Journal of Chromatography A, 1218 (2011) 2264–2275

Contents lists available at ScienceDirect

Journal of Chromatography A

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

Multi-class determination of around 50 pharmaceuticals, including 26 antibiotics, in environmental and wastewater samples by ultra-high performance liquid chromatography–tandem mass spectrometry

Emma Gracia-Lor, Juan V. Sancho, Félix Hernández*

Research Institute for Pesticides and Water, University Jaume I, Avda. Sos Baynat, E-12071 Castellón, Spain

ARTICLE INFO

Article history:
Received 2 November 2010
Received in revised form 9 February 2011
Accepted 10 February 2011
Available online 18 February 2011

Keywords:
Pharmaceuticals
Ultra-high performance liquid chromatography
Tandem mass spectrometry
Multi-class analysis
Surface water and wastewater
Matrix effects

ABSTRACT

A multi-class method for the simultaneous quantification and confirmation of 47 pharmaceuticals in environmental and wastewater samples has been developed. The target list of analytes included analgesic and anti-inflammatories, cholesterol lowering statin drugs and lipid regulators, antidepressants, anti-ulcer agents, psychiatric drugs, ansiolitics, cardiovasculars and a high number (26) of antibiotics from different chemical groups. A common pre-concentration step based on solid-phase extraction with Oasis HLB cartridges was applied, followed by ultra-high performance liquid chromatography–tandem mass spectrometry (UHPLC–MS/MS) measurement. All compounds were satisfactorily determined in just one single injection, with a chromatographic run time of only 10 min. The process efficiency (combination of the matrix effect and the extraction process recovery) for the 47 selected compounds was evaluated in nine effluent wastewater (EWW) samples, and the use of different isotope-labelled internal standards (ILIS) was investigated to correct unsatisfactory values. Up to 12 ILIS were evaluated in EWW and surface water (SW). As expected, the ILIS provided satisfactory correction for their own analytes. However, the use of these ILIS for the rest of pharmaceuticals was problematic in some cases. Despite this fact, the correction with analogues ILIS was found useful for most of analytes in EWW, while was not strictly required in the SW tested. The method was successfully validated in SW and EWW at low concentration levels, as expected for pharmaceuticals in these matrices (0.025, 0.1 and 0.5 μg/L in SW; 0.1 and 0.5 μg/L in EWW). With only a few exceptions, the instrumental limits of detection varied between 0.1 and 8 pg. The limits of quantification were estimated from sample chromatograms at the lowest spiked levels tested and normally were below 20 ng/L for SW and below 50 ng/L for EWW. The developed method was applied to the analysis of around forty water samples (river waters and effluent wastewaters) from the Spanish Mediterranean region. Almost all the pharmaceuticals selected in this work were detected, mainly in effluent wastewater. In both matrices, analgesics and anti-inflammatories, lipid regulators and quinolone antibiotics were the most detected groups.

1. Introduction

In recent years, there has been a growing interest to investigate the impact on the environment of a wide group of compounds so-called "emerging" or "new" unregulated contaminants. Under this expression, different groups of analytes that are considered of concern for the environment are included (algal and cyanobacterial toxins, nanomaterials, drugs of abuse, surfactants, disinfection by-products, hormones and other endocrine disrupting compounds, pharmaceuticals and personal care products, etc.) and their presence has been investigated in different environmental matrices [1]. Their consumption around the world is continuously increasing and they are normally detected in environmental and urban wastewater.

In contrast to other compounds, e.g. pesticides, emerging contaminants are still not regulated in the environment to guarantee the quality of the water. Among the wide group of emerging contaminants, pharmaceuticals are one of the major concern (especially antibiotics) because of their wide consumption and their potential negative effect on the water quality and living organisms. The improvement of analytical methodologies in terms of sensitivity, selectivity and scope of the method is of great interest to have realistic reliable data on their presence in the environment.

After human and/or veterinary consumption, pharmaceuticals are excreted mainly in unchanged form as the parent compound, although many of them are partially metabolized. Consequently,

* Corresponding author. Tel.: +34 964 387366; fax: +34 964 387368.
E-mail address: felix.hernandez@qfa.uji.es (F. Hernández).

0021-9673/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2011.02.026

both parents and metabolites enter into urban wastewater and are matter of concern from the analytical point of view. Most of these compounds are not completely removed during wastewater treatments and they can finally arrive at surface and ground waters [2]. The low pharmaceutical concentrations typically present (low ng/L) seem not to cause adverse effects on humans and in the aquatic environment, but not reliable data are currently available about long-term risk derived from their continuous input in the natural environment. Among pharmaceuticals, the presence of antibiotics in water causes more concern because they can induce bacterial resistance, even at low concentrations, through their continuous exposure [3,4]. Recent studies have reported that quinolone and fluoroquinolone cause the development of genotoxicity based on an *in vitro* bioassay [5] and the quinolone ciprofloxacin has effects on plankton and algae grown at environmental relevant concentrations [1].

Highly sensitive methods are required to determine the low levels normally present in environmental matrices. At present, the combination of ultra high-performance (or pressure) liquid chromatography (UHPLC) with (fast) tandem MS is surely the most suitable approach nowadays. This hyphenated technique provides the sensitivity and selectivity required in this type of analysis. The growing trend on using UHPLC coupled with MS/MS can be inferred from the evolution of scientific papers published during the last decade [6]. Recently, the use of UHPLC–QTOF MS has been proposed for rapid screening of antibiotics. This technique has been proven to be an efficient approach for detection and safe identification of these compounds in water [7].

Despite the high sensitivity reached by MS/MS analyzers, the majority of applications still need a pre-concentration step for the accurate analyte determination at sub-ppb levels. In the vast majority of methods, off-line solid phase extraction (SPE) mode is applied for this purpose [2,8–12], although automated on-line SPE coupled to LC–MS is an increasing trend [8,13–15].

Most of methods recently developed pursue the simultaneous determination of multi-class compounds because many compounds from very different therapeutical classes are found when monitoring environmental waters [4,9,16]. Obviously, multi-class methods provide more information about the occurrence of pharmaceuticals than single group analysis, with reduced analysis time and cost. However, the development of these methods involves a compromise in the selection of experimental conditions (i.e. LC separation, MS detection and sample preparation) [8]. Firstly, the LC chromatographic conditions should be optimized to enhance resolution and to minimize undesired co-elution. Secondly, a compromise between sensitivity and selected dwell times should be found to maintain satisfactory peak shape for all selected compounds. For this purpose, the MS/MS method is usually divided into different elution-time windows that contain different selected reaction monitoring (SRM) transitions with appropriate dwell times [6]. However, this restriction is nowadays changing as recent triple quadrupole instruments allow working with dwell times as low as of 0.001 s without affecting the method sensitivity. Moreover, new acquisition softwares make this task easier selecting automatically the most suitable dwell time for each compound based on time overlapping and chromatographic peakwidth. Finally, the sample procedure applied should assure the simultaneous efficient recovery of all selected compounds. This aspect is problematic in wide-scope multi-class methods, as pharmaceuticals belonging to different therapeutical groups can have rather different physico-chemical properties. As extraction efficiency is affected by several variables such as the type of sorbent used, sample pH or sample volume loaded, it has to be carefully tested for successful results. Finally, a satisfactory compromise should be reached along the overall analytical method for the simultaneous analysis of all target compounds.

A drawback associated to LC–MS/MS methods deals with matrix effects, which are attributed to the presence of undesirable sample components that co-elute with the analytes altering the ionization process. The consequence of matrix effects is the suppression or enhancement of the signal, which can affect both identification and quantification of analytes. Matrix effects depend on each analyte/matrix combination, but also on the sample preparation applied, the chromatographic separation, mass spectrometry instrumentation and the ionization conditions [17]. It is not possible to predict whether the combination of these conditions will affect the analyte signal or not; therefore, the evaluation of matrix effect should be included in the validation process of the method considering the different matrices studied (e.g. surface water, effluent water and influent water). Several strategies have been proposed to solve matrix effects, including modifications of the sample pre-treatment, the chromatographic or MS conditions and the calibration techniques [17]. In the field of antibiotics analysis, some of these approaches have being reviewed by our own group [18]. The use of isotope-labelled internal standards (ILIS) is, by far, the most used to face pharmaceutical analysis [10–12].

The goal of this paper is to develop a rapid, accurate and sensitive analytical strategy based on the use of UHPLC–MS/MS for the simultaneous determination (quantification and confirmation) of 47 pharmaceuticals that belong to the most representative therapeutical groups. A high number of antibiotics (around 30) have been added to the target list of our previous method [10] in order to have wider and more realistic knowledge of the presence of pharmaceuticals in the environment. Several ILIS have been tested to correct unsatisfactory values associated to matrix effects and/or potential losses associated to the SPE step.

