precision. The quadrupoles Q1 and Q3 were set at low and unit resolution, respectively. For confirmation of target analytes, the EU guidelines for LC–MS/MS analysis were considered (Commission Decision 2002/657/EC) [14]. The criteria required were as follows – the acquisition of two SRM transitions for each compound, the retention time and the monitoring of the SRM ratio (which is the relationship between the abundance of transitions selected for identification and for quantification, SRM2/SRM1) – in order to get a suitable confirmation and thus avoiding overestimations or false positive findings in quantitative analysis. Table 2 shows the values of the optimized parameters and the SRM transitions used for the confirmation and quantification of all the compounds studied. The most intense SRM transition was selected for quantitation purposes (SRM1). AB SCIEX Analyst software 1.5.1 was used for data acquisition and processing.

2.3.2. IDA conditions

An additional experiment was developed for those compounds of which further structural information was necessary for their confirmation. In this case, an IDA method was programmed combining SRM as the survey scan and two EPI scans as dependent scans, in the same injection. The survey scan contained 22 SRM transitions, which corresponds to the most intense for each substance in the SRM method (see SRM1, in Table 2). IDA parameters included the acquisition of two ions whose peak height exceeded 500 counts per second with no exclusion after dynamic background subtraction of

Table 2
Values of the optimized parameters with the developed method by LC–QLIT-MS/MS.

Compounds	tr (min)	Precursor ion (*m/z*)	DP	SRM 1 *quantitation*	CE1	SRM 2 *confirmation*	CE2	SRM2/SRM1 (C.V., %)
Nicotine	2.3	163.1	125	117.0	35	130.0	27	0.8 (4)
Cotinine	2.7	177.0	125	80.0	36	98.0	26	0.4 (7)
Morphine	3.5	286.1	125	201.0	36	229.0	31	0.6 (1)
Paraxanthine	6.0	181.2	100	124.0	25	69.2	40	0.2 (5)
Codeine	6.1	300.2	150	215.0	32	199.0	40	0.7 (5)
Ephedrine	6.3	166.1	125	148.1	16	117.0	27	0.3 (7)
Acetylmorphine	6.5	328.0	150	211.1	31	193.1	39	0.8 (7)
Amphetamine	6.6	136.2	136	91.0	26	119.0	12	0.4 (7)
MDA	6.7	180.1	125	163.1	15	135.0	26	0.3 (8)
Caffeine	6.8	195.1	125	138.0	25	110.0	30	0.3 (7)
Ethylmorphine	6.8	314.0	150	229.1	37	183.0	38	0.5 (7)
MDMA	6.9	194.2	125	163.1	14	135.1	26	0.4 (16)
Ketamine	7.0	238.0	125	125.1	30	220.1	20	0.5 (10)
Benzoylecgonine	7.1	290.2	150	168.1	25	105.0	40	0.4 (7)
Ethylamphetamine	7.1	164.0	125	91.0	23	119.0	15	0.3 (10)
MDEA	7.2	208.2	125	163.1	15	135.0	28	0.4 (11)
Phenylephrine	7.3	168.2	200	91.0	29	150.1	10	0.2 (12)
Heroin	7.5	370.0	125	268.2	40	328.0	31	0.4 (5)
Cocaine	7.7	304.1	125	182.0	25	82.1	38	0.4 (17)
Methamphetamine	7.8	150.2	188	91.0	24	119.0	13	0.3 (6)
EDDP	8.8	278.0	150	249.2	19	234.1	21	0.7 (4)
Methadone	9.1	310.2	150	265.1	19	105.0	35	0.7 (9)

DP: declustering potential (V); CE: collision energy (eV); EP: entrance potential (10 V); CXP: collision cell exit potential (5 V).

the survey scan. EPI scans were performed with Q1 set at low resolution and the linear ion trap scanning from 80 to 450 amu at a scan rate of 10,000 amu/s. The dynamic fill-time option was selected on the ion trap. In this fashion, two EPI scans were monitored in each SRM-IDA-EPI cycle, at two different CEs (25–40 eV) and with a DP of 125 V. The complete SRM-IDA-EPI cycle time was 1.58 s. The spectra generated by pure solvent solutions at 10 and 100 $\mu g\,L^{-1}$ concentrations, acquired at each EPI condition, were stored in a mass spectral library, which enabled the confirmation of organic compounds in real samples by searching in the library. For this methodology, the confirmation criteria applied for identification of the target compounds in water samples were: the presence of the characteristic SRM transition at the correct retention time and good agreement between the reference spectrum of the library and the spectrum obtained in the samples or a fit value higher than 70%.

