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RESEARCH PAPER

Multianalyte method for the determination of pharmaceuticals in wastewater samples using solid-phase extraction and liquid chromatography–tandem mass spectrometry

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Abstract A fast and sensitive multianalyte/multiclass high-performance reversed-phase liquid chromatography–tandem mass spectrometry (LC–MS/MS) method was developed and validated for the simultaneous analysis of 89 pharmaceuticals in influent and effluent wastewater samples. The method developed consists of solid-phase extraction (SPE) using a hydrophilic–lipophilic-balanced polymer followed by LC–MS/MS with electrospray ionization in both positive mode and negative mode. The selected pharmaceuticals belong to different classes—analgesic/anti-inflammatory drugs, antibiotics, antiepileptics, β-adrenoceptor-blocking drugs, lipid-regulating agents, statins, and many others. The influence of the mobile phase composition on the sensitivity of the method, and the optimum conditions for SPE in terms of analyte recovery were extensively studied. Chromatographic separation was performed on an Atlantis T3 (100 mm×2.1 mm, 3-μm) column with a gradient elution using methanol–0.01 % v/v formic acid as the mobile phase in positive ionization mode determination and methanol–acetonitrile–1 mM ammonium formate as the mobile phase in negative ionization mode determination. Recoveries for most of the compounds ranged from 50 to 120 %. Precision, expressed as relative standard deviations, was always below 15 %, and the method detection limits ranged from 1.06 ng/L (4-hydroxyomeprazole) to 211 ng/L (metformin). Finally, the method developed was applied to the determination of target analytes in wastewater samples obtained from the Psyttalia wastewater treatment plant, Athens, Greece. Although SPE of pharmaceuticals from wastewater samples and their determination by LC–MS/MS is a well-established technique, the uniqueness of this study lies in the simultaneous determination of a remarkable number of compounds belonging to more than 20 drug classes. Moreover, the LC–MS/MS method has been thoroughly optimized so that maximum sensitivity is achieved for most of the compounds, making the proposed method a valuable tool for pharmaceutical analysis in influent and effluent wastewater at the sub-nanogram per liter level.

Keywords Pharmaceuticals · Wastewater · Solid-phase extraction · Liquid chromatography–tandem mass spectrometry · Mobile phase optimization

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Introduction

Pharmaceuticals are classified as emerging environmental contaminants, and are regarded as being potentially hazardous since many of them are ubiquitous, persistent, and biologically active compounds with recognized endocrine-disruption functions [1–4]. In recent years, much attention has been paid to the presence of pharmaceuticals in wastewater, surface water, and drinking water. Pharmaceuticals, along with their metabolites, are introduced into the aquatic environment mainly through excreta, disposal of expired or unused medicine, aquaculture, and animal feeding [2–4]. Incomplete removal of these pharmaceutical compounds in wastewater treatment plants (WWTPs) can lead to their release into natural aquatic

environments [3–5]. Studies have shown that combinations of pharmaceutical compounds exert a much stronger toxic effect than the weak toxic effects related to exposure to each compound individually [6, 7].

The rise of pharmaceutical consumption worldwide makes absolutely essential the intensive study of their occurrence in the environment along with their short-term and long-term effects. Consequently, advanced analytical methods are needed in order to monitor the presence of pharmaceuticals in wastewater, surface water, and drinking water.

Several analytical methods have been reported in the literature for the determination of pharmaceutical compounds in water samples from different sources [8–33]. Most recent analytical methods focus on multiresidue analysis [8–22], since the wide number of contaminants has generated the need for fast and sensitive analytical methods which can simultaneously determine multiple classes of drugs in one analytical procedure. There have been some methods, however, focusing on the determination of specific therapeutic classes, paying special attention to antimicrobials owing to their potential for antibiotic resistance [23–25].

The separation technique that has been mainly implemented in the analysis of pharmaceuticals is liquid chromatography (LC) because of their polar and nonvolatile character. Gas chromatography has been used in some cases [26–29], but it often needs a time-consuming derivatization step, and is not compatible with thermolabile compounds.

High-performance LC (HPLC) coupled with triple-quadrupole (QqQ) mass spectrometry (MS) is the most extensively applied method for analyzing pharmaceutical residues in various environmental samples owing to its versatility, specificity, and selectivity, making possible the detection of target compounds in the low nanogram per liter range.

Since typical environmental concentrations of pharmaceuticals are in the sub-microgram per liter range, preconcentration prior to detection is imperative. For water samples, solid-phase extraction (SPE) is the method of choice for sample preparation, and both off-line and online SPE–LC–MS/MS as well as solid-phase microextraction have been used in the environmental analysis of pharmaceuticals [30, 31]. Recently, an electro-mediated microextraction approach for determination of pharmaceuticals and personal care products has been reported, as has a stir bar sorptive extraction approach [32, 33].

A challenge is presented in the simultaneous extraction and analysis of several classes of compounds with a wide range of polarities, solubilities, pK_a values, K_{ow} values, and stability under acidic and basic conditions. Often, compromises have to be made that may affect identification or quantification, such as reduced recoveries, elevated detection limits, extended chromatograms of up to 50 min, and the monitoring of only one product ion. In light of these concerns, the aim of this work was the development of a simple, reliable, and sensitive multiresidue analytical method based on off-line SPE followed by LC–electrospray ionization (ESI)–MS/MS (QqQ) for the simultaneous analysis of an extended list of 89 pharmaceuticals in wastewaters.

To the best of our knowledge, this is the first time that such a high number of compounds belonging to many different therapeutic classes have been determined simultaneously in wastewater samples using a QqQ mass analyzer (QqQ-MS). An extended mobile phase optimization was performed by comparing the performance of different modifiers (formic acid and acetic acid in positive ionization mode determination and ammonium formate and ammonium acetate in negative ionization mode determination) in different concentrations and in combination with different organic eluents for all the therapeutic classes. Very interesting results arose from this study revealing chromatographic behavior patterns for different groups of analytes. Overall, 16 mobile phases were tested in positive ionization mode determination and eight were tested in negative ionization mode determination for all 89 analytes, with this being the most thorough study reported in the literature where either only one group of compounds is examined or a much smaller number of mobile phases is tested [34–36].

Sample preparation was also optimized by investigating the optimal SPE parameters for the simultaneous extraction and cleanup of the target analytes. Many different SPE protocols were tested and the final method was validated. The final method has excellent performance criteria, indicating its significant value in wastewater analysis, but also in pharmaceutical analysis in biological and food samples and forensics.

Materials and methods

Chemicals and reagents

All the analytes studied are presented in Table S1. All pharmaceutical standards were of high-purity grade (more than 90 %), and were purchased from Sigma-Aldrich (Steinheim, Germany). Sulfadoxine and sulfaclozine were donated by the National Laboratory of Residue Analysis of Food of Animal Origin of the Hellenic Ministry of Rural Development and Food. Acetonitrile and methanol of LC–MS grade were purchased from Merck (Darmstadt, Germany), as was hydrochloric acid (37 %), whereas formic acid (99 %) and ammonium formate were from Fluka (Buchs, Switzerland). Ammonia (25 %) was purchased from Panreac (Barcelona, Spain). Distilled water was provided by a Milli-Q purification apparatus (Direct-Q UV; Millipore, Bedford, MA, USA). Strata-X cartridges (200 mg/6 mL), Strata-X-C cartridges (200 mg/6 mL), and RC syringe filters (4-mm diameter, 0.2-μm pore size) were provided by Phenomenex (Torrance, CA, USA), and the glass fiber filters used (pore size 0.7 μm) were from Millipore (Cork, Ireland).

About 10 mg of each individual standard was accurately weighed and placed in a 10-mL volumetric flask. Penicillins, cephalosporins, macrolides, and metformin were dissolved in Milli-Q water, whereas all other analytes were dissolved in methanol. In quinolone standard solutions, 50 μL of formic acid was added to enhance solubility. Stock solutions of each compound at 1,000 μg/mL were produced and stored at −20 °C. A multicomponent solution of the 89 compounds was obtained by diluting the stock solutions in methanol to a final concentration of 10 μg/mL, and it was also stored at −20 °C. All working solutions and calibration standards were prepared by gradient dilution of the multicomponent solution, in concentrations ranging from 1 ng/mL to 1 μg/mL.