2. Experimental

2.1. Reagents and chemicals

Reference standards of acetaminophen (paracetamol), salicylic acid, ibuprofen, 4-aminoantipyrine, omeprazole, ketoprofen, naproxen, bezafibrate, diclofenac, gemfibrozil, pravastatin sodium and enalapril maleate salt were purchased from Sigma–Aldrich (St Louis, MO, USA). Pantoprazole, lorazepam, alprazolam, venlafaxine hydrochloride, risperidone, simvastatin and paroxetine hydrochloride were from LGC Promochem (London, UK). Atorvastatin and olanzapine were supplied by Toronto Research Chemicals (Ontario, Canada). Antibiotic reference standards of sulfamethoxazole, sulfamethazine, sulfadiazine and sulfathiazole were from Across Organics (Geel, Belgium). Enrofloxacin, moxifloxacin and ciprofloxacin were from Bayer Hispania (Barcelona, Spain). Sarafloxacin, marbofloxacin and pefloxacin were provided by Fort Dodge Veterinaria (Gerona, Spain), Vetoquinol Industrial (Madrid Spain) and Aventis Pharma (Madrid, Spain), respectively. The rest of antibiotics were supplied by Sigma–Aldrich. All reference standards presented purity higher than 93%.

Isotopically labelled compounds of omeprazole-d_3, acetaminophen-d_4, diclofenac-d_4, salicylic acid-d_3 and ibuprofen-d_3 were from CDN Isotopes (Quebec, Canada); atorvastatin-d_5, paroxetine hydrochloride-d_4 and olanzapine-d_3 from Toronto Research Chemicals; sarafloxacin-d_8 hydrochloride trihydrate from Sigma–Aldrich and sulfamethoxazole-$^{13}C_6$ and trimethoprim-$^{13}C_3$ were from Cambridge Isotope Laboratories (Andover, MA, USA).

HPLC-grade methanol (MeOH) and HPLC-grade acetonitrile (ACN) were purchased from Scharlab (Barcelona, Spain). HPLC-grade water was obtained from distilled water passed through a Milli-Q water purification system (Millipore, Bedford, MA, USA). Formic acid (HCOOH, content >98%), ammonium acetate (NH_4Ac,

reagent grade) and sodium hydroxide (NaOH, >99%) were supplied by Scharlab (Barcelona, Spain).

Individual stock solutions of pharmaceuticals were prepared dissolving 25 mg, accurately weighted, in 50 mL methanol, obtaining a final concentration of 500 mg/L. For antibiotics, individual stock solutions were prepared dissolving 50 mg of solid standard in 100 mL ACN, except quinolones that were dissolved in MeOH. The addition of 100 μL of 1 M NaOH was necessary for the proper dissolution of the acidic analytes like quinolones. Stock solutions were stored at −20 °C.

Individual stock solutions of ILIS were prepared in methanol. A mix working solution at 100 μg/L (for those ionizing in positive mode) and at 1 mg/L (for ILIS ionizing in negative mode) was prepared in MeOH and used as surrogate.

An intermediate mixed solution containing all antibiotics at a concentration of 5 mg/L was obtained after mixing individual stock solutions and diluting with MeOH. Another intermediate solution containing the rest of pharmaceuticals was prepared also in MeOH following a similar procedure. From intermediate solutions, a mixture of all compounds at a concentration of 500 μg/L was prepared in MeOH. Working solutions were subsequently prepared from the mixed solution by diluting the appropriate volume with HPLC-grade water. All standard solutions (stock, intermediate and working solutions) were stored in amber glass bottles at −20 °C in a freezer. Cartridges used for SPE were Oasis HLB (60 mg) from Waters (Milford, MA, USA).

2.2. Liquid chromatography

UHPLC analysis were carried out using an Acquity UPLC system (Waters Corp., Milford, MA, USA), equipped with a binary solvent manager and a sample manager. Chromatographic separation was performed using an Acquity UPLC HSS T3 column, 1.8 μm, 100 mm × 2.1 mm (i.d.) (Waters) at a flow rate of 0.3 mL/min. The column was kept at 60 °C and the sample manager was maintained at 5 °C. Mobile phase consisted of a water/methanol, both 0.1 mM NH_4Ac and 0.01% HCOOH, gradient. The methanol percentage was changed linearly as follows: 0 min, 5%; 7 min, 90%; 8 min, 90%; 8.1 min; 5%. Analysis run time was 10 min. The sample injection volume was 20 μL.

2.3. Mass spectrometry

A TQD (triple quadrupole) mass spectrometer with an orthogonal Z-spray-electrospray interface (Waters Corp., Milford, MA, USA) was used. Drying gas as well as nebulising gas was nitrogen generated from pressurized air in a N_2 LC–MS (Claind, Teknokroma, Barcelona, Spain). The cone gas and the desolvation gas flows were set at 60 L/h and 1200 L/h, respectively. For operation in MS/MS mode, collision gas was Argon 99.995% (Praxair, Valencia, Spain) with a performance of 2×10^{-3} mbar in the T-Wave collision cell. Capillary voltages of −3.0 kV (negative ionization mode) and 3.5 kV (positive ionization mode) were applied. The interface temperature was set to 500 °C and the source temperature to 120 °C. A scan time of 0.01 s was selected.

Masslynx 4.1 (Micromass, Manchester, UK) software was used to process quantitative data.

2.4. Recommended procedure

The SPE method was based on our previous work developed for the determination of 20 pharmaceuticals [10]. The procedure was as follows: 100 mL water sample were spiked with the ILIS mix working solution to give a final concentration of 0.1 μg/L for each ILIS determined in positive mode and of 1 μg/L for those ILIS determined in negative mode. Oasis HLB (60 mg) cartridges were conditioned with 3 mL MeOH and 3 mL HPLC-grade water before use. Then, samples were passed through the cartridge and, after drying under vacuum, analytes were eluted with 5 mL methanol. The extract was evaporated to dryness under a gentle nitrogen stream at 40 °C and reconstituted with 1 mL MeOH–water (20:80, v/v). Finally, 20 μL were injected in the UHPLC–MS/MS system under the conditions shown in Table 1. Quantification was made using calibration standards prepared in solvent, based on relative responses analyte/ILIS, or on absolute responses, depending on whether ILIS was used for correction or not. ILIS were used to correct for matrix effects and/or SPE potential errors as shown in Tables 2–4.

2.5. Validation study

Method accuracy (estimated by means of recovery experiments) and precision (expressed as repeatability in terms of relative standard deviation (RSD)) were studied by recovery studies in surface water (SW) and effluent wastewater (EWW) spiked at different concentrations (25, 100 and 500 ng/L for SW; 100 and 500 ng/L for EWW). All experiments were performed in quintuplicate. Recovery values between 70% and 120%, with RSD lower than 20% were considered as satisfactory.

The limit of quantification (LOQ) was estimated for a signal-to-noise (S/N) ratio of 10 from the sample chromatograms at the lowest validation level tested, using the quantification transition. Rearding EWW, adequate blanks samples were not found for several analytes. In these cases, LOQ values were estimated from quantified levels present in non-spiked blanks. The instrumental limit of detection (LOD) was estimated for S/N = 3 from the chromatograms of standards at the lowest concentration level tested in the calibration curve.

The linearity of the method was studied by analyzing standard solutions in triplicate at seven concentrations in the range from 1 to 100 μg/L. Satisfactory linearity using weighed ($1/X$) least squares regression was assumed when the correlation coefficient (r) was higher than 0.99 and residuals lower than 30% without significant trend, based on relative responses (analyte peak area/ILIS peak area), except for those compounds that were quantified without ILIS (absolute response).

2.6. Application to real samples

Around 40 samples of SW (18 samples) and EWW (19 samples) were collected in polyethylene high-density bottles in selected sites of the Spanish Mediterranean area (Castellon and Valencia provinces). Samples were stored at <−18 °C until analysis. Before analysis, samples were thawed at room temperature. Wastewater samples consisted on 24-h composite urban wastewater samples and were collected from different wastewater treatment plants (WWTPs).

3. Results and discussion

In this work, 47 pharmaceuticals from the most representative therapeutical groups were studied (see Table 1). Among them, 21 compounds are widely consumed in human medicine in Spain [19]. The rest are antibiotics and were selected due to their potential negative effect on living organisms of the aquatic environment. All analytes corresponded to parent compounds except salicylic acid and 4-aminoantipyrine, the metabolites of acetylsalicylic acid and dipyrone, respectively. These metabolites were selected based on information reported in scientific literature on their occurrence in surface and wastewater [11,20–22] and on the human metabolism of their parent pharmaceuticals [23–25].

Table 1
MS/MS optimized conditions for selected compounds.

