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Multi-residue analysis of pharmaceuticals in wastewater by ultra-performance liquid chromatography–quadrupole–time-of-flight mass spectrometry

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Abstract

In this work, a new multi-residue method using ultra-performance liquid chromatography (UPLC) quadrupole-time-of-flight mass spectrometry (Q–TOF–MS) was developed for screening and confirmation of 29 pharmaceutical compounds belonging to different therapeutical classes: analgesics and antiinflammatories, lipid regulating agents cholesterol lowering statin agents, psychiatric drugs, anti ulcer agents, histamine H2 receptor antagonist, antibiotics and beta-blockers. UPLC uses columns packed with 1.7 μm particles and enables elution of sample components in much narrower, more concentrated bands, resulting in better chromatographic resolution and increased peak height. The typical peak width was 5–10 s at base, permitting very good separation of all compounds in 10 min, which represented an approximate three-fold reduction in the analysis time in comparison to conventional high-performance liquid chromatography (HPLC). Unequivocal identification of target pharmaceutical compounds was based on accurate mass measurement of the molecular ions in the TOF mode and by performing collision induced dissociation (CID) in the Q–TOF mode in order to generate accurate mass measurement of the product ions. Using lock mass correction the accurate masses calculated for the product ions deviated from the theoretical masses by 0.2 to 1.3 mDa (root mean square (RMS) value = 0.67) and 0.7–6.4 ppm (RMS = 3.53), respectively. Quantitation was carried out working in the TOF mode using the narrow window extracted ion chromatograms (nwXICs) of each compound (extracted using a 20 mDa window) yielding relative standard deviation (RSD) from 0.5 to 5.3% (run-to-run) and from 2.1 to 9.1% (day-to-day) and instrumental detection limits (IDLs) from 1 to 200 pg. Analysis of wastewater treatment plant (WWTP) samples gave method detection limits (MDLs) ranging from 10 to 500 ng/L. The UPLC–Q–TOF method was successfully applied to analyze pharmaceutical residues in WWTP samples.

Keywords: Quadrupole–time-of-flight mass spectrometry; Pharmaceuticals; Environmental analysis; Ultra-performance liquid chromatography

1. Introduction

Pharmaceutically active substances are a class of new, so-called "emerging" contaminants that have raised great concern in the last years [1]. Human and veterinary drugs are continuously being released in the environment mainly as a result of the manufacturing processes, the disposal of unused or expired products, and the excreta. The reason why these substances are interesting as environmental contaminants is that they are developed with the intention of performing a biological effect and they often have the same type of physico-chemical behavior as other harmful xenobiotics (persistence in order to avoid the substance to be inactive before having a curing effect, and lipophilicity in order to be able to pass membranes) [2].

The prerequisite for proper risk assessment and monitoring of waste, surface and drinking water quality is the availability of a multi-residual analytical method that permits measurement at low nanogram per liter level. Currently, the main advances in improving sensitivity and specificity of environmental analyses of pharmaceutical residues are due to the application of LC/MS and LC/MS/MS. Advanced LC–MS/MS technology allows the determination of a broad range of compounds and thus permits comprehensive assessment of pharmaceuticals as environmental contaminants. Recently, a significant number of analytical methods, mainly based on single quadurpole and triple quadrupole (QqQ) LC–MS, have been developed for the analysis of different therapeutical classes including antibiotics, non-steroidal anti-inflammatory drugs, β-blockers, lipid regulating agents and

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Table 1
Target compounds

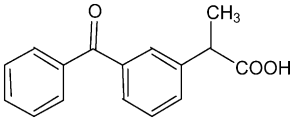
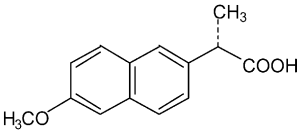
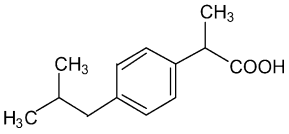
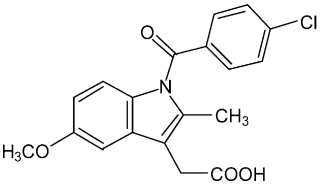
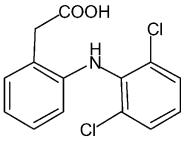
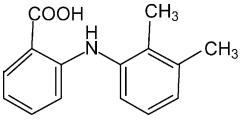
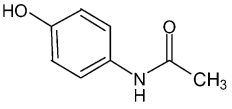
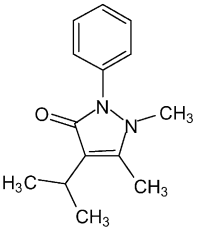
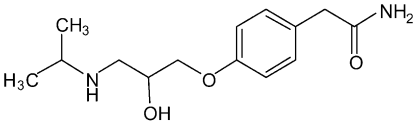
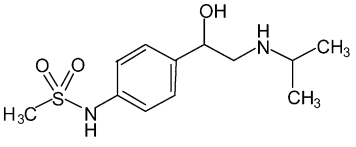
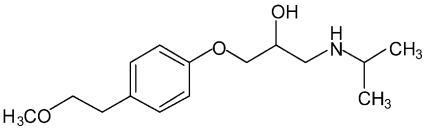
Group of compounds	Target compound	Structure	Elemental formula
Analgesic and antiinflammatories	Ketoprofen		$C_{16}H_{14}O_3$
	Naproxen		$C_{14}H_{14}O_3$
	Ibuprofen		$C_{13}H_{18}O_2$
	Indomethacin		$C_{19}H_{16}ClNO_4$
	Diclofenac		$C_{14}H_{11}Cl_2NO_2$
	Mefenamic acid		$C_{15}H_{15}NO_2$
	Acetaminophen		$C_8H_9NO_2$
	Propyphenazone		$C_{14}H_{18}N_2O$
β-blockers	Atenolol		$C_{14}H_{22}N_2O_3$
	Sotalol		$C_{12}H_{20}N_2O_3S$
	Metoprolol		$C_{15}H_{25}NO_3$

Table 1 (*Continued*)

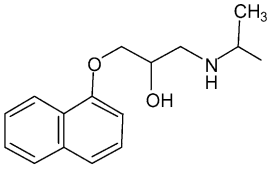
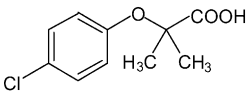
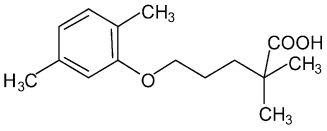
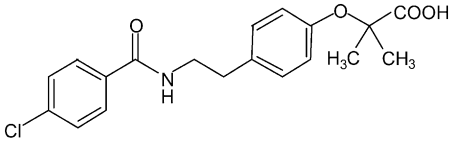
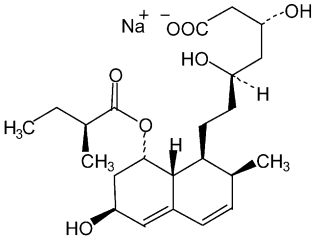
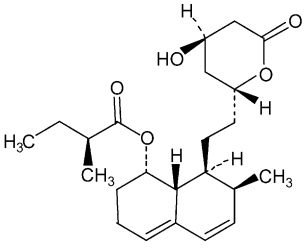
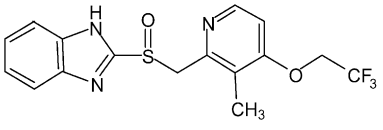
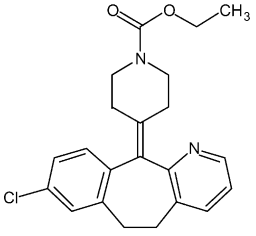
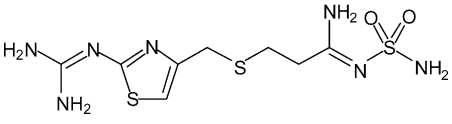
Group of compounds	Target compound	Structure	Elemental formula
	Propranolol		$C_{16}H_{21}NO_2$
Lipid regulator and cholesterol lowering drugs	Clofibric acid		$C_{10}H_{11}ClO_3$
	Gemfibrozil		$C_{15}H_{22}O_3$
	Bezafibrate		$C_{19}H_{20}ClNO_4$
	Pravastatin		$C_{23}H_{35}NaO_7$
	Mevastatin		$C_{23}H_{34}O_5$
Antiulcer agent	Lansoprazole		$C_{16}H_{14}F_3N_3O_2S$
Histamine H_1 and H_2 receptor antagonists	Loratadine		$C_{22}H_{23}ClN_2O_2$
	Famotidine		$C_8H_{15}N_7O_2S_3$