Sample pretreatment and SPE

Wastewater and sludge samples were collected from the Athens (Greece) WWTP, which serves a population of 3,700,000. Twenty-four-hour flow-proportional composite samples of sewage influents and secondary effluents were collected on eight consecutive days in April 2011. Wastewater samples were collected in 1.5-L plastic bottles and were transferred directly after sampling to the laboratory. There they were filtered on glass fiber filters and stored in a freezer (−20 °C). All samples were analyzed within 1 week from their sampling. As previously reported [37], most of the antibiotics have adequate stability in water samples when preserved at −20 °C for 1 week. Fifty milliliters of the sample was acidified to pH 2.5 with hydrochloric acid, and 1 mL of 5 % (w/v) EDTA was added to obtain an EDTA concentration of 0.1 % (w/v). Other pH values (4 and 7) were also tested, with and without the addition of EDTA, with the combination of pH 2.5 with EDTA being the most efficient. The addition of this chelating agent improves the extraction recovery of some antibiotics, especially that of tetracyclines [13, 38]. The sample was loaded in a Strata-X cartridge which had been previously conditioned with 6 mL of methanol and 6 mL of ultrapure water, under gravity. The cartridge was then washed with 6 mL of ultrapure water and was vacuum-dried for approximately 30 min before analyte elution. Analytes were eluted with 3 mL of methanol twice, and the eluate was evaporated to dryness under a gentle steam of nitrogen at 40 °C. Reconstitution of the analytes was performed in 500 μL of 0.05 % formic acid–methanol (75:25, v/v), followed by vortex stirring for 30 s. Finally, the extract was filtered through a 0.2-mm RC syringe filter, and then the samples were transferred to a glass vial for immediate ultra-high-performance LC–MS/MS analysis. A schematic of the sample preparation protocol is presented in Fig. 1. The extraction was also performed in Strata-X-C cartridges, but they showed very poor recovery of the analytes.

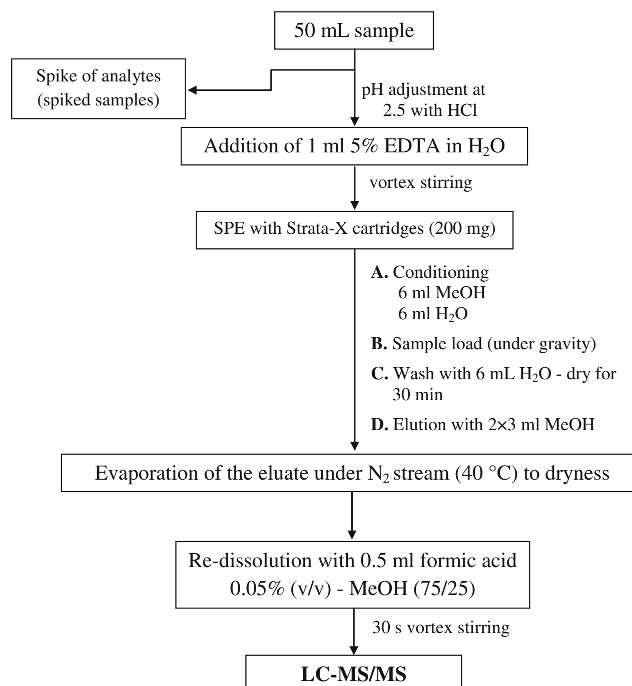

Fig. 1 The proposed method. *LC* liquid chromatography, *MS*/*MS* tandem mass spec

To examine the recovery efficiency of the extraction, at least one sample spiked with all pharmaceuticals (same matrix) was analyzed with each batch of real samples.

LC–MS/MS analysis

A Thermo Scientific Accela ultra-high-performance LC system was connected to a TSQ Quantum Access QqQ instrument (Thermo Scientific, San Jose, CA, USA). An Atlantis T3 C_{18} column (100 mm×2.1 mm, 3 μm, Waters) protected by a guard column was used at a constant flow rate of 100 μL/min. Two chromatographic runs were performed in order to determine all analytes in each sample, one in positive ionization mode and one in negative ionization mode. The mobile phase for the positive ionization mode detection consisted of water containing 0.01 % (v/v) formic acid (solvent A) and methanol (solvent B), whereas for the negative ionization mode detection, the mobile phase consisted of water containing 1 mM ammonium formate (solvent A), methanol (solvent B), and acetonitrile (solvent C). Many different mobile phases with different concentrations of modifiers (formic acid, acetic acid, ammonium formate, and ammonium acetate) and different organic solvents (methanol and acetonitrile) were tested in order to increase the sensitivity and the selectivity of the determination, with the ones mentioned above proving to be the most effective.

The gradient elution programs for both runs are presented in Tables S2 and S3. The time necessary for the reequilibration of the analytical column was 15 min in both cases, the column

was thermostated at 30 °C, and the full loop injection volume of the extract was set at 10 μL.

For MS, the mass spectra and the optimum collision energy and tube lens voltage were obtained for each compound separately by direct infusion of individual standard solutions at a concentration of 1 mg/L in formic acid–methanol (75:25, v/v) or ammonium formate–methanol (75:25, v/v), depending on whether the determination was performed in positive ionization mode or negative ionization mode. The ESI parameters for each determination are also presented in Tables S2 and S3.

Multiple reaction monitoring (MRM) was used, and detailed parameters for MRM acquisition are presented in Table 1. Two transitions were selected for identification, and the most intense one was used for quantification.

Each chromatographic run was divided into several scan events with a scan time of 20 ms for each transition. An LC–MS/MS chromatogram for 89 target compounds is presented in Fig. S1.

Quantification and method validation

For each compound, the MRM transition with the highest intensity was used for quantification (quantifier), whereas the other transition was used for confirmation (qualifier). The analytes were considered confirmed in unknown samples if the retention time did not differ by more than ±0.4 min from that of the reference standard [39] and if the quantifier/qualifier ratio in the extracted samples was within ±20 % of the ratio in the reference standards [40].

For some compounds, such as NSAIDs, metformin, some quinolones, and diuretics, real blank samples could not be obtained. To overcome this obstacle, SPE was performed in both effluent and influent wastewater samples. The first eluate was collected, which was analyte free for most of the compounds, and was used for the validation experiments.

Calibration standards were prepared at eight different concentrations, both in solvent and in blank extracts obtained as described previously (matrix-matched calibration curves), to cover the respective dynamic range for all analytes (1, 2, 5, 10, 20, 50, 100, and 200 ng/mL). Linear calibration curves typically displayed correlation coefficients greater than 0.99. Also, standard addition curves were constructed by analyzing wastewater samples spiked with the pharmaceuticals at concentrations of 0.1, 0.2, 0.5, 1, and 2 ng/mL.

Extraction recoveries of target compounds were calculated for wastewater samples using samples spiked at a concentration of 0.5 ng/mL (six replicates). The absolute recovery for each compound was assessed by comparing the integrated peak areas of an extracted spiked sample (*B*) with those of a matrix-matched standard solution which consists of a wastewater sample spiked with the analytes after SPE and evaporation (*A*):

$$\mathrm{Recovery}(\%) = B \Big/ A \times 100. \quad (1)$$

Relative recoveries were calculated on the basis of standard addition quantification, and overall recoveries were calculated by dividing the slope of the standard addition curve by the matrix-matched calibration curve slope.

Matrix effects were calculated by subtracting 1 from the ratio of the peak area of the matrix-matched standard solution (*A*) to that of the standard solution (*C*), and then multiplying the result by 100:

$$\mathrm{Matrix\,effect}(\%) = \left[\left(A \Big/ C\right) - 1\right] \times 100. \quad (2)$$

The signal is enhanced if the value is positive, whereas it is suppressed if the value is negative, and a signal enhancement or suppression effect is considered as significant if the matrix effect exceeds −20 % and 20 %, respectively.

