Investigation of illicit drugs and pharmaceuticals in waters by liquid chromatography-high resolution mass spectrometry

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Abstract

Mass spectrometry (MS) hyphenated to chromatography has been increasingly used in the environmental field, as it allows the best performance currently attainable for the investigation of a wide range of organic pollutants. When dealing with emerging contaminants, a clear trend has been observed towards the use of liquid chromatography-mass spectrometry (LC-MS) techniques, from tandem (low resolution) MS to high resolution (HR) MS. HRMS allows targeted and untargeted analysis, feasible thanks to the full-spectrum acquisition at high mass accuracy with good sensitivity. With the same instrument, target, suspect and non-target screening can be performed, as well as retrospective analysis and discovery of transformation/ degradation products. This paper gives a general overview on the use of HRMS in LC-based methods directed towards the investigation of illicit drugs and pharmaceuticals in the aqueous environment. Both time-of-flight and Orbitrap mass analyzers are considered, and the benefits of using ultra-high performance liquid chromatography (UHPLC) in combination to HRMS are discussed.

Keywords

Illicit drugs; pharmaceuticals; high resolution mass spectrometry; time of flight; Orbitrap; liquid chromatography; transformation products; screening of emerging contaminants

1.- INTRODUCTION

The increasing number of emerging contaminants, such as pharmaceuticals (both human and veterinary) and personal care products (PPCPs), or illicit drugs, detected in the water cycle can be attributed to the growth of the human population, the shift towards the use of more hydrophilic compounds in consumer applications, and undoubtedly the improvements in selectivity and sensitivity of modern analytical techniques. Pharmaceuticals and illicit drugs (PIDs) are continuously excreted or disposed into the sewer systems as the unaltered parent compound or as metabolites. Subsequently, they often end up in environmental waters, as a consequence of incomplete elimination by wastewater treatment plants (WWTPs) [1]. There is justified concern over the possible impact of these pharmacologically active compounds on the environment, especially over the long-term toxicological effects on living organisms and the combined effect of exposure to multiple compounds, particularly antibiotics [2]. In addition, a large number of transformation products (TPs), in many cases unknown, can be formed in the water cycle and should be taken into account to know the overall contribution of these contaminants in the environment [1]. The presence of PIDs and their metabolites in water and sediments has spurred researchers to set up monitoring studies to evaluate their fate [2,3], as well as their removal and transformation in WWTPs [4–6]. An interesting field that has emerged from the analysis of wastewater is the so-called sewage-based epidemiology (SBE) approach, directed towards the estimation of illicit drug use of a population on the basis of the determination of appropriate biomarkers in influent wastewater. SBE is a promising and complementary tool to existing population surveys and other conventional approaches for the estimation of illicit drug use. SBE requires the application of sophisticated analytical methodologies able to accurately quantify illicit drugs in urban raw wastewater [7–9].

The very low concentrations generally found for PIDs, and/or their metabolites (commonly sub-µg/L levels), in combination with the complexity and unknown composition of the different aqueous matrices to be analyzed makes the use of highly sensitive and selective analytical methodologies necessary. The majority of the methods developed until now are based on liquid chromatography (LC) coupled to tandem mass spectrometry (LC-MS/MS), particularly with triple quadrupole (QqQ) analyzers [2,10]. The medium-high polarity of most PIDs justifies the predominance of this technique. The use of MS/MS under selected reaction monitoring (SRM) mode facilitates the accurate quantification of target analytes at trace levels. Furthermore, the acquisition of two SRM transitions, together with retention-time data and measurement of ion intensity ratios, gives sufficient information for safe identification. Typically, the most sensitive transition is selected for quantification and at least one additional transition is acquired to render a reliable confirmative method. Yet, the MS source and conditions of the analyzer need to be optimized for the determination, carefully considering the specificity of transitions to avoid potential false negatives or positives [11].

The current trend in analytical chemistry is the development of LC-MS based methods which seek the simultaneous determination of many compounds in a single run, providing considerable information about their occurrence, and reducing analysis time and cost [10,12–14]. In spite of the high sensitivity and selectivity reached, LC-MS/MS has some limitations regarding multi-class analysis, especially when broadening the scope of the method to a large number of compounds. In MS/MS methods, the acquisition time of each transition restricts the number of target analytes able to be monitored. Although last-generation QqQ instruments have low dwell times and allow to notably increase the number of transitions acquired within a run, there is still a limitation when dealing with thousands of contaminants that may be potentially

present in waters. Thus, the application of target LC-MS/MS methods is clearly insufficient to have a realistic and extensive overview of pollutants present in the samples. This is because in target LC-MS/MS methods, compounds other than the selected analytes are commonly ignored, even if they are present at high levels in the sample.

High resolution mass spectrometry (HRMS) transcends this major limitation of targeted MS/MS analysis. HRMS instruments (such as time-of-flight (TOF) and Orbitrap) provide high quality information by combining sensitive full spectrum data with high mass resolution and mass accuracy [15,16]. In theory, the presence of an unlimited number of compounds can be investigated, without requiring the pre-selection of analytes or even without having reference standards available. With the ever-improving technology of LC-MS systems, it is easy to overlook the equally important chromatographic aspect. However, an efficient chromatographic separation is essential to avoid or minimize matrix interferences, and to get reliable identifications. Ultra-high performance (pressure) liquid chromatography (UHPLC) has emerged as an innovative and powerful separation technique based on the use of columns containing stationary phase packings with particles size smaller than conventional HPLC [17]. It has led to evident improvements of chromatographic separations, an important and relevant aspect in LC-MS analysis. Shorter chromatographic run times and improved sensitivity are common advantages derived from the use of UHPLC, but some other aspects may also be considered, as highlighted in this review. This article discusses the application of LC-HRMS for the investigation of PIDs and metabolites/TPs in water samples, and the role that UHPLC can play within this field of analysis.

2.- HIGH RESOLUTION MASS SPECTROMETRY

HRMS circumvents the main drawback of targeted SRM analysis, i.e., the missing of non pre-selected compounds even if they are present at high concentrations in the samples. HRMS instruments (as TOF and Orbitrap) provide high quality data by combining sensitive full spectrum mass data with high mass resolution and mass accuracy. Since acquisition is not targeted, any ionisable compound in the sample can be, in principle, detected and investigated. Furthermore, hybrid instruments such as quadrupole-TOF (QTOF) and Linear Trap Quadrupole Orbitrap (LTQ-Orbitrap) offer additional information on compound confirmation and/or structure elucidation [18].

Several studies employ a post-targeted approach, which involves an initial data inspection, based on the use of the exact mass of known substances in a customized database. Hundreds of compounds can be investigated on the basis of their theoretical exact mass, compared with the accurate mass measurements, which greatly increase the reliability of identification [19]. Moreover, the presence of compounds initially not considered, such as new substances and TPs, can be investigated from full-scan accurate-mass data acquired at any time without the need of additional analysis [20–23]. This fact is advantageous, as in many occasions the samples may have already been discarded or the analytes degraded; therefore additional sample injections may not be possible. This retrospective analysis enables the screening to be further widened, by only reprocessing raw data. The main benefit of using HRMS is that it eschews the need for reference standards, as tentative identifications of suspect compounds can be made on the basis of the information provided by this technique. Obviously, reference standards are required for the ultimate confirmation, but they may be acquired in a final stage when solid well-founded evidence exists on the presence of the compound in the sample. In this way, laboratories do not need to acquire all reference standards before

131 analysis, with the subsequent problems of availability (e.g. TPs), costs and expiry dates
132 [24].

133 Non-target analysis may also be explored when using HRMS. A true "unbiased"
134 non-target screening, without any *a priori* information on the compounds to be detected
135 is an analytical challenge, as the process needs expertise, is complex, and is time-
136 consuming [15,25,26]. An intermediate situation between target and true non-target
137 analysis is the application of "biased" non-target approaches, where, for example, the
138 formation of "unknown" TPs from a given parent compound are computationally
139 predicted [27,28], or are tentatively identified by performing laboratory experiments
140 [22,29–32]. Other options, like searching for common fragments or mass defect filtering
141 are also feasible and have demonstrated their utility in investigating the presence of
142 related compounds. Here, the number of chemically meaningful structures, which can
143 be assigned to an unknown peak, is limited to structures showing a close relationship
144 with the parent compound [15].