Table 1 (*Continued*)

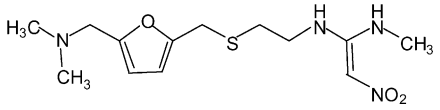
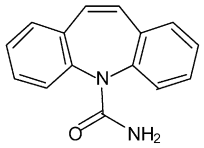
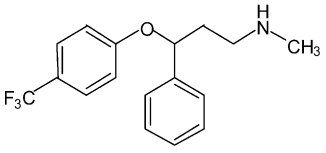
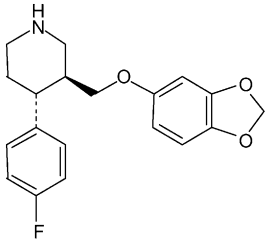
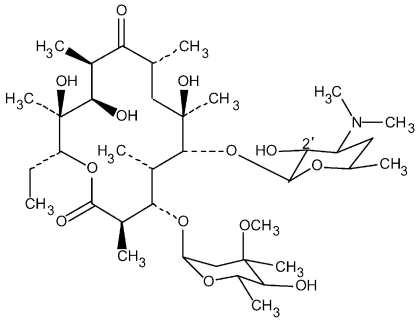
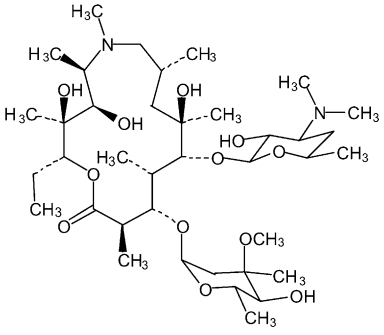
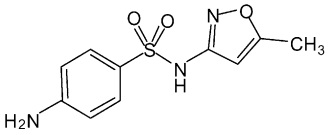
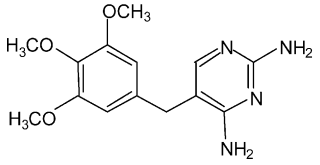
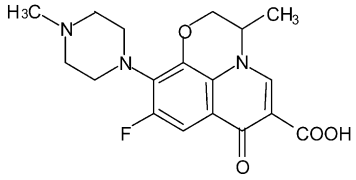
Group of compounds	Target compound	Structure	Elemental formula
	Ranitidine		$C_{13}H_{22}N_4O_3S$
Psychiatric drugs	Carbamazepine		$C_{15}H_{12}N_2O$
	Fluoxetine		$C_{17}H_{18}F_3NO$
	Paroxetine		$C_{19}H_{20}FNO_3$
Antibiotics	Erythromycin		$C_{37}H_{67}NO_{13}$
	Azithromycin		$C_{38}H_{72}N_2O_{12}$
	Sulfamethoxazole		$C_{10}H_{11}N_3O_3S$

Table 1 (*Continued*)

Group of compounds	Target compound	Structure	Elemental formula
	Trimethoprim		$C_{14}H_{18}N_4O_3$
	Ofloxacin		$C_{18}H_{20}FN_3O_4$

psychiatric drugs [3]. However, using LC-triple quadruple MS operating under multiple reaction monitoring (MRM) mode the qualitative information needed to support the structural elucidation of analytes is lost. In the full-scan mode, this information can be obtained, but the lack of sensitivity and the lack of compound databases and mass-spectral libraries often present an obstacle to the efficient structural elucidation. Orthogonal-acceleration time-of-flight (oaTOF) MS, and a combination of quadrupole and oaTOF–MS (Q–TOF) coupled to LC, have proved to be a powerful tool for the identification of trace constituents of complex mixtures and/or for confirming their presence [4]. Such instruments enable accurate mass measurement with accuracies of <5 ppm, which dispel interpretation ambiguities. Although environmental applications are still scarce, several authors reported on the application of LC–Q–TOF for screening, confirmation and quantitative analysis of target environmental contaminants, such as pharmaceuticals [5,6], phenols [7] and pesticides [8]; for screening and elucidation of unknown contaminants present in environmental waters [9,10] and for characterization of phtotodegradation or microbial degradation products of selected contaminants [11–14].

Due to very high selectivity of tandem MS detection, the importance of good chromatographic separation is often neglected. A poor chromatographic separation will lead to ion suppression and isobaric interferences that cannot be overcome by a specific MS detection, especially in the trace level analysis where failure to completely separate compounds from each other and from the matrix components may result in the non-detection of low concentration compounds or their equivocal structural assignment. Conventional approach is the application of high-performance liquid chromatography (HPLC) in a gradient reversed phase mode typically using columns with 5 μm particles. A novel approach to chromatographic separation is ultra-performance liquid chromatography (UPLC). UPLC uses columns packed with sub-2 μm particles, which enable elution of sample components in much narrower, more concentrated bands, resulting in better chromatographic resolution and increased peak capacity [15,16]. Reducing the particle diameter from 5 or 3 μm (typical HPLC columns) to 1.7 μm (UPLC) results in a multi-fold increase in linear velocity (speed) and efficiency (peak capacity). However, reducing the particle size by a factor of three results in an increase in the backpressure by a factor of 27. Therefore, to achieve the benefits of operating at higher linear velocities it is necessary to run at higher pressures (10,000–15,000 psi) and to use specially designed instruments.

In this work we used Acquity UPLC from Waters Corporation (Milford, MA, USA) coupled to a Q–TOF–MS. The method was developed for screening and confirmation of the identity of 29 pharmaceutical compounds belonging to different therapeutical classes including analgesics and antiinflammatories, lipid regulating agents cholesterol lowering statin agents, psychiatric drugs, anti ulcer agents, histamine H2 receptor antagonist, antibiotics and beta-blockers (the full list is given in Table 1). Target compounds were selected according to the information found in the literature on their occurrence and ubiquity in the aquatic environment, as well as their high human use and consumption worldwide [1,3,17].

The objective of the work was to explore the possibilities of combining the advantages of UPLC, such as improved chromatographic resolution and increased peak capacity through rapid elution at short column with 1.7 μm particles, with a high capture rate mass spectrometry (Q–TOF) with accurate mass measurement that enables the removal of interpretation ambiguities. Analysis was based on two complementary approaches: use of the TOF mode for quantitative analysis and the Q–TOF mode, performing collision induced dissociation (CID) of predefined precursor ions to collect product ion spectra for identification and confirmation purposes. The developed method was used for the unequivocal analysis of target pharmaceutical compounds in wastewater and, up to our knowledge this work presents the first application of the UPLC in the analysis of environmental contaminants.

2. Experimental

2.1. Chemicals and reagents

All pharmaceutical standards used were of the highest purity available (>90%). Ibuprofen, naproxen, ketoprofen, diclofenac and gemfibrozil were kindly supplied by Jescuder (Rubí, Spain). Azythromycin dehydrate, erythromycin hydrate, indomethacine, acetaminophen, mefenamic acid,

clofibric acid, bezafibrate, mevastatin, carbamazepine, fluoxetine hydrochloride, lansoprazole, loratadine, famotidine, ranitidine hydrochloride, sulfamethoxazole, trimethoprim, ofloxacin, atenolol, metoprolol, propanolol hydrochloride and sotalol hydrochloride were purchased from Sigma–Aldrich (Steinheim, Germany). Propyphenazone, pravastatin and paroxetine hydrochloride were from LGC Promochem (London, UK). Isotopically labelled compounds, used as internal standards, were [^{13}C]phenacetin obtained from Sigma–Aldrich, [$^{2}H_3$]mecoprop from Dr. Ehrenstorfer (Augsburg, Germany), and [$^{2}H_3$]ibuprofen, [$^{2}H_7$]atenolol and [$^{2}H_{10}$]carbamazepine obtained from CDN Isotopes (Quebec, Canada).