Precision was evaluated as the relative standard deviation (RSD) of six replicate measurements performed on the same day. The expanded uncertainty can be calculated as two times the RSD obtained for a confidence level of 95 % since the uncertainty associated with the repeatability of measurements for true samples is usually considered as the main element of the uncertainty estimate [41]. A detailed estimation of expanded uncertainty for each analyte was beyond the scope of this study. Specificity was evaluated by comparing blank Milli-Q water samples (n=3) and Milli-Q water samples spiked with the analytes at 0.5 ng/mL. The evaluation of specificity was done separately for each analyte, and no peaks should be present in the blank samples at the retention time of the analyte.

The method detection limit (MDL) was calculated as 3.3 times the standard deviation of the peak area of the analyte in six replicates of a blank effluent sample spiked at a low concentration (0.1 ng/mL) divided by the slope of the standard addition curve. The method quantification limit was calculated as ten times this ratio. Instrument detection limits were also calculated for each compound. The standard deviation of the peak area of each compound in six injections of a standard solution at a low concentration (1 ng/mL) was divided by the corresponding slope of the standard calibration curve. In every case, nonspiked samples were measured in parallel, and their signal was subtracted from the signal of the spiked samples.

Quality assurance/quality control

The application of quality assurance/quality control procedures is very important and necessary to ensure that reliable results are obtained. Several procedures were followed during

Table 1 Multiple reaction monitoring parameters and retention times for all compounds determined

	Compound	ESI	Precursor ion	Product ion 1	CE (eV)	Product ion 2	CE (eV)	Tube lens voltage (V)	Retention time (min)
Penicillins	Amoxicillin	+	366.2	348.9	8	114	22	68	11.8
	Ampicillin	+	350.2	106	20	160	12	87	14.9
	Cloxacillin	+	436.2	114	33	276.7	13	84	23.0
	Dicloxacillin	+	470.1	310.8	15	114	33	101	24.0
	Oxacillin	+	402.1	242.9	13	114.1	32	65	22.7
	Penicillin V	+	351.2	160	11	114.1	31	64	22.6
	Penicillin G	+	335.1	176.1	15	160	10	87	18.4
Macrolides	Azithromycin	+	749.7	591.1	29	157.9	37	127	15.0
	Clarithromycin	+	748.9	158	30	590.5	20	123	19.1
	Erythromycin	+	734.4	158.1	30	576.3	20	130	18.1
	Tiamulin	+	494.4	192	21	119	33	101	17.8
	Tylosin	+	916.8	173.9	36	772.2	28	148	18.2
Cephalosporins	Cefaclor	+	368.1	173.9	14	118.1	32	81	14.8
	Cefadroxil	+	364.1	114.1	19	134	29	97	12.2
	Cephalexin	+	347.8	158	6	106.1	30	113	14.4
	Cefazolin	+	455	323	11	155.9	17	86	17.2
Quinolones	Ciprofloxacin	+	332.1	288	18	314	22	85	14.2
	Danofloxacin	+	358	96	25	314	20	85	14.3
	Difloxacin	+	399.9	356	20	299	27	85	14.5
	Enrofloxacin	+	360.3	245	25	316.8	20	85	14.2
	Flumequine	+	262.1	244	20	201.9	30	85	20.5
	Oxolinic acid	+	262.1	244	18	158	31	79	18.7
	Marbofloxacin	+	363.1	320	15	71.9	20	85	13.4
	Norfloxacin	+	320.3	276	16	233	23	91	14.1
	Ofloxacin	+	362.2	317.9	19	260.9	27	120	13.8
	Sarafloxacin	+	386	342	18	299	27	85	14.7
Tetracyclines	Oxytetracycline	+	461	425.8	19	442.6	12	90	14.8
	Chlortetracycline	+	479	444	20	462	15	90	16.7
	Doxycycline	+	445	427.4	19	267	35	90	17.9
	Tetracycline	+	445	410	18	426.4	12	90	14.9
Sulfonamides	Sulfachloropyridazine	+	284.9	92.1	28	155.9	14	87	16.1
	Sulfaclozine	+	284.9	92.1	28	156	15	87	17.8
	Sulfadimidine	+	279	185.9	17	124.1	26	87	15.3
	Sulfadimethoxine	+	311	156	17	108.1	29	87	17.9
	Sulfadoxine	+	311	156	17	108.1	27	87	16.4
	Sulfadiazine	+	251	156	15	92.2	27	87	13.5

Table 1 (continued)

	Compound	ESI	Precursor ion	Product ion 1	CE (eV)	Product ion 2	CE (eV)	Tube lens voltage (V)	Retention time (min)
	Sulfaguanidine	+	215	156	14	92.2	14	87	7.4
	Sulfisoxazole	+	268	156	13	92.2	27	87	14.8
	Sulfamoxole	+	268	156	13	92.2	28	87	16.4
	Sulfamonomethoxine	+	281	156	13	92.2	29	87	15.5
	Sulfamethoxypyridazine	+	281	156	13	92.2	29	87	16.4
	Sulfamerazine	+	265	156	16	172	16	87	14.5
	Sulfamethizole	+	271	155.9	14	92.2	28	87	15.2
	Sulfamethoxazole	+	253.9	155.8	16	108	25	87	16.1
	Sulfapyridine	+	250	156	15	184	17	87	14.0
	Sulfaquinoxaline	+	301	156	18	92.2	30	87	18.2
	Sulfathiazole	+	255.9	155.9	15	92.2	26	87	13.7
Amphenicols	Chloramphenicol	-	321	256.8	13	152.1	19	90	9.4
	Florfenicol	-	356	336	11	185	18	90	7.8
	Thiamphenicol	-	354	290	11	185	19	90	6.4
Other antibiotics	Trimethoprim	+	290.9	230	25	122.9	30	87	13.2
	Lincomycin	+	407.3	126.2	30	359.2	17	99	13.1
NSAIDs	Acetylsalicylic acid	-	179	137	11	93.2	21	43	12.4
	Diclofenac	+	296.1	214.9	19	249.9	12	72	24.6
	Diclofenac	-	294	249.8	14	213.9	21	64	13.2
	Ibuprofen	-	205.1	161.3	10			65	12.0
	Ketoprofen	+	255.1	209	13	105.1	23	106	22.0
	Ketoprofen	-	253.1	209	10			79	8.9
	Mefenamic acid	-	240.1	196	19	191.1	26	78	11.0
	Meloxicam	-	350.1	285.8	16	146	23	67	8.4
	Naproxen	-	229	169	10			76	10.2
	Niflumic acid	+	283.1	264.9	22	244.9	28	97	25.9
	Niflumic acid	-	281	236.9	20	176.9	35	76	12.7
	Salicylic acid	-	137	93.1	18	65.3	32	45	4.2
Steroids	Betamethasone acetate	+	435	415	6	397	10	90	22.6
	Cortisol	+	363.2	308.7	16	268.8	21	102	20.4
	Cortisone	+	361.2	163.1	23	145.1	24	102	21.1
	Methylprednisolone	+	375.3	339	8	357	10	71	22.0
	Progesterone	+	315.2	109.2	28	97.2	24	79	25.1
Nitroimidazoles	Metronidazole	+	172	128	13	82.3	25	69	13.5
	Ronidazole	+	201	140.1	10	55.5	21	73	13.5
Xanthines	Theophylline	+	181.1	124.1	17	96.2	22	79	14.7

Table 1 (continued)

