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Rapid automated screening, identification and quantification of organic micro-contaminants and their main transformation products in wastewater and river waters using liquid chromatography–quadrupole-time-of-flight mass spectrometry with an accurate-mass database

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ABSTRACT

In this study we have developed and evaluated an analytical method for a rapid automated screening and confirmation of a large number of organic micro-contaminants (almost 400) and also the quantification of the positive findings in water samples of different types (surface and wastewaters) using liquid chromatography–electrospray quadrupole-time-of-flight mass spectrometry (LC–QTOFMS) based on the use of an accurate-mass database. The created database includes data not only on the accurate masses of the target ions but also on the characteristic in-source fragment ions, isotopic pattern and retention time data. This customized database was linked to commercially available software which extracted all the potential compounds of interest from the LC–QTOFMS raw data of each sample and matched them against the database to search for targeted compounds in the sample. The detailed fragmentation information has also been used as a powerful tool for the automatic identification of unknown compounds and/or transformation products with similar structures to those of known organic contaminants included in the database. The database can be continually enlarged. To confirm identification of compounds which have no fragment ions (or fragments with low intensity/relative abundance) from in-source CID fragmentation or isomers which are not distinguished within full single mass spectra, a "Targeted MS/MS" method is developed. Thereafter, these compounds can be further analyzed using the collision energy (CE) in QTOF-MS/MS mode. Linearity and limits of detection were studied. Method detection limits (MDLs) in effluent wastewater and river waters were, in most cases, lowers or equal to 5 and 2 ng/L, respectively. Only 15 compounds had MDLs between 5 and 50 ng/L in effluent wastewater matrix. We obtained a linearity of the calibration curves over two orders of magnitude. The method has been applied to real samples and the results obtained reveal that most of the pharmaceutically active compounds contained in the created database were present in the water samples with concentrations in the range of ng/L and μg/L levels and in most of the samples between 2 and 15 pesticides of the 300 contained in the database were also detected. In addition to the compounds included in the database, some degradation products were found, thus revealing the method as a useful tool for the analysis of organic micro-contaminants in waters.

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1. Introduction

Contamination of water resources by micro-contaminant residues is one of the major challenges for the preservation and sustainability of the environment. Although anti-pollution measures taken over the past half-century [1] have dramatically reduced the presence of many known contaminants in water, the number of potentially hazardous chemicals that can reach the environment is very large and new substances are constantly being developed and released. The focus for water pollution research has recently been extended from "priority" contaminants to the so-called "emerging contaminants" or "new environmental contaminants", many of which have been unknown until recently.

An important group of emerging contaminants are the pharmaceutically active substances. The relatively recent awareness of the impact of pharmaceutical products on the environment has been reflected in literature since the 1990s through the exponentially increasing number of studies concerning the emerging class of water pollutants [2–11]. This rising interest is not only concomitant with the widespread and growing use of these compounds consumption, but also with the improvements in analytical

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techniques allowing detecting traces (ng/L or less) in any type of water.

In addition to the analysis of emerging contaminants, analysis of the regulated contaminants must not be neglected. These have to be continuously monitored, as is the case with pesticides.

Because of the potentially adverse effects of the presence of organic micro-contaminants in the environment, data concerning the concentration, fate and behaviour of these contaminants in the environment is urgently needed. With this purpose in mind, analytical methods for a rapid, sensitive and selective determination of a broad range of compounds in complex environmental matrices are required. Multi-residue analytical methodologies are becoming the required tools, as they provide greater knowledge about the contamination of the waters [2,3] and they reduce the overall analysis time, field sampling and cost. Papers related to multi-residue analytical methodologies have increased over recent years, although most of them are focused on target analysis method. The scope of such methods rarely exceeds several tens of analytes, and it is quite unusual to find analytical methods applied to more than 100 organic micro-contaminants. This means that a large number of compounds and their degradation products fall outside of any control. The analysis of the transformation/degradation products represents an important challenge for environmental analysts. There is great concern over these products, which can be even more toxic, more dangerous and be at higher concentrations than the initial compounds themselves [4,5]. Regarding these, limited information is currently available.

Up to date, polar micro-contaminant residue analysis in waters has been accomplished by liquid chromatography–tandem mass spectrometry (LC–MS/MS) in the selected reaction monitoring (SRM) mode [2,6–8]. This approach has a severe limitation – the number of compounds that can be screened in a single run [9,10]. Up to 150–200 compounds (depending on the scan speed/dwell-time) can be analyzed in a run by LC–MS/MS in the SRM mode with a dedicated chromatographic method. In addition, when increasing the number of compounds included in the SRM method, the possibility of finding common or overlapped transitions for coeluting isobaric compounds rises. Besides, another major limitation of these SRM methods is that they are blind to compounds not defined in the SRM method (non-target analysis) so that no or scarce information on possible non-target/unknown organic micro-contaminants or their degradation products is available when using these techniques. The information provided by methods using the SRM mode is often insufficient in assessing the quality of wastewater and environmental waters, given that only a limited number of analytes are recorded. There is, therefore, a need for methods offering rapid and reliable screening of a large number of compounds.

Unlike gas chromatography/mass spectrometry (GC/MS) reverse-search methods, where library searching is possible (e.g. the large library of the National Institute for Standards and Testing (NIST)), one of the major shortcomings traditionally reported in the use of LC–MS is the unavailability of commercial libraries allowing a rapid screening of the samples as can be performed in GC/MS [11]. The universal applicability of mass spectral libraries has been hampered by the scarce reproducibility of in-source collision-induced dissociation (CID) spectra and the difficulty of interchanging spectra acquired with instruments from different manufacturers [9,10]. In contrast, accurate mass measurements are almost specific and universal for every target analyte regardless of the instrumentation used. In this sense, liquid chromatography–electrospray-time-of-flight mass spectrometry (LC–TOFMS) is a cost-effective technique for performing routine accurate mass analysis based on target databases [13]. The main features of LC–TOFMS instruments are accurate mass analysis capabilities and high sensitivity in "full-scan" acquisition mode so that micro-contaminants can be detected in complex matrices at low nanogram levels. Unambiguous identification is accomplished by means of accurate mass measurements from (de)protonated molecules, in-source CID fragment ions, and isotope signature matching [13–15]. In addition, LC–TOFMS provides satisfactory analytical performance for quantitation purposes, as has been demonstrated so far in the literature [15,16].

Since LC–TOFMS has the ability to record an unlimited number of compounds because it operates in full-scan mode, this technique is very convenient for the development of screening strategies based on the use of accurate-mass databases [13,17]. When coupled to a quadrupole or ion trap mass filter, QTOFMS or IT-TOF-MS permit MS/MS or MS^n analysis with accurate mass measurements for both the precursor and product ion, which constitutes a higher order mass identification than those afforded by nominal mass measurements obtained by other types of mass analyzers.

This work reports the development and evaluation of a method for a rapid automated screening, identification and quantification of organic micro-contaminants in waters using LC–QTOFMS, based on the use of an accurate-mass database. The database created includes accurate masses of the target ions, their characteristic in-source fragment ions, isotopic signature information, and retention time data. This database was linked to software which extracts all the compounds of interest from the LC–QTOFMS raw data of each sample and matches them against the database to search for targeted compounds in the sample. The number of compounds that can be screened in a run can easily be upgraded (non-target capabilities), thus enabling the reevaluation of the recorder data. The detailed fragmentation information has also been used as a powerful tool for the automatic identification of unknown compounds and/or transformation products with similar structure to known organic contaminants included in the database.

While the LC–QTOF instrument used as a TOF-MS system provides screening and quantification of both unknown and targeted organic contaminants, LC–QTOF, working in MS/MS mode, was required to confirm identification of compounds which have no fragment ions (or fragments with low intensity/relative abundance) from in-source CID fragmentation and also isomers which were not distinguished with full single mass spectra – this was done thanks to the valuable information given by the full product ion spectra at accurate masses.

2. Experimental

2.1. Chemicals and reagents

The 300 pesticides included in this study were purchased from Dr. Ehrenstorfer GmbH (Ausburg, Germany) or Riedel-de-Haën (Seelze, Germany) at analytical grade (purity >97%). The group of pharmaceutically active compounds and some of their more relevant metabolites comprise 87 organic pollutants belonging to different therapeutical groups, all of them were purchased from Sigma–Aldrich (Steinheim, Germany), Merck (Mollet del Vallés, Spain) and LGC Promechem (Barcelona, Spain) at analytical grade (purity >95%). Individual stock standard solutions of the target compounds were prepared in methanol, at a concentration between 1 and 2 mg/mL, and stored at −20 °C. Working standard solutions, at different concentrations, were prepared by appropriate dilution of the stock solutions in MeOH:water, 10:90 (v/v). HPLC-grade acetonitrile and methanol were supplied by Merck (Darmstadt, Germany). Formic acid (purity, 98%) was obtained from Fluka (Buchs, Switzerland). Water used for LC–MS analysis was generated in a Direct-Q™ 5 Ultrapure Water System from Millipore (Bedford, MA, USA) with a specific resistance of 18.2 MΩ cm. The reagents used in the preparation of the simulated effluent wastewater (pep-

tone, meat extract, urea, K_2HPO_4, $CaCl_2{\cdot}2H_2O$, $MgSO_4{\cdot}7H_2O$ and NaCl) were provided by Panreac. SPE commercial cartridges packed with Oasis™ HLB (divinylbenzene/N-vinylpyrrolidone copolymer, 200 mg, 6 cm^3) were purchased from Waters (Mildford, MA, USA).

2.2. Sampling and sample preparation

Effluent samples (200 mL) from four different sewage treatment plants (STPs) were collected. They are representative of different activities (urban, agricultural, and industrial). All plants apply a pretreatment for solid removal, a primary treatment to eliminate suspended material, an activated sludge biological treatment, and final clarification. Integrated samples representative of 24 h of work treatment in the STP, were taken at hourly intervals. Sampling was carried out by an automatic device (0.5 L/3 h). Effluent samples were collected using pre-rinsed amber glass bottles and sent to the laboratory in Almería for analysis.

River samples (400 mL) were collected from three different zones (north, centre and south) all located in the centre of Spain (Madrid). This area is the most developed and densely populated part of Spain. Its total area is about 8050 km^2 and it has a population of about 6 million people. The streams run through several residential and commercial areas therefore urban and industrial wastewaters represent a significant input into the rivers, but agricultural areas were also taken into account. Four rivers were the subject of research in this study. Grab water samples (1 L) were collected in clean amber glass bottles. Before sample collection, each bottle was pre-rinsed with sample three times. Samples were sent in boxes packed with ice to the laboratory in Almería for analysis.

All samples were filtered through a 0.7 μm glass fiber filter (Teknokroma, Barcelona, Spain) and extracted within 48 h in all the cases. A solid-phase extraction (SPE) procedure was applied to the wastewater samples using commercial Oasis HLB (divinylbenzene/N-vinylpyrrolidone copolymer) cartridges (200 mg, 6 cm^3) from Waters (Mildford, MA, USA). An automated sample processor ASPEC XL fitted with an 817 switching valve and an external 306 LC pump from Gilson (Villiers-le-Bel, France) was used for this purpose. The Oasis HLB cartridges were preconditioned with 6 mL of MeOH and 5 mL of deionized water HPLC-grade (pH adjusted to 8 with 20% NH_4OH) at a flow rate of 1 mL/min. After the conditioning step, 200 mL aliquots of effluent wastewater and 400 mL of river water (pH adjusted to 8, when necessary) were loaded into the cartridge. Samples were previously spiked with 10 μL of 10 mg/L solution of the surrogate standards ^{13}C-caffeine and ibuprofen-d^3. Samples were passed through the cartridges at a flow rate of 10 mL/min and then rinsed with 5 mL of deionized water prior to the elution. After that, the cartridges were dried by nitrogen stream for approximately 5 min to remove excess water and finally the analytes retained were eluted with 2× 4 mL of MeOH at 1 mL/min. The extracts were evaporated until almost dryness using a Turbo-Vap from Zymark (Hopkinton, Massachusetts), with the water temperature at 35 °C. The samples were then reconstituted with 1 mL of MeOH:water, 10:90 (v/v) and were then filtered directly into an analysis vial using a 0.45 μm PTEF syringe filter (Millipore, USA). Before effluent wastewaters analysis, a 1:1 dilution with MeOH/water (10:90) was applied.