Individual stock standard solutions were prepared on a weight basis in methanol and stored at −20 °C. A mixture of all pharmaceutical standards was prepared by appropriate dilution of individual stock solutions. Further dilutions of this mixture were prepared in methanol–water (25:75, v/v) before each analytical run and were used as working standard solutions. Stock solutions of internal standards were also prepared in methanol, except for [$^{2}H_3$]mecoprop which was dissolved in acetone, and were stored at −20 °C. A mixture of these standards, used for internal standard calibration, was also prepared by diluting the individual stock solutions in methanol.

HPLC-grade methanol, acetonitrile and water (LiChrosolv) were supplied by Merck (Darmstadt, Germany).

The cartridges used for solid phase extraction were Oasis HLB (60 mg, 3 mL) from Waters Corporation (Milford, MA, USA).

2.2. UPLC–Q–TOF

UPLC was performed on a Waters Acquity UPLC system (Waters Corp., Milford, MA, USA), equipped with a binary solvent delivery system, an autosampler and a TUV detector. The chromatography was performed on a 5 cm × 2.1 mm Waters Acquity C_{18} 1.7 μm column. The injection volume was 10 μL. Compounds analyzed in the positive ion (PI) mode were eluted off the column with a mobile phase consisting of (A) 5 mM aqueous NH_4Ac/acetic acid (pH 4.8) and (B) acetonitrile–methanol (2:1, v/v) at 400 μL/min. The elution started at 5% B for one min and then was linearly increased 60% B in 7 min, further increased to 90% B in 2 min and kept isocratic for 1.5 min. Compounds analyzed under negative ion (NI) conditions were eluted by (A) water and (B) methanol. The elution gradient was linearly increased from 5% B to 90% B in 6 min, then increased to 95% B in 2 min and kept isocratic for 1 min. Total run time, including the conditioning of the column to the initial conditions was 14 min.

The mass spectrometry was performed on a QTof-Micro (Waters Corp., Milford, MA, USA). The nebulization gas was set to 500 L/h at a temperature of 350 °C, the cone gas set to 50 L/h, and the source temperature set to 120 °C. The capillary and cone voltages were set to 3000/2700 (PI/NI) and 30 V, respectively. The Q-Tof-Micro instrument was operated in wide pass quadrupole mode, for MS experiments, with the TOF data being collected between *m/z* 80–800 (PI) and 70–500 (NI) with a low collision energy of 10 eV. Data were collected in the centroid mode, with a scan accumulation time of 1 s. The MS/MS experiments were performed using a variable collision energy (10–30 eV) which was optimized for each individual compound. All analyses were acquired using an independent reference spray via the LockSpray interference to ensure accuracy and reproducibility; Val-Tyr-Val was used as the lock mass (*m/z* 378.2029) under NI conditions, while terfenadine (*m/z* 472.3215) was used under PI conditions at a flow rate of 10 μL/min. The LockSpray frequency was set at 11 s, and data for the reference compound were averaged over 10 spectra/min. The accurate mass and composition for the precursor ions and for the fragment ions were calculated using software MassLynx incorporated in the instrument.

2.3. Sample preparation

The method was optimized using river water and wastewater treatment plant (WWTP) influent and effluent from different locations in Spain and Croatia. Water samples were collected using amber glass bottles prerinsed with ultra-pure water. Wastewater samples were vacuum filtered through 1-μm glass fiber filters followed by 0.45 μm nylon membrane filters (Teknokroma, Barcelona, Spain). Otherwise, river waters were only filtered using the 0.45-μm filters. Five-hundred milliliter of well water, 200 mL of effluent and 100 mL of influent wastewaters were measured.

For the preconcentration step, a Baker vacuum system (J.T. Baker, The Netherlands) was used. First, SPE cartridges (Oasis HLB, 60 mg, 3 mL) were conditioned with 5 mL of methanol followed by 5 mL of deionized water (HPLC grade) at a flow rate of 1 mL/min. After the conditioning step, water samples (500 mL of ground and river water, 200 mL of WWTP effluent and 100 mL of WWTP influent) were percolated through the cartridges at a flow rate of 10 mL/min. Finally, the cartridge was rinsed with 5 mL of HPLC-grade water. The cartridge was then dried under vacuum for 15–20 min, to remove excess of water. Elution was performed with 2 × 4 mL of methanol at 1 mL/min. The extract was evaporated under nitrogen stream and reconstituted with 1 mL methanol–water (25:75, v/v). Finally, 10 μL of a 10 ng/μL mixture of the internal standards [$^{2}H_3$]mecoprop, [$^{2}H_3$]ibuprofen, [^{13}C]phenacetin, [$^{2}H_7$]atenolol and [$^{2}H_{10}$]carbamazepine were added in the extract for internal standard calibration.

2.4. Validation of the analytical procedure

Quantitation was carried out using the TOF mode, by extracting the narrow window extracted ion chromatogram (nwXIC) of the molecular ion for each compound (typically extracted using a 20 mDa window). For further confirmation of the identity of the detected compounds a CID of molecular ions were performed using the Q–TOF mode and product ion spectra were collected. Positive identification of the target compounds was based on: (a) accurate mass measurement of the base ion with an error <5 ppm; (b) accurate mass of at least one specific product ion; and (c) LC retention time of the analyte compared to that of a standard (±2%).

The reproducibility and repeatability of the method were evaluated from run-to-run experiments (five successive injections of a standard solution) and day-to-day experiments (five successive days). The precision of the method (in terms of peak areas) was expressed as the relative standard deviation (RSD) of replicate measurements.

The instrumental detection limits (IDLs) were estimated from the injection of a standard solution successively diluted until reaching a concentration level corresponding to a signal-to-noise ration of three. The method detection limit (MDL) was defined and determined as the minimum detectable amount of an analyte in the spiked wastewater extract giving a signal-to-noise ratio of three.

Linear dynamic range was determined by injecting a standard mixture of pharmaceuticals in the wide concentration range (10 ng/L–5 mg/L). Calibration curves were generated from nwXICs obtained in the TOF mode using linear regression analysis and the concentration range that gave good fit ($r^2 > 0.99$) was established for each compound.

3. Results and discussion

3.1. Performance of UPLC

The major benefit from the use of the 1.7 μm particles is the increased column efficiency that resulted in narrow peaks and an improved separation. By using a 50 mm × 2.1 mm Acquity C_{18} column at a flow rate of 400 μL/min the average peak width was 5–10 s at base, giving a peak capacity for 10-min separation of approximately 30–60. In comparison, with HPLC, using a 5 μm, 125 mm × 2 mm i.d. C_{18} column, the average peak width was 30–60 s at the base giving a total peak capacity of approximately 10–20 for the same 10-min separation (for experimental details on HPLC see Gros et al. [18]). The column with an internal diameter of 2.1 mm was found advantageous over 1.0-mm column in terms of better separation of the target compounds. Based on Van Deemter equation that describes the relationship between linear velocity and column efficiency the optimal operating linear velocity for 2.1-mm column is achieved at flow rates of 400–1000 μL/min [19]. Elution with the same gradient conditions at different flow rates showed that the optimal performance, which is based on a compromise between the speed, separation efficiency, peak width and column backpressure, is obtained using 400 μL/min flow rate. Higher flow rate resulted in more narrow peaks; however the backpressure increased close to a limit value of 15,000 psi. At a flow rate of 400 μL/min a backpressure remained below 10,000 psi, although a gradual increase was observed by time. By the course of this study in total 400 injections were performed; approximately a half of them corresponding to pure standard solutions and a half to wastewater or river water extracts (all filtered using the 0.45-μm filters), resulting in an gradual increase in a backpressure in the order of 2000 psi. However, it should be noted that we used the first generation UPLC instrument, which at the moment when the study was performed was not equipped with a guard precolumn/filter, which is now available.