	Compound	ESI	Precursor ion	Product ion 1	CE (eV)	Product ion 2	CE (eV)	Tube lens voltage (V)	Retention time (min)
Angiotensin receptor blockers	Valsartan	+	436.2	207	28	291	16	99	23.4
Statins	Atorvastatin	+	559.4	440.1	22	250	42	123	23.3
	Simvastatin	+	419.3	199	14	224.9	19	103	27.4
β-Blockers	Atenolol	+	267.2	145	26	190	18	94	11.6
	Metoprolol	+	268.2	191.1	17	133.1	25	96	14.6
	Propranolol	+	260	183.1	19	155.2	25	99	16.6
Analgesics	Paracetamol	+	152.1	93.2	22	110.2	15	84	13.6
	Caffeine	+	195.1	138.2	18	110.2	22	87	15.5
	Tramadol	+	264.3	58.4	15	246	8	66	14.6
Antiulcer drugs	Ranitidine	+	315.2	176	17	102.2	31	79	11.6
	Cimetidine	+	253.1	159.1	13	95.2	29	73	11.8
	4-Hydroxyomeprazole	+	330.1	182	23	149.1	25	90	18.0
Antiepileptic drugs	Carbamazepine	+	237.1	194.1	19	193.1	32	114	20.5
Antidiabetic drugs	Metformin	+	130.1	71.4	20	85.3	13	50	2.9
Diuretics	Furosemide	-	329	284.8	17	204.8	25	58	8.1
	Hydrochlorothiazide	-	295.9	268.6	19	204.8	22	139	5.0
	Indapamide	-	364	188.8	28	232.8	21	72	10.5
	Triamterene	+	254.1	237	26	104.1	36	93	14.5
Fibrates	Clofibric acid	-	212.9	127	17	85.4	12	64	8.4
	Gemfibrozil	-	249.1	121.0	19			83	12.1

CE collision energy, *ESI* electrospray ionization

the development and validation of the proposed method and its application to wastewater samples. Procedural blank samples were prepared with nonspiked ultrapure water in order to rule out any possible cross-contamination during the process. An instrumental blank and a calibration solution at 100 ng/mL were analyzed at the beginning, after every ten samples, and at the end of each sequence to monitor the instrumental performance and potential cross-contamination during LC–MS/MS detection.

Results and discussion

LC–ESI-MS/MS analysis

Eighty-nine pharmaceutical compounds of different polarities belonging to 21 therapeutic classes were determined in two chromatographic runs following the proposed method. This is one of the most concise SPE–LC–MS/MS studies reported so far in the literature, where either a smaller number of compounds were determined or the target analytes belonged to a more limited number of drug classes [9, 12–15, 17–19].

Selection and tuning of the precursor and product ions as well as analyte-dependent parameters, such as collision energy and tube lens voltage, were performed by direct infusion of individual pharmaceutical solutions at a concentration of 1 mg/L in 0.1 % formic acid–methanol (75:25, v/v) or 1 mM ammonium formate–methanol (75:25, v/v). The mass spectra for all analytes, individually, were obtained, and after the selection of the precursor ion for each analyte, optimization of the tube lens voltage was performed automatically. After the optimum tube lens voltage had been found, a breakdown curve was constructed giving the product ions and the collision energy for which each one gave the highest abundance. These parameters are later used in MRM method.

The final operational conditions are compiled in Table 1. Diclofenac, ketoprofen, and niflumic acid were ionized in both negative mode and positive mode, and positive ionization was selected in all cases owing to the better sensitivity achieved with this mode. Only one MRM transition was acquired in negative ionization mode for ketoprofen, naproxen, ibuprofen, and gemfibrozil, which have a specific fragmentation with only one strong product ion at m/z 253, 229, 205, and 249, respectively.

The selected precursor ions in positive ESI mode and negative ESI mode were either the protonated ($[M+H]^+$) or the deprotonated ($M-H]^-$) molecular ions. The mass spectra of the amphenicols show a typical isotopic pattern due to the two chlorine atoms in their molecules [42].

As examples, the mass spectra and breakdown curves for 4-hydroxyomeprazole in positive ionization mode and furosemide in negative ionization mode are presented in Figs. S2–S5. To optimize the chromatographic separation, a series of preliminary experiments were performed, testing different mobile phases consisting of methanol or acetonitrile as the organic phase and water with different mobile phase additives, such as formic acid, acetic acid, ammonium acetate, and ammonium formate at various concentrations. The analysis of the whole set of analytes in a single chromatographic run is desired; however, this could not be done in practice as it was not possible to avoid co-elution of analytes measured in positive and negative mode. Polarity switching during the chromatogram resulted in decreased sensitivity, and it was not possible to separate the chromatogram into different segments for each polarity. Hence, two separate chromatographic runs were performed for each ESI mode.

Several patterns of chromatographic behavior for different groups of analytes were encountered during method development (see Figs. S6, S7). For the final method, a compromise between sensitivity, selectivity, and peak shape was necessary.

Different acidic additives (formic acid and acetic acid) were used as they are known to promote the protonation of basic molecules and a signal increase in the positive ESI interface [43]. For most of the analytes, sensitivity was decreased when formic acid or acetic acid was added to the organic phase, with the exception of simvastatin, 4-hydroxyomeprazole, and triamterene, which showed slightly increased signal. Penicillins, β-blockers, and steroids showed significant increase of sensitivity when methanol was used as the organic phase, whereas only tetracyclines exhibited an important improvement of sensitivity and peak shape when acetonitrile was used. Therefore, methanol with no additive was selected as the organic phase of the mobile phase.

Most analytes exhibited the highest sensitivity when no additive was added to the aqueous phase or when a low concentration of formic acid (0.01 %) was present. However, the sensitivity of steroids increased when the amount of acid was increased, and penicillins and cephalosporins exhibited higher signals when acetic acid was used; however, double peaks appeared for the latter classes. As a result, water with 0.01 % formic acid was selected since the addition of a low concentration of acid resulted in a great improvement in peak shape and sensitivity. The effect of the mobile phase's pH in the determination of some pharmaceuticals was also evaluated in accordance with Rainville et al. [36], indicating a decrease in the signal-to-noise ratio when strong acidic conditions were used.

In negative ionization mode, the most effective mobile phase was 1 mM ammonium formate–methanol with a constant portion of 5 % acetonitrile during the whole run which promoted the elution of NSAIDs and increased their sensitivity. Satisfactory results were also obtained with no additive in the aqueous phase, but irregular peak shapes and unstable retention times were reported. The sensitivity and retention times of NSAIDs were also acceptable with the presence of 10 % acetonitrile in the mobile phase. All the results of the

mobile phase optimization are presented in Figs. S6 and S7. This study constitutes the most rigorous mobile phase optimization study reported in the literature so far, providing valuable information on the retention and ionization behavior of a great number of pharmaceutical compounds.

Solid-phase extraction

SPE is a widely used technique for the extraction of pharmaceuticals from water samples [8–20]. Optimization of the extraction procedure was done with the aim of achieving satisfactory recoveries for the widest group of compounds in a single extraction step. Different parameters that affect the recovery of target compounds (type of sorbent, pH of the sample, addition of EDTA) were studied.

First, the extraction efficiency of two cartridges was tested using HPLC-grade water spiked with the analytes. Strata-X (200 mg) and Strata-X-C (200 mg) cartridges were used. Strata-X cartridges was tested at three pH values (2.5, 4, and 7), with and without the addition of EDTA, whereas Strata-X-C cartridges were tested only at pH 2.5 with the addition of EDTA. Strata-X-C is a cation-exchange mixed-mode polymeric sorbent, which is capable of both ion-exchange and reversed-phase interactions; therefore, Strata-X-C sorbent is designed for the extraction of basic and neutral compounds [44]. However, the strong cation exchange sorbent proved to be suitable for the extraction of a very limited number of compounds, in agreement with previous reports [8, 11, 15, 44]. Thus, Strata-X was selected as the extraction sorbent, since it showed versatility and efficiency in the extraction of analytes with a wide range of polarities and acid/base character, owing to its hydrophilic–lipophilic balance.

To evaluate the effect of sample pH, three pH values were tested (2.5, 4, and 7). The most satisfactory data were obtained when the sample pH was adjusted to 2.5, since most of the target compounds are acidic compounds and are therefore neutralized at low pH. The addition of EDTA, as mentioned before, enhances the extraction recovery of some antibiotics as it prevents their rapid chelation with metal ions [13, 38]. It proved to have great influence on the recovery of tetracyclines and sulfonamides, and consequently, 0.1 % (w/v) EDTA was added to the sample prior to the extraction. Methanol has proved to be an efficient solvent for the elution of contaminants from different SPE cartridges, and therefore was chosen for the elution of the target analytes [11, 45]. All the results from the sample preparation optimization are presented in Fig. S8. Compared with other extraction methods (such as liquid–liquid extraction), the method described is environmentally friendly and has the advantages of speed, simplicity, and frugal use of organic solvent (12 mL per sample). Furthermore, instead of acetonitrile, methanol was chosen for the SPE procedure as a more environmentally friendly alternative.