2.4. Method validation

Linearity, sensitivity (detection and quantitation limits, LODs and LOQs, respectively), repeatability and reproducibility were established to determine the accuracy and precision of the LC–ESI-MS/MS method. Direct injection of all samples at pH 3 was the procedure selected for carrying out all the validation studies. Because it was impossible to obtain blanks, the samples were previously analyzed and the presence of the target compounds taken into account. For assessment of matrix effects, standard calibration curves prepared in water, effluent, influent and river samples were compared. The dilution of each sample with acetonitrile prior to its filtration and analysis was the strategy applied in order to minimize matrix effects. Furthermore, matrix-matched calibration curves were used for quantitative determinations, in order to minimize ion suppression/enhancement effects: a consequence of the presence of sample matrix components. Each point was obtained as the average of three injections. The linearity in the response was studied by using matrix-matched calibration solutions prepared by spiking each matrix at six concentration levels, ranging from the quantitation limit of each analyte to 100 μg/L in wastewater extracts, or 1 μg/L in river extracts, depending on the concentration level usually present in the samples. Intra- and inter-day variability, determined as relative standard deviation (R.S.D.), were obtained from repeated analysis ($n = 5$) of spiked effluent samples at 50 μg/L, from run to run over 1 day (repeatability) and over 5 days (reproducibility), respectively. The instrumental detection limits (IDLs) were estimated from the injection of a standard solution successively diluted until reaching a concentration level corresponding to a signal-to-noise ratio of 3 (SRM2). The quantification limits (LOQs) were determined experimentally by extrapolation: these were the minimum detectable amounts of analyte which showed a signal-to-noise ratio of 10 for the SRM1 transition, from spiked extracts in the three different matrices. On the other hand, while in the SRM method, the detection limits (LODs) were determined experimentally by extrapolation as the minimum analyte concentrations which exhibited $S/N \geq 3$ for the SRM2 transition, in the IDA method, the LODs were considered to be the minimum amount of analyte concentrations which exhibited $S/N \geq 3$ for the SRM1 transition, which provided an EPI spectrum recognized by the mass spectral library with a sufficiently high degree of confidence (fit value > 70%) [16].

3. Results and discussion

3.1. Performance of the analytical procedure

In light of the results, direct injection of all samples at pH 3 was the pH selected for carrying out all the validation studies. This was because no significant difference in results at the three pHs tested (3, 6 and 8) was found (data not include) and, additionally, this was the pH used to prevent degradation of analytes during storage. Sewage and surface water were analyzed in order to evaluate the performance of the developed methods for the analysis of drugs of abuse in water samples, without prior sample pre-concentration. Intra- and inter-day variability were calculated as %RSD and ranged from 4% to 16% ($n = 5$) for all analytes. The method was shown to be linear, with correlation coefficients higher than 0.99, ranging from the quantitation limit of each analyte to 100 μg/L in wastewater extracts or 1 μg/L in river extracts, depending on the concentration level usually present in the samples.

3.1.1. Detection and quantification limits

Quantification limits obtained from the two operation modes (SRM and IDA) developed in this study were similar since, in both modes, the same transition was used to carry out the quantifi-

Table 3
Validation results of the two analytical methods. LOQ and LOD (ng L^{-1}) obtained in all matrices studied.

Compounds	IDL (pg inyected)	Influent			Effluent			Surface water		
		MRM mode		IDA mode	MRM mode		IDA mode	MRM mode		IDA mode
		LOQ	LOD	LOQ	LOQ	LOD	LOQ	LOQ	LOD	LOQ
Nicotine	0.10	250	200	200	200	150	170	200	130	170
Cotinine	0.10	210	180	180	200	170	150	10	7	7
Morphine	0.50	60	30	50	50	20	40	10	7	8
Paraxanthine	0.50	550	550	100	450	450	80	100	100	35
Codeine	0.10	40	20	30	40	20	30	30	15	30
Ephedrine	0.10	50	50	40	50	50	40	40	35	30
Acetylmorphine	0.10	75	40	50	65	40	40	30	20	20
Amphetamine	2.50	500	500	200	500	500	200	500	500	200
MDA	1.50	400	400	150	400	400	150	110	100	40
Caffeine	0.10	200	200	30	200	200	25	100	80	20
Ethylmorphine	0.10	100	55	70	85	50	60	30	20	20
MDMA	5.00	680	680	100	600	600	90	600	600	55
Ketamine	0.10	50	30	25	50	30	25	25	15	10
Benzoylecgonine	0.05	20	10	13	10	5	10	7	3	2
Ethylamphetamine	0.10	50	50	10	50	50	10	20	20	7
MDEA	0.10	50	20	30	50	20	30	20	10	10
Phenylephrine	4.00	500	500	80	500	500	80	400	350	50
Heroin	0.10	90	50	60	80	40	40	40	30	25
Cocaine	0.02	10	3	10	3	1	3	0.5	0.5	0.3
Methamphetamine	5.00	700	700	300	700	700	300	700	650	300
EDDP	0.10	40	30	40	30	20	30	30	20	20
Methadone	0.10	10	5	10	10	5	10	2	1	2

IDL: instrumental detection limit; LOQ: detection limit; LOD: quantification limit.

cation. The LOQs achieved ranged from 10 ng/L to 700 ng/L for wastewater and 0.5–700 ng/L for river water. However, for those compounds for which the second transition showed a difference in intensity up to 10 times lower than the first transition, or a ratio SRM2/SRM1 lower than 0.3, the LOQs obtained in IDA mode were of up one order of magnitude lower. Such was the case with caffeine, amphetamine, ephedrine and their respective metabolites, in all of matrices studied (Table 3). Thus, LOQ values obtained in wastewater from the IDA method developed in this work achieved ng L^{-1} levels which were quite similar to those most commonly reported in the literature. The differences in sensitivity, ranged from 1 to 100 times higher than those reported when using the habitual sample pre-concentration, as was the case with amphetamine and its metabolites; or even up to 8 times lower to those reported in previous publications, as was the case with nicotine, cotinine and paraxanthine. These differences in sensitivity might be related to the matrix effect associated with the sample pre-concentration process and the application of a more highly sensitive mass analyzer. Although, in general, LOD values were poorer than those obtained with the pre-concentration steps, an adequate identification of most analytes detected in the samples was achieved by the SRM method developed in this work. Besides, the use of the IDA method allowed the determination of some target compounds and metabolites, which the second transition did not detect at lower concentrations. The IDA method allowed us to obtain enough information for their confirmation in some of the samples by searching the library. On the other hand, as expected, LODs obtained in wastewater were higher than those for river water due to the matrix complexity that influences ionization effectiveness and increases noise. However, there were no significant differences in LOQ and LOD values between effluent and influent matrices (see Table 3).