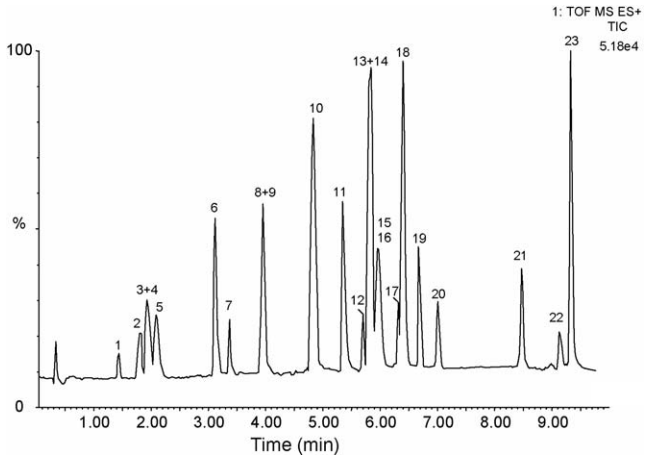


Fig. 1. UPLC–TOF total ion chromatogram showing the separation of 23 pharmaceutical compounds analyzed in PI mode (100 ng/mL standard solution). Peak assignments listed in Table 2.

To optimize the chromatographic separation, a serial of preliminary experiments was performed testing different mobile phases consisting of methanol, acetonitrile or mixture of acetonitrile and methanol as an organic phase and water with different mobile phase additives (ammonium acetate, formic acid or acetic acid at various concentrations). The optimal separation of 23 compounds detected in PI mode was achieved using a gradient elution with 5 mM aqueous NH_4Ac/acetic acid (pH 4.8) and acetonitrile–methanol (2:1, v/v). An example of the total ion chromatogram (TIC) showing the UPLC separation of 23 compounds detected in PI mode is shown in Fig. 1. Total analysis in the PI mode (including the equilibration to the initial mobile phase conditions) was 14 min, which represented an approximate three-fold reduction in the analysis time compared to HPLC (45-min run) [18]. For 12 compounds detected in NI mode (several compounds gave good signals in both ion modes) the best separation was achieved using methanol–water in a 6-min gradient (TIC is shown in Fig. 2). With exception of several compounds that co-eluted, very good separation was achieved;

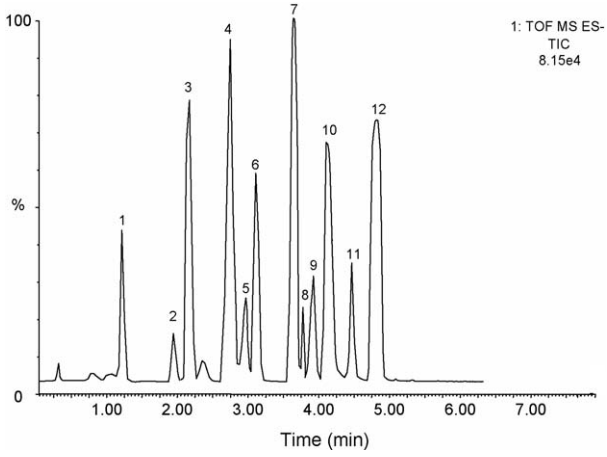


Fig. 2. UPLC–TOF total ion chromatogram showing the separation of 12 pharmaceutical compounds analyzed in NI mode (100 ng/mL standard solution). Peak assignments listed in Table 2.

peaks were narrow, which resulted in better chromatographic resolution and increased signal-to-noise ratio.

3.2. Q–TOF screening and confirmation

As a first step in the analysis of pharmaceutical residues in environmental and waste water samples UPLC–TOF chromatograms were recorded containing full scan spectral data. The *m/z* of the target analytes was extracted from the TIC and the accurate mass of the compound was obtained. In all cases (the only exception was pravastatine which formed the sodium adduct) the accurate mass of the protonated molecule $[M+H]^+$ and deprotonated molecule $[M-H]^-$, respectively for the PI and the NI mode were used for both confirmation and quantification purposes. Measuring environmental contaminants in complex matrices is a difficult task since the signal of the analyte can be altered by the presence of impurities. To increase the selectivity of TOF measurements a narrow accurate mass interval was used to reconstruct the chromatographic traces. XICs were typically extracted using a 20 mDa mass window. Reducing the mass window from 100 to 20 mDa resulted in an almost 15-fold increase of the signal-to-noise ratio and in almost complete loss of the interferences from the isobaric contaminant ions, as shown in Fig. 3 for carbamazepine in an urban wastewater.

The accurate mass data of the molecular ions were then processed through the software MassLynx, which provided the elemental formula and the mass errors (i.e. differences between measured masses and the theoretical values). Table 2 lists the exact mass measurements and mass errors obtained in the TOF mode for molecular ions. The errors obtained were between 0.7 and 4.4 ppm (root mean square (RMS) value 2.02) and 0.2–1.2 mDa (RMS = 0.72), which is within the limits of the widely accepted accuracy threshold of 5 ppm. However, it should be noted that in the case of concentrated compounds the peaks became distorted by depletion causing shifts in accurate mass. Therefore, the spectra were thoroughly examined for saturation and the mass measurements were taken off center to reduce intensity to maximum 500 counts. If necessary the samples were diluted and re-analyzed.

For further confirmation of the identity of the detected compounds a Q–TOF experiment under CID conditions was performed obtaining product ion spectra that are matched with the ones obtained for a standard solution. CID was performed for predefined base ions (protonated and deprotonated molecules, respectively in the PI and the NI mode) at different collision energies that were optimized for each individual compound in a separate experiment. Table 3 lists the accurate mass data for main fragments obtained at optimized conditions (cone voltage and collision energy). An interpretation of the fragmentation

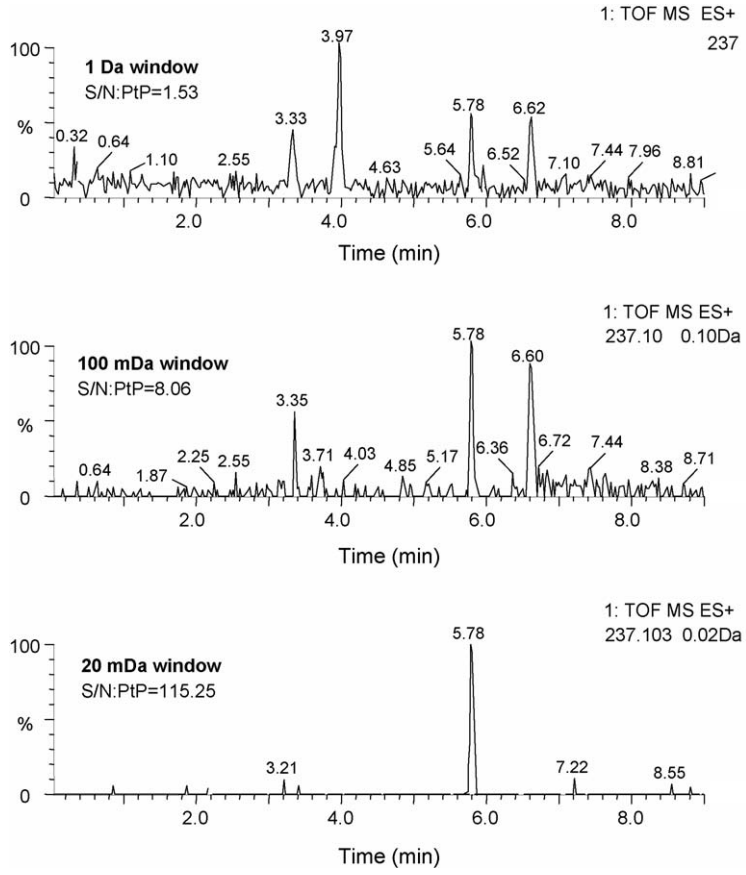


Fig. 3. Enhanced selectivity of UPLC–TOF analysis corresponding to reconstructed ion chromatogram of carbamazepine (*m/z* 237.103) in an urban wastewater sample with varying mass windows.