145 Krauss *et al.* performed an overview on the state-of-the-art and future trends of
146 LC-high resolution MS applied to the environmental analysis of polar micropollutants
147 [15], showing illustrative systematic workflows for different approaches: quantitative
148 target analysis with reference standards, suspect screening without reference standards,
149 and non-target screening of unknowns (**Figure 1**).

150 After a compound (based on a suspect or non-target approach) is discovered, the
151 following step is to confirm its identity. Usually, this process involves the acquisition of
152 full product ion spectra after re-analysing the sample by MS/MS systems, i.e. hybrid
153 QTOF or LTQ-Orbitrap, in order to match the observed accurate-mass product ions
154 with the chemical structure of the suspect/candidate(s). In order to obtain fragmentation

155 information in a single run, some hybrid analyzers allow the acquisition of full scan
156 spectra with and without applying collision energy in a sequential fashion. Using this
157 acquisition mode, named as MS^E by Waters, or High Collision Dissociation (HCD) by
158 Thermo [33,34], two separate acquisition functions are sequentially measured in full
159 scan mode. The first one without applying collision energy in the cell (low energy
160 function, LE), obtaining a conventional full spectrum where intact (de)protonated
161 molecules/adducts are commonly observed, followed by a second one (high energy
162 function, HE) where a fixed or collision energy ramp is applied in order to induce ion
163 fragmentation. In this way, fragmentation information is obtained in advance for all
164 compounds in a single run without the need for re-injecting the sample in MS/MS
165 mode.

166 Despite its qualitative potential, HRMS typically shows lower sensitivity than
167 QqQ instruments operating in SRM mode, and quantitative LC-HRMS applications are
168 more limited. However, Orbitrap and the latest TOF instruments show improved
169 sensitivity and wider linear dynamic range, similar to that of QqQ, and this has
170 prompted their use for both quantification and identification/confirmation in a single run
171 [20,35,36].

172

173 **3.- CONTRIBUTIONS OF UHPLC**

174 The most important development in LC in recent years is UHPLC. This
175 technique uses chromatographic columns packed with particles with diameters below 2
176 μm, which significantly increase efficiency even at high mobile phase flow rate,
177 achieving faster separations [17,37]. Conventional HPLC runs can be relatively long,
178 particularly to avoid or minimize co-elution of matrix interferences that may lead to

179 difficulties in terms of identification. To this end, UHPLC can be of additional value.
180 First, it shortens the run time, and secondly it facilitates attaining sufficient
181 chromatographic resolution to minimize co-elution of compounds with close *m/z* values.
182 In addition, a remarkable increase in detectability can be reached as a consequence of
183 the narrower and higher peaks provided by this technique.

184 The combination of UHPLC with MS appears to be a suitable approach that
185 fulfils sensitivity, selectivity and peak-assignment certainty. However, due to the very
186 narrow peaks produced by UHPLC (commonly 1-6 seconds), coupling with MS devices
187 may be critical. For this reason, specific quadrupole-based instruments that show
188 improved acquisitions rates were launched for UHPLC hyphenation. The short dwell
189 times (low millisecond range) offered by modern QqQ instruments allow an easy
190 coupling to UHPLC due to the narrow chromatographic peaks obtained. Initially,
191 UHPLC found many applications in the field of pesticide residues, where a large
192 number of pesticides could easily be separated and measured by MS/MS in less than 10
193 minutes [38–40]. Obviously, UHPLC-MS/MS has also been applied in other fields,
194 including the quantitative determination of PIDs in water [14,41–43].

195 Aside from triple quadrupole (QqQ) mass analyzers, TOF instruments afford fast
196 full-spectral acquisition rates at good sensitivity and high mass-resolution (10,000-
197 40,000 FWHM depending on the instrument) with high mass accuracy (typically lower
198 than 5 ppm). The scan speed attainable by recent accurate-mass TOF analyzers falls in
199 the range of 10 to 100 scans/s, more than enough to follow the narrow chromatographic
200 peaks obtained under UHPLC separations, without compromising mass resolution and
201 sensitivity.

Modern TOF analyzers represent a promising alternative, particularly for screening purposes, to the well-established QqQ instruments, as they can deal with the large number of compounds to be searched in environmental research. The improved chromatographic resolution and detectability achieved by UHPLC separations has been particularly essential when using QTOF mass analyzers in MS^E acquisition mode. As fragmentation is promoted in HE function, without pre-selecting any precursor ion, recognizing which ions are fragment ions and which are not becomes mandatory to avoid spectral interferences that would complicate the identification process. To this aim, UHPLC has become a valuable tool for choosing ions obtained from the high energy function that perfectly co-elute with the intact (de)protonated molecule from the low energy function, and assigning them as potential fragment ions. In this way, an additional MS/MS acquisition can be avoided, speeding up the screening and confirmation steps following the MS^E approach.

In order to illustrate the benefits of UHPLC separations along this process, **Figure 2a** shows the low and high-energy mass spectra for a wastewater sample extract obtained after solid phase extraction (SPE). The LE mass spectrum (bottom) shows several abundant ions, which are expected to be the "precursor ions" of the fragments observed in the HE mass spectrum (top). In order to correlate the potential (de)protonated molecules with their fragment ions, extracted ion chromatograms (XICs) were obtained for all ions (at LE and HE) and chromatographic peak shapes and retention times were evaluated. As can be seen in **Figure 2b**, two co-eluting compounds appeared in this sample, but based on the improved chromatographic profile achieved by UHPLC their fragment ions could be easily differentiated. Thus, the fragment ions observed in HE with *m/z* 119 and 91 were assigned to the "precursor" *m/z* 192, while the ions *m/z* 207 and 159 were assigned to *m/z* 297.

A new type of HRMS analyzer, Orbitrap, was invented by Alexander Makarov in 2003 [44,45]. This device shows high mass resolution (>100,000 FWHM), high mass accuracy (<5ppm) and acceptable dynamic range (10^3). The main drawback is its scanning speed, which is inverse of mass resolution. For example, only one scan per second can be acquired when using a resolution of 100,000. This obviously affects the number of points per chromatographic peak and therefore its correct chromatographic peak shape when coupled to UHPLC where peak widths are only a few seconds. Oppositely, when a faster scanning speed is selected (e.g. 10 scans/s), resolution decreases dramatically (e.g. 10,000 FWHM). Thus, a compromise between achievable resolution and adequate chromatography must be found [46,47]. Nevertheless, several examples of the use of UHPLC columns combined with Orbitrap mass analyzers can be found [48–51]. Unfortunately, either the mass resolution used is not reported or the chromatographic peak widths estimated from the chromatograms shown are unacceptably large (20-60 s) for a genuine UHPLC separation. Apparently, only Pinhancos *et al.* [48] worked in pseudo-UHPLC conditions, reporting chromatographic peaks of around 10 s width. In summary, up to now, there are not sufficient data reported to support the efficient combination of UHPLC with Orbitrap in this field. Further improvements in the scan speed of these mass analyzers without significantly affecting the mass-resolving power are expected in the near future, which will make a successful coupling to true UHPLC separations possible.

4.- APPLICATIONS OF LC-HRMS TO THE INVESTIGATION OF PIDs IN WATERS

In this section, different approaches are presented for the investigation of PIDs in waters by LC-HRMS, including target, suspect and non-target screening, as well as the characterization of TPs of PIDs subjected to degradation under laboratory controlled conditions. **Tables 1** and **2** show a literature overview on LC-(Q)TOF MS and LC-(LTQ)Orbitrap MS applications, respectively.

4.1.- Screening of PIDs in water samples

LC-HRMS has extraordinary potential for screening PIDs in waters. The most efficient and rapid way is to perform target screening of a high number of compounds on the basis of large (in-house) databases. In this target approach, theoretical exact masses of analytes are extracted from full-spectrum acquisition data, reconstructing accurate-mass chromatograms, where the presence of analytes in the samples can be depicted as a chromatographic peak. Mass accuracy is critical for identification purposes. Normally, mass errors below 5 ppm are observed in routine analysis with the new instruments commercially available. The accurate mass of the (de)protonated molecule (on occasions adducts), the information on characteristic fragment ion(s), the isotopic pattern, and retention time matching with reference standards, enable the unambiguous identification of PIDs in environmental samples..