Compound	Therapeutic group	Polarity (ES)	LOD (pg)	MW	Q Transition	Cone (V)	C.E. (eV)	q_1 transition	C.E. (eV)	Q/q
Acetaminophen	Analgesic and anti-inflammatories	+	3.9	151.1	152.1 > 110.1	30	15	152.1 > 93.0	25	7.5
4-Aminoantipyrine		+	0.2	203.3	204.2 > 56.0	30	20	204.2 > 83.0	15	9.6
Diclofenac		–	5.9	295.0	294.1 > 250.1	30	10	296.1 > 252.1	30	1.3
Ibuprofen		–	86.0	206.1	205.1 > 161.1	30	10	–	–	–
Ketoprofen		–	7.4	254.1	253.2 > 209.2	20	5	–	–	–
Naproxen		–	6.6	230.1	185.2 > 170.1	30	10	229.2 > 170.1[a]	20	7.8
Salicylic acid		–	56.6	138.0	137.1 > 93.0	30	15	–	25	–
Atorvastatin	Cholesterol lowering statin drugs and lipid regulators	+	1.2	558.3	559.4 > 440.3	45	20	559.4 > 250.2	45	0.9
Simvastatin		+	1.0	418.3	419.5 > 285.3	30	10	419.5 > 199.2	20	1.7
Pravastatin		–	18.5	424.2	423.4 > 321.2	40	15	423.4 > 101.1	30	1.3
Bezafibrate		–	1.8	361.1	360.2 > 274.1	30	15	362.2 > 276.2	20	2.2
Gemfibrozil		–	3.6	250.2	249.3 > 121.0	30	15	249.3 > 127.0	10	14.6
Paroxetine	Antidepressants	+	3.8	329.1	330.3 > 70.1	50	20	330.3 > 44.1	30	0.6
Venlafaxine		+	0.4	277.2	278.3 > 58.0	30	15	278.3 > 260.3	15	1.2
Omeprazole	Anti-ulcer agents	+	0.7	345.1	346.3 > 198.1	30	10	346.3 > 136.1	35	2.4
Pantoprazole		+	0.3	383.1	384.3 > 138.1	25	10	384.3 > 200.2	35	1.3
Olanzapine	Psychiatric drugs	+	1.6	312.1	313.3 > 256.2	45	25	313.3 > 84.1	25	1.3
Risperidone		+	0.6	410.2	411.3 > 191.2	50	30	411.3 > 82.1	60	16.8
Alprazolam	Ansiolitics	+	0.7	308.1	309.2 > 281.2	60	25	309.2 > 205.2	40	1.2
Lorazepam		+	2.7	320.2	321.2 > 275.1	40	20	323.2 > 277.1	20	1.2
Enalapril	Cardiovasculars	+	0.3	376.2	377.4 > 234.2	35	20	377.4 > 91.1	55	1.2
Erithromycin	Macrolide antibiotics	+	0.1	733.5	734.4 > 158.1	35	30	734.4 > 576.3	25	3.9
Clarithromycin		+	0.7	747.5	590.3 > 158.1	55	25	748.3 > 158.1[b]	30	1.5
Tylosin		+	0.2	915.5	916.9 > 174.2	50	35	916.9 > 101.0	40	4.2
Roxithromycin		+	0.2	836.5	679.8 > 158.1	60	30	837.9 > 158.1[b]	40	1.1
Moxifloxacin	Quinolone antibiotics	+	5.2	401.2	402.3 > 364.3	35	25	402.3 > 384.3	20	0.2
Norfloxacin		+	2.2	319.1	320.1 > 276.1	45	20	320.1 > 302.1	20	0.4
Pefloxacin		+	13.0	333.1	334.4 > 233.4	45	25	334.4 > 316.4	20	0.5
Ofloxacin		+	0.9	361.1	362.1 > 318.1	45	20	362.1 > 261.0	30	0.9
Marbofloxacin		+	13.5	362.1	363.4 > 320.4	35	20	363.4 > 345.4	20	0.7
Ciprofloxacin		+	28.9	331.1	332.1 > 231.1	45	40	332.1 > 314.1	20	1.3
Enrofloxacin		+	1.3	359.2	360.4 > 245.4	45	25	360.4 > 316.4	20	0.6
Sarafloxacin		+	4.4	385.1	386.4 > 299.3	40	30	386.4 > 368.4	20	0.7
Flumequine		+	0.6	261.1	262.3 > 202.3	35	30	262.3 > 244.3	20	0.5
Oxolinic acid		+	0.7	261.2	262.3 > 244.3	35	20	262.3 > 216.2	30	162
Nalidixic acid		+	0.4	232.1	233.2 > 187.2	20	25	233.2 > 215.2	20	0.8
Pipedimic acid		+	13.6	303.1	304.0 > 217.0	45	25	304.0 > 276.0	20	0.5
Sulfamethoxazole	Sulfonamide antibiotics	+	0.2	253.1	254.0 > 91.9	40	30	254.0 > 155.9	20	2.1
Sulfamethazine		+	0.2	278.1	279.3 > 92.0	40	30	279.3 > 186.2	15	0.7
Sulfadiazine		+	0.3	250.1	251.2 > 65.1	30	50	251.2 > 92.0	20	1.9
Sulfathiazole		+	0.5	255.0	256.2 > 156.0	30	15	256.2 > 92.0	25	1.3
Lincomycin	Lincosamide antibiotics	+	0.4	406.2	407.1 > 126.1	40	30	407.1 > 359.2	20	17
Clindamycin		+	0.6	424.2	425.1 > 126.0	45	30	427.1 > 126.0	25	4.5
Furaltadone	Other antibiotics	+	0.2	324.1	325.3 > 100.2	25	30	325.3 > 281.3	10	2.1
Furazolidone		+	0.6	225.0	226.3 > 139.2	35	15	226.3 > 122.1	20	1.2
Trimethoprim		+	0.8	290.1	291.1 > 230.1	50	25	291.1 > 261.1	25	1.8
Chloramphenicol		–	5.5	322.0	321.3 > 152.3	30	15	321.3 > 257.1	10	1.6
Acetaminophen-d_4		+	–	155.1	156.1 > 114.1	35	20	–	–	–
Diclofenac-d_4		–	–	299.0	300.1 > 256.1	30	10	–	–	–
Ibuprofen-d_3		–	–	209.1	208.2 > 164.2	20	10	–	–	–
Salicylic acid-d_4		–	–	142.1	141.1 > 97.0	30	20	–	–	–
Atorvastatin-d_5		+	–	563.3	564.4 > 445.2	45	25	–	–	–
Simvastatin-d_6		+	–	424.3	425.5 > 285.3	20	10	–	–	–
Paroxetine-d_4		+	–	333.2	334.3 > 74.1	40	30	–	–	–
Olanzapine-d_3		+	–	315.2	316.3 > 256.2	45	25	–	–	–
Omeprazole-d_3		+	–	348.1	349.1 > 198.1	30	10	–	–	–
Sarafloxacin-d_8		+	–	393.2	394.4 > 303.3	35	30	–	–	–
Sulfamethoxazole-$^{13}C_6$		+	–	259.2	260.2 > 98.2	30	30	–	–	–
Trimethoprim-$^{13}C_3$		+	–	293.3	294.1 > 233.1	40	20	–	–	–

ES, electrospray ionization; MW, monoisotopic molecular weight; Q, quantification; q, confirmation; C.E., collision energy.
[a] In this case an in-source fragment was used as precursor ion and the cone voltage was lowered to 20 V.
[b] In this case an in-source fragment was used as precursor ion and the cone voltage was lowered to 40 V.

3.1. MS and MS/MS optimization

Full-scan and MS/MS mass spectra of analytes were obtained from infusion of 1 mg/L methanol/water (50:50, v/v) individual standard solutions at a flow rate of 10 μL/min. The compounds investigated belong to various chemical groups and showed rather different ionization behaviour. The majority of the compounds (38 out of 47) were determined under positive ionization and the rest (9 out of 47) under negative ionization. All compounds showed an abundant $[M+H]^+$ or $[M-H]^-$ ion, except naproxen, roxithromycin and clarithromycin that showed better sensitivity when using an in-source fragment as precursor ion by increasing the cone voltage.

The two most sensitive SRM transitions were selected for each compound: the most abundant was used for quantification (Q) whereas the second one was for confirmation (q). This allowed us to reach the minimum number of identification points (IPs) required (3 IPs for legally registered compounds) for a safe confirmation [26].

Table 2
Average process efficiency and RSD values obtained from nine different EWW samples, spiked at 500 ng/L level and collected from three WWTPs.