2.3. Liquid chromatography–quadrupole-time-of-flight-mass spectrometry

The method for the analysis of water samples was developed by a liquid chromatography–electrospray ionization-quadrupole-time-of-flight-tandem mass spectrometry (LC–ESI-QTOF-MS/MS) system, in positive and negative ionization modes. The analytes were separated using a HPLC system (consisting of vacuum degasser, autosampler, and binary pump) (Agilent 1200 Series, Agilent Technologies) equipped with a reversed-phase XDB-C_{18} analytical column of 4.6 mm × 50 mm and 1.8 μm particle size (Agilent Technologies, Santa Clara, CA). Gradient LC elution was performed with 0.1% formic acid and 5% MilliQ water in acetonitrile as mobile phase A and 0.1% formic acid in water (pH 3.5) as mobile phase B. For the analysis in positive mode, the optimized chromatographic method held the initial mobile phase composition (10% A) constant for 1 min, followed by a linear gradient to 100% A up to 12 min, and kept for 5 min at 100% A. A 7-min post-run time back to the initial mobile phase composition was used after each analysis. The flow rate used was 0.6 mL/min. Compounds analyzed in negative mode were separated using acetonitrile as mobile phase A and HPLC-grade water as mobile phase B at a flow rate of 0.6 mL/min. The LC gradient was the same used in positive mode. The volume of injection was 20 μL in both modes.

The HPLC system was connected to a quadrupole-time-of-flight mass spectrometer (Agilent 6530 Series Accurate Mass QTOFMS, Agilent Technologies, Santa Clara, CA). The instrument was operated in the 4 GHz high-resolution mode. Ions are generated using an electrospray ion source with Agilent Jet Stream Technology. Parameters for the Agilent Jet Stream Technology are the superheated nitrogen sheath gas temperature (400 °C) and flow rate (12 L/min). Electrospray conditions were the following: capillary, 4000 V; nebulizer, 40 psi; drying gas, 10 L/min; gas temperature, 325 °C; skimmer voltage, 65 V; octapoleRFPeak, 750 V; fragmentor (in-source CID fragmentation), 90 V. The mass axis was calibrated using the mixture provided by the manufacturer over the m/z 70–3200 range. A sprayer with a reference solution was used as continuous calibration in positive ion using the following reference masses: 121.0509 and 922.0098 m/z (resolution: 19500 ± 500 at 922.0098 m/z). With the electrospray source in negative (ESI−), reference masses were 112.985587 and 966.000725 m/z (resolution: 23,900 ± 500 at 966.000725 m/z). For this work, the QTOF-MS instrument was used as a TOF-MS system working in the MS mode and also in the MS/MS mode for identification confirmation of isomer compounds or compounds with only one transition. The full-scan and MS/MS data recorder was processed with Applied Biosystems/MDS Sciex Analyst QS software (Frankfurt, Germany) with accurate mass application-specific additions from Agilent MSD TOF software and with Agilent MassHunter Workstation Software (version B.02.00).

2.4. Method validation

Because of the impossibility in obtaining blanks, the validation procedure of the method was carried out using simulated effluent wastewater. The simulated effluent wastewater consists of peptone (32 mg/L^{-1}), meat extract (22 mg/L^{-1}), urea (6 mg/L^{-1}), K_2HPO_4 (28 mg/L^{-1}), $CaCl_2{\cdot}2H_2O$ (4 mg/L^{-1}), $MgSO_4{\cdot}7H_2O$ (2 mg/L^{-1}) and NaCl (7 mg/L^{-1}) which derives to an initial DOC (dissolved organic carbon) of 25 mg/L^{-1} [18]. To minimize matrix effects, matrix-matched calibration curves were used for quantitative determinations.

The recovery studies were determined as the average of three analyses of a fortified simulated effluent wastewater extract (the most complex matrix) at a concentration level of 0.5 μg/L, before and after extraction.

The linearity in the response was studied using matrix-matched calibration solutions spiked with the analytes at six different concentration levels ranging from the limit of determination to 2.5 μg/L. Experimental data fitted a linear mode, $y = a + bx$, in the concentration range studied.

Precision of the analytical method, determined as relative standard deviation (RSD), was obtained from repeated injection ($n = 5$)

of a spiked extract during the same day (repeatability) and on different days (reproducibility).

The method detection limits (MDLs) were determined applying the screening method to matrix-matched solutions at low concentration levels: 5, 50 and 100 ng/L.

3. Results and discussion

3.1. Off line-SPE LC–QTOF-MS analysis

A LC–QTOF analytical method was developed which allowed the reliable screening and identification of a large number of compounds (almost 400) and also the quantification of 87 pharmaceuticals (the SPE method was only tested for the pharmaceuticals) in water samples of different types, making it possible to find non-target compounds in the sample while, at the same time, to enlarge the database continually.

The application of the instrument parameters described in Section 2 allowed the separation of the analytes in a total analysis time of 17 min in positive and negative modes.

Data on active pharmaceutical compounds included in the database are shown in Table 1. The investigated compounds are listed in alphabetical order together with detailed information of retention times (RT), theoretical masses, elemental compositions of the protonated molecule and their characteristic fragments or characteristic isotope profile in QTOF-MS mode. A Targeted MS/MS method was developed for confirming the identification of isomers not distinguished with full single mass spectra, compounds which have no fragments and/or characteristic isotope profile (no atoms of chlorine, bromine, or sulfur in their structures). The collision energy (CE) optimized for each compound is also include in Table 1.

The sample treatment protocol used is a very important issue. This step is somehow a bottleneck for the development of a universal screening procedure. Conflictive compounds not recovered in a generic sample treatment step, because need special analysis conditions (i.e. amoxicillin, tamoxifen, cefotaxime or salicylic acid) might not be sought or have a low recovery with the used screening method. The analytical methodology selected as sample treatment for the screening procedure was a conventional sample extraction with Oasis HLB SPE cartridges, which have been extensively used in the bibliography for the determination of a wide range of analytes in waters offering satisfactory results [2,3,7,11].

Optimization of the extraction procedure was made with the aim of reaching good recoveries from the widest group of compounds in a single extraction step. The use of the optimum SPE cartridge can have significant effects on recoveries, for multi-residue methodologies that determine simultaneously different groups of contaminants of widely varying structures, the universality of the sample preparation step is necessary and non-selective hydrophobic sorbents are used for this purpose. High rates of recovery for most of the compounds were obtained using Oasis HLB SPE cartridges with hydrophilic and lipophilic balance characteristics, which provide the excellent wetting properties of the hydrophilic N-vinylpyrrolidine monomer, and the best conditions for the simultaneous extracting of acidic analytes from water without acidification of the sample, together with neutral analytes over a wide polarity range. This is of great importance when performing a multi-residue analysis, because the risk of acidic hydrolysis of other compounds is not enhanced. Furthermore, no clean-up step is needed for the removal of humic and fluvic acid.

The effect of pH on the extraction efficiency is a very important parameter. Because of the different polarity of the compounds, experiments were performed adjusting the pH of the samples at three different values: 5, 7 and 8. The results showed that, as a general rule, neutral and basic pH yielded better results for the majority of analytes, with slight differences in favour of pH 8, which was finally selected. Recoveries (the mean of three replicates ± relative standard deviation) at the selected pH for the target compounds are presented in Table 2. As can be observed, recoveries were higher than 70% in the majority of cases (68 compounds). Lower recoveries (<50%) were obtained for eight compounds, although in all cases relative standard deviations observed were lower than 20%. Only 2 analytes were not recovered: amoxicillin and tamoxifen. These values can be considered acceptable taking into consideration the wide range of polarities involved and the good reproducibility obtained.

3.2. The accurate-mass database developed for pharmaceuticals and pesticides

The 300 pesticide database created by our group in a previous work [13] was revised and enlarged by almost a hundred pharmaceutical compounds. The possibility to continually update and extend the accurate-mass database to include new compounds is especially interesting.

The selected 87 pharmaceuticals were injected individually in the LC–QTOF, working in the MS mode, at a concentration of 100 ng/mL. For the automatic screening method, the retention time, the theoretical exact mass, and the elemental composition of each compound were collected on an Excel sheet. In addition, the mass spectrum of each compound was carefully investigated, and the characteristic fragment ions (with relative abundance higher than 10%, using the default 190 V fragmentor voltage) of each compound were also included in the database (Table 1). This file was put into csv format for use by the Agilent QTOF automated data analysis software. The csv file is searched automatically by the LC–QTOF-MS instrument at the end of the sample run and a report is generated on compounds that were found in the database.

3.2.1. Automatic screening method using the pharmaceutical and pesticide database

As previously stated, samples were first analyzed in full-scan mode, using the QTOF instrument as a TOF-MS system. The analytes from the chromatographic column are ionized and passed through the first quadrupole and into the TOF without CID. The automated screening method consists of two steps:

(1) Extraction of the compounds using the “find compounds by molecular feature extraction (MFE)” algorithm software (Qualitative Mass Hunter).
(2) Database searching.

The first step is a search for compounds by molecular feature. The MFE software examines the whole chromatogram at once in order to search and group all ions that can be logically associated with a real chromatographic peak and that may represent a “feature” of a molecule. The resulting data file is cleaned of extraneous background noise and unrelated ions and the MFE then creates a compound list of all peaks in the data file that it has determined represents real molecules. This algorithm is fast and generates good results with appropriate settings.

In a second step, the csv Excel file created is employed as a database. The resulting list of molecular features or potential compounds of interest extracted from the raw data in the first step are matched with the data of the target compounds included in the database in order to identify the compounds in the data file.

Both steps should be carefully examined, and parameters affecting the performance must be tuned according to the application, which involves the detection of compounds at concentration levels which can differ up to 3 orders of magnitude. Due to the complexity of the samples some filters should be applied to reduce the total number of compound extracted in the first step. We

Table 1
Accurate-mass database including retention times (RT), fragmentation and elemental compositions of the studied pharmaceuticals and its fragments. Isobaric species have been highlighted in bold and each pair marked with the same number.