3.1.2. Matrix effect

Matrix effects can compromise quantitative analysis by LC–ESI-MS. For this reason they were carefully considered during quantitative investigations. Co-eluting residual matrix components present in the sample can affect the ESI source, resulting in either signal suppression or enhancement that leads to erroneous results. Several different approaches have been proposed in the literature to account for matrix effects including: (i) matrix-matched standards calibration, (ii) sample dilution or (iii) the use of stable-isotopically labelled internal standards [17,18]. However, acquiring sufficient isotopic-labelled internal standards for multi-component analysis is almost impossible. Therefore, matrix-matched standard curves are commonly applied in multi-component quantitative analyses for compensation of matrix effects. In our study, we have minimized the matrix effects by diluting all samples with acetonitrile pure before filtration and analysis. Furthermore, to overcome possible matrix effect problems, matched calibration curves were used for quantitative determinations rather than surrogate standards: this was mainly because appropriate deuterated standards were not available. However, the more the samples are diluted, the greater the decrease in analyte concentrations. In our case, matrix ionization suppression effects were observed for all compounds, except morphine, which showed a signal-enhancement effect. For wastewater matrices, the signal suppression was small for most compounds (ranging from 2% to 25%). But nicotine and its metabolite, cotinine, presented an intermediate suppression effect (33–44%); and only one compound, morphine showed a strong matrix ion enhancement effect in sewage water of over 100%. With surface water matrices, all compounds exhibited signal suppression effects lower than 22% (see Fig. 2). As expected, matrix effects were more important in wastewater samples than in surface water, for most compounds. However, there were no significant differences, in values obtained for effluent and influent matrices. In general, as shown in Fig. 2, matrix ionization suppression effects decreased with increasing chromatographic retention time. The more polar compounds (first eluting), nicotine, cotinine and morphine, were the analytes which showed higher percentage values. This fact might be related to the highest matrix effects occurring at the beginning of the chromatography process, between 0.5 and 4 min, due to matrix co-eluting interferences affecting the first compounds to be eluted. Results demonstrated that the direct sample injection method allowed a drastic reduction in the matrix effects, compared to results in previous publications [20,21] because, with this method, it is possible to avoid pre-concentration interference.

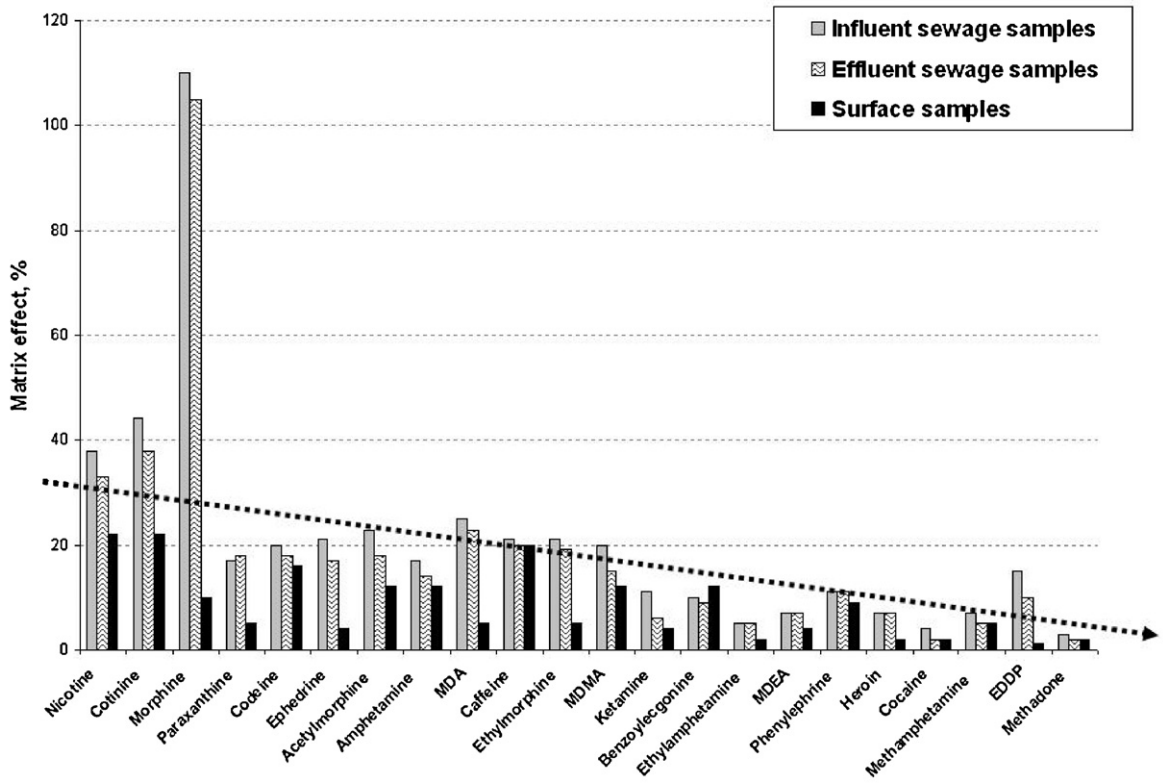

Fig. 2. Matrix effects for all selected analytes, in surface water, influent and effluent sewage water, by direct sample injection. Compounds ordered in relation to their chromatographic retention time. All compounds presented signal ion suppression effects, with the exception of morphine, which showed a signal-enhancement effect.