4.1.1. (Q)TOF MS applications

TOF and/or hybrid QTOF analyzers are most commonly used in this field. To the best of our knowledge, the first works dealing with the analysis of pharmaceuticals in the environment by LC-QTOF MS were reported in 2003. Marchese *et al.* [52] compared the potential of QqQ and QTOF for the LC-MS determination of 5 pharmaceuticals in river water samples. According to the authors, the selectivity was

much better on the QTOF system than on QqQ because of the high resolving power of the TOF analyser, permitting high-accuracy fragment ion selection and minimizing interferences from environmental matrices. In the same year, Ferrer *et al*. showed the benefits of the enhanced resolving power of LC-QTOF MS when investigating polar organic contaminants in complex environmental matrices, as isobaric interferences could be separated from the analyte signals [53-54]. After these pioneering works, the capability of (Q)TOF MS for screening, quantification and/or confirmation of pharmaceuticals in water samples has been regularly compared to that of LC-MS/MS with QqQ [11,55–57], which resulted in a general agreement on the strong and weak points of these techniques. The elevated mass resolution and selectivity of TOF instruments diminish the problem of isobaric interferences [55], making TOF MS highly useful not only for identification and confirmation purposes, but also to study metabolic routes and degradation pathways [57]. The main limitation of TOF instruments has traditionally been its lower sensitivity compared to QqQ instruments, and this makes the latter more appropriate for analysis at low ng L^{-1} analyte concentrations [11]. In terms of dynamic range and limits of detection (LODs), the quantitative performance of QqQ instruments still present better features than TOF MS [55].

Several reviews have been published in the last decade dealing with the use of LC-HRMS for the determination of PIDs in environmental analysis. The reading of these reviews is recommended for those researchers interested in this field [2,3,10,13,18,58–63].

QTOF MS has been used for the unequivocal confirmation of the identity of pharmaceuticals previously detected and quantified by LC-MS/MS in SRM mode using

297 either hybrid quadrupole linear ion trap (QTRAP) [64] or QqQ [65]. The possibility of
298 performing retrospective analysis has allowed the revision of recorded chromatograms
299 for new compounds, metabolites or TPs in the samples, increasing the scope of the
300 method along the monitoring programme [21,64]. LC-MS/MS and LC-(Q)TOF MS can
301 be seen as complementary techniques. On one hand, LC-MS/MS is the first choice for
302 quantification in pre-target analysis due to its good sensitivity and precision. On the
303 other hand, QTOF provides accurate mass measurements, being ideal for post-target
304 screening and confirmation. The most suitable strategy seems to be an automated
305 screening and identification by LC-(Q)TOF instruments, followed by quantification by
306 LC-MS/MS [65].

307 The use of (Q)TOF instruments for quantification of target analytes in
308 environmental samples has been rather limited; however in the last few years there has
309 been an increased use of quantitative analysis since the limitations of lower sensitivity
310 and linear dynamic range have mostly been solved. This has contributed to extending
311 the use of LC-(Q)TOF MS for quantitation of target pharmaceuticals [66,67] and drugs
312 [35] or alcohol biomarkers [68] in water.

313 The qualitative field is undoubtedly where HRMS can take full advantage of its
314 capabilities derived from sensitive accurate-mass full-spectrum acquisition. This offers
315 the possibility to investigate the presence of compounds once the sample analysis has
316 been performed and MS data acquired (i.e. without pre-selection of analytes), being
317 especially suitable for screening/identification of contaminants and confirmation of
318 presumed positive samples reported by other techniques.

319 LC-(Q)TOF MS has successfully been applied for the investigation of
320 pharmaceuticals belonging to different therapeutic classes in surface water [55,69,70],

sea water [67] and wastewater [55,66,71,72], and also for illicit drugs and their metabolites in wastewater [35,73]. As a consequence of the intrinsic characteristics of HRMS analyzers, their use enables screening for a large number of contaminants with high sensitivity within one run, with the obvious restrictions derived from the chromatographic and ionization processes in the LC-MS as well as from sample pre-treatment. LC-(Q)TOF MS instruments have been applied for comprehensive screening of many pollutants of different chemical families, including PIDs [36,64,73,74] in water samples. In some cases, LC-(Q)TOF MS has also been used as a complementary tool for target LC-QqQ MS based methods allowing the detection of analytes different to those selected in previous QqQ methods [75].

The easy reviewing step and the useful information provided by TOF MS gives high confidence to the identification of the compounds detected, even without reference standards being available. In these cases, tentative identification may be possible based on the presence of the (de)protonated molecule *i.e.* when a chromatographic peak is detected at its accurate-mass. Subsequently, the Collision Induced Dissociation (CID) fragments/product ions (or characteristic isotopic ions) are then evaluated [16]. For this purpose, different possibilities are available, such as comparing experimental MS(/MS) spectra or the main fragment ions with those reported in the literature (massbank, METLIN public library), or justifying the accurate-mass fragments taking into account the structure of the molecule. For the latter, the use of specialized software (for example, MassFragment) can be of help.

As an example, **Figure 3** illustrates the detection and tentative identification of the veterinary anthelminthic drug levamisole, recently also recognized as an adulterant in cocaine, in an effluent wastewater by using UHPLC-QTOF MS, operating in MS^E

mode. The protonated molecule of levamisole was detected in the LE function (**Figure 3a, bottom**), with 3.4 ppm mass error. As the reference standard was not available at the laboratory, the accurate mass of the fragment ions was justified using the MassFragment software (Waters) in order to advance towards a tentative identification. To minimize spectral interferences that would complicate the identification process, recognizing which ions are and which are not fragments became mandatory. To this end, UHPLC was valuable for choosing perfectly co-eluting ions (see chromatographic peak at 3.86 min, **Figure 3b**). The elemental composition for the two fragments detected in the HE function (**Figure 3a, top**) (*m/z* 178.0689 and 123.0267) was calculated, obtaining errors lower than 1 ppm in relation to the theoretical exact masses predicted. In addition to all information available, tentative identification of levamisole was supported by the MS/MS product ions reported in the literature, where the two fragments (*m/z* 178 and 123) observed had been previously reported using an LTQ-Orbitrap with a resolution of 7,500 [76]. The final acquisition of the reference standard allowed the ultimate confirmation of this compound in the wastewater sample.

The interesting possibilities offered by hybrid QTOF MS, including the option of working in MS^E mode, have led to a drastic increase in the number of contaminants being included in the search, with more than one thousand compounds included in some cases [16,75] by applying suspect screening approaches. This opens up a new scenario in screening strategies, favoring a wider and more realistic overview when investigating organic contaminants or residues in different applied fields.

4.1.2. (LTQ) Orbitrap MS applications

The development of the Orbitrap analyser in 2003 have spurred researchers to explore its potential for accurate mass screening, identification and quantification of

illicit drugs and pharmaceuticals with potential for abuse in wastewaters [77,78], surface waters [79] and drinking water [48,79] with good recoveries and RSD values. Similarly to QTOF, Orbitrap has also been used for the confirmation of PIDs previously found by other techniques [80] and for wide-scope target screening. A multiresidue method has been developed for target and suspect screening (*i.e.* compounds which did not form a part of the originally target screening list but were expected to be present in samples) of more than 180 organic contaminants, including pharmaceuticals, in lake sediments [81]. Orbitrap also enables a retrospective analysis of the full-scan data, without the need for additional analysis [20,48,49]. Assessing steps included retention time prediction, isotope patterns, ionization efficiency and fragmentation pattern. Product ions of the MS/MS spectrum of a suspect compound were compared with the spectrum of a reference standard or with a predicted fragmentation pattern. This allowed the tentative identification of transformation products of triclosan and triclocarban. In another work, 42 groundwater samples were screened for 249 known chemicals and 386 unidentified chemicals (i.e. accurate masses and retention times) [82]. Nearly 400 chemicals were observed in the samples, of which 82 were known and more than 300 were of unknown identity.