Table 2
Retention times and accurate mass measurements of molecular ions of target compounds in a standard solution

Peak no.	Compound	Retention time (min)	Elemental composition	Experimental mass (*m/z*)	Theoretical mass (*m/z*)	Error	
						mDa	ppm
PI mode							
1	Acetaminophen	1.43	$[M+H]^+$ $C_8H_{10}NO_2$	152.0717	152.0712	0.5	3.6
2	Sotalol	1.83	$[M+H]^+$ $C_{12}H_{21}N_2O_3S$	273.1285	273.1273	1.2	4.4
3	Famotidine	1.91	$[M+H]^+$ $C_8H_{16}N_7O_2S_3$	338.0531	338.0527	0.3	1.0
4	Atenolol	1.93	$[M+H]^+$ $C_{14}H_{23}N_2O_3$	267.1711	267.1708	0.2	0.9
5	Ranitidine	2.09	$[M+H]^+$ $C_{13}H_{23}N_4O_3S$	315.1500	315.1491	0.9	2.9
6	Trimethoprim	3.12	$[M+H]^+$ $C_{14}H_{19}N_4O_3$	291.1466	291.1457	0.9	3.0
7	Ofloxacine	3.36	$[M+H]^+$ $C_{18}H_{21}FN_3O_4$	362.1509	362.1516	−0.7	−2.0
8	Sulphametaxozole	3.91	$[M+H]^+$ $C_{10}H_{12}N_3O_3S$	254.0605	254.0599	0.6	2.2
9	Metoprolol	3.95	$[M+H]^+$ $C_{15}H_{26}NO_3$	268.1921	268.1912	0.8	3.1
10	Azithromycin	4.83	$[M+H]^+$ $C_{38}H_{73}N_2O_{12}$	749.5155	749.5163	−0.9	−1.1
11	Propranolol	5.35	$[M+H]^+$ $C_{16}H_{22}NO_2$	260.1659	260.1650	0.8	3.3
12	Pravastatin	5.70	$[M+Na]^+$ $C_{23}H_{35}NaO_7$	447.2349	447.2358	−1.0	−2.2
13	Carbamazepine	5.80	$[M+H]^+$ $C_{15}H_{13}N_2O$	237.1033	237.1028	0.5	2.2
14	Propyphenazone	5.84	$[M+H]^+$ $C_{14}H_{19}N_2O$	231.1502	231.1497	0.5	2.0
15	Erythromycin	5.88–6.00	$[M+H]^+$ $C_{37}H_{68}NO_{13}$	734.4701	734.4690	1.0	1.4
16	Bezafibrate	5.93	$[M+H]^+$ $C_{19}H_{21}ClNO_4$	362.1165	362.1159	0.6	1.6
17	Ketoprofen	6.32	$[M+H]^+$ $C_{16}H_{15}O_3$	255.1017	255.1021	−0.4	−1.6
18	Paroxetine	6.40	$[M+H]^+$ $C_{19}H_{21}FNO_3$	330.1499	330.1505	−0.6	−2.0
19	Lansoprazole	6.40	$[M+H]^+$ $C_{16}H_{15}F_3N_3O_2S$	370.0841	370.0837	0.4	1.1
20	Fluoxetine	7.00	$[M+H]^+$ $C_{17}H_{19}F_3NO$	310.1421	310.1418	0.2	0.7
21	Mefenamic acid	8.47	$[M+H]^+$ $C_{15}H_{16}NO_2$	242.1191	242.1180	1.0	4.1
22	Loratadine	9.19	$[M+H]^+$ $C_{22}H_{24}ClN_2O_2$	383.1519	383.1526	−0.7	−1.9
23	Mevastatin	9.33	$[M+H]^+$ $C_{23}H_{35}O_5$	391.2496	391.2484	1.2	2.9
NI mode							
1	Acetaminophen	1.22	$[M-H]^-$ $C_8H_8NO_2$	150.0559	150.0555	0.4	2.6
2	Famotidine	1.92	$[M-H]^-$ $C_8H_{14}N_7O_2S_3$	336.0365	336.0371	−0.6	−1.8
3	Clofibric acid	2.11	$[M-H]^-$ $C_{10}H_{10}ClO_3$	213.0321	213.0319	0.3	1.2
4	Naproxene	2.69	$[M-H]^-$ $C_{14}H_{13}O_3$	229.0873	229.0865	0.8	3.6
5	Ketoprofen	2.78	$[M-H]^-$ $C_{16}H_{13}O_3$	253.0858	253.0865	−0.7	−2.6
6	Bezafibrate	3.10	$[M-H]^-$ $C_{19}H_{19}ClNO_4$	360.0997	360.1003	−0.6	−1.6
7	Diclofenac	3.67	$[M-H]^-$ $C_{14}H_{10}Cl_2NO_2$	294.0093	294.0089	0.4	1.5
8	Ibuprofen	3.83	$[M-H]^-$ $C_{13}H_{17}O_2$	205.1234	205.1229	0.5	2.7
9	Indomethacin	3.89	$[M-H]^-$ $C_{19}H_{15}ClNO_4$	356.0700	356.0690	1.0	2.9
10	Mefenamic acid	4.12	$[M-H]^-$ $C_{15}H_{14}NO_2$	240.1032	240.1025	0.7	3.1
11	Lansoprazole	4.38	$[M-H]^-$ $C_{16}H_{13}F_3N_3O_2S$	368.0676	368.0681	−0.5	−1.2
12	Gemfibrozil	4.95	$[M-H]^-$ $C_{15}H_{21}O_3$	249.1500	249.1491	0.9	3.7

pattern and the accurate mass measurement of the obtained product ions complemented the elemental composition, thus alleviating existing ambiguities. An example of the analysis of selected pharmaceuticals in an urban wastewater is shown in Fig. 4. Traces shown on the left panel correspond to nwXICs of the $[M+H]^+$ extracted with a mass window of 20 mDa for carbamazepine (*m/z* 237.103), phenylphenazone (*m/z* 231.150), erythromycin (*m/z* 734.468), azithromycin (*m/z* 749.516), trimethoprim (*m/z* 291.146) and acetaminophen (*m/z* 152.071). In a separate experiment using the Q–TOF mode those ions were used as precursor ions to obtain accurate mass product spectra (shown on the right panel). The accurate mass measurements and elucidation of product ions were consistent with previous tentative identification based on QqQ measurements [3]. Mass errors for the fragment ions were slightly higher than those for ionized molecules, i.e. data obtained in the TOF mode. The mass errors of 49 product ions for which the elemental compositions were determined were in the range from 0.7 to 6.4 ppm (RMS = 3.53).

3.3. Quantitative analysis

Quantitation was carried out using nwXIC (20 mDa window) of the molecular ion for each compound obtained in the TOF mode. The RSD values obtained from run-to-run experiments (five successive injections of a standard solution) ranged from 0.5 to 5.3% and for day-to-day (five successive days) from 2.1 to 9.1%. The variability of the chromatographic retention times was lower than 2% in all cases.

For the majority of compounds analyzed the IDL ranged from 1 to 50 pg injected with the exception of several compounds that gave IDL higher than 100 pg (Table 4). Several compounds, such as trimethoprim, metoprolol, mefenamic acid and clofibric acid were exceptionally sensitive giving IDL of 1–5 pg. Such good sensitivity combined with the high quality structural information contained in the full scan spectral data, makes UPLC–Q–TOF a powerful tool to be used in studies on the fate and behavior of selected pharmaceuticals in the environment, as well as in the occurrence studies.