Compound name	RT (min)	*m/z* calculated	Ion	Elemental composition	QTOF-MS fragment ions[a]	
					m/z calculated	Elemental composition
4-AAA	5.60	246.1237	$[M+H]^+$	$C_{13}H_{16}N_3O_2$	204.1132	$C_{11}H_{14}N_3O$
4-DAA (3)	4.75	232.1445	$[M+H]^+$	$C_{13}H_{18}N_3O$	113.1073	$C_6H_{13}N_3$
4-FAA (3)	5.69	232.1081	$[M+H]^+$	$C_{12}H_{14}N_3O_2$	204.1132	$C_{11}H_{14}N_3O$
Acetaminophen	4.49	152.0706	$[M+H]^+$	$C_8H_{10}NO_2$	110.0601	C_6H_8NO
Amidotrizoate	1.50	614.7769	$[M+H]^+$	$C_{11}H_{10}I_3N_2O_4$	360.968	$C_{11}H_{10}IN_2O_4$
Amoxicillin	2.31	366.1118	$[M+H]^+$	$C_{16}H_{20}N_3O_5S$	349.0853	$C_{16}H_{17}N_2O_5S$
Atenolol	3.03	267.1703	$[M+H]^+$	$C_{14}H_{23}N_2O_3$	225.1234	$C_{11}H_{17}N_2O_3$
Azithromycin	6.14	749.5158	$[M+H]^+$	$C_{38}H_{73}N_2O_{12}$	591.4215	$C_{30}H_{59}N_2O_9$
Caffeine	4.73	195.0877	$[M+H]^+$	$C_8H_{11}N_4O_2$	138.0662	$C_6H_8N_3O$
Carbamazepine 10,11-epoxide	7.48	253.0972	$[M+H]^+$	$C_{15}H_{13}N_2O_2$	180.0808	$C_{13}H_{10}N$
					210.0914	$C_{14}H_{12}NO$
					236.0706	$C_{15}H_{10}NO_2$
Carbamazepine	7.88	237.1023	$[M+H]^+$	$C_{15}H_{13}N_2O$	194.0964	$C_{14}H_{12}N$
Cefotaxime	5.98	456.0642	$[M+H]^+$	$C_{16}H_{18}N_5O_7S_2$	396.0431	$C_{14}H_{14}N_5O_5S_2$
Ciprofloxacin	5.79	332.1405	$[M+H]^+$	$C_{17}H_{19}FN_3O_3$	288.1507	$C_{16}H_{19}FN_3O$
Clomipramine (8)	8.37	315.1623	$[M+H]^+$	$C_{19}H_{24}ClN_2$	317.1593	$C_{19}H_{24}[^{37}Cl]N_2$
Clotrimazole	7.99	277.0779	$[M+H]^+$	$C_{19}H_{14}Cl$	279.0749	$C_{19}H_{14}[^{37}Cl]$
Diazepan	9.52	285.0789	$[M+H]^+$	$C_{16}H_{14}ClN_2O$	287.0759	$C_{16}H_{14}[^{37}Cl]N_2O$
Famotidine	4.63	338.0522	$[M+H]^+$	$C_8H_{16}N_7O_2S_3$	189.0263	$C_5H9N_4S_2$
					259.0794	$C_8H_{15}N_6S_2$
Fenofibrate	13.21	361.1201	$[M+H]^+$	$C_{20}H_{22}ClO_4$	233.0364	$C_{13}H_{10}ClO_2$
Fenofibric acid	10.30	319.0732	$[M+H]^+$	$C_{17}H_{16}ClO_4$	233.0364	$C_{13}H_{10}ClO_2$
Indomethacin	10.36	358.0841	$[M+H]^+$	$C_{19}H_{17}ClNO_4$	360.0811	$C_{19}H_{17}[^{37}Cl]NO_4$
Ketoprofen	9.60	255.1016	$[M+H]^+$	$C_{16}H_{15}O_3$	209.0961	$C_{15}H_{13}O$
Lansoprazole	7.15	370.0832	$[M+H]^+$	$C_{16}H_{15}F_3N_3O_2S$	252.0301	$C_9H_9F_3NO_2S$
Loratadine	8.21	383.1521	$[M+H]^+$	$C_{22}H_{24}ClN_2O_2$	385.1491	$C_{22}H_{24}[^{37}Cl]N_2O_3$
Mefenamic Ac.	11.52	242.1176	$[M+H]^+$	$C_{15}H_{16}NO_2$	224.1070	$C_{15}H_{14}NO$
Methylprednisolone	8.02	375.21541	$[M+H]^+$	$C_{22}H_{31}O_5$	339.19429	$C_{22}H_{27}O_3$
					357.20588	$C_{22}H_{29}O_4$
Metronidazole	3.45	172.0717	$[M+H]^+$	$C_6H_{10}N_3O_3$	128.0455	$C_4H_6N_3O_2$
Mevastatin	11.43	391.2479	$[M+H]^+$	$C_{23}H_{35}O_5$	271.1693	$C_{18}H_{23}O_2$
					289.1798	$C_{18}H_{25}O_3$
Nadolol (7)	5.35	310.2013	$[M+H]^+$	$C_{17}H_{28}NO_4$	254.1387	$C_{13}H_{20}NO_4$
Naproxen (2)	9.20	231.1016	$[M+H]^+$	$C_{14}H_{15}O_3$	185.0961	$C_{13}H_{13}O$
Nicotine	1.19	163.1230	$[M+H]^+$	$C_{10}H_{15}N_2$	132.0808	$C_9H_{10}N$
Norfloxacin	5.59	320.1405	$[M+H]^+$	$C_{16}H_{19}FN_3O_3$	276.1507	$C_{15}H_{19}FN_3O$
					302.1300	$C_{16}H_{17}FN_3O_2$
Ofloxacin	5.22	362.1511	$[M+H]^+$	$C_{18}H_{21}FN_3O_4$	318.1613	$C_{17}H_{21}FN_3O_2$
Omeprazole	6.13	346.1220	$[M+H]^+$	$C_{17}H_{20}N_3O_3S$	136.0757	$C_8H_{10}NO$
					198.0583	$C_9H_{12}NO_2S$
Paraxanthine (1)	3.55	181.0720	$[M+H]^+$	$C_7H_8N_5O_2$	124.0506	$C_5H_5N_4O$
Pentoxifylline (6)	6.08	279.1452	$[M+H]^+$	$C_{13}H_{19}N_4O_3$	181.0720	$C_7H_9N_4O_2$
Primidone	6.57	219.1128	$[M+H]^+$	$C_{12}H_{15}N_2O_2$	162.0914	$C_{10}H_{12}NO$
Ranitidine (8)	2.18	315.1486	$[M+H]^+$	$C_{13}H_{23}N_4O_3S$	176.0488	$C_5H_{10}N_3O_2S$
					270.0907	$C_{11}H_{16}N_3O_3S$
Salbutamol	3.69	240.1594	$[M+H]^+$	$C_{13}H_{22}NO_3$	148.0757	$C_9H_{10}NO$
					166.0863	$C_9H_{12}NO_2$
					222.1489	$C_{13}H_{20}NO_2$
Simvastatin	12.51	419.2792	$[M+H]^+$	$C_{25}H_{39}O_5$	285.1849	$C_{19}H_{25}O_2$
					303.1955	$C_{19}H_{27}O_3$
Sotalol	4.53	273.1268	$[M+H]^+$	$C_{12}H_{21}N_2O_3S$	255.1162	$C_{12}H_{19}N_2O_2S$
Sulfadiazine	5.13	251.0597	[M+H]+	$C_{10}H_{11}N_4O_2S$	156.0114	$C_6H_6NO_2S$
Sulfamethazine (6)	6.17	279.0910	$[M+H]^+$	$C_{12}H_{15}N_4O_2S$	124.0869	$C_6H_{10}N_3$
					186.0332	$C_6H_8N_3O_2S$
Sulfamethoxazole	7.36	254.0594	$[M+H]^+$	$C_{10}H_{12}N_3O_3S$	156.0114	$C_6H_6NO_2S$
Sulfapyridine	5.60	250.0645	$[M+H]^+$	$C_{11}H_{12}N_3O_2S$	156.0114	$C_6H_6NO_2S$
Sulfathiazole (9)	5.76	256.0209	[M+H] +	$C_9H_{10}N_3O_2S_2$	156.0114	$C_6H_6NO_2S$
Terbutaline	2.77	226.1438	[M+H] +	$C_{12}H_{20}NO_3$	152.0706	$C_8H_{10}NO_2$
Tetracycline	5.41	445.1606	$[M+H]^+$	$C_{22}H_{25}N_2O_8$	428.1340	$C_{22}H_{22}NO_8$
Theobromine (1)	1.91	181.0720	$[M+H]^+$	$C_7H_9N_4O_2$	138.0662	$C_6H_8N_3O$
Theophylline (1)	2.84	181.0720	$[M+H]^+$	$C_7H_9N_4O_2$	124.0506	$C_5H_6N_3O$
Venlafaxine (5)	6.34	278.2115	$[M+H]^+$	$C_{17}H_{28}NO_2$	260.2009	$C_{17}H_{26}NO$
Bezafibrate	7.58	360.1008	$[M-H]^-$	$C_{19}H_{19}ClNO_4$	274.0662	$C_{15}H_{13}ClNO_2$
Chlorophene	10.24	217.0426	$[M-H]^-$	$C_{13}H_{10}ClO$	219.0396	$C_{13}H_{10}[^{37}Cl]O$
Clofibric acid (10)	6.47	213.0324	$[M-H]^-$	$C_{10}H_{10}ClO_3$	85.0301	$C_4H_5O_2$
					126.9958	C_6H_4ClO
Diclofenac	9.55	294.0094	$[M-H]^-$	$C_{14}H_{10}ClNO_2$	296.0064	$C_{14}H_{10}[^{37}Cl]ClNO_2$
					298.0034	$C_{14}H_{10}[^{37}Cl]2NO_2$
Fenoprofen	9.22	241.0870	$[M-H]^-$	$C_{15}H_{13}O_3$	197.0973	$C_{14}H_{13}O$
					211.0757	$C_{14}H_{11}O_2$
Furosemide	5.95	329.0040	$[M-H]^-$	$C_{12}H_{10}ClN_2O_5S$	204.98436	$C_6H_6ClN_2O_2S$
					285.0129	$C_{11}H_{10}ClN_2O_3S$

Table 1 (*Continued*)

Compound name	RT (min)	*m*/*z* calculated	Ion	Elemental composition	QTOF-MS fragment ions[a]	
					m/*z* calculated	Elemental composition
Gemfibrozil	10.52	249.1496	[M−H]⁻	$C_{15}H_{21}O_3$	121.0661	C_8H_9O
Hydrochlorothiazide	4.34	295.9572	[M−H]⁻	$C_7H_7ClN_3O_4S_2$	297.9542	$C_7H_7[^{37}Cl]N_3O_4S_2$
					299.9500	$C_7H_7[^{37}Cl]N_3O_4[^{34}S]S$
					301.9458	$C_7H_7[^{37}Cl]N_3O_4[^{34}S]_2$
					297.9530	$C_7H_7ClN_3O_4[^{34}S]S$
					299.9488	$C_7H_7ClN_3O_4[^{34}S]_2$
Ibuprofen	9.91	205.1234	[M−H]⁻	$C_{13}H_{17}O_2$	161.1336	$C_{11}H_{18}$
Indomethacin	9.51	356.0695	[M−H]⁻	$C_{19}H_{15}ClNO_4$	312.0798	$C_{17}H_{16}ClNO_2$
Ketoprofen	8.07	253.0870	[M−H]⁻	$C_{16}H_{13}O_3$	209.0975	$C_{15}H_{13}O$
MCPP (10)	5.16	213.0324	[M−H]⁻	$C_{10}H_{10}ClO_3$	141.01143	C_7H_6ClO
Pravastatin	6.65	423.2388	[M−H]⁻	$C_{23}H_{35}O_7$	321.17096	$C_{18}H_{25}O_5$
Salicylic acid	1.29	137.0244	[M−H]⁻	$C_7H_5O_3$	93.03407	C_6H_5O

Compound name	RT (min)	*m*/*z* calculated	Ion	Elemental composition	QTOF-MS/MS fragment ions		
					CE	*m*/*z* calculated	Elemental composition
4-AA	2.18	204.1132	[M+H]⁺	$C_{11}H_{14}N_3O$	15	56.0495	C_3H_6N
						83.0604	$C_4H_7N_2$
						94.0651	C_6H_8N
						159.0917	$C_{10}H_{11}N_2$
4-MAA	1.95	218.1288	[M+H]⁺	$C_{12}H_{16}N_3O$	13	56.0495	C_3H_6N
						97.076	$C_5H_9N_2$
						159.0917	$C_{10}H_{11}N_2$
Acetanilide	5.57	136.0757	[M+H]⁺	$C_8H_{10}NO$	15	77.0386	C_6H_5
						94.0651	C_6H_8N
Amitriptyline (5)	7.34	278.1903	[M+H]⁺	$C_{20}H_{24}N$	23	91.0542	C_7H_7
						105.0701	C_8H_9
						191.0855	$C_{15}H_{11}$
						233.1325	$C_{18}H_{17}$
Antipyrine	5.34	189.1023	[M+H]⁺	$C_{11}H_{13}N_2O$	31	56.05	C_3H_6N
						77.03686	C_6H_5
						106.0651	C_7H_8N
						147.0917	$C_9H_{11}N_2$
Citalopram Hydrobr.	6.74	325.1711	[M+H]⁺	$C_{20}H_{22}FN_2O$	23	109.0448	C_7H_6F
						234.0714	$C_{16}H_9FN$
						262.1027	$C_{18}H_{13}FN$
Clarithromycin	7.38	748.4842	[M+H]⁺	$C_{38}H_{70}NO_{13}$	19	116.107	$C_6H_{14}NO$
						158.1176	$C_8H_{16}NO_2$
						590.3899	$C_{30}H_{56}NO_{10}$
Codeine	3.43	300.1594	[M+H]⁺	$C_{18}H_{22}NO_3$	37	58.0651	C_3H_8N
						181.0648	$C_{13}H_9O$
						199.0754	$C_{13}H_{11}O_2$
						215.1067	$C_{14}H_{15}O_2$
Cotinina	1.19	177.1023	[M+H]⁺	$C_{10}H_{13}N_2O$	22	80.0495	C_5H_6N
						98.06	C_5H_8NO
						146.06	C_9H_8NO
Cyclophosphamide (4)	6.57	261.0321	[M+H]⁺	$C_7H_{16}Cl_2N_2O_2P$	6	106.0418	C_4H_9ClN
						120.0209	$C_3H_7NO_2P$
						140.0028	$C_4H_8Cl_2N$
						142.0185	$C_4H_{10}Cl_2N$
Erythromycin	6.94	734.4685	[M+H]⁺	$C_{37}H_{68}NO_{13}$	18	83.0491	C_5H_7O
						116.107	$C_6H_{14}NO$
						158.1176	$C_8H_{16}NO_2$
						576.3749	$C_{29}H_{53}NO_{10}$
Fluoxetine (7)	7.78	310.1413	[M+H]⁺	$C_{17}H_{19}F_3NO$	15	44.0495	C_2H_6N
						148.1121	$C_{10}H_{14}N$
Ifosfamide (4)	6.38	261.0321	[M+H]⁺	$C_7H_{16}Cl_2N_2O_2P$	6	78.0103	CH_5NOP
						92.026	C_2H_7NOP
						153.9819	$C_3H_6ClNO_2P$
						182.0132	$C_5H_{10}ClNO_2P$
Ketorolac (9)	8.32	256.0968	[M+H]⁺	$C_{15}H_{14}NO_3$	12	105.0335	C_7H_5O
						178.0499	$C_9H_8NO_3$
						210.0913	$C_{14}H_{12}NO$
Lincomycin	3.32	407.2211	[M+H]⁺	$C_{18}H_{34}N_2O_6S$	20	126.1277	$C_8H_{16}N$
						359.2196	$C_{11}H_{27}N_{12}S$
Mepivacaine	4.86	247.1805	[M+H]⁺	$C_{15}H_{23}N_2O$	20	70.0651	C_4H_8N
						98.0964	$C_6H_{12}N$
						150.0913	$C_9H_{12}NO$
Metoprolol	5.73	268.1907	[M+H]⁺	$C_{15}H_{26}NO_3$	23	74.06	C_3H_8NO
						116.107	$C_6H_{14}NO$
						133.0648	C_9H_9O
						159.0804	$C_{11}H_{11}O$

Table 1 (*Continued*)