Quantification and method validation

Linearity, recoveries, precision, sensitivity, specificity, and the calculation of matrix effects were considered as the criteria for the validation of the analytical method developed. Table 2 summarizes the method validation data for influent and effluent wastewater samples.

The calibration curves, matrix-matched curves, and standard-addition curves were linear in the range investigated, with correlation coefficients greater than 0.99 for all compounds. The specificity of the method was also acceptable. Seven sets of compounds—azithromycin/clarithromycin, oxolinic acid/flumequine, tetracycline/doxycycline, sulfamethoxypyridazine/sulfamonomethoxine, sulfachloropyridazine/sulfaclozine, sulfadoxine/sulfadimethoxine, and sulfamoxole/sulfisoxazole—have similar molecular ions at *m/z* 749, 262, 445, 281, 285, 311, and 268, respectively. However, these compounds can be easily distinguished on the basis of the retention time, as shown in the chromatogram of the analytes (see Fig. S1).

The absolute recoveries achieved for all target compounds ranged from 50 to 107 %, and only atorvastatin, simvastatin, cimetidine, ranitidine, gemfibrozil, metformin, and sulfaguanidine exhibited absolute recoveries below 50 %. Metformin and sulfaguanidine are very polar compounds not efficiently retained in the Strata-X sorbent, and the low recoveries of statins have been related to their instability, owing to a possible interconversion of the lactone and acidic forms [46, 47]. However, the low recoveries for some compounds are a widely reported phenomenon [8, 13, 14, 19], and they are considered acceptable, as the sensitivity and reproducibility of the method were satisfactory [8]. Overall recoveries were calculated, and are presented in Table 2 for both influent and effluent wastewater samples. The overall recovery is of great value since it indicates the recovery of each analyte within the whole working range of concentrations.

To ensure correct quantification, method precision was determined as the RSD (%), by analyzing six replicates ($n=6$) of wastewater samples spiked at 1 ng/L with the method described during the same laboratory day (method repeatability). The precision limit of less than 15 % RSD was met for all analytes, indicating the good precision of the method developed.

Calculated MDLs were between 1.06 ng/L (0.016 pg on column for 4-hydroxy omeprazole) and 211 ng/L (2.11 pg on column for metformin). These MDLs are comparable with those reported in previous studies and are adequate for wastewater analysis, since high concentrations are expected for most of these pollutants [9, 11, 12, 16, 38].

The influence of matrix effects in the quantitative LC–MS/MS analysis has been widely observed and studied [38, 48]. The ESI source is highly susceptible to other components present in the matrix affecting the amount of charged ions

Table 2 Performance and validation data of the analytical method in influent wastewater (*IWW*) and effluent wastewater (*EWW*)

Compounds	EWW			IWW			MDL (ng/L)	MQL (ng/L)	IDL	
	Absolute recovery (%)	RSD (n=6; %)	Overall recovery (%)	Absolute recovery (%)	RSD (n=6; %)	Overall recovery (%)			ng/mL	pg injected
Penicillins										
Amoxicillin	79.4	6.5	127	110.8	5.8	115	4.6	13.8	0.15	1.5
Ampicillin	82.3	6	117	74.4	8.3	119	24	72.7	0.25	2.5
Cloxacillin	62.6	7.4	59.3	85.7	6.4	91.2	31	93	0.082	0.82
Dicloxacillin	71.6	7.9	61.2	105.9	12	68.8	35	106	0.094	0.94
Oxacillin	64.3	9.4	70.3	87.3	9.3	95.7	17	51.5	0.11	1.1
Penicillin V	76.9	13	75.2	88.9	13	64.8	16	49.1	0.18	1.8
Penicillin G	98.9	14	80.1	114	11	110	37	111	0.16	1.6
Macrolides										
Azithromycin	93.9	7	104	111	15	62.5	19	58.5	0.23	2.3
Clarithromycin	93.1	12	69.2	103	12	68.9	1.9	5.76	0.032	0.32
Erythromycin	113	3.4	54.5	93.6	12	101	155	470	0.21	2.1
Tiamulin	77.5	1.3	75.2	76.8	8.2	93.7	9.8	29.6	0.014	0.14
Tylosin	79.6	6.6	89.5	90.7	12	74.6	28	84.8	0.13	1.3
Cephalosporins										
Cefaclor	76.7	5.8	106	108	6.5	117	3.8	11.5	0.16	1.6
Cefadroxil	88.2	13	92.4	91.4	8.9	120	8.3	25	0.14	1.4
Cephalexin	83.9	13	163	82.8	14	112	7.5	22.6	0.17	1.7
Cefazolin	97.1	14	103	72.7	11	80.3	4.4	13.3	0.14	1.4
Quinolones										
Ciprofloxacin	88.4	12	81.5	101	14	98.7	5.4	16.3	0.031	0.31
Danofloxacin	76.3	8.1	57.0	81.2	9.8	110	21	63	0.21	2.1
Difloxacin	88.4	11	77.3	70.4	3.8	95.6	9.9	29.8	0.012	0.12
Enrofloxacin	84.2	6.4	83.8	78.2	5.7	94.7	7.4	22.5	0.022	0.22
Flumequine	96.3	9.2	88.1	90.8	6.1	104	2.5	7.55	0.011	0.11
Marbofloxacin	83.9	5.6	79.1	79.8	6.1	92.0	5.1	15.5	0.044	0.44
Norfloxacin	109	4	76.5	85.1	10	94.9	7.2	21.7	0.033	0.33
Ofloxacin	119	11	78.0	86.6	2.9	99.9	1.7	5.18	0.034	0.34
Oxolinic acid	90.4	12	85.4	91.7	6.8	91.0	2.4	7.24	0.018	0.18
Sarafloxacin	80.1	9.5	78.9	66.2	11	100	1.9	5.61	0.016	0.16
Tetracyclines										
Chlortetracycline	83.4	10	71.7	84.6	14	75.2	7.4	22.5	0.058	0.58
Doxycycline	82.5	8.6	71.4	67.9	10	74.4	15	45.2	0.021	0.21
Oxytetracycline	99.4	9.2	74.6	85.6	9.4	93.4	7.0	21.3	0.009	0.09

Table 2 (continued)