3.2. Occurrence of drugs of abuse and their metabolites in wastewater

The number of positive samples, concentration ranges and mean concentrations for each drug of abuse and their metabolites in the water samples analyzed are summed up in Table 4. As can be seen, 6 out of the 22 compounds investigated were not detected in any water sample. These compounds were ketamine, heroin, two metabolites of morphine (acetylmorphine and ethylmorphine) and two metabolites of amphetamine (MDEA and MDMA). The most ubiquitous compounds were caffeine, nicotine, cocaine, ephedrine and their respective metabolites – present in all samples (except nicotine, $n = 12$) – followed by codeine, methadone, EDDP and morphine ($n = 13$–15).

The non-controlled drugs, caffeine, nicotine and their metabolites, paraxanthine and cotinine, were the major contributors to the total amount of drugs, both in influent and effluent samples. Caffeine and paraxanthine were detected in all sampling campaigns ($n = 15$), both in the input and output of the STP, at mean concentrations of 58.3 μg/L and 50.3 μg/L in influent waters, while in effluent samples, the mean levels found were 17.3 μg/L and 19.3 μg/L, respectively. Nicotine and cotinine were also detected in all samples, with the exception of nicotine in effluent samples ($n = 12$). These stimulant drugs showed concentrations of up to 23.3 μg/L and 17.3 μg/L for nicotine, and 27.7 μg/L and 9.5 μg/L for cotinine, in influent and effluent samples, respectively. Concentrations for caffeine and its metabolite were up to an order of magnitude higher than those values for nicotine and cotinine. Many data on concentration levels in STPs have been finding for all these compounds. The mean concentration values detected for nicotine and cotinine in treated effluent samples were in the range of those reported by Huerta-Fontela et al. [4] from 42 STPs in north-eastern Spain (3.5 μg/L and 2.4 μg/L, respectively). However, mean concentrations for caffeine and its metabolite were an order of magnitude higher than those reported in the same work in effluent samples (2.5 μg/L and 1.3 μg/L, respectively).

Regarding controlled drugs, cocaine, ephedrine and their respective metabolites were the next group of stimulant drugs detected at elevated concentrations during the monitoring. All of them were found in each analyzed sample, detected at relatively high concentrations, both in the inlet and outlet of the sewage plant. Concentration ranges in WWTP influents were 0.9–4.8 μg/L for ephedrine and 0.9–4.5 μg/L for phenylephrine. Regarding to data reported for untreated wastewater from STPs in the USA [10], the concentrations of ephedrine were comparable to the present study, ranged from 0.5 μg/L to 6.9 μg/L. However, these concentration values were higher than those reported by Postigo et al. [5] for ephedrine (0.2–0.7 μg/L) from input sewage of STPs in Europe. This difference in the results can be explained because the sewage plant evaluated in this work is located very close to an important hospital in the area, and furthermore, this compound is a legal therapeutic medication, widely used in hospitals as a bronchodilator as well as to counteract the hypotensive effects of anesthesia. On the other hand, cocaine and benzoylecgonine (BE) concentrations in WWTP influent, with mean concentration of 474 ng/L and 2541 ng/L, respectively, were in agreement with other previously published results in Spain [4,5]. However, concentration levels found in effluent samples – 171 ng/L for cocaine and 1010 ng/L for BE – were one magnitude order higher than those values reported in these same publications, indicating worse removal efficiency in the STP studied. Similarly, Castiglioni et al. [3] reported maximum values of 13.9 ng/L and 128.9 ng/L for cocaine and BE in effluent wastewater samples of Italy.

In relation to the other group, methadone and its metabolite, EDDP, were the tranquilizing drugs found at lower concentrations, both in influent and effluent samples, with mean concentrations of 45–18 ng/L for methadone and 138–64 ng/L for EDDP. Nevertheless, they were also quite ubiquitous, detected in most samples

Table 4
Concentration range and mean values found for each drug of abuse and their metabolites in the three different matrices studied, obtained by the SRM or IDA method.

Compounds	Influent			Effluent			River water		
	N° [a] (n = 15)	Concentration		N°[a] (n = 15)	Concentration		N° [a] (n = 6)	Concentration	
		Range (ng/L)	Mean (ng/L)		Range (ng/L)	Mean (ng/L)		Range (ng/L)	Mean (ng/L)
Stimulant drugs									
Nicotine	15	7683–23,325	15,416	12	295–17,385	4510	*3*	*175–215*	*193* [b]
Cotinine	15	4279–27,724	12,480	15	375–9530	4933	6	22–45	29
Caffeine	15	18,305–96,154	58,300	15	1207–53,200	17,302	6	475–515	493
Paraxanthine	15	18,272–89,712	50,381	15	15,714–43,200	19,310	6	100–130	110
Cocaine	15	40–820	474	15	12–496	171	3	5–87	44
Benzoylecgonine	15	851–4094	2541	15	487–2221	1010	6	10–530	142
Amphetamine	*5*	*212–1021*	*496* [b]	*4*	*215–325*	*225* [b]	*1*	*309*	*309* [b]
METH	*3*	*475–700*	*614* [b]	–	–	–	–	–	–
EAMP	*7*	*29–96*	*63* [b]	*4*	*22–44*	*30* [b]	*2*	*8–12*	*10* [b]
MDA	*1*	*266*	*266* [b]	–	–	–	–	–	–
MDEA	–	–	–	–	–	–	–	–	–
MDMA	–	–	–	–	–	–	–	–	–
Ephedrine	15	912–4817	3212	15	217–2749	1420	2	51–90	70
Phenylephrine	15	891–4505	2420	15	510–1992	1090	*2*	*201–481*	*341* [b]
Ketamine	–	–	–	–	–	–	–	–	–
Tranquilising drugs									
Morphine	13	90–275	152	13	60–155	73	2	12–19	16
Acetylmorphine	–	–	–	–	–	–	–	–	–
Ethylmorphine	–	–	–	–	–	–	–	–	–
Methadone	14	19–127	45	13	15–80	18	3	2–14	7
EDDP	13	64–542	138	13	49–90	64	2	31–40	35
Heroin	–	–	–	–	–	–	–	–	–
Codeine	14	234–1556	845	15	289–786	390	3	32–174	76