Emke *et al* [83] applied enantiomeric profiling in verifying sources of MDMA and amphetamine present in Dutch wastewater. The results from Orbitrap analysis showed that MDMA was usually present in wastewater due to its consumption as MDMA enriched with the R(−)-enantiomer. Therefore, the high mass loads of racemic MDMA detected during a sampling campaign in the Netherlands seemed to proceed from the direct disposal of unused MDMA, possibly as the result of a police raid at a nearby illegal production facility. HPLC separation was successfully performed using

393 Chiral-CBH column, 100 × 2 mm, 5 μm. Unfortunately, this approach is not yet feasible
394 for UHPLC, due to the lack of chiral sub-2μm stationary phases.

395 **4.2.- Screening of metabolites/TPs of PIDs in water samples**

396 After human or animal consumption, PIDs or veterinary drugs may be excreted
397 in the unchanged form, and/or as free or conjugated metabolites. Some of these
398 compounds are not completely removed during wastewater treatments and may finally
399 reach surface water and even ground water. Consequently, several authors have
400 included the main metabolites/TPs among the compounds monitored [21], but only a
401 few works have focused the screening on a notable number of TPs in aquatic
402 compartments. Different approaches have been proposed in the literature for the
403 investigation of metabolites/TPs, as shown below

404 **4.2.1. (Q)TOF MS applications**

405 Hernández *et al.* [21] studied the presence of 160 pharmaceutical metabolites in
406 wastewater samples that had been previously analysed only for parent compounds using
407 LC-QTOF MS (MS^E mode). The compounds investigated were selected after an
408 extensive search for TPs/metabolites reported in the literature and from lists of
409 commercially available reference standards. In a first step, the presence of the
410 metabolite ion (typically $[M+H]^+$ or $[M-H]^-$) at its accurate mass was evaluated.
411 Different strategies were applied in the tentative identification: a) when the reference
412 standard of the parent compound was available, and therefore accurate-mass fragment
413 ions of the pharmaceutical were known, the accurate-mass fragments of the suspected
414 metabolite were predicted, taking into account the structural differences between both
415 molecules; b) when fragmentation information on the parent was not available, chemical

structures of the accurate mass fragments were proposed using specialized software (MassFragment). In this case, information reported in the literature on product ions for the suspect metabolite or for the parent compound was essential (either based on nominal or accurate mass measurements). Following this strategy, several metabolites, such as clopidogrel carboxylic acid and N-desmethyl clarithromycin, were tentatively identified and subsequently confirmed after acquisition of the reference standards.

In recent work, Boix *et al.* [23] investigated the presence of 24 omeprazole metabolites (identified in previous excretion tests performed with healthy volunteers) in surface water and effluent wastewater by both UHPLC-QTOF MS and UHPLC-MS/MS. Up to nine metabolites were detected, the most frequent being an omeprazole isomer, which presented the same exact mass (*m/z* 346.1225), and also shared a major common fragment at *m/z* 198.0589. On the contrary, parent omeprazole was never detected in any of the water samples. The authors emphasized that monitoring the presence of omeprazole in the aquatic environment should be focused on the main metabolites as well as some of the major TPs identified in laboratory experiments, instead of the parent compound.

4.2.2. (LTQ) Orbitrap MS applications

Another strategy makes use of target lists of plausible TPs assembled using the University of Minnesota Pathway Prediction System (UM-PPS) for the computer-aided prediction of products of microbial metabolism and of metabolites/TPs reported in the literature. In this way, Kern *et al.* screened for potential biotransformation product structures of pharmaceuticals, in sludge-seeded batch reactors [27] or surface water [28] using HRMS (LTQ Orbitrap). These authors developed an identification procedure to efficiently examine a large number of proposed TPs for tentative identification without

reference standards [28]. The procedure was based on a series of steps, with each step reducing the number of potential false positive findings: a) mass error of the measurements was <5 ppm for all compounds; b) positive peak findings from the extracted chromatogram were discarded if the peak intensities in the extracted chromatogram were $<10^5$ (arbitrary units), or if a peak at similar retention and similar intensity was found in the blank sample; c) experimental gradient retention times of the target TPs were tested against an upper plausible limit; d) isotope pattern of the HRMS spectra was evaluated to confirm a target molecular formula; e) peaks with reasonable retention times and isotopic patterns were further checked for plausibility of a proposed TP in positive or negative ionization mode (compounds containing amino but no acidic groups, for instance, were considered to be analyzable in positive ionization mode only, whereas strong acids or sulfonates were expected to be detectable in negative mode); f) to further confirm the presence of the target compounds in the samples, HR-MS/MS at higher energy collision dissociation settings was used. Altogether, 19 TPs were identified [28], including some rarely reported TPs, such as biotransformation products of the pharmaceuticals venlafaxine and verapamil.

In another work, Helbling *et al.* [84] performed a preliminary identification of TPs formed within a biotransformation test system using HRMS with two independent post-acquisition data processing approaches: a) target screening at the exact masses of plausible TPs proposed by the UM-PPS for 12 selected parent compounds; b) screening for compound masses formed during biodegradation experiments based on background subtraction and exact mass filtering. With this strategy, previously unreported microbial TPs were tentatively identified for several pharmaceuticals.

463 Hollender *et al*. [85] evaluated the removal efficiency of 220 micropollutants at
464 a municipal WWTP upgraded with post-ozonation treatment followed by sand filtration.
465 Afterwards, a screening was conducted for known ozonation transformation products of
466 diclofenac, carbamazepine, and sulfamethoxazole as well as possible oxidation products
467 of benzotriazole and atenolol by LC-HRMS (Orbitrap).

468

469 **4.3. – Degradation/transformation of PIDs under laboratory-controlled conditions:**
470 **Elucidation of transformation products.**

471 Once PIDs enter the wastewater treatment process, they can either be completely
472 mineralized, transformed into metabolites/TPs, adsorbed onto the solid phase (e.g.
473 sewage sludge), or pass through the WWTP unaltered. Similarly, once released into the
474 environment via the discharge of treated wastewater, PIDs are subjected to different
475 processes, such as hydrolysis and photo-degradation by natural sunlight. In order to
476 evaluate the fate of PIDs in the water cycle, the removal of the parent compounds and
477 metabolites in the treatment processes must be taken into account, but also the possible
478 formation of TPs. The identification of TPs is complicated but important, not only to
479 provide a comprehensive risk assessment on drug residues in the environment (TPs can
480 be equally or even more toxic and dangerous than the parent pollutants), but also for
481 designing improved treatment technologies for organic contaminants. From the different
482 possibilities currently available, LC-HRMS has gained popularity and has become one
483 of the preferred techniques for elucidating TPs in the environment. Several reviews
484 have been published focused on the capabilities and potential of MS techniques to
485 determine pharmaceutical degradation products [4,5,86], although very little was
486 included on the use of HRMS, surely due to the time-frame reviewed.

The process of identification/elucidation of unknown TPs is arduous, and commonly starts with laboratory experiments to study the formation of TPs under controlled conditions simulating those that occur in the environment. The most feasible strategy relies on MS measurement of their accurate molecular mass and subsequent determination of the empirical formula, considering low mass errors (e.g. <5 ppm). The maximum and minimum parameters considered in the elemental composition calculation may be restricted on the basis of the structure of the parent compound, and the number of halogen atoms selected according to the observed isotopic pattern. Further accurate MS/MS measurement and observation of characteristic fragmentation pattern of the precursor ion provide valuable information for TPs identification. In some cases, HRMS alone is insufficient to identify the exact position of transformation, to differentiate isomers, or to provide the precise structure of transformation products. Thus, LC-HRMS can be combined with other techniques, such as nuclear magnetic resonance (NMR), or hydrogen/deuterium-exchange (H/D-exchange) for the final characterization of TP candidates of PIDs [87]. Although these techniques have little applicability in the environmental field as high analyte concentrations are required for measurement, they might be useful in laboratory experiments, where higher concentrations can be used.