Compounds	EWW			IWW			MDL (ng/L)	MQL (ng/L)	IDL	
	Absolute recovery (%)	RSD (n=6; %)	Overall recovery (%)	Absolute recovery (%)	RSD (n=6; %)	Overall recovery (%)			ng/mL	pg injected
Tetracycline	96.4	6.7	81.0	82.6	4.2	99.2	23	69.7	0.029	0.29
Sulfonamides										
Sulfaclozine	77.4	6.4	66.6	62.4	13	67.8	22	65.5	0.13	1.3
Sulfachloropyridazine	78.1	4.8	59.3	61.6	8.5	79.0	19	57.6	0.062	0.62
Sulfadimidine	73.6	3.6	65.8	72.9	5.4	66.2	12	37	0.021	0.21
Sulfadimethoxine	76.5	4	74.9	66.2	2.1	72.1	10	30.6	0.031	0.31
Sulfadoxine	80.4	2.4	62.7	71.0	12	66.3	19	57.3	0.02	0.2
Sulfadiazine	79.3	4.2	78.1	76.9	6.5	72.6	14	42.4	0.036	0.36
Sulfaguanidine	47.7 (103)[b]	1.3	38.5	53.7	11.0	42.2	8.6	26	0.068	0.68
Sulfisoxazole	62.9	3.7	63.5	62.1	2.7	61.5	14	41.2	0.042	0.42
Sulfamonomethoxine	70.3	7	66.7	67.5	11	64.7	7.7	23.3	0.039	0.39
Sulfamethoxypyridazine	79.2	5.4	66.6	67.6	11	63.1	6.6	19.9	0.061	0.61
Sulfamerazine	76.3	4.1	70.4	67.3	15	73.4	11	33.3	0.055	0.55
Sulfamethizole	69.8	1.6	65.5	57.9	12	62.9	22	66.7	0.021	0.21
Sulfamethoxazole	83.8	5	70.5	88.7	12	74.4	16	47.6	0.028	0.28
Sulfamoxole	73.3	3.6	63.9	69.5	8.1	69.3	17	52.4	0.037	0.37
Sulfapyridine	75.7	7.6	68.2	72.3	5.3	69.9	9.6	29.1	0.026	0.26
Sulfaquinoxaline	70.8	5.6	52.1	56.2	10	75.7	12	37.3	0.037	0.37
Sulfathiazole	71.3	6.9	70.7	63.8	7.9	66.2	18	55.5	0.031	0.31
Amphenicols										
Chloramphenicol	92.1	9.6	88.9	96.4	13	86.3	5.0	15.2	0.63	6.3
Florfenicol	88.3	13	102	94.5	7.1	81.4	1.4	4.33	0.051	0.51
Thiamphenicol	93.7	5.2	89.0	90.1	14	83.3	5.0	15.2	0.12	1.2
Other antibiotics										
Trimethoprim	98.5	2.8	76.5	96.5	9.3	107	1.7	5.24	0.011	0.11
Lincomycin	79.1	7.4	79.4	92.3	4.4	88.2	5.9	17.9	0.005	0.05
NSAIDs										
Acetylsalicylic acid	95.4	14	125	66.6	13	116	50	152	1.02	10.2
Diclofenac[a]	80.5	9.4	44.2	108	14	61.9	21	63.6	0.061	0.61
Ibuprofen	112	10	79.8	111	3.3	52.9	16	47	0.46	4.6
Ketoprofen[a]	89.3	12	80.6	81.7	9.0	85.3	3.8	11.5	0.029	0.29
Mefenamic acid	18.2 (98)[b]	14	24.5	29.2 (103)[b]	13	29.6	67	202	0.032	0.32
Meloxicam	79.0	12	52.0	85.8	14	68.1	6.5	19.8	0.011	0.11
Naproxen	52.4	7.3	87.0	87.2	5.7	99.4	8.0	24.2	0.13	1.3

Table 2 (continued)

Compounds	EWW			IWW			MDL (ng/L)	MQL (ng/L)	IDL	
	Absolute recovery (%)	RSD (n=6; %)	Overall recovery (%)	Absolute recovery (%)	RSD (n=6; %)	Overall recovery (%)			ng/mL	pg injected
Niflumic acid[a]	81.2	11	56.3	80.9	8.7	71.7	5.3	15.9	0.011	0.11
Salicylic acid	90.0	13	79.0	117	5.8	94.1	3.1	9.39	0.10	1
Steroids										
Betamethasone acetate	93.3	8.8	92.7	87.2	11	106	30	92.1	0.20	2
Cortisol	98.9	5	99.8	93.6	12	90.5	16	48.5	0.28	2.8
Cortisone	96.3	4.7	82.5	85.0	7.0	107	10	30.3	0.082	0.82
Methylprednisolone	107	13	108	91.9	10.9	110	19	56.4	0.18	1.8
Progesterone	59.1	9.2	56.7	70.4	12	82.5	8.2	24.8	0.039	0.39
Nitroimidazoles										
Metronidazole	96.4	7.1	81.6	93.6	8.4	102	2.4	7.39	0.012	0.12
Ronidazole	96.0	4.6	96.1	80.2	5.2	103	1.1	3.45	0.057	0.57
Xanthines										
Theophylline	100	9.8	77.9	107	5.5	110	5.5	16.7	0.063	0.63
Angiotensin receptor blockers										
Valsartan	80.5	6.5	63.7	103	9.2	64.1	8.9	26.8	0.064	0.64
Statins										
Atorvastatin	21.7 (112)[b]	0.8	33.3	38.3 (100)[b]	15	25.5	4.5	13.5	0.074	0.74
Simvastatin	10.1 (109)[b]	8	40.2	36.2 (111)[b]	15	61.8	28	84.2	0.089	0.89
β-Blockers										
Atenolol	96.7	5.3	73.9	109	2.9	88.7	6.2	18.7	0.052	0.52
Metoprolol	97.8	4.1	77.7	105	8.1	85.7	54	163	0.057	0.57
Propranolol	83.6	3.6	79.0	82.4	9.4	100	5.2	15.9	0.046	0.46
Analgesics										
Paracetamol	119	9.6	82.5	107	2.3	113	23	68.5	0.24	2.4
Caffeine	107	6	83.7	113	7.8	102	7.0	21.1	0.020	0.2
Tramadol	100	4.8	80.8	98.2	2.7	87.5	6.2	18.8	0.030	0.3
Antiulcer drugs										
Ranitidine	41.2 (105)[b]	13	51.1	55.9	15	35.8	7.7	23.3	0.039	0.39
Cimetidine	37.6 (105)[b]	13	63.2	49.8 (99.2)[b]	14	62.5	15	45.8	0.042	0.42
4-Hydroxyomeprazole	107	11	85.5	79.1	11	96.5	1.1	3.21	0.067	0.67
Antiepileptic drugs										
Carbamazepine	104	9.1	81.0	95.5	7.2	116	21	64.2	0.049	0.49
Antidiabetic drugs										
Metformin	9.5 (104)[b]	2.3	12.4	22.3 (101)[b]	8.8	6.6	211	639	0.13	1.3

Table 2 (continued)

Compounds	EWW			IWW			MDL (ng/L)	MQL (ng/L)	IDL	
	Absolute recovery (%)	RSD (n=6; %)	Overall recovery (%)	Absolute recovery (%)	RSD (n=6; %)	Overall recovery (%)			ng/mL	pg injected
Diuretics										
Furosemide	84.0	7.3	88.2	107.2	8.3		21	64.8	0.18	1.8
Hydrochlorothiazide	95.0	9.6	81.8	108.8	10	82.9	9.2	27.9	2.3	23
Indapamide	76.5	11	83.0	69.6	11	108	71	216	0.16	1.6
Triamterene	90.3	8.1	86.1	83.5	7.9	103	4.3	13.1	0.012	0.12
Fibrates										
Clofibric acid	88.5	5.8	100	83.6	14	105	6.0	18.2	0.28	2.8
Gemfibrozil	35.3 (111)[b]	10	41.5	42.7 (102)[b]	13	62.7	2.6	7.88	0.048	0.48

IDL instrument detection limit, *MDL* method detection limit, *MQL* method quantification limit, *RSD* relative standard deviation

[a] Positive ionization

[b] Relative recoveries based on standard addition calibration

getting into the detector. This may result in signal suppression or signal enhancement, making the assessment of these matrix effects extremely important in order to obtain reliable and accurate results. To evaluate the degree of ion suppression or ion enhancement in each target compound, matrix effects were calculated by comparing the peak areas from the analysis of matrix-matched standard solutions and standard solutions of the analytes in the same concentration. The matrix effects from influent and effluent samples from the Athens WWTP were calculated for three consecutive years (2011, 2012, and 2013), and the results are presented in Table S4.

More than 75 % of the compounds are subjected to signal suppression in effluent samples in all three years, and for influent wastewater the percentage is even higher. Previous studies also reported strong signal suppression for many pharmaceuticals [8, 9, 12, 13, 15]. In effluents, in 2011, 23 compounds exhibited matrix effects below ±20 %, which is considered insignificant, 27 compounds did so in 2012, and 31 compounds did so in 2013. In influents, only seven compounds exhibited insignificant matrix effects in 2011, 15 compounds did so in 2012, and 11 compounds did so in 2013. Thus, it is safe to conclude that an increase in matrix effect is observed as the matrix becomes more complex, an observation that is in agreement with previous reports [13]. The antibiotics azithromycin, sarafloxacin, and tetracycline exhibited very strong signal suppression, whereas strong signal enhancement was observed for the antibiotic oxolinic acid and the diuretic hydrochlorothiazide.