[a] N°: number of samples with concentrations higher than their LOQ value.
[b] Values in italics: data obtained by the IDA method.

(n = 13). These concentrations are in agreement with those reported previously by Castiglioni et al. [3], which were up to 39 ng/L for methadone and 80.8 ng/L for EDDP, in treated sewage.

Although amphetamine-type stimulants are consumed more than the opiate and cocaine group, according to UNODC, 2010 [1], no compound from these drug groups could be properly identified by the SRM method developed, in any of the samples analyzed. This was because they were presented in the samples at concentration levels below their LOQs, or at concentrations for which the second transition was not perceived. However, the use of the IDA method enabled a higher sensitivity in the detection of these compounds, obtaining more information for their positive confirmation in some sewage samples by searching in the library. Therefore, 4 and 2 amphetamine-compounds were detected in influent and effluent samples, respectively. Amphetamine, METH and EAMP, were detected in less than half of the wastewater samples, at mean concentrations of 496 ng/L, 614 ng/L and 63 ng/L, respectively, in influent waters. Chiaia and Banta-Green [10] reported concentrations of amphetamine and METH very similar to the values found in this study, up to 630 ng/L for amphetamine and 2200 ng/L for METH, from WWTPs municipal influent from USA. In effluent samples, the mean levels found were 225 ng/L for amphetamine and 30 ng/L for EAMP. MDA was detected in 1 influent sample (266 ng/L), while MDEA and MDMA were not detected in any sewage samples at all (see Table 4). In all cases, the EPI spectra acquired during the analysis of real samples were identified by the spectral library with a match quality of at least 70%. Fig. 3 shows an example of methamphetamine (METH) identification in an influent wastewater sample, using two methods developed in this work – the MRM and IDA methods. Using the MRM method, it was not possible to get a suitable identification of this compound. However, the IDA method allowed its positive confirmation in the same sample by searching in the library. As we can see, FIT values were higher than 88% for two spectra obtained under two different conditions (CE = 25 and 40 eV). Finally, if we compare the results obtained during the sampling campaign carried out in this study with other publications relating to wastewater [4,5,10], we will see that although the mean concentrations are slightly higher for most compounds detected, the magnitude order remained the same.

In order to evaluate the concentration changes of some illicit drugs over time, daily variability was evaluated by performing a sequential survey, which revealed some important fluctuations in the concentration levels, depending on the compounds. For that, composite influent samples were collected over seven consecutive days. Different variations in concentration levels were found over the week, depending on the substance. Most of them were detected at similar concentrations over the 7 consecutive days: methadone (ranging from 50 ng/L to 90 ng/L), morphine (150–275 ng/L) and codeine (700–930 ng/L). However, as Fig. 4 shows, cocaine and its metabolite, benzoylecgonine (BE), presented the highest influent concentration levels in the Saturday, Sunday and Monday samples, which suggested a preference for using this kind of drug at the weekend; with maximums of 0.8 μg/L and 4.1 μg/L, respectively, measured on the Sunday. Concentrations from Tuesday to Friday ranged from 50 ng/L to 300 ng/L for cocaine and 0.8–2 μg/L for BE, while from Saturday to Monday, the values jumped to 0.5–0.8 μg/L and 3.0–4.1 μg/L, respectively. In contrast, concentration levels of ephedrine and its metabolite, phenylephrine, detected over the 7 consecutive days, decreased by more than 50% in the Saturday and Sunday samples, coinciding with decreased activity at the hospital at the weekend – these two drugs are widely used to counteract the hypotensive effects of anesthesia in hospitals. Concentrations from Monday to Friday ranged from 3.3 μg/L to 4.8 μg/L for ephedrine and 1.7–4.5 μg/L for phenylephrine, with maximum levels on Tuesday.