Table 3
Elemental composition and accurate mass measurement for product ions obtained in a standard solution using the Q-TOF mode

Compound	Precursor ion (*m/z*)	Cone voltage (V)	Collision energy (eV)	Product ions				
				Elemental composition	Experimental mass (*m/z*)	Theoretical mass (*m/z*)	ΔmDa	Δppm
PI mode								
Acetaminophen	152	30	15	C_6H_8NO	110.0600	110.0606	−0.6	−5.4
				C_6H_5O	93.0335	93.0340	−0.5	−5.8
Sotalol	273	30	10	$C_{12}H_{19}N_2O_2S$	255.1180	255.1167	1.3	5.0
				$C_9H_{19}NO_3S$	213.0470	213.0460	1.0	4.9
Famotidine	338	25	10	$C_8H_{15}N_6S_2$	259.0809	259.0800	0.9	3.6
				$C_9H_9N_4S_2$	189.0274	189.0269	0.5	2.8
Atenolol	267	30	18	$C_{11}H_{12}NO_2$	190.0874	190.0868	0.6	3.1
				$C_{10}H_9O$	145.0658	145.0653	0.5	3.2
Ranitidine	315	20	15	$C_{11}H_{16}N_3O_3S$	270.0919	270.0912	−1.3	−5.0
				$C_5H_{10}N_3O_2S$	176.0485	176.0494	−0.9	−5.0
Trimethoprim	291	30	22	$C_{12}H_{14}N_4O$	230.1165	230.1168	−0.3	−1.1
				$C_{13}H_{17}N_4O_2$	261.1348	261.1352	−0.4	−1.3
Ofloxacine	362	30	20	$C_{17}H_{21}FN_3O_2$	318.1610	318.1618	−0.8	−2.5
Sulphametaxozole	254	30	25	$C_6H_6NO_2S$	156.0124	156.0119	0.5	3.0
				C_6H_6NO	108.0452	108.0449	0.3	2.4
Metoprolol	268	30	20	$C_{12}H_{15}NO$	116.1070	116.1075	−0.5	−4.6
				$C_{12}H_{15}O_2$	191.1070	191.1072	−0.2	−1.1
Azithromycin	749	30	25	$C_{30}H_{59}N_2O_9$	591.4229	591.4221	0.8	1.4
				$C_{30}H_{57}N_2O_8$	573.4123	573.4115	0.8	1.4
Propranolol	260	30	20	$C_{13}H_{11}O$	183.0818	183.0810	0.8	4.4
				$C_{12}H_{15}NO$	116.1082	116.1075	0.7	5.7
Pravastatin	447	30	20	$C_{18}H_{23}NaO_4$	327.1578	327.1572	0.6	1.7
Carbamazepine	237	30	25	$C_{14}H_{12}N$	194.0976	194.0970	0.6	3.2
				$C_{14}H_{11}$	179.0868	179.0861	0.7	4.0
Propyphenazone	231	15	20	$C_{11}H_{13}N_2O$	189.1032	189.1028	0.4	2.2
				$C_{12}H_{13}N_2O$	201.1032	201.1028	0.4	2.0
Erythromycin	734	30	15	$C_{29}H_{54}NO_{10}$	576.3739	576.3748	−0.9	−1.5
				$C_{29}H_{52}NO_9$	558.3642	558.3642	−0.4	−0.7
Paroxetine	330	20	20	$C_{12}H_{15}FN$	192.1181	192.1189	−0.8	−3.9
Lansoprazole	370	20	20	$C_9H_9NO_2F_3S$	252.0302	252.0306	−0.4	−1.6
				$C_9H_7NOF_3S$	234.0196	234.0200	−0.4	−1.9
Fluoxetine	310	30	10	$C_{10}H_{14}N$	148.1130	148.1126	0.4	1.6
Loratidine	383	30	25	$C_{20}H_{18}ClN_2O$	337.1113	337.1108	0.5	1.6
Mevastatin	391	20	7	$C_{18}H_{23}O_2$	271.1692	271.1698	−0.6	−2.2
NI mode								
Acetaminophen	150	20	20	C_6H_5NO	107.0377	107.0371	0.5	5.0
Clofibric acid	213	15	20	C_6H_4ClO	126.9958	126.9951	0.7	5.8
				$C_4H_5O_2$	85.0296	85.0290	0.5	6.4
Naproxene	229	15	10	$C_{12}H_{10}O$	170.0737	170.0732	0.5	3.1
				$C_{13}H_{13}O$	185.0971	185.0966	0.5	2.5
Ketoprofen	253	15	10	$C_{15}H_{13}O$	209.0959	209.0966	−0.7	−3.5
Bezafibrate	360	30	10	$C_{15}H_{13}ClNO_2$	274.0627	274.0635	−0.8	−2.9
				C_7H_5ClNO	154.0053	154.0060	−0.7	−4.3
Diclofenac	294	15	15	$C_{13}H_{10}Cl_2N$	250.0198	250.0190	0.9	3.5
Ibuprofen	205	15	10	$C_{12}H_{17}$	161.1326	161.1330	−0.4	−2.6
Indomethacine	356	15	10	$C_{18}H_{15}ClNO_2$	312.0801	312.0791	1.0	3.1
				$C_{17}H_{12}ClNO_2$	297.0563	297.0557	0.6	2.2
Mefenamic acid	240	30	15	$C_{14}H_{14}N$	196.1134	196.1126	0.8	4.0
Gemfibrozil	249	15	10	C_8H_9O	121.0658	121.0653	0.5	3.8
				$C_7H_{11}O_2$	127.0764	127.0759	0.5	3.9