It is of great importance to use the appropriate approach to correct the matrix effect when analyzing real samples. The use of isotope-labeled internal standards is a very powerful tool to compensate for signal alternations. However, adequate correction for matrix effects is only ensured when the isotope-labeled internal standard corresponding to the analyte is used [11, 22]. In this study, only a few deuterated internal standards were available for compounds with high recoveries, which could not compensate for the matrix effects and the extraction losses for all the other analytes. Therefore, the use of a one-point standard-addition calibrator sample was used in order to quantify accurately all the analytes in real samples, following an already published method [16].

Analysis of real samples

The applicability of the method was evaluated by analyzing effluent and influent wastewater samples from the Athens WWTP for eight consecutive days. Measurements of wastewater values such as biological oxygen demand, chemical oxygen demand, level of total suspended solids, pH, level of nitrates, and level of ammonium were initially performed in order to characterize our matrices. The results are presented in Table S5.

Table 3 Results from the analysis of wastewater samples from the Athens wastewater treatment plant

Compounds	Influents (ng/L)						Effluents (ng/L)				
	MDL	*N*>MDL	Mean	Median	Minimum	Maximum	*N*>MDL	Mean	Median	Minimum	Maximum
Penicillins											
Amoxicillin	4.57	8	29.4	32.1	15.7	39	8	28.8	26.7	18.8	51.3
Ampicillin	24	0	<24.0	<24.0	<24.0	<24.0	0	<24.0	<24.0	<24.0	<24.0
Cloxacillin	30.7	0	<30.7	<30.7	<30.7	<30.7	0	<30.7	<30.7	<30.7	<30.7
Dicloxacillin	34.9	0	<34.9	<34.9	<34.9	<34.9	0	<34.9	<34.9	<34.9	<34.9
Oxacillin	17	0	<17.0	<17.0	<17.0	<17.0	0	<17.0	<17.0	<17.0	<17.0
Penicillin V	16.2	0	<16.2	<16.2	<16.2	<16.2	0	<16.2	<16.2	<16.2	<16.2
Penicillin G	36.6	0	<36.6	<36.6	<36.6	<36.6	0	<36.6	<36.6	<36.6	<36.6
Macrolides											
Azithromycin	19.3	2	<19.3	<19.3	<19.3	64	8	171	166	123	245
Clarithromycin	1.9	8	1,377	1,004	671	2,683	8	1,153	1,131	900	1,476
Erythromycin	155	0	<155	<155	<155	<155	0	<155	<155	<155	<155
Tiamulin	9.77	0	<9.77	<9.77	<9.77	<9.77	0	<9.77	<9.77	<9.77	<9.77
Tylosin	28	0	<28.0	<28.0	<28.0	<28.0	0	<28.0	<28.0	<28.0	<28.0
Cephalosporins											
Cefaclor	3.8	8	214	193	164	291	1	<3.80	<3.80	<3.80	20.4
Cefadroxil	8.25	0	<8.25	<8.25	<8.25	<8.25	0	<8.25	<8.25	<8.25	<8.25
Cephalexin	7.46	0	<7.46	<7.46	<7.46	<7.46	0	<7.46	<7.46	<7.46	<7.46
Cefazolin	4.38	0	<4.38	<4.38	<4.38	<4.38	0	<4.38	<4.38	<4.38	<4.38
Quinolones											
Ciprofloxacin	5.39	8	1588	1384	1057	2881	8	1116	1277	523	1437
Danofloxacin	20.8	5	82.4	96.9	<20.8	157	5	163	200	<20.8	307
Difloxacin	9.85	1	<9.85	<9.85	<9.85	12.5	5	12.4	11.6	<9.85	24.9
Enrofloxacin	7.43	5	23	29.1	<7.43	40.2	6	37.5	49.8	<7.43	66.7
Flumequine	2.49	5	2.54	3.02	<2.49	3.53	5	2.57	3.07	<2.49	3.87
Norfloxacin	7.17	8	292	268	237	447	8	226	279	91.1	308
Ofloxacin	1.71	8	133	126	116	180	8	142	167	62	194
Oxolinic acid	2.39	8	27.5	20.7	11.4	85.7	8	33.8	23.5	20.9	102
Marbofloxacin	5.13	4	8.08	6.23	<5.13	18.2	5	11.5	9.43	<5.13	26.5
Sarafloxacin	1.85	6	15.3	16.8	<1.85	25.3	5	20.6	28	<1.85	38.1
Tetracyclines											
Chlortetracycline	7.42	8	30.8	28.7	23.6	43	8	58.2	53.9	40.6	85.8
Doxycycline	14.9	2	<14.9	<14.9	<14.9	324	7	149	169	7.45	209
Oxytetracycline	7.02	8	28.5	25.4	18.4	41.8	8	41.3	34	24.5	59.3
Tetracycline	23	7	27.4	29	<23.0	37.4	8	42.5	43.6	23.1	66.2
Sulfonamides											
Sulfaclozine	21.6	0	<21.6	<21.6	<21.6	<21.6	0	<21.6	<21.6	<21.6	<21.6
Sulfachloropyridazine	19	0	<19.0	<19.0	<19.0	<19.0	0	<19.0	<19.0	<19.0	<19.0
Sulfadimidine	12.2	0	<12.2	<12.2	<12.2	<12.2	0	<12.2	<12.2	<12.2	<12.2
Sulfadimethoxine	10.1	0	<10.1	<10.1	<10.1	<10.1	0	<10.1	<10.1	<10.1	<10.1
Sulfadoxine	18.9	0	<18.9	<18.9	<18.9	<18.9	0	<18.9	<18.9	<18.9	<18.9
Sulfadiazine	14	8	37.5	38.3	29.7	45.7	0	<14.0	<14.0	<14.0	<14.0
Sulfaguanidine	8.57	0	<8.57	<8.57	<8.57	<8.57	0	<8.57	<8.57	<8.57	<8.57
Sulfisoxazole	13.6	0	<13.6	<13.6	<13.6	<13.6	0	<13.6	<13.6	<13.6	<13.6
Sulfamonomethoxine	7.68	0	<7.68	<7.68	<7.68	<7.68	0	<7.68	<7.68	<7.68	<7.68
Sulfamethoxypyridazine	6.57	0	<6.57	<6.57	<6.57	<6.57	0	<6.57	<6.57	<6.57	<6.57
Sulfamerazine	11	0	<11.0	<11.0	<11.0	<11.0	0	<11.0	<11.0	<11.0	<11.0

Table 3 (continued)