3.3. Occurrence of drugs of abuse and their metabolites in surface water

In order to study the occurrence of several drugs of abuse and some of their metabolites in the environment, surface water river samples were analyzed. The Henares River basin comprises a densely populated area with large contributions from urban and industrial zones. The river was sampled at six sites, before and after

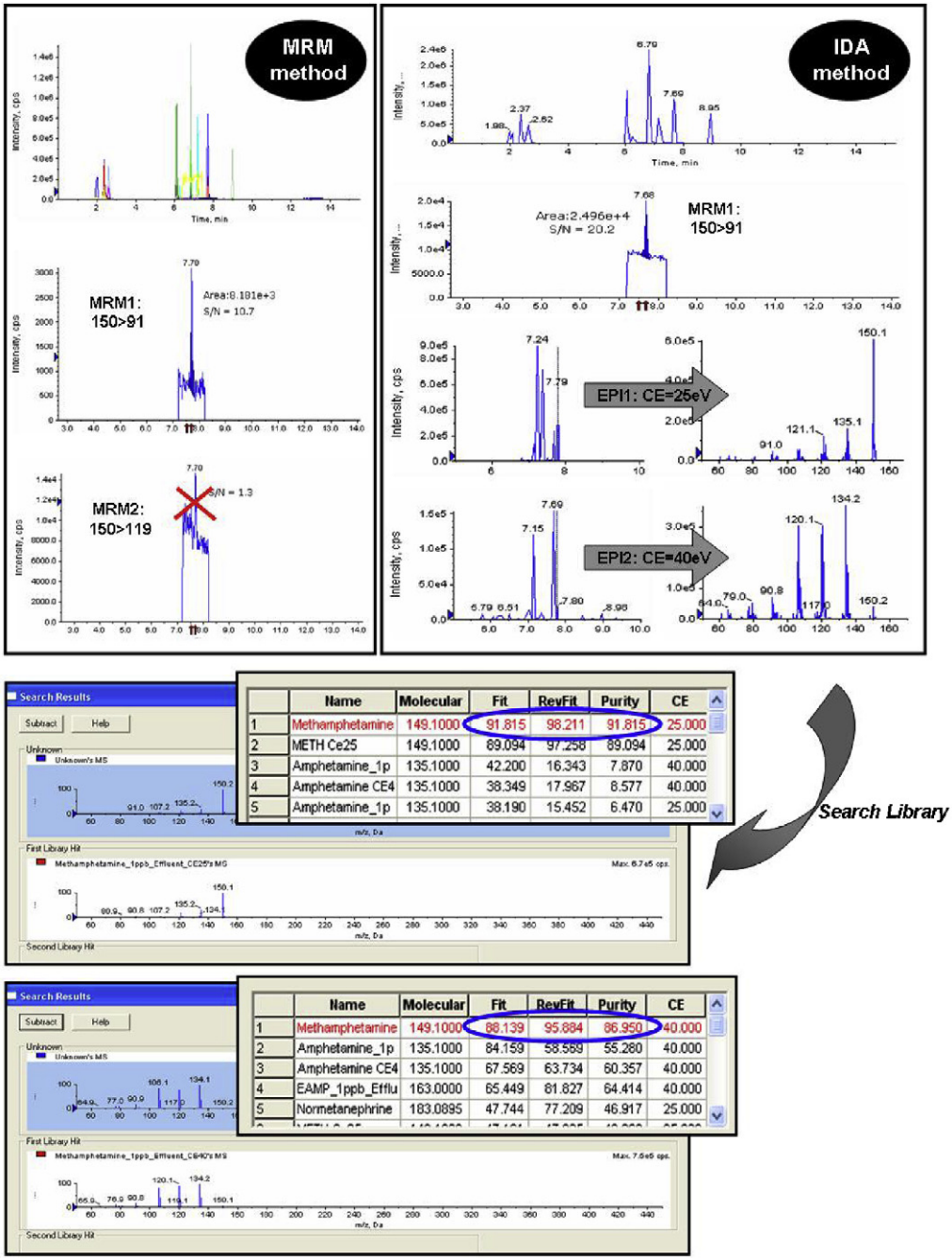

Fig. 3. Identification of methylamphetamine in influent wastewater, by the MRM and IDA methods.

the location of an important WWTP and a densely populated area (site 4), 36 km, 24 km and 6 km, before the plant (site 1, 2 and 3, respectively), 20 m after it (site 5) and 1.15 km further downstream (site 6). A summary of the results (number of positive samples, mean and concentration range) measured for target drugs of abuse in surface water is shown in Table 4. Each result was obtained as the average of two injections. More than half of the target compounds were detected in at least one of the samples collected, ranging from 10 ng/L to 493 ng/L. As expected, the numbers of compounds and concentration levels detected in surface water were lower than in sewage water, due mainly to the dilution factor applied.

Caffeine and its metabolite, paraxanthine, were detected at all sampling points at concentrations between 475–515 ng/L and 100–130 ng/L, respectively. Cotinine was also detected in all samples but at concentrations lower than those found for caffeine and paraxanthine (22–45 ng/L). However, its precursor, nicotine was only detected at half of the sampling sites (points 3–5) at levels over its LOQ using the IDA method developed, at a mean concentration of 193 ng/L. Although concentration values found for nicotine, cotinine, caffeine and paraxanthine were lower than the mean values reported by Huerta-Fontela et al. [19] in a river in north-eastern Spain (595, 331, 1926 and 1756 ng/L, respectively), the levels detected in this work were of the same magnitude order to those found in a previous monitoring campaign performed in the same area [22].