Considerable time and effort is required to interrogate the intensive LC-HRMS data to identify unknowns. However, normally the TPs are not complete unknowns as they hold a certain structural relationship with their parent compound. Some works only focus the research on the peaks visible in the Total Ion Chromatogram (TIC), and this implies that other not visible peaks, which may correspond to TPs, would be lost. To avoid this problem, detection can be improved by applying spectral and chromatographic search algorithms, such as MetaboLynx (Waters),

512 Analyst/MetaboliteID (Applied Biosystems), Xcalibur/MetWorks (Thermo Fisher) or
513 MassHunter (Agilent). Such algorithms search XICs for expected metabolites based on
514 predicted or unpredicted molecular changes relative to the parent compound, and thus
515 aid in the detection and identification of TPs, particularly those buried in spectral noise.
516 The software compares mass spectral chromatograms of a control-sample versus
517 analyte-sample (i.e. metabolised, stressed or treated sample), and automates the
518 detection, identification and reporting of metabolites [88-90].

519 PIDs can be degraded in natural environments or in WWTP by both abiotic
520 (advanced oxidation, photolysis and hydrolysis) or biotic (biodegradation) processes
521 [87]. Different degradation/transformation experiments have been performed under
522 laboratory-controlled conditions, trying to simulate the probable processes occurring in
523 the aquatic environment and/or in WWTPs. Some representative examples are discussed
524 below.

525 **4.3.1. – Hydrolysis experiments**

526 Hydrolysis is a process occurring naturally in the environment. Pérez-Parada *et*
527 *al.* [91] performed solely hydrolysis degradation experiments on amoxicillin in both
528 alkaline and acidic aqueous media. Four compounds were identified as main TPs by
529 LC-QTOF MS. Data showed that although this antibiotic is not present as such in
530 environmental samples, different TPs might occur. However, most papers reported on
531 hydrolysis, studied this process together with other degradation experiments [22,29,30]
532 .

533 **4.3.2. – Photodegradation experiments**

534 Photodegradation by sunlight may constitute a relevant natural attenuation
535 process for pollutant residues that have been discharged from wastewater treatment

536 facilities. It is considered one of the most important processes responsible for the
537 degradation of contaminants in environmental systems [92-94]. Understanding the
538 degradation pathways is essential to predict the fate and environmental impact of
539 organic contaminants in waters. Photolytic reactions lead to multiple reaction products
540 that may be more toxic than the parent compound [95], retain the properties of the
541 parent compound (for example, antibiotic activity) [96], or lose antimicrobial activity
542 and/or toxicity [95].

543 LC-TOF MS has been used to elucidate the photo-TPs of several
544 pharmaceuticals in water exposed to direct solar irradiation. Analytes studied included
545 sulfa-drugs containing six-membered heterocyclic substituents (*i.e.* sulfamethazine,
546 sulfamerazine, sulfadiazine, sulfachloropyridazine, and sulfadimethoxine) [88] and the
547 non-steroidal anti-inflammatory drug diclofenac [97]. In the latter case, the authors
548 combined LC-TOF MS with GC-MS to detect and identify a complete range of TPs, as
549 both techniques provided complementary information. Similarly, the photochemical fate
550 of the prodrug enalapril and its active metabolite enalaprilat [98], as well as sildenafil
551 (Viagra®) and its human metabolite N-demethylsildenafil [90], have been investigated
552 in aqueous media under the influence a sunlight simulator. Accurate mass
553 measurements were combined with complementary data sets from distinct instruments:
554 QTRAP mass spectrometer exploiting its MS^3 capabilities [98] or LC-APCI-QqQ MS
555 and H/D-exchange experiments [90]. Plausible chemical structures were finally
556 postulated for several photo-TPs

557 The aqueous environmental fate of pharmaceuticals have also been studied using
558 LTQ Orbitrap MS. Calza *et al.* [31,32] investigated the degradation of the antibiotics
559 lincomycin and clarithromycin, and the antiepileptic drug carbamazepine, whereas
560 Zonja *et al.* [51] evaluated the phototransformation of the antiviral zanamivir in surface

561 waters using simulated and natural sunlight. Several species were formed under
562 irradiation, and were characterized by evaluating MS and MS^n spectra. **Figure 4** shows
563 an MS^2 spectrum of a dihydroxyl derivative of lincomycin. The presence of a product
564 ion at *m/z* 407.1836, due to the loss of methanol from the N-hydroxymethyl moiety,
565 combined with *m/z* 142.1219, is indicative of the existence of a hydroxyl group in the
566 ortho position, and this allowed the tentative structure elucidation. In the end, eight
567 transformation products from carbamazepine, three from clarithromycin, and two from
568 lincomycin were detected in natural river water [99].

569 Illicit drugs have also been subjected to degradation experiments, although less
570 studied than pharmaceuticals. Postigo *et al.* described the transformation of the
571 synthetic opioid methadone [100] and cocaine [101] in distilled water and simulated
572 effluent wastewater after natural solar irradiation and two solar photocatalytic processes:
573 heterogeneous photocatalysis with titanium dioxide and homogeneous photocatalysis by
574 photo-Fenton. Phototransformation intermediates generated during each treatment were
575 investigated and characterized by UHPLC-QTOF MS/MS. Identity confirmation was
576 possible for some of them with the analysis of commercially available analytical
577 standards.

578 The group of Hernández *et al.* performed laboratory controlled degradation
579 experiments for several PIDs in surface water. Hydrolysis, photo-degradation under
580 ultraviolet (UV) irradiation and simulated sunlight, and also chlorination experiments,
581 were carried out. The TPs formed were investigated by LC–QTOF under MS^E mode.
582 Studies were directed towards the degradation of cocaine and its main metabolite
583 benzoylecgonine [30], THC-COOH (the main metabolite of cannabis and commonly
584 selected as biomarker for the investigation of its consumption) [29] and omeprazole
585 (one of the most consumed pharmaceuticals world-wide for treating gastric diseases)

[22]. 6 TPs from cocaine and 10 TPs from benzoylecgonine were tentatively identified. Regarding THC-COOH, one hydrolysis, 8 chlorination, 3 ultraviolet photo-degradation and 7 sunlight photo-degradation TPs were tentatively identified. In addition, 17 TPs were identified for omeprazole. In a subsequent step, influent and effluent sewage water, and surface water samples, were retrospectively screened using UHPLC-QTOF MS and UHPLC–QqQ MS for the TPs previously identified in the laboratory experiments. In addition to some known compounds, several TPs that had not been reported in the literature yet were found in the samples, illustrating the usefulness of the degradation experiments performed.

As an illustrative example, **Figure 5** shows the detection and identification of an omeprazole photo-TP (OTP 5) after 14 days of solar irradiation of a spiked surface water. A chromatographic peak was observed at 5.68 min (**Figure 5a**, **bottom**), while it was absent in the blank control sample **(Figure 5a, top)**. According to its accurate mass (*m/z* 330.1284, **Figure 5b, bottom**), the elemental composition of the protonated molecule was assigned as $C_{17}H_{20}N_3O_2S^+$ (+2.4 ppm mass error), which would imply the loss of one oxygen atom from the omeprazole molecule. The fragment ions at *m/z* 297.1497 ($C_{17}H_{19}N_3O_2^{+\bullet}$, 6.7 ppm mass error) and *m/z* 282.1240 ($C_{16}H_{16}N_3O_2^{+}$, -1.1 ppm) (**Figure 5b, top**), were assigned to thiol [•SH] and subsequent methyl [•CH_3] radical losses, respectively, and were related with the presence of a sulfide group [23]. Thus, an oxygen loss from the sulfur atom in the original omeprazole structure was suggested for this TP. This compound, identified in laboratory experiments, was later found in the water samples analyzed.

4.3.3. – Advanced oxidation processes (AOP) experiments

Great efforts have been made in recent years to develop additional (or alternative) processes for wastewater treatment. An interesting review on removal of

611 pharmaceuticals from water has been recently published, and is recommended for
612 additional information on this issue [102]. Although ozonation leads to the elimination
613 of many organic compounds in aqueous solution, this is not necessarily accompanied by
614 total mineralization [103]. In most cases, degradation by-products generated in the
615 process persist after the parent compounds have been totally eliminated. Recently, it has
616 been reported that ozonation may release oxidation intermediates with enhanced toxicity
617 for aquatic life [104,105]. This fact highlights the need to characterize reaction mixtures
618 in order to identify persistent and possibly toxic compounds.