Compounds	Influents (ng/L)						Effluents (ng/L)				
	MDL	*N*>MDL	Mean	Median	Minimum	Maximum	*N*>MDL	Mean	Median	Minimum	Maximum
Sulfamethizole	22	0	<22.0	<22.0	<22.0	<22.0	0	<22.0	<22.0	<22.0	<22.0
Sulfamethoxazole	15.7	8	218	222	156	280	8	160	162	140	169
Sulfamoxole	17.3	0	<17.3	<17.3	<17.3	<17.3	0	<17.3	<17.3	<17.3	<17.3
Sulfapyridine	9.6	8	44.7	48	32.4	57	8	26	23.5	22	36.5
Sulfaquinoxaline	12.3	0	<12.3	<12.3	<12.3	<12.3	0	<12.3	<12.3	<12.3	<12.3
Sulfathiazole	18.3	0	<18.3	<18.3	<18.3	<18.3	0	<18.3	<18.3	<18.3	<18.3
Amphenicols											
Chloramphenicol	5	1	<5.00	<5.00	<5.00	15.8	0	<5.00	<5.00	<5.00	<5.00
Florfenicol	1.43	0	<1.43	<1.43	<1.43	<1.43	0	<1.43	<1.43	<1.43	<1.43
Thiamphenicol	5	0	<5.00	<5.00	<5.00	<5.00	0	<5.00	<5.00	<5.00	<5.00
Other antibiotics											
Trimethoprim	1.73	8	194	183	133	309	8	134	132	119	154
Lincomycin	5.92	8	17.4	18.7	8.37	26.4	8	16.6	17.5	11.7	20.9
NSAIDs											
Acetylsalicylic acid	50	8	11,657	8,366	5,908	25,900	8	2,420	1,928	392	7,421
Diclofenac[a]	21	8	738	712	514	1,001	8	874	880	761	987
Ibuprofen	15.5	8	1,269	1,306	526	1,928	0	<15.5	<15.5	<15.5	<15.5
Ketoprofen[a]	3.84	8	197	195	134	229	8	57.6	56.5	41.3	73.7
Mefenamic acid	66.6	8	51,335	16,771	9,581	129,427	8	992	866	360	1,850
Meloxicam	6.54	1	<6.54	<6.54	<6.54	121	2	<6.54	<6.54	<6.54	218
Naproxen	8	8	942	866	741	1,363	8	142	137	112	176
Niflumic acid[a]	5.26	8	497	479	420	675	8	554	569	423	632
Salicylic acid	3.1	8	5,684	1,774	272	16,044	8	265	164	121	591
Steroids											
Betamethasone acetate	30.4	0	<30.4	<30.4	<30.4	<30.4	0	<30.4	<30.4	<30.4	<30.4
Cortisol	16	0	<16.0	<16.0	<16.0	<16.0	0	<16.0	<16.0	<16.0	<16.0
Cortisone	10	8	60.2	56.1	27.5	112	0	<10.0	<10.0	<10.0	<10.0
Methylprednisolone	18.6	0	<18.6	<18.6	<18.6	<18.6	0	<18.6	<18.6	<18.6	<18.6
Progesterone	8.17	0	<8.17	<8.17	<8.17	<8.17	0	<8.17	<8.17	<8.17	<8.17
Nitroimidazoles											
Metronidazole	2.44	8	219	118	28	490	8	173	170	159	186
Ronidazole	1.14	8	13.4	15.4	3.05	22.4	8	4.69	4.83	2.44	6.77
Xanthines											
Theophylline	5.5	8	1,009	1,021	796	1,314	8	38.9	38.1	30.5	49.8
Angiotensin receptor blockers											
Valsartan	8.86	8	8,702	8,581	7,238	10,313	8	624	560	412	1,072
Statins											
Atorvastatin	4.46	8	194	175	132	298	8	13.7	12.9	9.77	17.9
Simvastatin	27.8	3	147	<27.8	<27.8	914	2	<27.8	<27.8	<27.8	39.1
β-Blockers											
Atenolol	6.16	8	1,297	1,312	1,047	1,517	8	540	529	484	597
Metoprolol	53.9	8	333	334	256	410	8	373	377	338	413
Propranolol	5.24	8	45.7	43.5	19.6	82.2	8	68.7	66.9	58.1	82.7
Analgesics											
Caffeine	6.97	8	49,769	50,211	42,521	58,032	8	464	312	258	807
Paracetamol	22.6	8	29,635	25,198	10,555	81,016	8	1,926	822	124	7,420
Tramadol	6.21	8	455	402	357	689	8	630	625	582	696

Table 3 (continued)

Compounds	Influents (ng/L)						Effluents (ng/L)				
	MDL	*N*>MDL	Mean	Median	Minimum	Maximum	*N*>MDL	Mean	Median	Minimum	Maximum
Antiulcer drugs											
Cimetidine	15.1	8	72.6	71.6	49.7	100	8	30.2	30.8	19.8	43.6
Ranitidine	7.68	8	1,018	797	270	1,995	8	953	1,059	505	1,377
4-Hydroxyomeprazole	1.06	7	32.4	17.5	<1.06	73.2	8	64.5	66.4	45.5	81.9
Antiepileptic drugs											
Carbamazepine	21.2	8	533	372	318	1,713	8	461	466	427	501
Antidiabetic drugs											
Metformin	211	8	135,149	135,782	97,036	176,417	8	2,448	1,911	885	4,806
Diuretics											
Furosemide	21.4	8	1,704	1,566	1,371	2,583	8	1,201	1,137	854	1,666
Hydrochlorothiazide	9.22	8	707	550	386	1,378	8	1,149	1,136	482	2,158
Indapamide	71.2	0	<71.2	<71.2	<71.2	<71.2	0	<71.2	<71.2	<71.2	<71.2
Triamterene	4.31	5	4.9	4.98	<4.31	9.18	8	8.06	7.64	6.79	9.93
Fibrates											
Clofibric acid	6	0	<6.00	<6.00	<6.00	<6.00	1	<6.00	<6.00	<6.00	6.38
Gemfibrozil	2.6	8	348	200.2	114.7	752.5	7	12.3	9.17	1.3	35

[a] Positive ionization

Among the 89 analytes, 40 were detected in all influent and effluent samples (40 %), and 56 of them (63 %) were determined at least once in the samples. The highest average and maximum concentrations were detected, in influent wastewater samples, for the antidiabetic drug metformin (maximum concentration 176.5 μg/L) and the stimulant caffeine (maximum concentration 58.0 μg/L). Metformin is one of the most prescribed drugs worldwide, but a small amount of field data have been reported since it is difficult to include it in a multiresidue method, owing to its strong polar character. It has been determined in concentrations ranging up to 100 μg/L [49–51]. Caffeine, being by far the most widely consumed nonprescription, human legal drug worldwide, is frequently detected in wastewater [8, 52]

The antihypertensive valsartan (maximum concentration 8.7 μg/L) and the analgesics and anti-inflammatories acetylsalicylic acid (11.6 μg/L) (and its metabolite salicylic acid with maximum concentrations of 5.7 μg/L), mefenamic acid (51.3 μg/L), and paracetamol (29.6 μg/L) also exhibited remarkably high concentrations in influent wastewater samples. Relatively high concentrations were also detected for the antiulcer drug ranitidine, the β-blocker atenolol, the diuretic furosemide, the antimicrobials ciprofloxacin and clarithromycin, and also theophylline. Even though the concentrations found in influent wastewater samples were much higher than those found in effluent wastewater samples, significant levels were still detected in treated wastewater, especially in the cases of paracetamol, acetylsalicylic acid, furosemide, metformin, ciprofloxacin, and clarithromycin, where the concentrations were above 1 μg/L.

Paracetamol and ranitidine were detected in similar levels in wastewater samples from a WWTP in Croatia [8] and a WWTP in Spain [11], where also salicylic acid was detected in high concentrations. Valsartan was detected in comparable concentrations in wasterwater samples from Brussels [10] and Spain [12]. Gros et al. [15] also reported high concentrations of caffeine, salicylic acid, valsartan, and atenolol in Girona's (Catalonia, Spain) WWTP. In general terms, the results obtained are similar to those reported in the literature, and the concentrations of the detected pharmaceuticals are in good agreement with those reported in WWTPs in Croatia, Spain, China, and Wales [8, 11, 12, 15, 16, 38, 53]. There is a distinct difference regarding the anti-inflammatory mefenamic acid, which was detected at very high concentrations in the Athens WWTP, as well as for ranitidine, which was found at concentrations higher than those reported in the literature.

All the determined concentrations of the compounds are presented in Table 3. Mean, median, minimum, and maximum concentrations of the analytes are reported in influent and effluent wastewater samples.

Conclusions

A novel analytical method using HPLC–positive/negative ESI MS/MS for the sensitive, fast, and cost-effective simultaneous

analysis of 89 pharmaceuticals in wastewater samples was presented in this study. The compounds analyzed belong to more than 20 different classes of pharmaceuticals (analgesic/anti-inflammatory drugs, antibiotics, antiepileptics, β-adrenoceptor-blocking drugs, lipid-regulating agents, etc.) with different physicochemical properties.

The method developed consists of a single SPE step, simplifying considerably sample preparation. A thorough optimization of the LC–MS/MS parameters (mass spectra, mobile phase optimization) and SPE parameters (pH, sorbent, addition of EDTA) was performed, resulting in maximum sensitivity, selectivity, and recoveries of the target compounds. The final method was validated and matrix effects were evaluated throughout three consecutive years. Application of the method to the analysis of wastewater samples from the Athens WWTP revealed a widespread occurrence of pharmaceuticals at the nanogram per liter level and the microgram per liter level.

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