As regards controlled drugs, the most abundant compounds were the respective metabolites of cocaine and ephedrine – BE and phenylephrine. Both substances were among the most abundant and frequently detected stimulant drugs in all the sewage samples analyzed. BE was found at all sampling sites at a mean

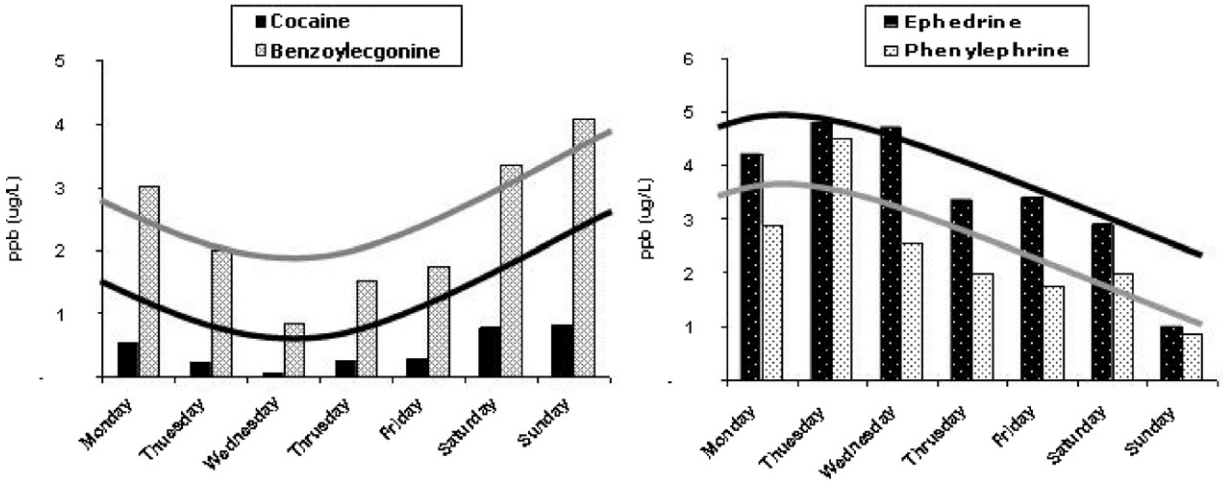

Fig. 4. Evaluation of the concentration changes of some illicit drugs over time from composite influent samples collected over seven consecutive days.

concentration of 142 ng/L. However, its precursor, cocaine, was only detected at three points (points 3–5) between 5 ng/L and 87 ng/L. Ephedrine and phenylephrine were detected in less than half of the river water samples, at levels up to 90 ng/L and 481 ng/L, respectively. These concentrations were in agreement with the maximum values reported in Spanish rivers [5,19] and with those published cocaine and BE levels found in Italian surface water [2]. Although amphetamine was found at a high concentration, 309 ng/L, it was only detected in one sample (point 4), while EAMP was detected twice but at low levels, ranging from 8 ng/L to 12 ng/L. In order to confirm both substances, it was necessary to use the IDA method, because they presented in the water samples at concentration levels below their respective LOQs achieved using the SRM method.

On the other hand, codeine, morphine, methadone and its metabolite EDDP, were the tranquilizing drugs detected at levels over their respective LOQs, at mean concentrations of 76 ng/L, 10 ng/L, 7 ng/L and 35 ng/L, respectively. Although these values were higher than the mean levels reported by Berset et al. in Switzerland [23], they were of the same order of magnitude.

Finally, Fig. 5 illustrates the total load of substances found along the river. The highest total load of drugs of abuse and metabolites was detected at sampling point 4. This is because it is a densely populated area and furthermore, it receives the discharges from an important WWTP (above 2600 ng/L). The next highest total load was detected at point 5, situated 20 m downstream of this zone (above 1470 ng/L). The remaining points presented stable total load levels (below 900 ng/L), mainly due to the contribution of non-controlled drugs, caffeine, nicotine and their metabolites.

3.4. WWTP removal efficiency

The removal efficiency for drugs of abuse and their metabolites during their passage through a municipal STP with secondary treatment was investigated. Analysis of the corresponding daily composite samples, both influent and effluent, were performed over seven consecutive days and over two consecutive months. Elimination efficiency for each compound was calculated as the mean removal percentage of each sampling campaign: considered as being 100% eliminated when the analyte was quantified in the influent water but not in the corresponding effluent. Fig. 6 shows the mean concentration and mean removal percentages estimated for each compound detected at a STP in south-eastern Spain. As expected, concentrations of drugs of abuse and their metabolites were lower in effluents than in influents. A comparison of the influent and effluent concentrations of each of the different substances revealed that out of all of them, morphine, codeine, EDDP and EAMP were not extensively degradated in the STPs, with elimination percentages below 54%.

Nicotine, caffeine and their respective metabolites, cotinine and paraxanthine, showed acceptable removal efficiencies: ≥70% for nicotine/caffeine, and ≥60% for cotinine/paraxanthine. Cocaine and its metabolite, BE, were also efficiently removed with percentages higher than 60%. However, removal rates reported for these compounds vary, between the different STPs studied by various authors. However, in general, the values obtained in this work were lower than those previously reported, which were up to 99% [4]. The estimated removal efficiencies for ephedrine and phenylephrine were 57% and 55%, respectively. This ephedrine value is in agreement with that published by Postigo et al. [5].