619 Gómez-Ramos *et al.* [105] identified six TPs using LC-QTOF MS after
620 ozonation of the antibiotic sulfamethoxazole under different operational conditions. In a
621 recent study [50], several illicit drugs were subjected to ozonation to estimate their
622 removal as a function of ozone dose and to identify the significant oxidation TPs. Once
623 the potential TPs were identified by LC- LTQ-Orbitrap, their structures were elucidated
624 by carrying out $HRMS^n$ experiments (up to MS^4).

625 **4.3.4. – Biodegradation experiments**

626 The use of membrane bioreactor (MBR) technology for wastewater treatment
627 presents advantages compared to conventional activated sludge (CAS) process, such as
628 enhanced biological performance, complete retention of solids, and smaller footprint
629 [106].

630 To date, a number of abiotic PID degradation products have been identified, but
631 published studies dealing with the structural elucidation of these degradates resulting
632 from microbial transformation are still scarce. UHPLC-QTOF MS has been used for
633 screening and structural elucidation of biodegradation products, previously generated in
634 small-scale laboratory batch reactors, of the β-blocker atenolol and the hypoglyaemic

635 agent glibenclamide [107], the antimicrobial trimethoprim [89] and the analgesic
636 diclofenac [87]. In some cases, QTOF MS has been combined with multiple-stage
637 fragmentation studies and H/D-exchange experiments using IT-MS for final elucidation
638 [89] or with HPLC-QqLIT MS for confirmation of bio-TPs in wastewater samples
639 [107]. In the case of diclofenac, pre-processing based on isotopic cluster analysis was
640 performed, since the parent compound contained two chlorine atoms in its chemical
641 structure. In this way, the number of resulting peaks could be reduced to only those
642 attributed to chlorinated diclofenac bio-TPs [87].

643 Helbling *et al.* [84] used HRMS Orbitrap data from a single bioreactor to
644 identify compound masses formed during the biotransformation experiment. For each
645 sample, a two-dimensional matrix of masses (*m/z vs* intensity) was found and their
646 corresponding intensities were extracted. A script in Visual Basic was written that
647 compared the mass-intensity matrices between t= 0 (sample withdrawn immediately
648 after spiking with the compound of interest) and each t> 0 (samples withdrawn from the
649 reactors at time points), and used a series of mass filters to reduce the number of
650 extracted masses to a list of masses of candidate TPs. Candidate TP masses were further
651 confirmed or rejected following manual inspection of MS spectra for the relative
652 abundance of ^{13}C and/or ^{37}Cl monoisotopic masses and/or adduct masses. Additionally,
653 data-dependent MS/MS acquisitions were triggered when peaks were detected in full-
654 scan at the exact masses of any parent compound or candidate TP. Comparison of
655 MS/MS fragments between each parent compound and TP further confirmed or rejected
656 each candidate mass as an actual TP. Following this strategy, a dehydrogenation
657 product of bezafibrate (not predicted by UM-PPS) was tentatively identified.

Degradation of sulfamethazine by the white-rot fungus *Trametes versicolor* has been also assessed. Four degradation intermediates produced by fungal cultures or purified laccase were identified using UHPLC–QTOF MS [108].

4.4.- Non-target analysis of water samples

4.4.1. – Unbiased non-target analysis

The fact that HRMS provides full-spectrum acquisitions at accurate masses opens the possibility to investigate non-target compounds in environmental samples. A genuine non-target analysis pursues the investigation (*i.e.* detection and identification/elucidation) of unknowns, which means that the analyst does not have any information on the compounds to be investigated, *i.e.* an unbiased analysis is pursued. The term "unknown" does not necessarily mean that the compound discovered in the analysis is new or not previously reported. It may be a very well-known compound that was not specifically searched in analysis, and therefore was treated as an unknown [109].

It was early recognized that non-target analysis in environmental waters using LC-HRMS is a challenging task and successful identification of non-targets relies heavily on the (free) availability of chemical databases for finding candidates [25]. In fact, two unidentified components in the pioneer work reported by Ibáñez *et al.* [25] were subsequently characterized thanks to the use of the commercial chemical database SciFinder® which allowed the candidate chemical structures to be found [110].

A non-target analysis would require a visual inspection of the TIC to find potential components that might be present in the sample. Following this idea, Terzic *et al.*[111] developed a comprehensive analytical procedure based on UHPLC-QTOF MS for the identification of non-target polar contaminants in aquatic sediments from a small

682 water course highly influenced by wastewater discharges from pharmaceutical industry.
683 The chromatograms, recorded in TIC mode, were systematically examined by manually
684 generating mass spectra of each individual chromatographic peak. Only major peaks
685 (>10% of the full scale intensity) were subjected to the identification process as non-
686 target analytes. The first identification step was the calculation of the possible elemental
687 composition of the (de)protonated molecules from the mass spectra recorded, followed
688 by a database search (using Chemspider, Merck Index and European chemical
689 Substances Information System (ESIS), and an in-house database) for possible
690 candidate compounds. For some contaminants, for which the reference standard was
691 available, the information on accurate mass together with information on retention time
692 was sufficient for a reliable identification. For unknown compounds, the ultimate
693 assignment of their identity was achieved by MS/MS experiments, followed by
694 comparing the observed fragmentation patterns with the main product ions expected for
695 the selected candidates. Different pharmaceuticals were successfully identified and
696 confirmed with reference compounds. However, eight prominent peaks remained
697 unidentified.

698 One of the limitations of non-target analysis is that compounds of low-
699 abundance may not be apparent by visual inspection or, on the contrary, intense peaks
700 may not necessarily be associated to a single component or to “relevant” organic
701 contaminants. Powerful software with chromatographic peak deconvolution capabilities
702 is required to detect multiple components and to produce pure spectra for each
703 individual component. The non-target analysis workflow commonly starts with
704 automated peak detection by exact mass filtering from the chromatographic run,
705 followed by assignment of an elemental formula to the exact mass of interest, and a
706 subsequent search of plausible structures in available chemical databases for the

determined elemental formula [15]. This is a laborious, difficult and time-consuming task when the unknown compounds are present at trace levels in complex-matrix samples.

4.4.2. – Biased non-target analysis

An intermediate situation between target and non-target analysis is what some authors call a semi-non target approach, based, for example, on the use of a chlorine mass-filter technique in accurate mass and high resolution systems [112]. Following this strategy, that only applies to the investigation of chlorine-containing compounds, the antidepressant lamotrigine and its major metabolite (2-N-glucuronide) were identified in environmental water samples [112].

In non-target analysis, the use of large mass spectra libraries can notably facilitate the identification of unknowns. The main difficulty is the lack of LC-MS standardized libraries, as a consequence of the differences in the ionization efficiency between the existing interfaces together with the variability in the results depending on the mobile phase composition or the cone voltage applied. Under these circumstances, it is more convenient to build home-made libraries to simplify the searching. Obviously, the higher the number of compounds included in the library, the wider the possibilities to detect as many contaminants as possible in the samples. With the software currently available, it is possible to build both empirical and/or theoretical libraries [16,113,114]. Home-made empirical libraries offer the possibility to include fragmentation and retention time information, which greatly aid the identification process. However, commonly not many compounds are included, due to the need to inject reference standards. Instead, theoretical mass spectra libraries, based on a database with solely the molecular formulae, can easily be built including a large number of compounds. Díaz *et*

731 *al*. [16,113] combined UHPLC-QTOF MS (MS^E) with specialized software
732 (ChromaLynx^{TM}) for non-target screening of environmental water and wastewater.
733 After chromatographic peak deconvolution, the software discriminated ions coming
734 from organic compounds present in the sample from background ions. In a second step,
735 a library search was performed to match the experimental deconvoluted mass spectra
736 with the existing entries in the available libraries (theoretical and empirical). Finally, the
737 formula from the library hit was submitted to an elemental composition calculator and
738 the most intense ions were either confirmed or rejected based on accurate mass criteria
739 and isotopic pattern.