Compound	Polarity (ES)	t_R (min)	Before correction		After correction		ILIS used
			Process efficiency (%)	RSD (%)	Process efficiency (%)	RSD (%)	
Acetaminophen	+	2.40	26	18	103	3	Acetaminophen-d_4
Sulfadiazine	+	2.51	14	26	50	25	Acetaminophen-d_4
Furaltadone	+	2.55	59	23	68	16	Trimethoprim-$^{13}C_3$
Sulfathiazole	+	2.59	68	18			–
Pipedimic acid	+	2.84	36	23	104	22	Acetaminophen-d_4
Marbofloxacin	+	2.97	70	25	117	9	Sulfamethoxazole-$^{13}C_6$
Trimethoprim	+	2.98	80	13	85	11	Trimethoprim-$^{13}C_3$
Lincomycin	+	3.00	88	13			–
Olanzapine	+	3.14	82	28	97	15	Olanzapine-d_3
Ofloxacin	+	3.15	51	26	89	25	Sulfamethoxazole-$^{13}C_6$
Pefloxacin	+	3.19	101	20			–
Norfloxacin	+	3.20	40	17			–
Furazolidone	+	3.23	43	26	84	18	Sulfamethoxazole-$^{13}C_6$
Ciprofloxacin	+	3.30	37	25	98	19	Sulfamethoxazole-$^{13}C_6$
Enrofloxacin	+	3.43	128	14	108	12	Sarafloxacin-d_8
4-Aminoantipyrine[a]	+	3.43	–	–			–
Sulfamethazine	+	3.46	96	13			–
Sulfamethoxazole	+	3.60	41	25	84	7	Sulfamethoxazole-$^{13}C_6$
Sarafloxacin	+	3.65	60	18	72	13	Sarafloxacin-d_8
Salicylic acid	–	4.32	22	6	96	15	Salicylic acid-d_4
Moxifloxacin	+	4.40	108	33			–
Chloramphenicol	–	4.48	70	14			–
Risperidone	+	4.50	144	11	122	5	Sarafloxacin-d_8
Venlafaxine	+	4.62	165	7			–
Clindamycin	+	5.14	90	10			–
Enalapril	+	5.35	83	10			–
Paroxetine	+	5.39	89	24	99	8	Paroxetine-d_4
Nalidixic acid	+	5.45	64	20			–
Oxolinic acid	+	5.55	47	21	106	12	Atorvastatin-d_5
Flumequine	+	5.55	35	25	83	14	Atorvastatin-d_5
Omeprazole	+	5.56	129	14	115	3	Omeprazole-d_3
Tylosin	+	5.72	55	11	112	21	Sulfamethoxazole-$^{13}C_6$
Erithromycin	+	5.74	54	17	114	22	Sulfamethoxazole-$^{13}C_6$
Pantoprazole	+	5.80	99	14			–
Pravastatin	–	6.12	55	17	91	18	Diclofenac-d_4
Clarithromycin	+	6.26	43	12	82	18	Sulfamethoxazole-$^{13}C_6$
Roxithromycin	+	6.32	70	14			–
Ketoprofen	–	6.32	37	19	55	16	Diclofenac-d_4
Lorazepam	+	6.40	89	11			–
Alprazolam	+	6.46	60	14			–
Naproxen	–	6.47	35	21	55	17	Diclofenac-d_4
Bezafibrate	–	6.56	56	15	94	17	Diclofenac-d_4
Atorvastatin	+	6.99	45	13	106	5	Atorvastatin-d_5
Diclofenac	–	7.19	49	25	86	16	Diclofenac-d_4
Ibuprofen	–	7.35	86	12	116	6	Ibuprofen-d_3
Gemfibrozil	–	7.75	42	18	71	2	Diclofenac-d_4
Simvastatin	+	8.13	37	25	100	8	Simvastatin-d_6

ES, electrospray ionization; t_R, retention time.
[a] Not estimated due to the high analyte levels found in the "blank" samples.

Only one transition could be monitored for ibuprofen, salicylic acid and ketoprofen, due to their poor fragmentation.

Non-specific transitions (i.e. loss of water) were avoided in order to improve the selectivity of the method and to decrease the possibilities for occurrences of false positives or false negatives. Thus, for quinolone antibiotics, although the transition corresponding to the neutral loss of H_2O was the most sensitive, the second most sensitive transition was selected for quantification (Q), i.e. $[M+H–CO_2–C_2H_5N]^+$ for sarafloxacin and pipedimic acid, $[M+H–H_2O–C_2H_5N–C_3H_4]^+$ for pefloxacin and ciprofloxacin, and $[M+H–CO_2]^+$ for the rest of quinolones, in similarity to previous works [5,15]. The exception was oxolinic acid, where the $[M+H–H_2O]^+$ ion was chosen for quantification because it was much more sensitive than the rest.

For sulfonamide antibiotics, the $m/z = 92$ product ion, corresponding to the amide ring ($NH_2–C_6H_4$), was chosen for the four compounds belonging to this therapeutic group. This ion was the most abundant fragment in the case of sulfamethazine and sulfamethoxazole, while for sulfadiazine and sulfathiazole it was selected for confirmation. This product ion has been already reported by other authors [27,28].

Most of analgesic and anti-inflammatory compounds were normally in ESI negative mode. For these compounds, the neutral loss of CO_2 $[M–H-44]^-$ was the main product ion observed.

Regarding ILIS, only one transition was monitored. In the particular case of diclofenac-d_4, the transition 300.1 > 256.1 was chosen in order to avoid the mass overlap between the natural analyte (isotope peak due to the presence of two chlorine atoms; $2Cl^{37}$) and the ILIS signal, which would have occur if the transition 298.1 > 254.1 was chosen.

Dwell times of 10 ms were selected to assure enough data points per chromatographic peak (at least 10 points) to have satisfactory peak shape. All SRM transitions (around 100) were divided along eight overlapping windows. This favourable overlapping was pos-

Table 3
Method validation in effluent wastewater (EWW). Recovery (%) before and after correction with ILIS and relative standard deviation (RSD %) for five replicates.

Compound	100 ng/L		500 ng/L		LOQ (ng/L)
	Before correction	After correction	Before correction	After correction	
Acetaminophen[a]	38 (6)	120 (7)	33 (4)	104 (7)	88
Sulfadiazine	27 (10)	80 (10)	23 (2)	65 (7)	45
Furaltadone	42 (3)	74 (2)	46 (0)	65 (2)	5
Sulfathiazole	95 (3)	–	99 (3)	–	9
Pipedimic acid	32 (1)	129 (2)	18 (2)	71 (8)	91
Marbofloxacin	[b]	–	48 (6)	81 (7)	110
Trimethoprim[a]	55 (10)	86 (7)	63 (5)	85 (4)	9
Lincomycin	90 (1)	–	96 (2)	–	2
Olanzapine[a]	68 (4)	72 (8)	89 (7)	102 (12)	48
Ofloxacin	60 (15)	147 (1)	45 (6)	92 (7)	13
Pefloxacin	112 (2)	–	121 (5)	–	50
Norfloxacin	35 (5)	–	34 (4)	–	25
Furazolidone	40 (1)	75 (5)	41 (6)	83 (1)	23
Ciprofloxacin	67 (3)	147 (1)	42 (6)	92 (7)	46[d]
Enrofloxacin	116 (3)	88 (5)	120 (6)	100 (10)	21
4-Aminoantipyrine	[c]	–	81 (4)	–	23[d]
Sulfamethazine	91 (3)	–	92 (5)	–	0.8
Sulfamethoxazole[a]	59 (2)	106 (2)	46 (8)	90 (6)	13[d]
Sarafloxacin[a]	49 (8)	59 (7)	42 (11)	49 (13)	25
Salicylic acid[a]	18 (18)	92 (15)	22 (12)	101 (6)	79
Moxifloxacin	[c]	–	108 (5)	–	114
Chloramphenicol	73 (15)	–	75 (9)	–	19
Risperidone	160 (0)	150 (3)	141 (3)	127 (4)	3
Venlafaxine	230 (3)	–	218 (4)	–	7
Clindamycin	94 (2)	–	91 (1)	–	6
Enalapril	97 (7)	–	79 (3)	–	6
Paroxetine[a]	[b]	–	82 (14)	89 (9)	170
Nalidixic acid	73 (1)	–	72 (2)	–	6
Oxolinic acid	32 (3)	70 (6)	38 (4)	85 (5)	10
Flumequine	24 (7)	53 (9)	25 (2)	60 (8)	9
Omeprazole[a]	253 (3)	108 (7)	238 (3)	114 (9)	18
Tylosin	49 (8)	86 (13)	43 (5)	86 (3)	2
Erithromycin	60 (4)	108 (8)	51 (5)	104 (3)	8
Pantoprazole	111 (5)	–	100 (1)	–	4
Pravastatin	37 (14)	66 (20)	42 (7)	77 (4)	33[d]
Clarithromycin	62 (4)	125 (3)	42 (4)	85 (6)	3
Roxithromycin	59 (4)	–	59 (4)	–	17
Ketoprofen	18 (4)	48 (12)	36 (3)	70 (6)	51[d]
Lorazepam	115 (5)	–	107 (2)	–	46[d]
Alprazolam	54 (4)	–	56 (2)	–	4
Naproxen	46 (4)	85 (6)	35 (9)	66 (8)	20[d]
Bezafibrate	59 (2)	101 (6)	57 (4)	106 (7)	10[d]
Atorvastatin[a]	56 (5)	115 (2)	53 (5)	110 (5)	4[d]
Diclofenac[a]	67 (8)	106 (10)	48 (2)	97 (4)	49[d]
Ibuprofen[a]	[c]	–	71 (18)	122 (16)	150[d]
Gemfibrozil	43 (2)	101 (7)	54 (3)	105 (2)	4
Simvastatin[a]	42 (4)	109 (12)	38 (4)	98 (17)	24

Correction with ILIS made as shown in Table 2.
[a] Correction made with the analyte-labelled IS.
[b] Not estimated due to the poor sensitivity.
[c] Not estimated due to the high analyte levels found in the "blank" sample.
[d] LOQ determined from the "blank" sample chromatogram (non-spiked).

sible due to the low positive-to-negative-switching time (20 ms) attainable by the triple quadrupole analyzer used in this work.