Compound name	RT (min)	m/z calculated	Ion	Elemental composition	QTOF-MS/MS fragment ions		
					CE	m/z calculated	Elemental composition
Paroxetine	7.01	330.1500	$[M+H]^+$	$C_{19}H_{21}FNO_3$	24	70.0651	C_4H_8N
						123.0605	C_8H_8F
						151.039	$C_8H_7O_3$
						192.1183	$C_{12}H_{15}FN$
Propyphenazone (2)	8.06	231.1492	$[M+H]^+$	$C_{14}H_{19}N_2O$	28	56.0495	C_3H_6N
						189.1022	$C_{11}H_{13}N_2O$
						201.1022	$C_{12}H_{13}N_2O$
Propanolol	6.36	260.1645	$[M+H]^+$	$C_{16}H_{22}NO_2$	22	74.06	C_3H_8NO
						116.107	$C_6H_{14}NO$
						155.0855	$C_{12}H_{11}$
						183.0804	$C_{13}H_{11}O$
Tamoxifen	8.94	372.2322	$[M+H]^+$	$C_{26}H_{30}NO$	26	72.0808	$C_4H_{10}N$
						91.0542	C_7H_7
						129.0699	$C_{10}H_9$
Trans3hy.cotinine	1.02	193.0972	$[M+H]^+$	$C_{10}H_{13}N_2O_2$	20	80.0495	C_5H_6N
						106.0651	C_7H_8N
						134.06	C_8H_8NO
						149.0709	$C_8H_9NO_2$
Trimethoprim	4.97	291.1452	$[M+H]^+$	$C_{14}H_{19}N_4O_3$	26	123.0665	$C_5H_7N_4$
						230.1176	$C_{14}H_{16}NO_2$
						261.0982	$C_{12}H_{13}N_4O_3$
						275.1139	$C_{13}H_{15}N_4O_3$

[a] Isotopic signature information has been included in some compounds that have no fragments.
4-AAA: N-acetyl-4-aminoantipiryne; 4-DAA: 4-dimethylaminoantipiryne; 4-FAA: N-formyl-4-aminoantipiryne; MCPP: mecoprop; 4-AA: 4-aminoantipiryne; 4-MAA: 4-methylaminoantipyrine.

selected a peak filter higher or equal to 100 counts for the ion extraction and the compound filter had a relative abundance higher or equal to 0.01% and an absolute abundance higher or equal to the height of 1000 counts. These values provide 100% positives on spiked samples tested, keeping the total number of features extracted as low as possible. In the second step, the defined search criteria are accurate mass and retention time tolerances. We selected a ±0.15 min RT window and a tolerance of 5 ppm. Satisfactory results were obtained with the combination of these two values.

An example of an effluent wastewater sample processed using the automatic screening method with the developed database is shown in Fig. 1. The MFE produced 463 features or potential compounds. It must be taken into account that the number of features does not correspond to the total number of chemical species, because both in-source fragment ions and isotopic signals are considered features. In the second step the accurate mass of each of these compounds at their retention time was subsequently searched against the user-created exact mass and retention time database of almost 400 compounds with their fragment ions and isotopic signals. Fifty-one of the 463 potential compounds had mass and RT matches with the target species included in the database, according to the database search parameters selected (3 ppm tolerance with a ±0.3 min RT window). Fig. 1 shows the TIC and the database search results. As can be observed, 26 compounds have been detected, including five pesticides, 16 pharmaceuticals and five of their major metabolites or degradation products. Fifteen of the compounds have additional information of fragment ions from in-source CID fragmentation and indomethacin has information about the chlorine isotopic profile, very useful for identification purposes. The carbamazepine and ketoprofen fragments, included in the database, do not appear in the list, maybe because the concentration levels of these compounds in the sample is close to the detection limit, and in this situation the fragments could be too low in abundance to be detected. Seventeen of the compounds detected have no in-source fragments or characteristic isotope profiles (highlighted in bold): therefore these compounds are then identified using MS/MS (QTOF) analysis with the same instrument.

In the report, a score column is also included. The score value is calculated by the software taking into account not only the accurate masses but also the isotopic distribution: thus, the higher the value, the more plausible the elemental composition. Compounds with scores below a defined relevance threshold – that we have established as being 60, following our experience in this work – have to be carefully checked manually to define the compound responsible. In Fig. 1 the compounds with a score below 60 are marked in gray, most of them are fragments: this could be due to the lower intensity/relative abundance of some fragments or concentration levels of the compounds approaching the limits of detection. In this situation, the identification might fail because the concentration is so low that any background interference that is overlapped with our target could alter the isotopic distribution and also could give poor accurate mass measurements.

3.2.2. Identification with the accurate-mass database

3.2.2.1. Mass accuracy. One of the main attributes of TOF instruments, making them an attractive analytical technique, is their accurate mass measurement, which gives the elemental composition of parent and fragment ions. The reliability of the screening method depends heavily on the ruggedness of the TOF instrument in order to provide consistently accurate mass measurements within a fixed mass error tolerance. Typically, the measurement of accurate masses within 5 ppm is widely accepted for the verification of the elemental composition [20]. The Q-TOF system used for this work has demonstrated mass accuracy values of <2 ppm in most cases, regardless of the matrices or the concentration level. Only large concentrations or very sensitive compounds may yield higher errors due to the saturation of the detector. To achieve such accurate mass measurement, the Q-TOF instrument uses automatic internal referencing, two compounds of known mass are introduced continuously in the ion source and the software automatically calibrates the mass axis of every spectrum.

The mass accuracy can be used to identify unknown compounds and differentiate isobaric compounds (different compounds with the same nominal mass but different elemental composition, and thus, different exact masses). With low-resolution mass spectrometry it is not possible to distinguish isobaric species. From the

Table 2
Analytical parameters of the proposed LC–QTOF-MS method for the analysis of target compounds in wastewater effluent and river water.

Compound name	Recovery (RSD, %)$n=3$	Linearity		MDL (ng/L)
		Range (ng/L)	r^2	
Positive ionization				
4-AA	112 (7)	5–1000	0.9467	<5
4-AAA	94 (11)	5–1000	0.99	<5
4-DAA	77 (5)	5–250	0.9725	<5
4-FAA	90 (12)	5–1000	0.9857	<5
4-MAA	101 (7)	5–1000	0.9682	<5
Acetaminophen	42 (12)	5–2500	0.9869	<5
Acetanilide	112 (15)	5–250	0.9841	<5
Amidotrizoate	41 (12)	100–2500	0.857	50–100
Amitriptyline	74 (9)	2–250	0.92274	<5
Amoxicillin	–(–)	50–1000	0.9596	5–50
Antipyrine	114 (8)	5–1000	0.9921	<5
Atenolol	83 (11)	5–125	0.9423	<5
Azithromycin	73 (11)	5–125	0.998	<5
Caffeine	100 (16)	5–2500	0.9988	<5
Carbamazepine 10,11-epoxide	127 (12)	5–2500	0.9934	<5
Carbamazepine	88 (2)	5–1000	0.9915	<5
Cefotaxime	38 (11)	50–2500	0.997	5–50
Ciprofloxacin	70 (13)	5–250	0.9682	<5
Citalopram hydrobromide	86 (13)	5–250	0.9	<5
Clarithromycin	86 (8)	5–250	0.9458	<5
Clomipramine	51 (16)	5–250	0.9443	<5
Clotrimazole	34 (11)	5–250	0.9669	<5
Codeine	120 (23)	5–250	0.9872	<5
Continine	100 (8)	5–1000	0.9558	<5
Cyclophosphamide	84 (8)	5–2500	0.9943	<5
Diazepan	90 (14)	5–500	0.9796	<5
Erythromycin	107 (8)	5–250	0.9095	<5
Famotidine	107 (8)	5–125	0.9809	<5
Fenofibrate	20 (3)	1–500	0.9968	<5
Fenofibric acid	70 (11)	50–2500	0.9885	5–50
Fluoxetine	72 (13)	5–125	0.9376	<5
Ifosfamide	81 (18)	5–2500	0.994	<5
Indomethacin	81 (12)	50–2500	0.9968	5–50
Ketoprofen	100 (12)	5–2500	0.9997	<5
Ketorolac	83 (2)	5–2500	0.9954	<5
Lansoprazole	91 (12)	5–2500	0.9995	<5
Loratadine	76 (19)	5–125	0.9694	<5
Lincomycin	75 (22)	5–250	0.9915	<5
Mefenamic acid	67 (13)	50–2500	0.9975	5–50
Mepivacaine	91 (6)	5–250	0.93	<5
Methylprednisolone	95 (5)	5–2500	0.9918	<5
Metoprolol	100 (5)	5–125	0.9123	<5
Metronidazole	52 (14)	50–2500	0.9873	5–50
Mevastatin	93 (10)	5–2500	0.9986	<5
Nadolol	86 (23)	5–125	0.8684	<5
Naproxen	98 (3)	5–125	0.9751	<5
Nicotine	86 (16)	5–250	0.983	<5
Norfloxacin	52 (13)	5–1000	0.9946	<5
Ofloxacin	102 (5)	5–250	0.9927	<5
Omeprazole	74 (6)	5–2500	0.9854	<5
Paraxanthine	45 (13)	5–2500	0.9925	<5
Paroxetine	55 (18)	5–125	0.9358	<5
Pentoxifylline	82 (9)	5–2500	0.9973	<5
Primidone	88 (10)	5–2500	0.9961	<5
Propyphenazone	79 (14)	5–125	0.9753	<5
Propanolol	95 (9)	5–125	0.9586	<5
Ranitidine	109 (8)	5–125	0.9565	<5
Salbutamol	95 (7)	5–125	0.9129	<5
Simvastatin	76 (19)	50–500	0.9925	<5
Sotalol	89 (13)	5–125	0.9521	<5
Sulfadiazine	22 (15)	5–1000	0.9947	<5
Sulfamethazine	98 (7)	5–2500	0.9905	<5
Sulfamethoxazole	57 (2)	5–2500	0.9989	<5
Sulfapyridine	62 (14)	5–1000	0.9968	<5
Sulfathiazole	83 (14)	50–2500	0.9947	5–50
Tamoxifen	–(–)	5–125	0.9759	<5
Terbutaline	82 (15)	5–125	0.9286	<5
Tetracycline	25 (11)	50–2500	0.9987	5–50
Theobromine	49 (18)	50–2500	0.9924	5–50
Theophylline anhydrous	62 (16)	50–2500	0.9924	5–50
Trans-3′-hydroxycotinine	71 (10)	5–2500	0.9979	<5
Trimethoprim	104 (13)	5–500	0.9547	<5
Venlafaxine	79 (16)	5–250	0.977	<5

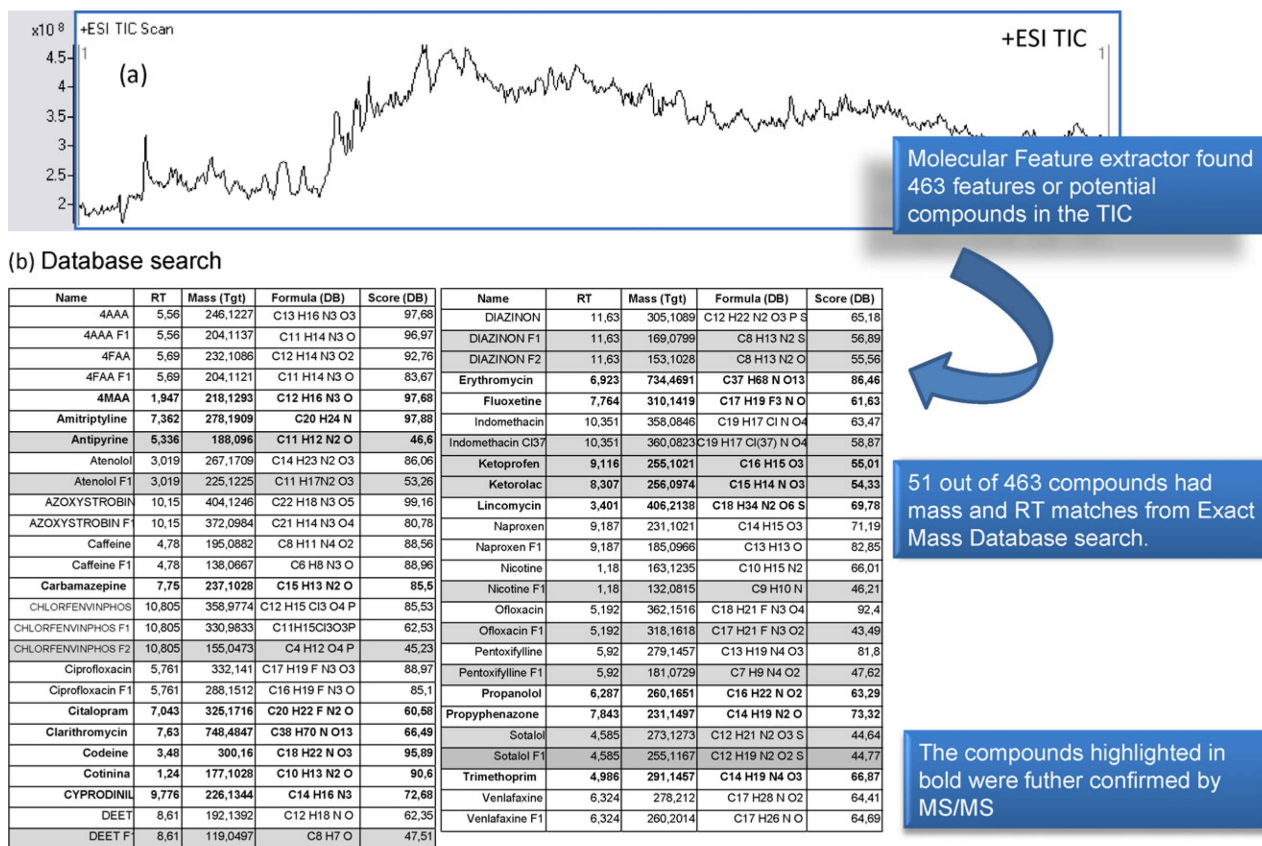
Table 2 (*Continued*)