740 In a similar way, Gómez-Ramos *et al*. [115] proposed a systematic strategy to
741 simplify the identification of organic contaminants TPs, based on characteristic
742 fragmentation undergone by the parent compound during MS/MS fragmentation, and on
743 the relationship with the transformations experimented by these chemicals in the
744 environment or during water treatment processes. A database containing accurate-mass
745 information of 147 compounds (including pharmaceuticals, drugs and some relevant
746 metabolites) and their main fragments generated by MS/MS was created using LC–
747 QTOF MS/MS. The developed database was applied for the tentative identification of
748 TPs and related unexpected compounds in eight wastewater effluent samples. The
749 approach comprised three stages: (a) automatic screening, consisting on extraction of
750 compounds using “molecular feature extraction” algorithm and database search, (b)
751 identification of possible TPs (those reported compounds which yielded a good match
752 in accurate mass but presented different retention time were initially considered as
753 potential TPs or fragments of potential TPs), and (c) confirmation by MS/MS analysis
754 of the TPs tentatively identified in the previous step. Eight degradation products, from
755 the pharmaceuticals acetaminophen, amoxicillin, carbamazepine, erythromycin and

azithromycin were tentatively identified. Three of them were confirmed by analysis of the corresponding analytical standards.

4.4.3. – The "All In One" approach

In recent years, there has been a move towards the "All In One" approach, which consists on the combination of suspect and non-target screening with (quantitative) target analysis. Thus, LC–QTOF MS has been proposed for simultaneous quantitative screening of target analytes and qualitative analysis of non-target compounds [116]. For 10 target compounds, the use of specialized software allowed processing mass spectral data using LC retention time, accurate mass (error) and spectral purity score. Satisfactory results were obtained in terms of sensitivity and linearity. A second identification approach was presented based on library searching for compounds not included in the analytical method as target, but which were present in a commercial accurate-mass MS/MS library (including approximately 1,200 pharmaceuticals and personal care products). The spectra acquired were automatically searched by spectral comparison using the MS/MS library to assist in compound identification. Following this, ketorolac, trazodone, fluconazole, metformin and venlafaxine could be identified in river water samples. Furthermore other compounds not included in the library were also identified by screening the peaks of highest intensity in the samples and by analysis of the full scan TOF MS, isotope pattern and MS/MS spectra. This was the case of the histaminergic loratadine.

Nurmi *et al.* [117] evaluated the performance of UHPLC-TOF MS in the identification of emerging contaminants in spiked wastewater, using target and non-target analysis. The method was satisfactory for target analysis with information about the retention times. For those compounds lacking the retention time (suspect screening),

780 a four-stage process for identification was developed: the number of candidate
781 compounds was reduced by using the accurate mass of selected compounds in two steps
782 (30 mDa nw-XIC (stage 1) and ± 5 mDa mass error (stage 2)), structure–property
783 relationships (i.e. retention time prediction (stage 3)) and isotope patterns of the
784 analytes (stage 4). Non-target analysis was tested by applying a theoretical mass spectra
785 library for a wastewater sample spiked with six pharmaceuticals. The results showed a
786 high number of false identifications. In addition, manual processing of the data was
787 considered laborious and ineffective. Finally, the target analysis was applied to a real
788 wastewater sample. The analysis revealed the presence of six compounds that were
789 afterwards confirmed with reference standards. Three psycholeptics (nordiazepam,
790 oxazepam and temazepam) could be tentatively identified.

791 Hogenboom *et al.* applied similar approaches but using a LTQ- Orbitrap mass
792 spectrometer [118]. Full-scan accurate mass measurements were compared with
793 theoretical exact masses of known environmental microcontaminants and/or with their
794 self-created accurate mass MS and MS^n database, containing about 3,000 water
795 pollutants. Database included, amongst others, the theoretical mass, retention time,
796 retention time relative to two internal standards and elemental composition of product
797 ions. For accurate masses not found in the mass database, a (possible) elemental
798 composition was proposed, and searched in databases, such as Chemfinder or
799 Chemspider, to find out whether the unknown compound was ever patented, studied or
800 commercialized. MS^n measurements were performed to obtain information on the
801 fragment ions generated in the LIT (nominal mass of product ions) within the same
802 analysis. The structures found in the libraries were evaluated based on the fragmentation
803 patterns observed in the simultaneously acquired product-ion spectra. Confirmation of
804 the identity was done by comparing the retention time and fragmentation pattern to that

805 of a reference standard. If no information on the reference standard was available, the
806 theoretical log Kow was calculated based on the proposed structure and compared to the
807 retention time of the compound detected.

808 Very recently, Schymanski *et al.* [119] developed and applied a three-fold
809 approach: 1) (Semi)quantitative target analysis for 364 target compounds; 2) Suspect
810 screening, demonstrated for sulfur-containing surfactants (evidence used to support (or
811 reject) the tentative suspect identification included expected retention time behavior and
812 interpretation of the MS/MS spectra); 3) Non-target screening, to perform a
813 comprehensive characterization of polar compounds in wastewater effluents. A non-
814 target mass list for each sample was compiled with the enviMass software. Isotope and
815 adduct grouping of these non-target masses was subsequently performed using the
816 "non-target" R package. Finally, non-target identification was performed on selected
817 masses from the top 30 most intense peaks. The program system MOLGEN-MS/MS
818 (MOLecular structure GENeration) was used to calculate molecular formulas from the
819 exact mass and isotope patterns from the MS and MS/MS fragmentation information, if
820 available. MetFusion was used to perform parallel searches of compound databases and
821 spectral libraries and perform *in silico* fragmentation of the candidate structures. The
822 number of references per compound, retrieved from Chemspider, was also used to rank
823 candidates.

824 Systematic strategies with automated approaches are required to filter the
825 suspect compounds to be searched and select "relevant" peaks on which the
826 identification efforts should focus. Hug *et al.* [26] established a screening procedure
827 based on LC–HRMS to detect site-specific, suspected and formerly unknown
828 contaminants in a wastewater treatment plant effluent. Firstly, the effluent was screened

for the 98 target compounds using known retention time, exact mass and fragment ions. Fifteen target compounds were detected and quantified, among them seven pharmaceuticals. Secondly, accurate mass ion chromatograms and peak lists were generated from full scan data using MZmine. For suspect screening, an initial list of 2,160 site-specific and reported water contaminants was reduced to those amenable to LC–HRMS. Thus, only suspects containing at least one atom of nitrogen, phosphorus, oxygen, sulfur or any metal(loid) were considered ionizable by ESI. After searching MZmine peak lists for the exact masses of suspects (only those with intensity higher than 10^5 a.u.), presumably false positive detections were stepwise excluded by retention time prediction, the evaluation of isotope patterns, ionization behavior, and HRMS/MS spectra. Finally, in non-target analysis, masses for identification were selected using the R package "nontarget" based on distinctive isotope patterns (containing ^{37}Cl, ^{81}Br, ^{34}S or ^{15}N isotope peaks) and intensity (>10^6 a.u.). For the remaining masses, all possible formulae were calculated and checked using the Seven Golden Rules reducing the number to one or two molecular formulae, which were searched in the Chemspider database, generating candidate lists for each formula. Only candidates with >10 data sources were considered of commercial importance and necessitated an automated HRMS/MS evaluation using MetFrag software. To further confirm tentative identifications of compounds, deuterium exchange experiments were also conducted. Six suspected and five non-target chemicals were identified, of which two have not been previously reported as environmental pollutants. However, another five non-target compounds could not be confirmed by the reference standard of the most likely candidate structure.

5. CONCLUSIONS AND FUTURE TRENDS

HRMS provides high resolution, accurate mass and high full-scan sensitivity and selectivity, making it very attractive for both target and non-target screening. Other advantages are the possibility of performing retrospective data examination as well as tentative identification of compounds when reference standards are unavailable. HRMS is also very powerful in the identification of degradation/transformation products in laboratory experiments and the elucidation of unknown compounds.

Recent developments in HRMS have allowed the improvement of analytical methodologies applied in environmental mass spectrometry. The search for the "All-in-One" method and instrument will still continue in the coming years, as combining all desired features in just one method/instrument is an exciting issue: qualitative and quantitative analysis, with possibilities for structural elucidation of unknowns. Some recent configurations appearing in the market in the last few years (Triple TOF from AB Sciex, Xevo G2 QTOF from Waters, Q Exactive from Thermo, among others) are trying to meet this purpose: high sensitivity and selectivity, accurate mass, sufficient fragmentation to provide information on ion fragments, and satisfactory linear dynamic range. In the near future, a rapid growth of HRMS applications will surely occur, not only in environmental research but also in other fields like food-safety, toxicology and doping control analysis.