Mass spectrometry parameters selected, precursor and product ions, and instrumental LODs are shown in Table 1.

3.2. Chromatographic conditions

In this work, a UPLC HSS column (100 mm × 2.1 mm, 1.8 μm) was chosen for the separation of 59 compounds (47 analytes and 12 internal standards) in only 10 min. A larger column, compared to our previous work [10], was required for a satisfactory separation of higher number of analytes but maintaining similar chromatographic runs. As target compounds belong to different groups and have quite distinct physico-chemical characteristics, with different ionization behaviour (e.g. sensitivity for analytes determined in positive mode was normally better than in negative mode), it was necessary to find a compromise for their satisfactory separation using the same mobile phase. Methanol and acetonitrile with different modifiers (HCOOH and NH_4Ac at various concentrations) were tested for this purpose. A mobile phase containing both 0.1 mM NH_4Ac and 0.01% HCOOH, which was also used for the chromatographic separation of 20 pharmaceuticals [10] led to good peak shape and sensitivity for the wide majority of compounds. Therefore, this mobile phase (see Section 2.2) was selected as a compromise for the simultaneous chromatographic separation of both positive and negative ionized analytes.

3.3. Method validation

The linearity of the method was studied in the range 1–100 μg/L for all selected compounds. These values corresponded to 0.01–1 μg/L in the water samples taking into account the

Table 4
Method validation in surface water (SW). Recovery (%) before and after correction with ILIS and relative standard deviation (RSD %) for five replicates.

Compound	25 ng/L		100 ng/L		500 ng/L		LOQ (ng/L)
	Before correction	After correction	Before correction	After correction	Before correction	After correction	
Acetaminophen[a]	51 (3)	112 (1)	52 (8)	108 (8)	52 (3)	103 (2)	23
Sulfadiazine	53 (15)	–	59 (10)	–	49 (2)	–	4
Furaltadone	97 (9)	–	109 (3)	–	105 (5)	–	3
Sulfathiazole	99 (5)	–	101 (3)	–	101 (3)	–	2
Pipedimic acid	[d]	–	76 (6)	–	65 (5)	–	36
Marbofloxacin	66 (20)	–	71 (14)	–	101 (10)	–	19
Trimethoprim[a]	125 (4)	100 (3)	120 (5)	95 (3)	115 (1)	93 (2)	2
Lincomycin	72 (7)	–	90 (2)	–	94 (6)	–	2
Olanzapine[a]	73 (2)	95 (18)	119 (8)	103 (19)	76 (11)	98 (8)	9
Ofloxacin	66 (15)	–	73 (21)	–	108 (4)	–	2
Pefloxacin	108 (4)	–	116 (10)	–	120 (4)	–	13
Norfloxacin	109 (12)	–	97 (2)	–	117 (4)	–	11
Furazolidone	91 (9)	–	104 (5)	–	102 (7)	–	1
Ciprofloxacin	70 (6)	–	82 (5)	–	101 (1)	–	18
Enrofloxacin	120 (10)	–	117 (10)	–	156 (4)	–	9
4-Aminoantipyrine[b]	54 (4)	97 (11)	52 (11)	81 (22)	50 (5)	94 (9)	1
Sulfamethazine	109 (13)	–	123 (3)	–	109 (15)	–	0.5
Sulfamethoxazole[a]	106 (3)	108 (3)	104 (2)	94 (6)	99 (3)	99 (3)	3
Sarafloxacin[a]	74 (5)	77 (13)	63 (14)	66 (11)	71 (7)	73 (3)	10
Salicylic acid[a]	[d]	–	38 (18)	102 (15)	42 (16)	105 (13)	76
Moxifloxacin	[d]	–	121 (10)	–	149 (4)	–	55
Chloramphenicol	102 (11)	–	97 (1)	–	109 (4)	–	7
Risperidone[c]	139 (2)	118 (3)	128 (4)	122 (9)	137 (3)	115 (5)	2
Venlafaxine	133 (3)	–	135 (4)	–	142 (4)	–	3
Clindamycin	72 (14)	–	83 (1)	–	90 (7)	–	1
Enalapril	78 (4)	–	83 (3)	–	76 (4)	–	4
Paroxetine[a]	[d]	–	108 (11)	86 (6)	118 (12)	91 (4)	29
Nalidixic acid	86 (4)	–	95 (5)	–	101 (8)	–	3
Oxolinic acid	70 (5)	–	81 (2)	–	80 (9)	–	2
Flumequine	72 (15)	–	77 (9)	–	76 (12)	–	2
Omeprazole[a]	196 (5)	115 (13)	159 (2)	107 (3)	134 (10)	109 (3)	2
Tylosin	51 (6)	–	52 (6)	–	54 (5)	–	0.7
Erithromycin	63 (9)	–	67 (9)	–	73 (8)	–	0.4
Pantoprazole	118 (3)	–	115 (4)	–	114 (5)	–	2
Pravastatin	[d]	–	104 (8)	–	89 (2)	–	23
Clarithromycin	72 (14)	–	72 (11)	–	82 (6)	–	2
Roxithromycin	69 (8)	–	74 (10)	–	84 (16)	–	2
Ketoprofen	[e]	–	92 (14)	–	99 (10)	–	29
Lorazepam	111 (13)	–	118 (4)	–	99 (0)	–	8
Alprazolam	111 (6)	–	107 (2)	–	109 (1)	–	1
Naproxen	85 (17)	–	91 (8)	–	81 (3)	–	20
Bezafibrate	106 (7)	–	100 (0)	–	102 (2)	–	3
Atorvastatin[a]	14 (21)	91 (8)	21 (10)	103 (3)	21 (18)	97 (3)	3
Diclofenac[a]	116 (15)	103 (11)	112 (5)	99 (8)	104 (2)	103 (3)	4
Ibuprofen[a]	[d]	–	92 (16)	110 (15)	89 (21)	105 (17)	85
Gemfibrozil	105 (12)	–	103 (8)	–	97 (1)	–	9
Simvastatin[a]	[d]	–	30 (8)	102 (5)	34 (10)	106 (9)	18

[a] Correction made with the analyte-labelled IS.
[b] Correction made with acetaminophen-d_4.
[c] Correction made with sarafloxacin-d_8.
[d] Not estimated due to the poor sensitivity.
[e] Not estimated due to the high analyte levels found in the "blank" sample.

pre-concentration factor applied along the sample procedure. Calibration curves showed satisfactory correlation coefficients (greater than 0.99) and residuals were lower 30% for all compounds.

Instrumental LODs are shown in Table 1. For the majority of the compounds (24 out of 47) LODs were below 1 pg, and for 20 analytes varied from 1 to 20 pg. In the case of ciprofloxacin, the LOD could have been improved if the non-specific transition corresponding to the loss of water (the most sensitive) had been selected instead of 332 > 231 that was finally used for quantification. The LODs for ibuprofen and salicylic acid were higher notably than for the rest of compounds. The reason was their poor fragmentation, and that only a low sensitive transition could be monitored.

It is well known that matrix effects are one of the main drawbacks of LC–MS/MS methods when applied to environmental samples. These effects may considerably alter the signal of many analytes, affecting severely to the quantification process. These effects are more noticeable when analysing complex-matrix samples like wastewater. A detailed study of those variables that may affect the overall analytical process efficiency (i.e. the matrix effect and the extraction process) is required when an analytical method is developed. In the line of our previous works on LC–MS/MS analysis of wastewater samples [29] we have evaluated the overall process efficiency for the 47 selected compounds. The process efficiency (PE) represents the percentage of matrix effect (ME) and extraction process recovery (RE), and it is expressed as [30]:

$$\mathrm{PE}\ (\%) = \frac{\mathrm{ME}\ (\%) \cdot \mathrm{RE}\ (\%)}{100}$$

For this purpose, nine different EWW samples collected from three different WWTPs of the Castellon province (sample collection performed in autumn, winter and spring) were spiked at 500 ng/L for each individual compound as well as with the ILIS mix working solution (12 ILIS). "Blank" EWW samples, spiked only with the ILIS mix, were also processed to subtract the

Table 5
Summary of the results obtained for target pharmaceuticals.