Compound name	Recovery (RSD, %) $n = 3$	Linearity		MDL (ng/L)
		Range (ng/L)	r^2	
Negative ionization				
Bezafibrate	85 (7)	5–2500	0.9795	<5
Chlorophene	104 (9)	5–2500	0.9947	<5
Clofibric acid	75 (7)	5–2500	0.996	<5
Diclofenac	120 (4)	5–2500	0.9983	<5
Fenoprofen	105 (12)	50–2500	0.9195	5–50
Furosemide	83 (6)	50–2500	0.9842	5–50
Gemfibrozil	114 (11)	5–2500	0.9754	<5
Hydrochlorothiazide	96 (9)	5–2500	0.9765	<5
Ibuprofen	116 (13)	5–2500	0.9992	<5
Ketoprofen	100 (12)	50–2500	0.9996	5–50
MCPP	61 (7)	5–2500	0.9957	<5
Pravastatin	120 (11)	5–2500	0.9978	<5
Salicylic acid	10 (14)	5–250	0.9838	<5

MDL: method detection limit; 4-AAA: N-acetyl-4-aminoantipiryne; 4-DAA: 4-dimethylaminoantipiryne; 4-FAA: N-formyl-4-aminoantipiryne; 4-AA: 4-aminoantipiryne; 4-MAA: 4-methylaminoantipyrine; MCPP: mecoprop.

pharmaceuticals studied, we found 21 isobaric species (in Table 1 highlighted in bold, and each pair of isobaric species marked with the same number).

3.2.2.2. Retention time. Most of these isobaric compounds are resolved by their retention times, such as sulfathiazole–ketorolac, theophylline–paraxanthine–theobromine, fluoxetine–nadolol or ranitidine–clomipramine. This demonstrates the importance of providing retention time data when developing large-scale databases for automated screening. Besides this, when analyzing real samples, a greater number of potential interfering species might be expected, and the retention time can be used to get rid of most of them when performing screening of samples. As an example of this, in Fig. 1, an effluent wastewater sample processed using the automatic screening method is shown, in which 51 compounds were found using the exact mass and retention time criteria. However, if the retention time is not included in the database, 463 potential compounds are found, making the assignment of target compounds much more complicated and making the occurrence of false positives far likelier. Therefore, the addition of retention time provides a higher degree of specificity, mandatory for this kind of application. Another advantage of using retention time data is an important saving of time, without retention time the time devoted to performing the



Name	RT	Mass (Tgt)	Formula (DB)	Score (DB)
4AAA	5,56	246,1227	C13 H16 N3 O3	97,68
4AAA F1	5,56	204,1137	C11 H14 N3 O	96,97
4FAA	5,69	232,1086	C12 H14 N3 O2	92,76
4FAA F1	5,69	204,1121	C11 H14 N3 O	83,67
4MAA	**1,947**	**218,1293**	**C12 H16 N3 O**	**97,68**
Amitriptyline	**7,362**	**278,1909**	**C20 H24 N**	**97,88**
Antipyrine	**5,336**	**188,096**	**C11 H12 N2 O**	**46,6**
Atenolol	3,019	267,1709	C14 H23 N2 O3	86,06
Atenolol F1	3,019	225,1225	C11 H17N2 O3	53,26
AZOXYSTROBIN	10,15	404,1246	C22 H18 N3 O5	99,16
AZOXYSTROBIN F1	10,15	372,0984	C21 H14 N3 O4	80,78
Caffeine	4,78	195,0882	C8 H11 N4 O2	88,56
Caffeine F1	4,78	138,0667	C6 H8 N3 O	88,96
Carbamazepine	**7,75**	**237,1028**	**C15 H13 N2 O**	**85,5**
CHLORFENVINPHOS	10,805	358,9774	C12 H15 Cl3 O4 P	85,53
CHLORFENVINPHOS F1	10,805	330,9833	C11H15Cl3O3P	62,53
CHLORFENVINPHOS F2	10,805	155,0473	C4 H12 O4 P	45,23
Ciprofloxacin	5,761	332,141	C17 H19 F N3 O3	88,97
Ciprofloxacin F1	5,761	288,1512	C16 H19 F N3 O	85,1
Citalopram	**7,043**	**325,1716**	**C20 H22 F N2 O**	**60,58**
Clarithromycin	**7,63**	**748,4847**	**C38 H70 N O13**	**66,49**
Codeine	**3,48**	**300,16**	**C18 H22 N O3**	**95,89**
Cotinina	**1,24**	**177,1028**	**C10 H13 N2 O**	**90,6**
CYPRODINIL	**9,776**	**226,1344**	**C14 H16 N3**	**72,68**
DEET	8,61	192,1392	C12 H18 N O	62,35
DEET F1	8,61	119,0497	C8 H7 O	47,51

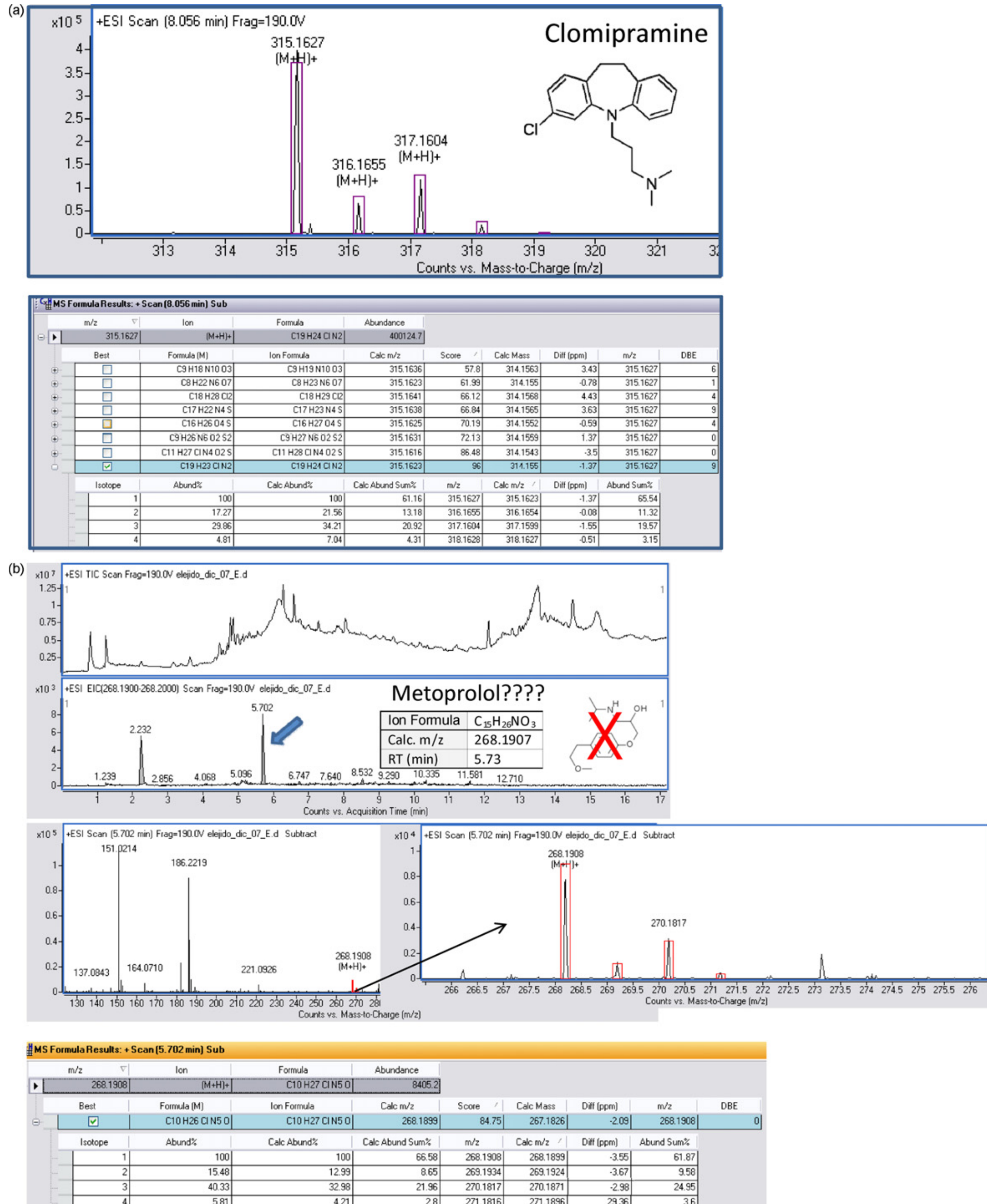
Name	RT	Mass (Tgt)	Formula (DB)	Score (DB)
DIAZINON	11,63	305,1089	C12 H22 N2 O3 P S	65,18
DIAZINON F1	11,63	169,0799	C8 H13 N2 S	56,89
DIAZINON F2	11,63	153,1028	C8 H13 N2 O	55,56
Erythromycin	**6,923**	**734,4691**	**C37 H68 N O13**	**86,46**
Fluoxetine	**7,764**	**310,1419**	**C17 H19 F3 N O**	**61,63**
Indomethacin	10,351	358,0846	C19 H17 Cl N O4	63,47
Indomethacin Cl37	10,351	360,0823	C19 H17 Cl(37) N O4	58,87
Ketoprofen	**9,116**	**255,1021**	**C16 H15 O3**	**55,01**
Ketorolac	**8,307**	**256,0974**	**C15 H14 N O3**	**54,33**
Lincomycin	**3,401**	**406,2138**	**C18 H34 N2 O6 S**	**69,78**
Naproxen	9,187	231,1021	C14 H15 O3	71,19
Naproxen F1	9,187	185,0966	C13 H13 O	82,85
Nicotine	1,18	163,1235	C10 H15 N2	66,01
Nicotine F1	1,18	132,0815	C9 H10 N	46,21
Ofloxacin	5,192	362,1516	C18 H21 F N3 O4	92,4
Ofloxacin F1	5,192	318,1618	C17 H21 F N3 O2	43,49
Pentoxifylline	5,92	279,1457	C13 H19 N4 O3	81,8
Pentoxifylline F1	5,92	181,0729	C7 H9 N4 O2	47,62
Propanolol	**6,287**	**260,1651**	**C16 H22 N O2**	**63,29**
Propyphenazone	**7,843**	**231,1497**	**C14 H19 N2 O**	**73,32**
Sotalol	4,585	273,1273	C12 H21 N2 O3 S	44,64
Sotalol F1	4,585	255,1167	C12 H19 N2 O2 S	44,77
Trimethoprim	**4,986**	**291,1457**	**C14 H19 N4 O3**	**66,87**
Venlafaxine	6,324	278,212	C17 H28 N O2	64,41
Venlafaxine F1	6,324	260,2014	C17 H26 N O	64,69

Fig. 1. Screening of a wastewater effluent sample by LC–QTOFMS using the automatic screening method with the user-created database: (a) total ion chromatogram; (b) database search results. The detected compounds with a score below 60 are marked in gray and the compounds which have no in-source fragments or characteristic isotope profile are highlighted in bold.

search of a sample would become a bottleneck in the screening procedure.

3.2.2.3. Isotopic pattern. In addition to the use of accurate mass and RT, database screening with isotope pattern recognition (not only monoisotopic masses) will increase the performance of the method and will also provide enhanced confirmation of the findings based on the isotopic signals. This is particularly useful for confirmatory purposes on those chemicals containing chlorine, bromine, sulfur or a large number of carbon atoms. The application of these filters allows a reduction in the number of proposed elemental compositions that would be fit for a certain mass accuracy window, given that their presence in the molecule produces a characteristic isotopic distribution.