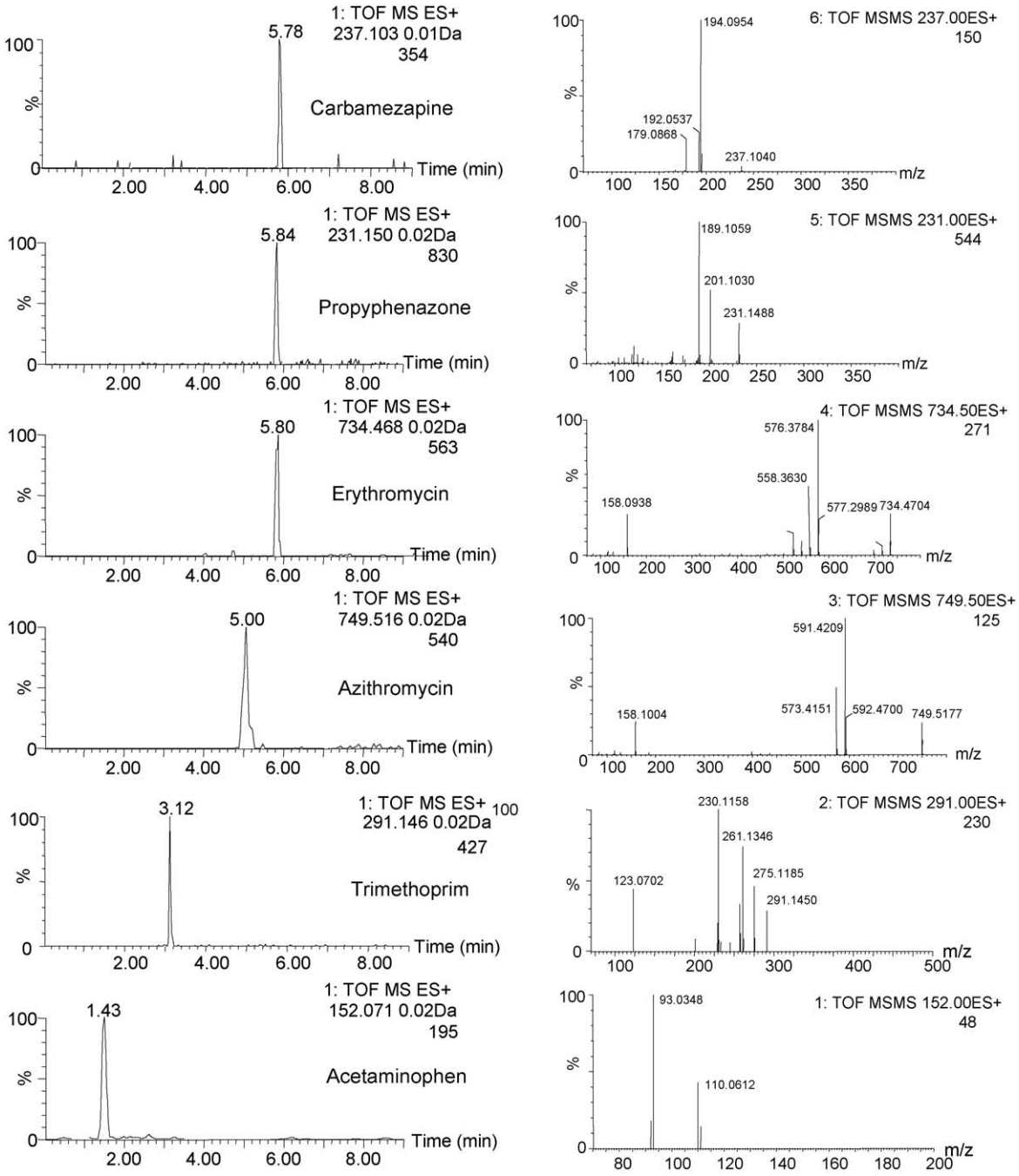


Fig. 4. Confirmation of several pharmaceuticals in an urban wastewater: (left panel) narrow window extracted ion chromatograms (nwXICs) of [M + H] obtained in the TOF mode for *m/z* 152.071 (acetaminophen), *m/z* 291.146 (trimethoprim), *m/z* 749.516 (azithromycin), *m/z* 734.468 (erythromcyn), *m/z* 231.150 (propyphenazone) and *m/z* 237.103 (carbamazepine); (right panel) product ion spectra obtained in the Q–TOF mode.

Analysis of WWTP samples yielded MDLs ranging from 10 to 500 ng/L (Table 4), which is approximately one order of magnitude higher than the MDLs obtained using a QqQ instrument in MRM mode [18]. However, having in mind that the method offers the advantage of accurate mass data and the fact that typical concentrations of pharmaceutical residues in WWTP influent and effluent samples are at high ng/L to low μg/L levels (generally low elimination is achieved in WWTP [20]) the UPLC–Q–TOF method is applicable to the quantitative analysis of WWTP samples, as shown below. For less contaminated waters (river, ground and drinking water) the MDLs might be not sufficient to detect low concentrations occurring in these samples, and the UPLC–Q–TOF method should be complemented with a sensitive quantitative analysis using a QqQ in MRM mode. The MDLs obtained by UPLC–Q–TOF are somewhat higher than those reported in the literature, mainly due to the differences in the target compounds (wide range of multi-class compounds versus selected compounds or one therapeutical group), sample matrix (waste water versus ground, surface and drinking water) and the instrumentation used. Using

Table 4
Instrumental detection limits (IDLs) and method detection limits (MDLs) for wastewater matrix obtained in the TOF mode (using 20 mDa XIC)

Compound	$IDL_{absolute}$ (pg injected) (ionization mode)	MDL (ng/L) WWTP influent[a]
Ketoprofen	40 (NI)	150
Naproxen	10 (NI)	55
Ibuprofen	50 (NI)	150
Indomethacine	30 (NI)	80
Diclofenac	10 (NI)	50
Mefenamic acid	5 (NI); 30 (PI)	20
Acetaminophen	10 (NI); 35 (PI)	50
Propyphenazone	10 (PI)	50
Clofibric acid	5 (NI)	25
Gemfibrozil	10 (NI	50
Bezafibrate	10 (NI); 50 (PI)	50
Pravastatin	100 (PI)	350
Mevastatin	30 (PI)	150
Carbamazepine	50 (PI)	100
Fluoxetine	200 (PI)	500
Paroxetine	15 (PI)	65
Lansoprazole	30 (PI); 50 (NI)	120
Loratadine	10 (PI)	50
Famotidine	50 (PI); 50 (NI)	200
Ranitidine	20 (PI)	75
Erythromycin	25 (PI)	100
Azithromycin	15 (PI)	70
Sulfamethoxazole	50 (PI)	150
Trimethoprim	1 (PI)	10
Ofloxacin	200 (PI)	500
Atenolol	10 (PI)	50
Sotalol	10 (PI)	50
Metoprolol	2 (PI)	15
Propranolol	20 (PI)	100

[a] Determined as signal-to-noise ratio of three for a spiked WWTP influent (preconcentration factor 100).

a Q–ToF Ultima API (Waters, Milford, MA, USA) Stolker et al. [5] claimed that the technique can be used for the screening and confirmation of selected analgesics, antibiotics, lipid regulators, β-blockers and anti-epileptics in surface, drinking and ground water at concentrations of 1–100 ng/L. Marchese et al. [6] used a QSTAR Pulsar Hybrid tandem-MS system (PE-Sciex, Concord, Canada) and reported quantification limits of 3 ng/L for the analysis of analgesics in drinking water.

In addition to very high price of Q–TOF instruments, the poor quantitative performance in terms of small dynamic range and in spite of improvements that have taken place over the last couple of years, is one of the reasons why their use in the field of environmental analysis is low [21]. A linear calibration range of our method was typically over two, and only for several compounds (trimethoprim, atenolol, propyphenazone, ranitidine and azithromycine) up to three orders of magnitude, which is generally at least one order of magnitude lower than the linearity of a QqQ instrument operating in MRM mode, but comparable with the previously reported for Q–TOF instruments [5,6].

3.4. Matrix effect in UPLC-Q–TOF

One of the limitations of LC–MS/MS is the susceptibility of atmospheric pressure ionization (API) interfaces to co-extracted matrix components, which typically results in the suppression or, less frequently, the enhancement of the analyte signal. In a previous study, that evaluated matrix effect in the HPLC–MS/MS (QqQ) of pharmaceutical residues in wastewaters, all studied compounds showed a large degree of matrix suppression [18]. The phenomenon was less pronounced in PI mode, with signal reduced by 15–50% in WWTP effluents and from 40 to 60% in WWTP influents. In NI mode, ion suppression ranged from 40 to 60% in WWTP effluent wastewaters, and over 60% for WWTP influent, reaching for some compounds values of 80% (ranitidine) and 90% (atenolol). In the HPLC–MS/MS (QqQ) analysis of β-blockers and lipid regulating agents Hernando et al. [22] reported on the signal reduction of up to 28% in tap and river water, up to 54% in WWTP effluents and up to 60% in WWTP influent samples as compared to the pure standard solution. Similar loss of signal intensity was reported in several other studies using LC–MS/MS [3,23].

In this work the evaluation of the extent of ion suppression/enhancement in UPLC–Q–TOF was conducted using spiked river water, WWTP effluent and WWTP influent extracts. The signal suppression was calculated for each individual compound as the percentage of signal intensity (XICs extracted using 20 mDa window) in a sample matrix versus the signal of the same concentration in the pure solvent (MeOH–H_2O, 25:75, v/v).

Fig. 5 shows the change in analyte signal in two samples for each matrix (WWTP influent, effluent and river water). In WWTP influent for the majority of the compounds the suppression of the signal was less than 30%, with some compounds showing suppression higher than 50% in one sample (atenolol, rantidine and mevastatin). Some compounds, such as pravastatin and famotidine, showed enhancement up to 35%. In WWTP effluent the signal reduction was generally lower than 20%, whereas in river water the reduction was limited to 10% for the majority of the compounds analyzed. These results are consistent with previously reported findings that MS detection proceeded by an efficient UPLC separation is less susceptible to matrix effects. Castro-Perez et al. [24] reported that UPLC may contribute to a reduction in ion suppression that result from co-elution of the metabolites and endogenous compounds and its application therefore helped to alleviate problems related with the analysis of complex matrices.

3.5. Analysis of real wastewater samples

To demonstrate the applicability of the proposed method samples from 5 WWTP (influent and effluent) and 10 river water samples from different locations in Spain and Croatia were analyzed. Analyte identification and confirmation was performed in compliance with the EU regulations (EU Commission Decision 2002/657/EC [25]), which requires at least three identification points (IPs) for the determination of pharmaceuticals in environmental samples. By determining accurate mass of 1 precursor and 1 product (MS/MS) a total of 4.5 IP are earned, which secured the identification of analytes present in real samples.