Compound	Surface water (n = 18)		Effluent wastewater (n = 19)	
	% positive findings	Maximum level (ng/L)	% positive findings	Maximum level (ng/L)
Acetaminophen	72	1968	21	201,000
4-Aminoantipyrine	50	811	84	2770
Alprazolam	0	–	47	7
Atorvastatin	11	42	74	209
Bezafibrate	39	49	79	312
Chloramphenicol	0	–	0	–
Ciprofloxacin	100	740	100	2292
Clarithromycin	56	91	74	247
Clindamycin	0	–	0	–
Diclofenac	61	358	84	690
Enalapril	44	88	16	236
Enrofloxacin	100	70	53	220
Erithromycin	44	78	74	82
Flumequine	83	20	11	41
Furaltadone	0	–	5	9
Furazolidone	0	–	0	–
Gemfibrozil	28	304	84	2008
Ibuprofen	22	2850	21	15,100
Ketoprofen	44	70	79	583
Lincomycin	50	47	79	142
Lorazepam	0	–	79	81
Marbofloxacin	0	–	0	–
Moxifloxacin	17	205	16	540
Nalidixic acid	39	14	6	60
Naproxen	44	285	79	710
Norfloxacin	100	54	89	310
Ofloxacin	100	400	100	925
Olanzapine	39	58	21	< LOQ
Omeprazole	0	–	32	30
Oxolinic acid	83	23	0	–
Pantoprazole	50	117	47	36
Paroxetine	0	–	0	–
Pefloxacin	22	64	5	112
Pipedimic acid	11	245	68	430
Pravastatin	0	–	16	69
Risperidone	0	–	0	–
Roxithromycin	11	12	42	18
Salicylic acid	61	1160	74	80,000
Sarafloxacin	89	55	16	52
Simvastatin	0	–	0	–
Sulfadiazine	0	–	0	–
Sulfamethazine	0	–	5	11
Sulfamethoxazole	28	33	84	432
Sulfathiazole	0	–	11	30
Trimethoprim	28	151	84	232
Tylosin	0	–	0	–
Venlafaxine	39	575	74	875

responses of possible target compounds. Their relative responses were quantified by internal standard calibration with standards in solvent.

Table 2 shows the average overall process efficiency for the nine samples, as well as the average RSD value before and after correction with an ILIS. For the wide majority of compounds, PE < 100% were obtained. This may be due to matrix effects (ion suppression) and/or compound losses during SPE process. A few compounds showed PE > 100%, which was surely due to matrix effects resulting in ionization enhancement, as it is not expected to obtain RE > 100% due to the presence of compounds released from the SPE cartridges and coeluting with the analytes producing signal enhancement. In the case of 4-aminoantipyrine, data could not be reported due to high concentrations found in the samples tested.

As only 14 out of 47 pharmaceuticals showed satisfactory recoveries (without using any ILIS), it seems clear that some correction is required to obtain successful results. Otherwise, non-accurate quantification would be made leading typically to concentrations lower than actually present in the samples. The use of ILIS is nowadays widely accepted for matrix effects correction in environmental and wastewater analysis. However, the large number of compounds analyzed in our multi-residue method made unfeasible to correct each analyte with its own ILIS. Then, we considered the possibility of correcting unsatisfactory values using 12 ILIS that were available at our laboratory. Those compounds which ILIS were available were quantified using their own labelled analyte. Under these circumstances, all showed satisfactory values, indicating that both the SPE step and/or matrix effects correction was appropriate. For the rest of analytes, the selection of an analogue ILIS to correct for unsatisfactory values was rather problematic because the results might considerably vary from one sample to other. The main criterion for selection of ILIS was based on retention time similarity between the analyte and the ILIS selected, because it is expected that both will be affected by similar co-extracted constituents of the matrix. However, the use of an ILIS eluting at close retention time did not always ensure adequate correction. For example, although tylosin, erithromycin, clarithromycin and omeprazole-d_3 had similar retention times, the correction with this ILIS was unsatisfactory; however, using sulfamethoxazole-$^{13}C_6$, recoveries increased above 80% and acceptable RSD values (around 20%) were obtained. Nevertheless, for roxithromycin, although belonging to the macrolide antibiotics group and presenting similar retention time, recov-

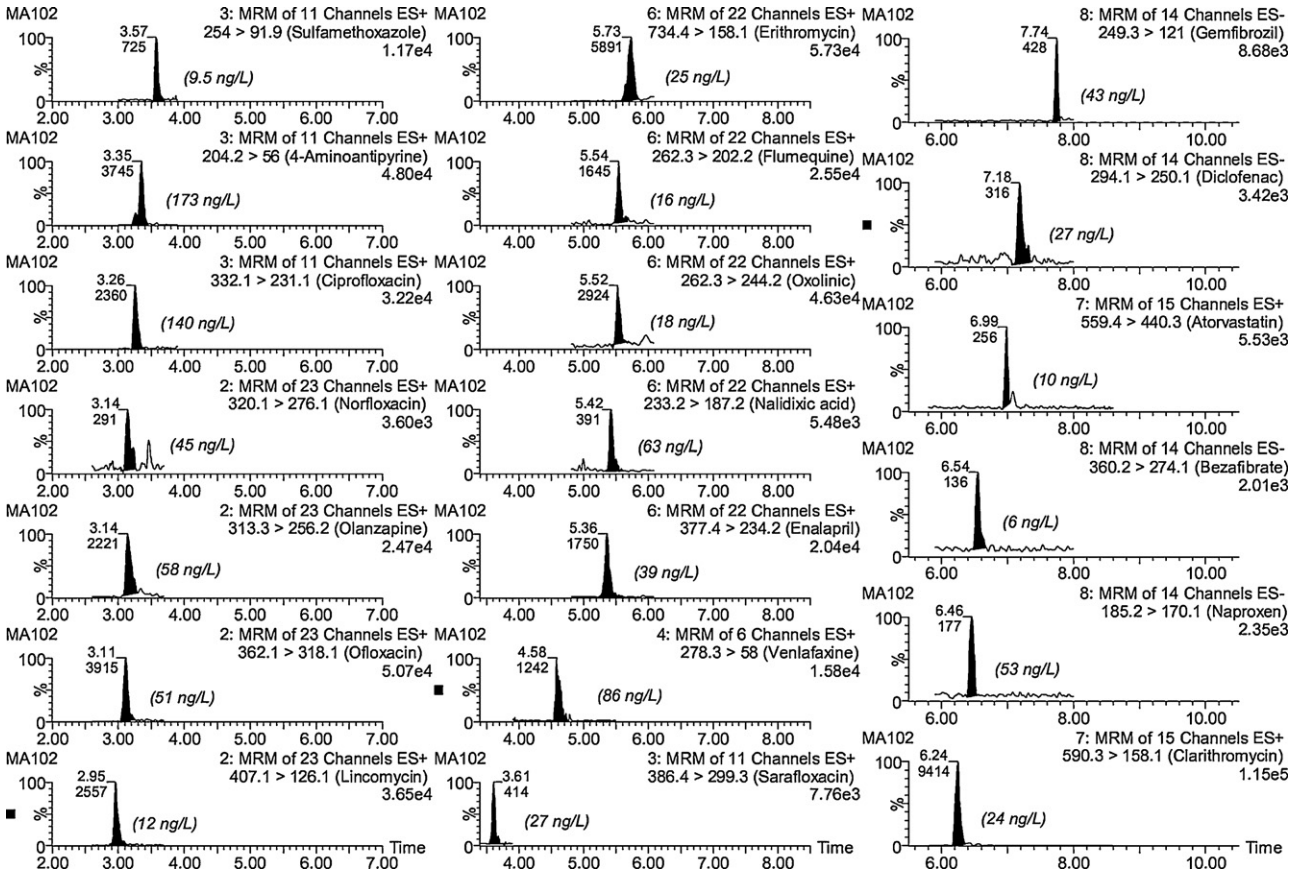

Fig. 1. UHPLC–MS/MS chromatograms (Q transition) for a surface water that was positive to 20 pharmaceuticals. ES+ and ES− was simultaneously applied within the same run.

eries and RSD were satisfactory making the correction with ILIS unnecessary. Another example is venlafaxine where undesirable enhancement was observed that could not be corrected with any of the available ILIS. For sulfadiazine, using acetaminophen-d_4 (the ILIS at the nearest retention time) process efficiency increased from around 15% up to around 50%. Although this approach did not fully compensate process efficiency values, it allowed improving quantitation. Despite our efforts to correct unsatisfactory data, three more compounds still presented recoveries below 60% (norfloxacin 40%, ketoprofen 55%, naproxen 55%). For negatively ionized compounds satisfactory recoveries were normally obtained using diclofenac-d_4 as ILIS.

Based on these results, method validation in EWW was carried out, using ILIS in the way shown in Table 2. Several pharmaceuticals were quantified without ILIS correction, 12 compounds were corrected with their own ILIS, and the rest using an "analogue" ILIS. "Blank" EWW samples were spiked at two different concentration levels (100 and 500 ng/L) in quintuplicate. At the lowest level tested, marbofloxacin and paroxetine could not be validated due the poor sensitivity, and 4-aminoantipyrine, moxifloxacin and ibuprofen were not validated due to the high concentrations found in the "blank" sample. As Table 3 shows, recoveries were satisfactory (between 70% and 120%) at the two spiking levels with some exceptions. For sarafloxacin, despite using its own ILIS, the recovery was slightly lower than expected, which is in the line of our previous experiments on process efficiency in EWW (see Table 2). It seems that by any unknown reason, this ILIS did not properly correct its own analyte. In some cases, sporadic unexpected values were observed when an analogue ILIS was used (e.g. risperidone and flumequine). Nevertheless, as the RSDs were satisfactory, the use of analogues ILIS was preferred in both cases. In agreement with the previous study of matrix effects, the recoveries were non satisfactory for venlafaxine (higher than 200%) and norfloxacin (around 35%). Ketoprofen and naproxen presented normally recoveries below 70% although slightly better than expected from the study of matrix effects.