Fig. 2(a) shows an example of elemental composition confirmation of the pharmaceutical clomipramine using the retention time, accurate mass measurements and isotopic pattern. Even with very high mass accuracy several chemical formulae candidates might be obtained depending on the mass reasons considered. To determine the most probable elemental composition, the use of isotopic pattern is quite a helpful tool in screening empirical formulae by overlaying the theoretical isotope abundances on the actual spectrum. Using a mass window of 5 ppm, eight elemental compositions of the ion 315.1627 are possible. According to these criteria,



MS Formula Results: + Scan (8.056 min) Sub

m/z	Ion	Formula	Abundance
315.1627	(M+H)+	C19 H24 Cl N2	400124.7

Best	Formula (M)	Ion Formula	Calc m/z	Score	Calc Mass	Diff (ppm)	m/z	DBE
☐	C9 H18 N10 O3	C9 H19 N10 O3	315.1636	57.8	314.1563	3.43	315.1627	6
☐	C8 H22 N6 O7	C8 H23 N6 O7	315.1623	61.99	314.155	-0.78	315.1627	1
☐	C18 H28 Cl2	C18 H29 Cl2	315.1641	66.12	314.1568	4.43	315.1627	4
☐	C17 H22 N4 S	C17 H23 N4 S	315.1638	66.84	314.1565	3.63	315.1627	9
☐	C16 H26 O4 S	C16 H27 O4 S	315.1625	70.19	314.1552	-0.59	315.1627	4
☐	C9 H26 N6 O2 S2	C9 H27 N6 O2 S2	315.1631	72.13	314.1559	1.37	315.1627	0
☐	C11 H27 Cl N4 O2 S	C11 H28 Cl N4 O2 S	315.1616	86.48	314.1543	-3.5	315.1627	0
☑	C19 H23 Cl N2	C19 H24 Cl N2	315.1623	96	314.155	-1.37	315.1627	9

Isotope	Abund%	Calc Abund%	Calc Abund Sum%	m/z	Calc m/z	Diff (ppm)	Abund Sum%
1	100	100	61.16	315.1627	315.1623	-1.37	65.54
2	17.27	21.56	13.18	316.1655	316.1654	-0.08	11.32
3	29.86	34.21	20.92	317.1604	317.1599	-1.55	19.57
4	4.81	7.04	4.31	318.1628	318.1627	-0.51	3.15

MS Formula Results: + Scan (5.702 min) Sub

m/z	Ion	Formula	Abundance
268.1908	(M+H)+	C10 H27 Cl N5 O	8405.2

Best	Formula (M)	Ion Formula	Calc m/z	Score	Calc Mass	Diff (ppm)	m/z	DBE
☑	C10 H26 Cl N5 O	C10 H27 Cl N5 O	268.1899	84.75	267.1826	-2.09	268.1908	0

Isotope	Abund%	Calc Abund%	Calc Abund Sum%	m/z	Calc m/z	Diff (ppm)	Abund Sum%
1	100	100	66.58	268.1908	268.1899	-3.55	61.87
2	15.48	12.99	8.65	269.1934	269.1924	-3.67	9.58
3	40.33	32.98	21.96	270.1817	270.1871	-2.98	24.95
4	5.81	4.21	2.8	271.1816	271.1896	29.36	3.6

Fig. 2. (a) Elemental composition confirmation of the antidepressant clomipramine by the retention time, accurate mass measurements and isotopic pattern. (b) Metoprolol false positive identification by isotopic pattern.

the best-fit (with mass accuracy of −1.37 ppm and isotopes) was $C_{19}H_{23}ClN_2$, the formula for clomipramine. In addition, the four isotope masses for the molecular ion all differed by less than 2 ppm. The table outlined in Fig. 2(a) shows that the experimental isotope abundances of the four isotopes match well with the calculated (theoretical) abundances. The boxes in the spectrum from Fig. 2(a), surrounding the isotopes, represent the theoretical isotope abundances. It should be remarked that although not being the closest match by accurate mass, the correct formula was highlighted as the first option since its score was the best (score 96). The score value is calculated by the software considering not only the accurate masses but also the isotopic distribution.

3.2.2.4. Score value. Taking into account the accurate masses and the isotopic distribution, the software calculates a score value, which is used to rank database search results. The scoring of the generated formulae is based on three factors:

- *Mass*: How well the measured mass (or m/z) compared to the value predicted from the proposed formula.
- *Abundance*: How well the abundance pattern of the measured isotope cluster compared with values predicted from the proposed formula.
- *Spacing*: How the m/z spacing between the lowest m/z ion and the A + 1 and A + 2 ions compared with the values predicted from the proposed formula.

The score is reported on a scale of 0 to 100. When a formula is available as a database entry or target compound, a combined score is calculated which is based on mass, isotope abundance and isotope spacing. The overall score for a formula is computed as a weighted average of individual probabilities. The default weighting factors values are:

$$W_{\text{mass}} = 100$$

$$W_{\text{abundance}} = 60$$

$$W_{\text{spacing}} = 50$$

The equation for computing the overall score is then:

$$\text{Score} = \frac{W_{\text{mass}} \times P_{\text{mass}} + W_{\text{abundance}} \times P_{\text{abundance}} + W_{\text{spacing}} \times P_{\text{spacing}}}{W_{\text{mass}} + W_{\text{abundance}} + W_{\text{spacing}}}$$

Even in the case where no favourable isotopic pattern was present (absence of Cl, Br or S atoms in the molecule), the score value is helpful in discriminating between potential elemental compositions for other compounds with a more favourable isotopic distributions. For example, in Fig. 2(b) a case of metoprolol false positive identification is shown. Metropolol elutes at a retention time of 5.73 min, with m/z 268.1907. If we manually extract this exact mass from the TIC of the wastewater sample in Fig. 2(b) a peak appears at 5.71 min with m/z 268.1908, which suggests the presence of metoprolol in the sample. However, the measured isotope pattern (boxes in the spectrum, insert in Fig. 2) shows the pattern that is typical for a chlorinate compound whereas metoprolol has no chlorine atoms in the molecule. Using a mass window of 5 ppm and the isotopic pattern from the TOF calculator, we get a unique formula ($C_{10}H_{26}ClN_5O$) with a good match (score 84.75). In such cases, it is important to confirm that metoprolol is present, or not, with further analysis using the QTOF-MS/MS mode because sometimes if the concentration level of the compound approaches the limit of detection, the identification might fail because the concentration is so low that any background interference that is overlapped with our target could alter the isotopic distribution. In this case, the MS/MS analysis confirmed that metoprolol was not present in this sample.

3.2.2.5. In-source fragment ions. The accurate-mass database created also includes data on the accurate masses of the characteristic in-source fragment ions. This information is essential due to the complexity of screening almost 400 compounds with similar features in complex matrices at low concentration levels. The fragmentation behaviour performed by the fragmentor voltage in TOF plays an important role in the combinative identification or confirmation procedure. Fragmentor voltage is crucial in providing characteristic fragment ions in the MS spectra resulting from collision-induced dissociation (CID) in-source. As with identification, the more characteristic the fragment ions obtained, the more reliable the structure confirmation is. We used a relatively high fragmentor voltage, aiming at obtaining additional information of fragment ions for confirmation purposes. The main drawback is a loss of sensitivity on the compounds, since the intensity of the molecular ion decreases as fragment ions are formed. As a balance between sensitivity and fragmentation, the fragmentor voltage was found to be optimum at 90 V.

Over 77% of the studied compounds, at least one characteristic fragment ion was contained (see Table 1). The use of fragmentation information is very useful not only as a complementary set of data to provide unambiguous confirmation of the findings but also to differentiate between high-resolution isobaric compounds and isomers. The QTOF instrument we used can resolve interferences in the range of 10–30 mDa (see Section 2) so that it could be used for resolving most of the isobaric species included in Table 1. However, some of the isobaric species have exact mass differences less than 10–30 mDa, which is the mass difference that can be discriminated by resolution of the analyzer on the m/z axis. Some of the isobaric species are isomers with the same elemental composition and accurate mass. Therefore, additional information included in the database must be employed to differentiate between these isobaric species when retention time is not enough. For this reason, it is very important to include information of fragment ions from in-source CID fragmentation in the exact mass database.

As an example to illustrate the usefulness of the fragmentation data included in the database, in Fig. 3(a) the mass spectra of two isobaric pharmaceuticals pentoxifylline and sulfamethazine are shown, which have very similar retention times, 6.081 and 6.173 min, respectively. In addition, pentoxifylline and sulfamethazine mass spectra showed the molecular ion at m/z 279.1452 and 279.0910, respectively. So, the exact mass difference and the retention time might not be enough to differentiate these compounds. We could not confirm the presence of both compounds, unless they possess characteristic fragment ions, which could assist their unambiguous confirmation. Indeed, the TOF-MS spectrum of pentoxifylline shown in Fig. 3(a), reveals a fragment ion at m/z 181.0725 and the sulfamethazine mass spectrum shows two fragment ions at m/z 124.0864 and m/z 186.0334, which could be used for confirmatory purposes in a complex matrix and to distinguish the two isobaric compounds. Fig. 3(b) shows the mass spectra of theobromine and theophylline as an example of two isomers that are compounds with the same elemental composition ($C_7H_9N_4O_2$) and accurate mass (m/z 181.0720). Besides, the retention times (1.091 min for theobromine and 2.84 min for theophylline) are very similar. In this case, they cannot be differentiated by isotopic profile. However, they can be distinguished because they have different fragmentation; theobromine fragments in-source yield an ion at m/z 138.0658, whereas theophylline characteristic fragment is m/z 124.0491. This is another example of how essential the fragmentation information for this application is.

One of the main advantages of TOF instruments, over QqQ instruments, is the acquisition of the full spectrum at high resolu-