Based on accurate mass measurement, the following target compounds were identified and confirmed in WWTP samples:

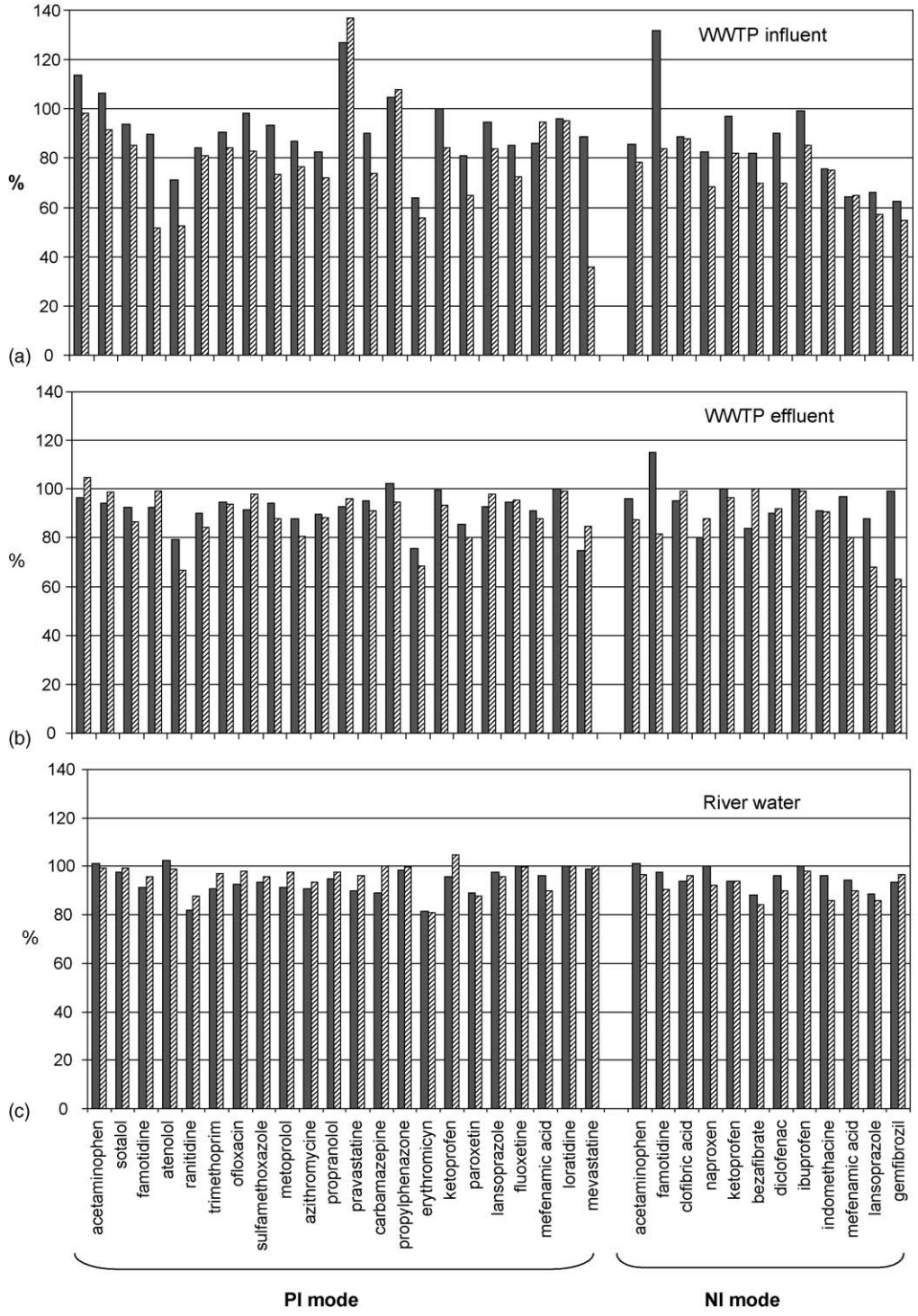


Fig. 5. The percentage of signal intensity (XICs extracted using 20 mDa window) in a sample matrix vs. the signal of the same concentration in the pure solvent (MeOH–H_2O, 25:75, v/v): (a) two samples of WWTP influent; (b) two samples of WWTP effluent; (c) two samples of river water. Compounds in the order of elution in PI and NI mode, respectively.

acetaminophen, atenolol, mevastatin, trimethoprim, and ibuprofen; all found in μg/L concentrations. Several compounds were detected in ng/L level (ketoprofen, diclofenac, sulfamethoxazole, propyphenazone, propranolol, azithromycin, erythromycin and carbamazepine), while other target compounds were not detected. The ranges of concentrations found in WWTP influent and effluent, respectively are listed in Table 5.

None of the target compounds were detected in river water samples, due to the rather high MDLs that were not sufficient to measure low concentrations found in those samples. LC–MS–MS (QqQ) analysis of the same samples resulted in the detection of a range of compounds at low ng/L levels. The most ubiquitous compounds belonged to the group of anti-inflammatories and analgesics, antibiotics, the lipid regula-

Table 5
Range of concentrations of target of pharmaceuticals found in influents and effluents of five urban WWTP (median value is given in parenthesis)

Compound	WWTP influent (μg/L)	WWTP effluent (μg/L)
Acetaminophen	<0.05–19.5 (5.4)	<0.05–6.2 (2.1)
Ketoprofen	0.15–0.96 (0.23)	0.20–0.75 (0.20)
Ibuprofen	<0.15–1.20 (0.54)	<0.15–1.05 (0.27)
Diclofenac	<0.05–0.50 (0.25)	<0.05–0.50 (0.32)
Propyphenazone	<0.05–0.85 (0.50)	<0.05–0.45 (0.20)
Mevastatin	<0.15–1.25 (0.55)	<0.15–0.85 (0.35)
Atenolol	<0.05–1.00 (0.23)	<0.05–1.20 (0.28)
Propranolol	<0.10–0.38 (0.12)	<0.10–0.52 (0.14)
Trimethoprim	0.040–0.65 (0.38)	<0.005–0.23 (0.11)
Sulfamethoxazole	<0.15–0.96 (0.45)	<0.15–0.80 (0.40)
Azithromycin	<0.07–0.45 (0.16)	<0.07–0.30 (0.14)
Erythromycin	<0.10–0.25 (0.13)	<0.10–0.28 (0.15)
Carbamazepine	<0.10–0.95 (0.40)	<0.10–0.60 (0.36)

tors being acetaminophen, trimethoprim, ibuprofen, ketoprofen, atenolol, propranolol, mevastatin, carbamazepine and ranitidine most frequently detected compounds [18].

4. Conclusions

The application of UPLC, with 1.7 μm particles, produced a significant increase in peak capacity for a 10-min separation of 29 pharmaceuticals. The typical peak widths generated by the UPLC system were in the order of 5–10 s, which resulted in better chromatographic resolution and increased signal-to-noise ratio. Maintaining the chromatographic resolution, the speed provided by the UPLC system allowed the reduction of the run time by a factor of three in comparison to conventional HPLC using 5 μm particles (14 min versus 45 min).

High data capture rate Q–TOF mass spectrometer offered the advantage of unequivocal identification of target pharmaceutical compounds based on accurate mass measurement of the precursor ions and their products. Accurate mass measurements of at least one product ion (two if available) provided qualitative information which was used to secure identification of analytes present in real samples, Mass errors for the molecular ions, obtained in the TOF mode, were generally lower than those obtained for fragments, i.e. data obtained in the Q–TOF mode. The mass errors of 49 product ions for which the elemental compositions were determined were in the range from 0.7 to 6.4 ppm.

The method showed some disadvantages in terms of sensitivity (as the MDL were generally one order of magnitude lower than those of a QqQ instrument working in MRM mode). Nevertheless, this work illustrated the quantitative possibilities of Q–TOF mass spectrometry in analysis of pharmaceutical residues in complex matrices.

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