For most pharmaceuticals, the method presented satisfactory precision with RSD values even below 15% in the two fortification levels. Regarding the LOQs, they were $\leq$10 ng/L for 20 out of 47 compounds. For another 20 analytes they were lower than 50 ng/L. For the remaining 7 compounds, the LOQs ranged from 79 to 170 ng/L (see Table 3).

Regarding the analysis of SW, with very few exceptions, we did not observe severe matrix effects on the samples tested. However, as 12 ILIS were available, we decided to use them for correction of their own analytes and to compensate for potential errors that might occur along sample treatment and/or unexpected matrix effects. The rest of pharmaceuticals were quantified without using ILIS with the exception of 4-aminoantipyrine and risperidone that were corrected using an analogue ILIS (see Table 4). The method was tested at three fortification levels. At the lowest concentration (25 ng/L), eight compounds could not be validated due to the low sensitivity or, as for ketoprofen, due to the high analyte concentration found in the "blank" SW sample. Recoveries and RSD were mostly satisfactory at the three levels assayed (25, 100 and 500 ng/L). Only two compounds (moxifloxacin and venlafaxine) showed values higher than 120% and another two (sulfadiazine and tylosin) yielded recoveries around 50%. The LOQs in SW were lower than 20 ng/L for the majority of compounds (39 out of 47). Similarly to EWW, the LOQs for salicylic acid, moxifloxacin and ibuprofen were among the highest, as a consequence of the poor sensitivity of the method for these compounds.

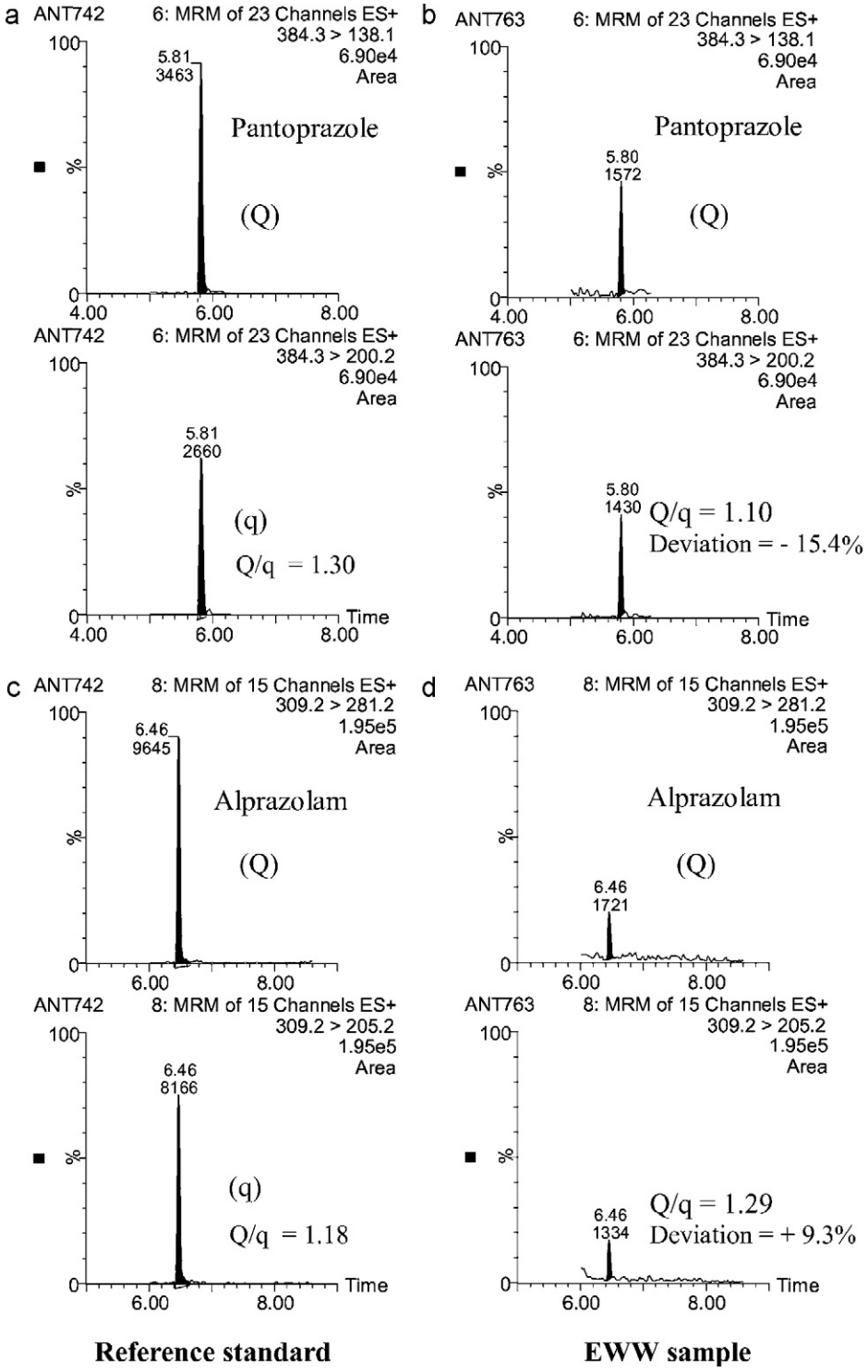

Fig. 2. Selected UHPLC–MS/MS chromatograms for (a) pantoprazole reference standard (1 μg/L), (b) effluent wastewater sample containing 0.005 μg/L of pantoprazole, (c) alprazolam reference standard (1 μg/L) and (d) effluent wastewater sample (estimated concentration 0.002 μg/L of alprazolam).

3.4. Application to environmental water samples

It is important to remark that matrix effects are a problematic issue in environmental analysis, particularly when dealing with multi-residue methods. This problem is not easy to solve, as discussed in the previous section and reported in the bibliography. The unavailability of true blank samples to perform a calibration in matrix, and the extreme difficulties to get the ILIS required for every analyte in a method for a large number of compounds, make necessary to find a realistic compromise between time, analytical efforts and amount and quality of information obtained. The composition of environmental water or wastewater is never the same. So, unexpected matrix effects could occur in every analysis, even if the method has been satisfactorily tested in similar matrices. Although the use of analogues ILIS can be a satisfactory solution in some particular cases, this approach does not ensure an appropriate correction in all samples analyzed as reported in this work or in previous articles [29,31,32]. In the absence of the own ana-

lyte ILIS, the most reliable option seems to be the application of the standard additions methods, or to perform an extensive clean-up of the extracts. However, both approaches are time-consuming and can increase the analytical errors due to the sample manipulation. The standard additions method, typically reported as one of the best ways to get accurate data, is not so easy to apply when analyzing ng/L levels. To obtain satisfactory data, it requires a previous analysis to have an estimation of the concentration level in sample in order to adjust the concentrations added. Then, it is necessary to have extreme care when obtaining the calibration with each sample, as calculating the concentration by extrapolation can lead to very high errors. Finally, the number of samples analyzed increases by a factor of 4–5, i.e. the points corresponding to the different additions made. By other side, to ensure an efficient clean-up when sample composition is highly variable and when a method is applied for a large number of compounds is rather complicated, as the method would end up being more restrictive, which is the opposite to that pursued in multi-residue methods.

Taking into account all previous considerations, quantitative data presented in this work should possibly be taken as estimated levels, with the exception of those analytes that are corrected with their own ILIS. Despite that QCs included in every sequence of samples analyzed were satisfactory, from a strict point of view no fully correction would be ensured in those cases where the own analyte ILIS could not be used. In the case that the concentrations reported had severe implications (levels above the maximum allowed in the legislation), which seems not to be the case with emerging contaminants that are still unregulated in water, a highly reliable quantification would be required in a second analysis.

The method developed was applied to 18 SW collected from different sampling points in Mediterranean rivers (Valencia region) and to 19 EWW from different WWTPs from this region (see Table 5).

In every sequence of analysis, the calibration curve was injected twice, at the beginning and the end of the sample batch. Moreover, quality control samples (QCs) were included in every sequence in order to assure the quality of the analysis. QCs consisted on SW or EWW that were spiked to 100 ng/L with all pharmaceuticals. They were analyzed following the same analytical procedure than the samples. QC recoveries in the range 70–120% were considered as satisfactory.

Confirmation of positive findings was carried out by calculating the peak area ratios between the quantification (Q) and confirmation (q) transition, and comparing them with the ion-ratio calculated from a reference standard. The finding was considered as true positive when the experimental ion-ratio was within the tolerance range [26] and the retention time of the compound in the sample within ±2.5% the retention time of the reference standard. Thus, the method fulfills the European Union guidelines and it ensures accurate identification of target analytes [26].

In SW, up to 31 pharmaceuticals were detected at least once. Analgesic and anti-inflammatories, cholesterol lowering statin drugs and lipid regulators, and quinolone antibiotics were the most detected groups. The highest concentrations corresponded to ibuprofen, acetaminophen and salicylic acid with maximum levels of 2.9, 1.9, and 1.2 μg/L, respectively. These compounds are included in the list of the 35 most consumed active principles with medical prescription in Spain [19], although they may also be acquired without medical prescription. These occurrences are in accordance with studies of other countries where a similar situation has been observed [21].