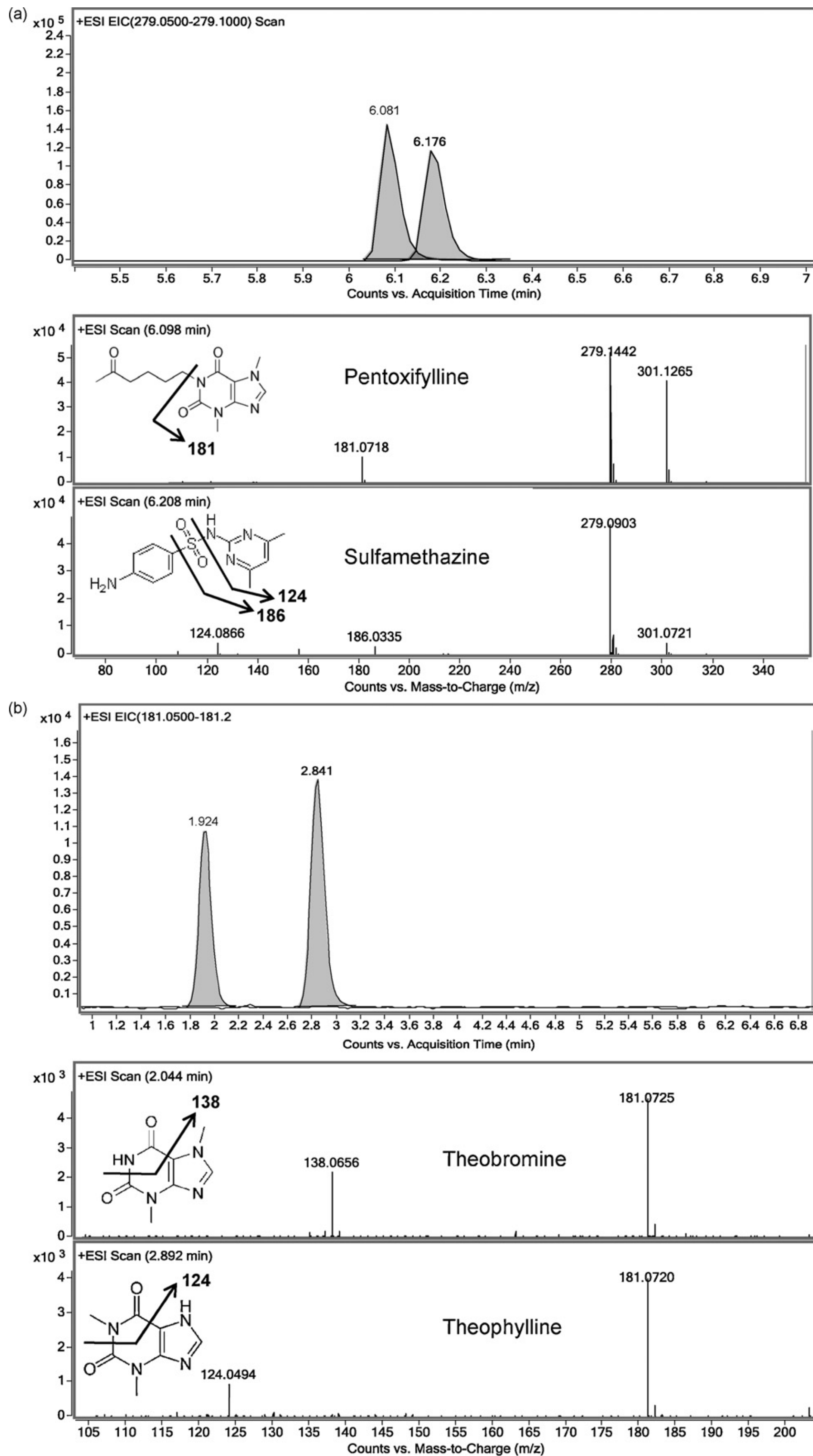
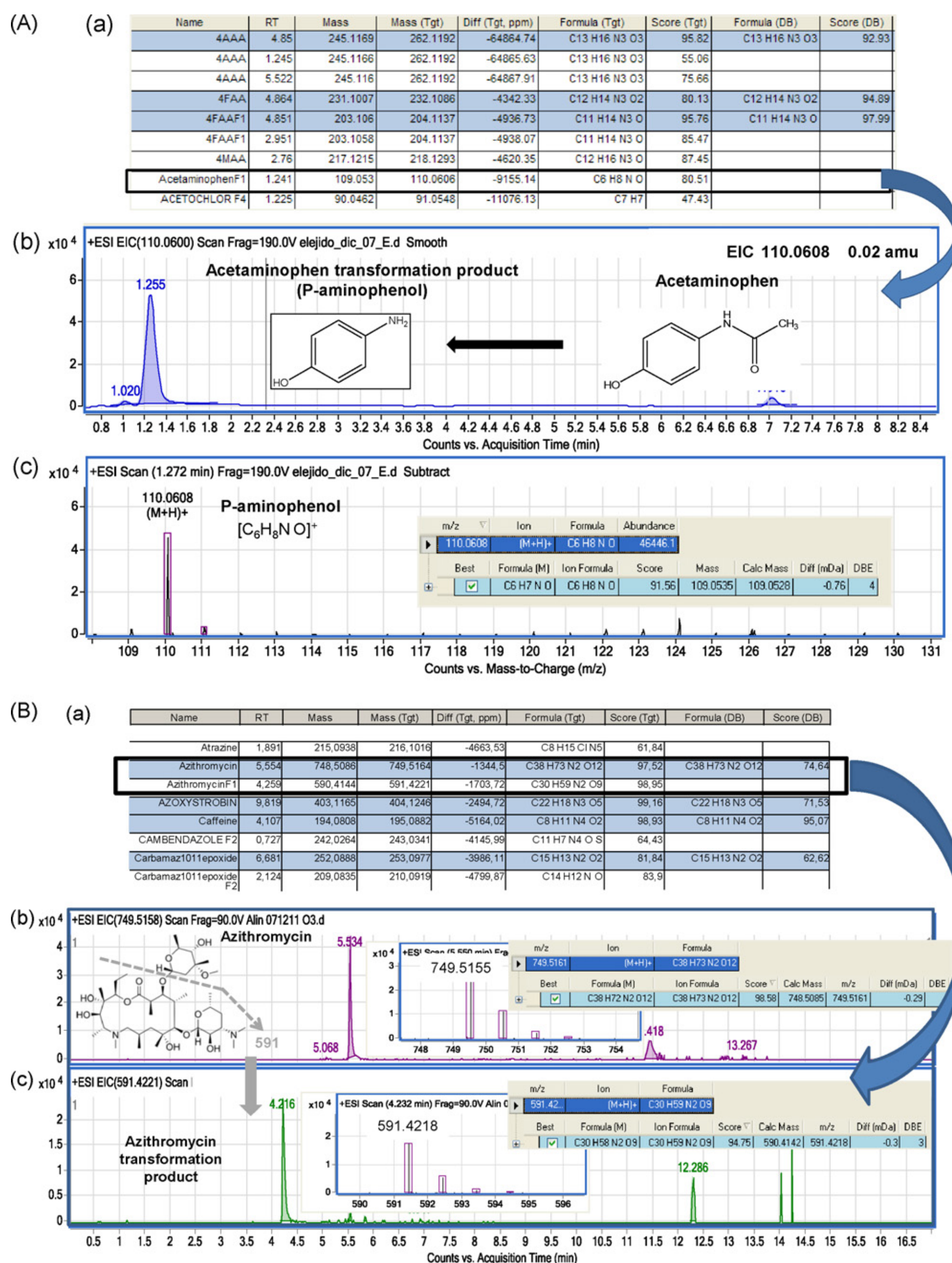


Fig. 3. (a) Differentiation of two ceolutining isobaric pharmaceuticals by in-source fragment ions. (b). Differentiation of two isomers pharmaceuticals by in-source fragment ions.

tion and mass accuracy, thus favouring the detection of additional compounds that might be present in the water samples.

3.2.3. Fragmentation information used for the identification of transformation products

In this way, fragmentation information could also be used as a powerful tool for the automatic identification of unknown compounds and/or transformation products with similar structure to known compounds included in the database. It has been suggested that organic pollutants are often transformed into degradation products in the same fashion as they are fragmented in the instrument [19]. The fragmentation pathways of the parent species can be used to predict possible degradation products, since the bonds that are easily cleaved are those that might be broken in reaction to ambient conditions. Taking into account this approach, we have investigated the fragment ions of the parent compound in the instrument (in-source CID fragmentation), eluting at a retention time different from the parent compound in order to identify possible degradation products of these compounds in the water samples. An example of this strategy, showing the potential of the exact mass database in identifying degradation products (which fall outside any control and are often more toxic, or are present in the environment at higher concentrations than the parent compounds) is shown in Fig. 4(A). In this example of an effluent wastewater sample processed using the automatic screening method developed, one of the most widely used analgesic–antipyretics in The United States and Europe – acetaminophen – does not appear in the report generated by the software. However, the characteristic fragment of this compound (highlighted in a box) appears at a retention time of 1.2 min. Acetaminophen elutes at 4.5 min:



Name	RT	Mass	Mass (Tgt)	Diff (Tgt, ppm)	Formula (Tgt)	Score (Tgt)	Formula (DB)	Score (DB)
4AAA	4.85	245.1169	262.1192	-64864.74	C13 H16 N3 O3	95.82	C13 H16 N3 O3	92.93
4AAA	1.245	245.1166	262.1192	-64865.63	C13 H16 N3 O3	55.06		
4AAA	5.522	245.116	262.1192	-64867.91	C13 H16 N3 O3	75.66		
4FAA	4.864	231.1007	232.1086	-4342.33	C12 H14 N3 O2	80.13	C12 H14 N3 O2	94.89
4FAAF1	4.851	203.106	204.1137	-4936.73	C11 H14 N3 O	95.76	C11 H14 N3 O	97.99
4FAAF1	2.951	203.1058	204.1137	-4938.07	C11 H14 N3 O	85.47		
4MAA	2.76	217.1215	218.1293	-4620.35	C12 H16 N3 O	87.45		
AcetaminophenF1	1.241	109.053	110.0606	-9155.14	C6 H8 N O	80.51		
ACETOCHLOR F4	1.225	90.0462	91.0548	-11076.13	C7 H7	47.43		

m/z	Ion	Formula	Abundance
110.0608	(M+H)+	C6 H8 N O	46446.1

Best	Formula (M)	Ion Formula	Score	Mass	Calc Mass	Diff (mDa)	DBE
✓	C6 H7 N O	C6 H8 N O	91.56	109.0535	109.0528	-0.76	4

Name	RT	Mass	Mass (Tgt)	Diff (Tgt, ppm)	Formula (Tgt)	Score (Tgt)	Formula (DB)	Score (DB)
Atrazine	1,891	215,0938	216,1016	-4663,53	C8 H15 Cl N5	61,84		
Azithromycin	5,554	748,5086	749,5164	-1344,5	C38 H73 N2 O12	97,52	C38 H73 N2 O12	74,64
AzithromycinF1	4,259	590,4144	591,4221	-1703,72	C30 H59 N2 O9	98,95		
AZOXYSTROBIN	9,819	403,1165	404,1246	-2494,72	C22 H18 N3 O5	99,16	C22 H18 N3 O5	71,53
Caffeine	4,107	194,0808	195,0882	-5164,02	C8 H11 N4 O2	98,93	C8 H11 N4 O2	95,07
CAMBENDAZOLE F2	0,727	242,0264	243,0341	-4145,99	C11 H7 N4 O S	64,43		
Carbamaz1011epoxide	6,681	252,0888	253,0977	-3986,11	C15 H13 N2 O2	81,84	C15 H13 N2 O2	62,62
Carbamaz1011epoxide F2	2,124	209,0835	210,0919	-4799,87	C14 H12 N O	83,9		

Fig. 4. (A) Screening of a effluent wastewater using LC–QTOF. (a) A part of the table with the compounds found by MFE. Compounds that are not marked in gray have not retention time matches with the target compounds included database. (b) Extracted ion chromatogram of the ion at m/z 110.0608. (c) Accurate mass spectrum of the peak at 1.25 min, which corresponds to the acetaminophen transformation product (p-aminophenol). (B) Screening of a effluent wastewater using LC–QTOF. (a) A part of the table with the compounds found by MFE. Compounds that are not marked in gray have not retention time matches with the target compounds included database. (b) Identification of azithromycin. (c) Identification of azithromycin transformation product.

therefore we can suspect this might be a degradation product of acetaminophen. Searching the bibliography, we found it could be the primary degradation product of acetaminophen, the p-aminophenol, a compound more toxic than the parent [20]. As can be seen in Fig. 4(A), the accurate mass analysis of the peak, found at 1.25 min, yields the same elemental composition (C_6H_8NO) as the characteristic fragment of acetaminophen and its main degradation product, with a very high mass accuracy of 0.76 and an score of 91.56. This clearly reveals that acetaminophen degrades in the same fashion as its fragment. Another example of the potential use of fragmentation–degradation relationships to detect transformation products and to understand the way pharmaceuticals are degraded in waters is shown in Fig. 4(B). In this case, the parent compound, the antibiotic azithromycin, is present in the sample at 5.5 min with its main fragment ion (*m*/*z* 591), the accurate mass analysis performed on this antibiotic is included in the figure. In the report of the sample, the same fragment of azithromycin can also be seen, but at a different retention time (4.2 min), the peak and the accurate mass analysis of this transformation product is shown in the figure, and obviously, the accurate mass analysis yields the same possible elemental composition as the azithromycin fragment ion. These identifications were accomplished without the use of standards, which may not be available for most of the transformation products, and may help to develop methods for analysis of organic contaminant residues in waters, including not only the compounds in the parent form but also transformation products – to provide a more comprehensive view of the true overall contamination present in samples.

3.3. QTOF-MS/MS analysis to confirm identification of compounds not properly identified by TOF-MS

While LC–QTOF instruments used as a TOF-MS system provide screening and identification of both unknown and targeted organic contaminants, and quantification (of pharmaceuticals and pesticides found in the waters), LC–QTOF or another MS/MS approach, is required to confirm identification of some compounds. This is the case for compounds which have no fragment ions (or fragments with low intensity/relative abundance) from in-source CID fragmentation and/or characteristic isotope profile, and also isomers not distinguished with full single mass spectra. A “Targeted MS/MS” method is developed for these compounds, which can subsequently be analyzed in a second injection using the collision energy (CE) in QTOF-MS/MS mode. In the method, the retention time and precursor ion for each target are entered. Comparison of the structure of the proposed compound with the fragments obtained can confirm its identity. Accurate-mass data and isotopic distributions for the precursor and product ions can be compared to spectral data of reference compounds, if available, obtained under identical conditions for final confirmation. In Table 1, the compounds, which need a posterior identification by MS/MS are listed. In total there are 23 compounds, 26% of the 87 pharmaceuticals included in the database. The product ion mass spectrum of each compound was carefully investigated, and the accurate mass of the characteristic fragment ions, generated in the collision cell, together with their elemental composition, are included in the table. We can also find the collision energy (CE) optimized for each compound in Table 1. As one can see, with QTOF-MS/MS mode, abundant fragmentation is obtained, in comparison to the instrument working with in-source fragment ions.