Conversely, the elimination rates for the amphetamine compounds were very different: the values ranged from 53% for EAMP, followed by 55% for amphetamine and finally, 100% for METH

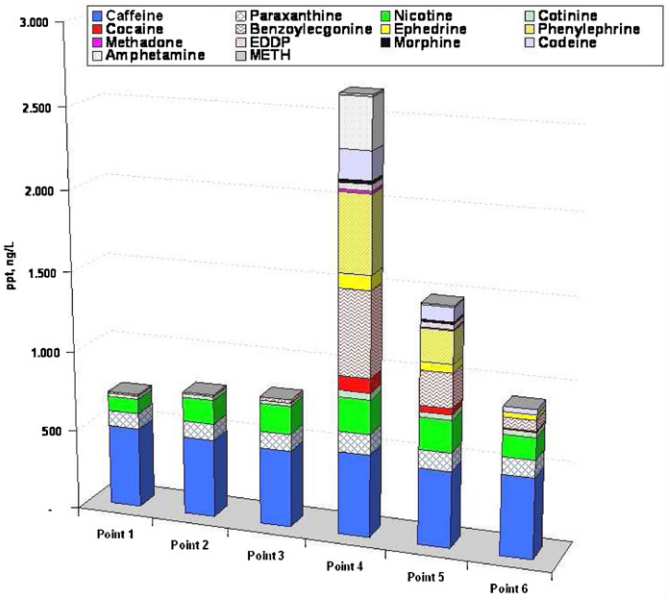

Fig. 5. Total load of the drugs of abuse and their metabolites detected along The Henares River, during the monitoring campaign carried out.

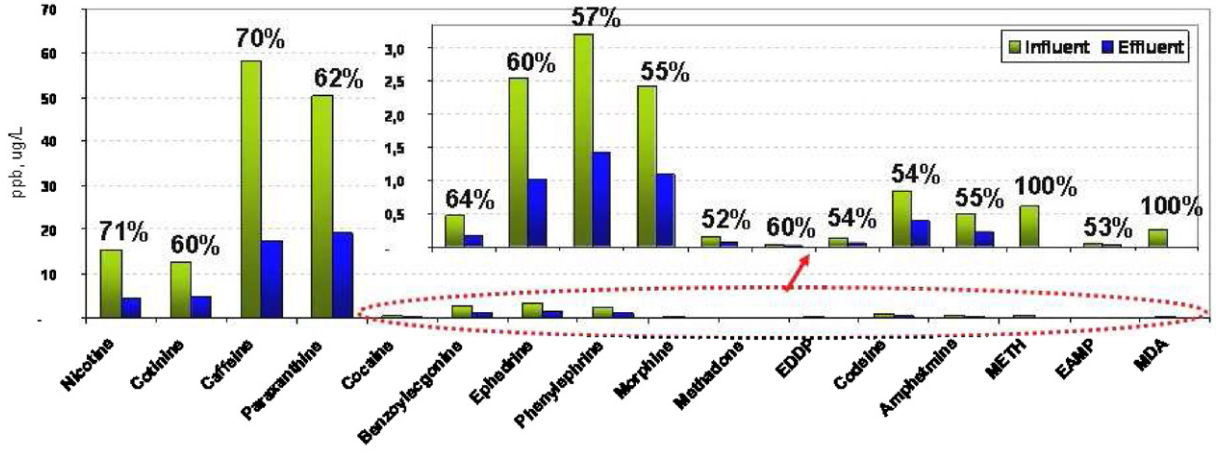

Fig. 6. Mean concentration (ng/L) of each drug of abuse and their metabolites detected in influent and effluent STP samples, along with their removal efficiency percentage.

(this was not detected in any treated effluent samples) and MDA (detected in only one influent sample). In comparison to data found here, Huerta-Fontela et al. [4] reported values ranged from 52% to more than 99% for amphetamine and from 44% to more than 99% for METH. Furthermore, similarly to this study, MDA was only detected in 3 out of 8 WWTPs, showing in two of these an elimination percentage higher than 99%. Finally, regarding the tranquilizing drugs, morphine, methadone, EDDP and codeine, the removal percentages ranged from 52% to 60%. In spite of the acceptable removal efficiencies found for most drugs of abuse and their metabolites, all of them were detected in treated effluents and even in river samples (see Table 4), except for METH and MDA.

4. Conclusion

The direct analysis of environmental samples without prior sample treatment is attractive because it is an environmentally friendly practice: it reduces the sample preparation steps as well as the amounts of solvent and water; it avoids the time-consuming characteristics of SPE and the need to measure recovery with an internal standard. Besides, the signal-enhancement/suppression problems generated by the matrix effect are decreased, avoiding sample pre-concentration – therefore, the overall robustness of the analysis is increased. In this study, a constant consumption of some drugs of abuse was observed during the investigated period (caffeine, nicotine, cocaine, ephedrine, codeine, morphine and methadone). Most of them were found both in wastewater as well as in surface water, which reveals the prevalence of these substances in the aquatic environment. The SRM method was sufficiently sensitive to directly quantify12 analytes in wastewater and 10 analytes in surface water, at concentrations less than 700 ng/L, without sample pre-concentration. However, the IDA method, which combines SRM and two analyses in full scan product ion mode (EPI) within the same chromatographic run, enabled us to achieve higher sensitivity. In this case, 16 and 14 target compounds were detected in sewage and river water samples, respectively, at concentrations less than 300 ng/L. Therefore, the IDA method conserves the quantitative performance of the SRM method and provides additional product ion spectra at low concentration levels (when the monitoring of two transitions is not possible) avoiding the reporting of false positive findings in cases where only one SRM was detected. However, sometimes the IDA mode shows a variable background noise.

All in all, a combination of both methods proved to be the best option for carrying out the analysis of these kinds of target compounds in environmental water, and achieved detection limits below the ng L^{-1} level, without the need for a preliminary pre-concentration step.

Acknowledgements

The authors wish to acknowledge the Spanish Ministry of Education and Science (Programa Consolider Ingenio 2010 CE-CSD2006-00044) for their economic support. M.J. Martínez Bueno acknowledges the research fellowship from the Junta de Andalucía (Spain) associated to the project (Ref. TEP2329).

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