Regarding EWW, a higher number of target compounds were detected at least once (37 out of 47). Some of them, as ciprofloxacin and ofloxacin, were detected in 100% of the samples. Compounds belonging to the cholesterol lowering statin drugs and lipid regulators group were also frequently detected, except for simvastatin which was not found in either SW or EWW. This compound is the fourth most consumed in Spain [19] and its absence might be explained by the transformation of the parent compound in the aquatic environment.

Omeprazole and lorazepam were not detected in SW; however, they were present in around 30% and 80% of the EWW samples, respectively. A similar situation was observed for trimethoprim and atorvastatin that were hardly found in SW, but were detected in around 80% of the EWW samples. This fact might be due to the dilution of pharmaceuticals when they reach surface water together with transformation processes. Similarly to SW, the highest concentrations in EWW were found for acetaminophen (around 200 μg/L), salicylic acid (80 μg/L) and ibuprofen (15 μg/L), which were notably higher than in SW possibly due to the dilution process suffered in SW. Quantification of the EWW samples with high analyte levels required an additional analysis previous dilution of the sample before the SPE step.

The majority of the EWW samples analyzed were positive for at least 20 out of 47 target compounds. We did not expect to find so many positives in SW as we presumed they were less affected by the presence of pharmaceuticals. However, around 50% of the SW samples contained at least 19 analytes. These data reinforce the need of applying multi-class methods to obtain a wider and realistic knowledge on the occurrence of pharmaceuticals in environmental water. As an illustrative example, Fig. 1 shows UHPLC–MS/MS chromatograms for a SW sample which was positive to 20 compounds, with concentrations varying from 6 ng/L (bezafibrate) to 173 ng/L (4-aminoantipyrine). The high sensitivity of the method allowed us the detection and confirmation of analytes at concentrations around, or even below, the LOQ level, as shown in Fig. 2 for a EWW sample.

4. Conclusions

In this paper, advanced UHPLC–MS/MS analytical methodology has been developed for the simultaneous quantification and confirmation of 47 pharmaceuticals in surface water and wastewater samples. The proposed methodology allows the extraction of all pharmaceuticals in a single SPE step and their simultaneous determination under positive and negative electrospray modes with a chromatographic run of only 10 min. Two SRM transitions have been acquired per compound for a reliable identification.

Special attention has been paid to the correct quantification of analytes, which is more problematic in wastewater due to the presence of co-extracted components of the sample that can produce severe matrix effects. The use of 12 ILIS has been tested to correct undesirable effects for the 47 selected compounds in nine EWW, collected from different WWTPs. Appropriate correction was ensured in all samples tested only when ILIS were used to correct their own analyte. Also a correction with analogue ILIS, different from the labelled analyte, was required for several compounds in EWW. However, matrix variability of environmental water and waste water makes this correction problematic. Therefore, the evaluation and correction of matrix effects should not be based on the behaviour of an analogue ILIS in only a few samples (e.g. one or two samples), but in several random samples that represent the matrix variability along the time. In addition, quality control samples should always be included in every sequence of analysis to test if the analogue ILIS leads to confident quantitative data actually.

The high number of target compounds and the rather different class of therapeutical groups made of this method one of the most advanced in relation to its wide scope. In addition, pharmaceuticals have been selected based on their wide consumption and/or potential negative effects (mainly antibiotics). Therefore, considering that positively and negatively ionized compounds are simultane-

ously determined in just one injection, this method can offer a more realistic overview of the water quality as regards pharmaceuticals contamination than most methods previously reported. The interest of increasing the number of analytes is clearly shown by the fact that almost all compounds selected have been detected in the water samples analyzed.

In the near future, the presence of metabolites will be investigated by using a quadrupole time-of-flight mass analyzer. This investigation might be on interest especially for those compounds that are not detected in water despite their frequent use, e.g. simvastatin.

Acknowledgements

This work has been developed under financial support of the Ministry of Education and Science, Research Project CTM-2006-07711 and CTQ-2009-12347.

The authors are very grateful to the Serveis Centrals d'Instrumentació Científica (SCIC) of University Jaume I for using Acquity and TQD instrument. E. Gracia-Lor acknowledges University Jaume I for her pre-doctoral grant. The authors acknowledge the financial support of Generalitat Valenciana as research group of excellence PROMETEO/2009/054.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2011.02.026.

References

[1] S.D. Richardson, Anal. Chem. 82 (2010) 4742.
[2] M.C. Moreno-Bondi, M.D. Marazuela, S. Herranz, E. Rodriguez, Anal. Bioanal. Chem. 395 (2009) 921.
[3] J. Hu, J. Shi, H. Chang, D. Li, M. Yang, Y. Kamagata, Environ. Sci. Technol. 42 (2008) 3415.
[4] M. Petrovic, M.D. Hernando, M.S. Díaz-Cruz, D. Barceló, J. Chromatogr. A 1067 (2005) 1.
[5] Y. Xiao, H. Chang, A. Jia, J. Hu, J. Chromatogr. A 1214 (2008) 100.
[6] D. Guillarme, J. Shcappler, S. Rudaz, J.-L. Veuthey, Trends Anal. Chem. 29 (2010) 15.
[7] M. Ibáñez, C. Guerrero, J.V. Sancho, F. Hernández, J. Chromatogr. A 1216 (2009) 2529.
[8] M. Petrovic, M. Farré, M. Lopez de Alda, S. Perez, C. Postigo, M. Köck, J. Radjenovic, M. Gros, D. Barcelo, J. Chromatogr. A 1217 (2010) 4004.
[9] M. Seifrtová, L. Nováková, C. Lino, A. Pena, P. Solich, Anal. Chim. Acta 649 (2009) 158.
[10] E. Gracia-Lor, J.V. Sancho, F. Hernández, J. Chromatogr. A 1217 (2010) 622.
[11] M. Gros, M. Petrovic, D. Barceló, Anal. Chem. 81 (2009) 898.
[12] A.L. Batt, M.S. Kostich, J.M. Lazorchak, Anal. Chem. 80 (2008) 5021.
[13] M.J. García-Galán, M.S. Díaz-Cruz, D. Barceló, Talanta 81 (2010) 355.
[14] A. García-As, P.A. Segura, L. Viglino, A. Fürtös, C. Gagnon, M. Prévost, S. Sauvé, J. Chromatogr. A 1216 (2009) 8518.
[15] O.J. Pozo, C. Guerrero, J.V. Sancho, M. Ibáñez, E. Pitarch, E. Hogendoorn, F. Hernández, J. Chromatogr. A 1103 (2006) 83.
[16] E. Zuccato, S. Castiglioni, R. Bagnati, M. Melis, R. Fanelli, J. Hazard. Mater. 179 (2010) 1042.
[17] F. Gosetti, E. Mazzucco, D. Zampieri, M.C. Gennaro, J. Chromatogr. A 1217 (2010) 3929.
[18] F. Hernández, J.V. Sancho, M. Ibáñez, C. Guerrero, Trends Anal. Chem. 26 (2007) 466.
[19] Prescription data: IT del Sistema Nacional de Salud. Vol. 33, no. 3/2009. http://www.msc.es/biblioPublic/publicaciones/recursos_propios/infMedic/docs/vol33_3NotasInteres.pdf.
[20] S. Marchese, D. Perret, G. D'Ascenzo, F. Pastori, Chromatographia 58 (2003) 263.
[21] C. Lacey, G. McMahon, J. Bones, L. Barron, A. Morrissey, J.M. Tobin, Talanta 75 (2008) 1089.
[22] B. Kasprzyk-Hordern, R.M. Dinsdale, A.J. Guwy, Talanta 74 (2008) 1299.
[23] M. Farré, M. Petrovic, D. Barceló, Anal. Bioanal. Chem. 387 (2007) 1203.
[24] L. Penney, C. Bergeron, B. Coates, A. Wijewickreme, J. AOAC Int. 88 (2005) 496.
[25] H. Ergün, D.A.C. Frattarelli, J.V. Aranda, J. Pharm. Biomed. Anal. 32 (2004) 479.
[26] European Union Decision 2002/657/EC, Off. J. Eur. Commun., L221 (2002) 8–36.
[27] E. Botitsi, C. Frosyni, D. Tsipi, Anal. Bioanal. Chem. 387 (2007) 1317.
[28] C. Hao, L. Lissemore, B. Nguyen, S. Kleywegt, P. Yang, K. Solomon, Anal. Bioanal. Chem. 384 (2006) 505.
[29] J.M. Marín, E. Gracia-Lor, J.V. Sancho, F.J. López, F. Hernández, J. Chromatogr. A 1216 (2009) 1410.
[30] B.K. Matuszewski, M.L. Constanzer, C.M. Chavez-Eng, Anal. Chem. 75 (2003) 3019.
[31] J.E. Renew, C.-H. Huang, J. Chromatogr. A 1042 (2004) 113.
[32] J.V. Sancho, O.J. Pozo, F.J. López, F. Hernández, Rapid Commun. Mass Spectrom. 16 (2002) 639.