In the cases mentioned in the previous section, the combined use of accurate mass measurements (if resolution is enough), retention time, isotopic pattern, along with characteristic fragmentation of each compound provides the unambiguous identification of each compound in a mixture of isobaric compounds. The only isobaric coeluting species that could not be resolved were iphosphamide and cyclophosphamide. Fig. 5 shows the chromatograms and the mass spectra of this pair of compounds, which are isomers: therefore they have the same accurate mass (*m*/*z* 261.0321), the same elemental composition and the same isotopic pattern. The retention time (6.54 for cyclophosphamide and 6.26 for iphosphamide) could not be used to distinguish them. Besides, as can be observed in the TOF-MS spectra, they have no in-source fragments. This pair of compounds, can be distinguished when QTOF in MS/MS mode is used. As can be seen in the figure, valuable fragmentation information is obtained and the existence of fragment ions different in both MS/MS spectra would differentiate the two pharmaceuticals. The precursor ion chosen for the MS/MS analysis was the $[M+H]^+$ exact mass (*m*/*z* 261.0321). The most characteristic fragments were the masses 153.9816 and 182.0131 for iphosphamide and the mass 140.0024 for cyclophosphamide. The accurate masses and relative intensities of the main ions in the sample are compared

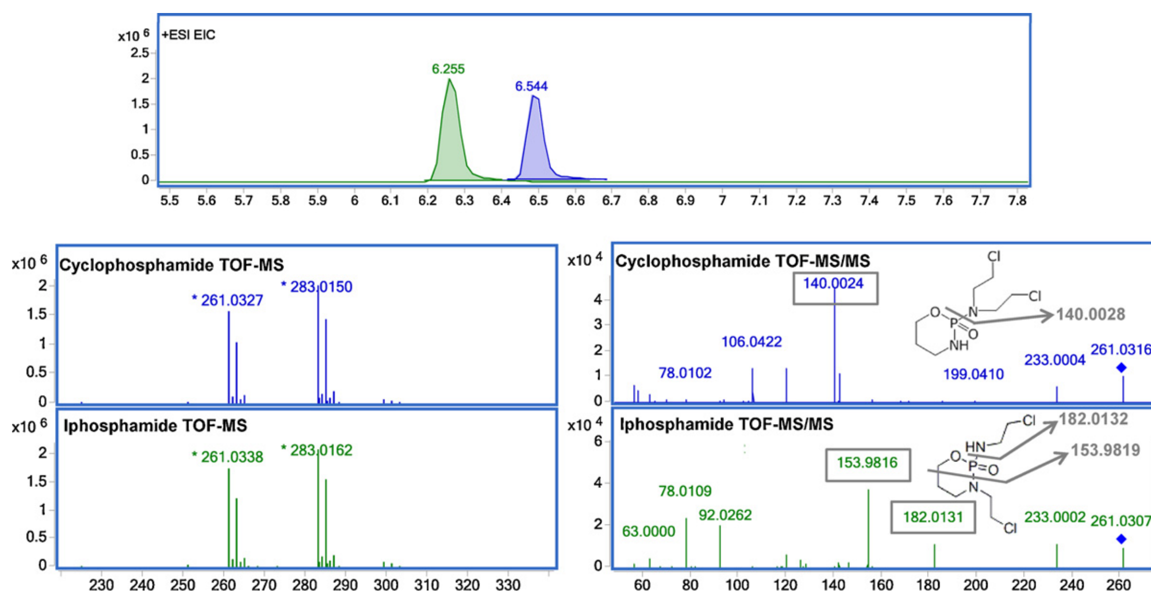


Fig. 5. Identification of isomers that cannot be distinguish with QTOF-MS using QTOFMS/MS.

with the theoretical ones. Mass accuracy obtained for all product ions presented errors lower than 1 ppm.

The acquisition of the full product ion spectra in QTOF allows the potential use of all fragments in order to get the correct confirmation without the need to specifically pre-select some of them, as in the case of QqQ.

3.4. Quantification of positive findings

The analytical method was evaluated to prove its quantification capability. In the validation procedure of the analytical method, the following criteria-sensitivity, linearity, recovery and precision were considered. In Table 2, analytical parameters of the proposed LC–QTOF-MS method are presented. The recoveries have been discussed in Section 3.1.

The sensitivity of the screening method is a key feature to assess the viability of the screening procedure. TOF instruments offer high selectivity and sensitivity under full-scan conditions compared to other analyzers. It has been reported that TOF instruments are around one order of magnitude less sensitive to some compounds when compared with a triple quadrupole instrument used in SRM mode [22]. However, the sensitivity achieved by the QTOF-MS instrument used in this work is improved using an electrospray ion source with Jet Stream Technology. This technology utilizes a super-heated sheath gas to collimate the nebulizer spray, dramatically increasing the number of ions that enter the mass spectrometer and concomitant improved signal to noise. The method limits of detection (MDLs) of the pharmaceuticals included in the database were calculated by the injection of a matrix-matched solution of simulated effluent wastewater at three different concentration levels: 5, 50 and 100 ng/L. Eighty-four percent of the compounds were automatically detected, using the developed screening method, in the 5 ng/L solution, having MDLs lower, or equal to, 5 ng/L. Only 15 compounds were not detected in the 5 ng/L extract, 14 of these compounds were found using the automatic screening method in the 50 ng/L extract, thus having MDLs between 5 and 50 ng/L. Amidotrizoate was the only pharmaceutical with an MDL higher than 50 ng/L. The sensitivity attained using this method is enough to detect the target compounds in the wastewater samples. In the case of surface waters, which are twice as concentrated as effluent wastewaters, the MDLs were also low enough to detect the organic contaminants in these matrices.

In addition to selectivity and sensitivity, the feasible linear dynamic range of the TOF response is important when applied for quantitative purposes. The Jet Stream Technology is very useful in improving sensitivity, but this high sensitivity can cause detector saturation, thus leading to large mass error and in some cases narrow dynamic ranges. As can be observed in Table 2, the QTOF instrument used in this work offered a linear dynamic range of about 2–3 orders of magnitude. Some compounds which are extremely sensitive, might lead to detector saturation at the 125 or 250 ng/L levels. In these cases, mass errors in the range 1–5 ppm might be expected if these compounds are present in the sample at these concentration levels. The QTOF instrument used saturates at concentrations lower than other TOF instruments [13,16,21], but the linear dynamic range for most of the compounds is enough to make possible successful quantitative applications in the analysis or organic contaminants in waters, seeing as the contaminant concentrations usually found in the real samples are within the linear ranges.

To assure the correct quantification of the analytes in the samples, precision in the chromatographic response was determined in terms of repeatability and reproducibility. The RSD obtained ranged from 0.4% to 19% and 3% to 22% for intra- and inter-day studies, respectively.

3.5. Application of the off line-SPE-LC–QTOF-MS method to real samples

The developed method was applied to the analysis of 4 wastewater effluent (WWE) samples from different municipal sewage treatment plants located in Spain and 4 river water (RW) samples collected from different rivers located in the centre of Spain (Madrid).

Results obtained are summarized in Table 3. All samples were first analyzed in full-scan mode with the automated screening method using the pharmaceutical and pesticide database. As shown in Table 3, most of the pharmaceutically active compounds contained in the created database were present in the WWE samples (between 67% and 79% of the 87 pharmaceuticals) and in the RW (between 56% and 62% of the 87): this pointed out their resistance to the conventional water treatment usually applied in wastewater treatment plants (WWTPs). In addition, in most of the WWE samples, between 5 and 15 pesticides were detected from the 300 (3–7%) contained in the database. The most common were diazinon, carbendazim, DEET, diuron, propiconazole, clorfenvinfos, cyprodinil, pirimethanil and azoxystrobin. In river water, between two and five pesticides were often detected, the most frequent being diazinon, carbendazim, DEET and diuron.

Table 3 also shows the range of concentrations measured for target compounds in WWE and RW. The level of concentration detected for pharmaceutically active compounds in WWE were in the range of low ng/L to more than 30 μg/L. River samples presented, in general, lower levels of target compounds, ranging from 4 ng/L to more than 1 μg/L.

The total load of target compounds in WWE samples was in the range of more than 30 μg/L to more than 100 μ/L, whereas in RW, it ranged from 4 to 8 μg/L.

It can be said that, in general, a reduced number of compounds represent more than 50% of the total load of the studied contaminants in the effluents and in the river waters. There are some compounds that are the main contributors in all urban effluent wastewater contamination: this is the case with the analgesic and antipyretic dipyrone metabolites 4-FAA and 4-AAA, the β-blocker atenolol, the diuretic hydrochlorothiazide, the lipid regulator gemfibrozil and the antibiotic ciprofloxacine. Other contaminants are omnipresent in all the effluent wastewaters, such as caffeine and its active metabolite; the diuretic furosemide, the antibiotic ofloxacin and the analgesic/anti-inflammatories: diclofenac, ibuprofen, codeine and naproxen. However, depending on the WWTP, they might or might not be among the main contributors to the total load. The same behaviour is observed in the river waters, there is a omnipresent group of pharmaceutically active compounds that are the main contributors in river water contamination: these are the dipyrone metabolites 4-FAA and 4-AAA, atenolol, nicotine, naproxen and caffeine. It is interesting to note that the dipyrone metabolites are those compounds present at higher concentrations in both effluents and in river waters; in effluents they were detected at concentrations higher than 20 μg/L, and in river waters in some cases at concentrations over 1 μg/L. This fact confirms that the monitoring of metabolites and transformation products is necessary in these studies.

Furthermore, the possibility for the identification of unknown compounds, especially metabolites and transformation products with similar structure to known compounds included in the database, as a result of accurate mass measurements and fragmentation information is a powerful tool. An example of this has been the identification of some important transformation products in the water samples, as we have previously discussed in Section 3.2.2.

Table 3
Occurrence and concentration levels detected of pharmaceuticals and pesticides in effluent wastewater and river water samples from Spain using the LC–QTOF-MS developed method.

Sample ($n = 5$)	Percentage of target compounds detected in the sample		Concentration range (ng/L)	Total load (ng/L)	Compounds with concentration higher than 1000 ng/L (WWE) and 100 ng/L (RW)
	Pharmaceuticals (87)	Pesticides (300)			
WWE 1	67%	3%	8–4868	37,798	4-AAA Caffeine Hydrochlorothiazide 4-FAA Atenolol Furosemide Naproxen
WWE 2	77%	6%	7–20,500	91,758	4-AAA 4-FAA Ranitidine Ciprofloxacin Naproxen 4-AA Gemfibrozil
WWE 3	79%	7%	11–36,364	114,434	Caffeine 4-AAA Ibuprofen 4-FAA Paraxanthine Hydrochlorothiazide 4-MAA 4-AA Atenolol Codeine Gemfibrozil
WWE 4	69%	3%	8–14,636	61,915	4-AAA 4-FAA Hydrochlorothiazide Ciprofloxacin Atenolol 4-MAA Ranitidine 4-AA Ofloxacin
RW 1	57%	2%	5–755	4491	4-AAA 4-FAA Atenolol Naproxen Nicotine
RW 2	62%	2%	5–689	6831	4-AAA Atenolol Nicotine 4-FAA Caffeine Naproxen Paraxanthine Codeine Carbamazepine Ranitidine Omeprazole
RW 3	58%	1%	4–3218	8036	Nicotine 4-AAA 4-FAA Atenolol Caffeine Naproxen Ciprofloxacin Ranitidine Omeprazole Ofloxacin Codeine
RW 4	56%	1%	5–514	4897	4-AAA 4-FAA Atenolol Nicotine Caffeine Carbamazepine Naproxen Sulfamethoxazole

WWE: wastewater effluent; RW: river water; WWE 1: Cantabria; WWE 2: Almería_1; WWE 3: Almería_2; WWE 4: Madrid; RW 1: Guadarrama (Madrid); RW 2: Jarama (Madrid); RW 3: Henares (Madrid); RW 4: Manzanares (Madrid); 4-AAA: N-acetyl-4-aminoantipiryne; 4-MAA: 4-methylaminoantipyrine; 4-FAA: N-formyl-4-aminoantipiryne; 4-AA: 4-aminoantipiryne.

4. Conclusions

The applicability and efficiency of the LC–QTOF-MS technique in automated screening, qualitative and quantitative analysis, based on the use on an accurate-mass database and a "Targeted MS/MS" method, has been demonstrated by the development of one of the first applications reported of this technique for the simultaneous determination of a large number of pharmaceutically active compounds and pesticides in wastewater effluent and river water samples. The method has been demonstrated to be a very simple, fast, and viable alternative for routine monitoring of organic contaminants in waters.

The accurate-mass database created includes data not only on the accurate masses of the target ions but also retention time data, the characteristic in-source fragment ions and/or characteristic isotope profile. This information is essential due to the complexity of screening over 400 compounds of similar features in complex matrix at low concentration levels.

The detailed fragmentation information has also been used as a powerful tool for the automatic identification of unknown compounds, and/or transformation products, with similar structure to known organic contaminants included in the database. This made it possible to identify important degradation products.

As well as the obvious advantage of using a TOF analyzer – allowing it to perform full-scan acquisition with sensitivity (detection limits in the ng/L range) and high mass accuracy (mass errors lower than 2 ppm) – it also makes the qualitative analysis easier, quicker and more accurate, because the monitoring of a specific mass of an analyte is not predefined before data acquisition. This fact is very useful in detecting the presence of an unlimited number of chemical constituents in a sample without re-analysis. Consequently, the method could be readily extended to include additional analytes.

A "Targeted MS/MS" method was developed to confirm the identity of a group of 23 compounds, which have no fragment ions (or fragments with low intensity/relative abundance) from in-source CID fragmentation and/or characteristic isotope profile, and also a pair of isomers (iphosphamide–cyclophophamide) which were not distinguished by the full single mass spectra.

The results obtained in the analysis of real samples with the developed method showed that most of the pharmaceutically active compounds contained in the created database were present in the WWE and RW samples with concentrations in the ng/L and μg/L levels range and in most of the water samples, between 2 and 15 pesticides out of the 300 contained in the database were also detected.

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