UHPLC has become a very popular technique, replacing HPLC separations in most recently developed methods. It has been successfully coupled to MS(/MS) analyzers working at nominal mass and also to HR TOFMS analyzers, with evident advantages deriving from its superior performance: short chromatographic runs, better sensitivity

and more efficient separations. However, its hyphenation to Orbitrap is still problematic due to the low scan rates attainable when selecting high resolving power.

The expected developments of HRMS will surely be related to an improvement in scan speed for Orbitrap, which will allow hyphenation to UHPLC and certainly increase the number of applications, and an improvement in mass resolving power for TOF. Advances in accurate-mass full-acquisition data processing using more powerful and user-friendly software are also likely. New hybrid analyzers incorporating Ion Mobility Spectrometry (IMS), either before or between mass analyzers, are appearing in the market, adding an orthogonal separation mechanism to classical mass-to-charge ratio. IMS will probably improve identification of coeluting isomers or confirmation of suspect compounds in complex samples.

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FIGURE CAPTIONS

Figure 1. Comparison of systematic workflows for (a) quantitative target analysis with reference standards, (b) suspects screening without reference standards, and (c) non-target screening of unknowns in environmental samples by using LC–high resolution (tandem) mass spectrometry. *Note that the *m/z* range of the extraction window for the exact mass filtering depends on the mass accuracy and the resolving power of the mass spectrometer used (reproduced with permission of [15])

Figure 2. (a) Low and high-energy (Q)TOF mass spectra obtained for a wastewater sample extract obtained by SPE; (b) XICs for the protonated molecule (at LE) and main fragment ions (at HE) for two co-eluting compounds.

Figure 3. Detection and identification of levamisole by UHPLC-QTOF MS in effluent wastewater (the reference standard was not available at the laboratory at the time of analysis): (a) LE (bottom) and HE (top) spectra of the compound eluting at 3.86 min. (b) Extracted-ion chromatograms (0.02 Da mass width) for the protonated molecule in LE function and different fragment ions in HE function. (×) indicates that this ion is not related to levamisole.

Figure 4. MS^2 spectra of a dihydroxy derivative of lincomycin (reproduced with permission of [32]).

Figure 5. Detection and identification of a TP of omeprazole (OTP 5) by LC–QTOF MS (MS^E) resulting from photodegradation. (a) XIC at *m/z* 330.1276 (0.02 Da mass window width) for analyte-sample (bottom) and control-sample (top), after 14 days of solar irradiation. (b) LE (bottom) and HE (top) spectra and justification of fragment ions observed.

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Table 1. Literature overview on LC-QTOF MS applications.

Author (year)	LC	Compound (no.) [a]	T/S/NT/D[a]	Matrix[b]	Observations[c]	Ref.
Marchese (2003)	HPLC	Pharmaceuticals (5)	T	SW	Quantification and confirmation (QqQ vs QTOF)	[52]
Stolker (2004a)	HPLC	Pharmaceuticals (13)	T	SW,GW, DW	Quantification (QqQ) and confirmation (QqQ vs QTOF)	[56]
Stolker (2004b)	HPLC	Pharmaceuticals (5)	T	SW, WW	Quantification and confirmation (QqQ vs QTOF)	[57]
Agüera (2005)	HPLC[d]	Diclofenac	D	A	Elucidation of Hy and PhN TPs by LC-TOF MS+GC-MS (13)	[97]
Boreen (2005)	Infusion[d]	Pharmaceuticals (5)	D	SW	Elucidation of PhN TPs of sulfa drugs by LC-UV+TOF MS+FT IR+^{1}H NMR + ^{13}C NMR (5)	[88]
Eichhorn (2005)	HPLC	Trimethoprim	D	WW	Elucidation of Bd TPs (2) by LC-QTOF MS+LC-IT MS+H/D exchange. Quantification by LC-MS	[89]
Ibáñez (2005)	HPLC	Unknown compounds	NT	SW,GW,WW	Screening and elucidation of unknown compounds	[25]
Petrovic (2006)	UHPLC	Pharmaceuticals (29)	T	SW,WW	Screening, quantification and confirmation	[71]
Pozo (2006)	HPLC	Antibiotics (16)	T	SW,GW	Quantification (QqQ) and confirmation (QqQ vs QTOF)	[11]
Martínez Bueno (2007)	HPLC[d]	Pharmaceuticals and metabolites (48), and other emerging contaminants (56T, 5S)	T,S,NT	WW	Quantification (QTRAP) and confirmation (QTRAP vs TOF)	[64]
Pérez (2007)	UHPLC	Enalapril and its metabolite enalaprilat	D	A	Elucidation of PhS TPs (3 from enalapril + 1from enalaprilat by QTOF+QqLIT MS)	[98]
Farré (2008)	UHPLC	Pharmaceuticals (28) + phytoestrogens (4)	T	SW,WW	Quantification and confirmation (QqQ vs QTOF)	[55]
Ibáñez (2008)	UHPLC[d]	Organic pollutants	NT	SW,WW	Screening and identification. Empirical library (104) vs Theoretical library (500)	[114]
Kosjek (2008)	UHPLC	Diclofenac	D	A, HPLC	Elucidation of Bd TPs (4). Isotopic cluster analysis	[87]
Radjenović (2008)	UHPLC	Atenolol and Glibenclamide	D	WW	Elucidation of Bd TPs (1 from atenolol+1from glibenclamide). 1 TP detected in WW by QqLIT MS.	[107]
Ibáñez (2009)	UHPLC	Pharmaceuticals (62)	T	SW, WW	Screening and confirmation	[69]
Lavén (2009)	HPLC	Pharmaceuticals (15)	T	WW	Screening, quantification, confirmation and removal efficiency	[72]
Ferrer (2010)	HPLC	Chlorine-containing compounds	Semi-NT	DW,GW,WW, SW, HPLC	Lamotrigine and its metabolite (2-N-glucuronide) detected in DW, SW and WW samples.	[112]

Author (year)	LC	Compound (no.) [a]	T/S/NT/D[a]	Matrix[b]	Observations[c]	Ref.
					Quantification	
Gómez (2010)	HPLC	Pharmaceuticals, transformation products and pesticides	NT	WW, SW	Screening, quantification and confirmation. Database containing pharmaceuticals (87) + pesticides (300)	[74]
López-Roldán (2010)	UHPLC[d]	Pharmaceutical (28) and estrogens (10)	T	SW	Quantification (QqQ) and confirmation (TOF)	[65]
Magnér (2010)	UHPLC	Pharmaceuticals (10)	T	SW,MW	Screening, quantification and confirmation	[67]
García-Galán (2011)	UHPLC	Sulfamethazine	D	A	Elucidation of TPs after Bd by fungus *Trametes versicolor* (4). Removal efficiency in SS by QqLIT-MS	[108]
Gómez-Ramos (2011a)	HPLC	Sulfamethoxazole	D	n.d.	Elucidation of Oz TPs (6)	[105]
Gómez-Ramos (2011b)	HPLC	Pharmaceuticals, illicit drugs, TPs and other organic pollutants	NT	WW	Screening and identification. Database containing 147 compounds. Identification of related and unexpected TPs	[115]
Hernández (2011a)	UHPLC	Pharmaceutical metabolites (160)	S	WW	Screening and identification (retrospective analysis)	[21]
Hernández (2011b)	UHPLC	Illicit drugs and metabolites (11 T + 65 S)	T,S	WW	Screening and identification	[73]
Nurmi (2011)	UHPLC[d]	Pharmaceuticals (16) and pesticides (68)	T	WW	Screening, quantification and confirmation	[120]
Pérez-Parada (2011)	HPLC	Amoxicillin	D	WW, SW	Elucidation of Hy TPs (4). 1 TP detected in SW samples	[91]
Postigo (2011a)	UHPLC	Methadone	D	A, HPLC	Elucidation of Hy, PhN and Pc TPs (6). 1 confirmed with ref st	[100]
Postigo (2011b)	UHPLC	Cocaine	D	A, HPLC	Elucidation of Hy, PhN and Pc TPs (14)	[101]
Terzic (2011)	UHPLC	Polar contaminants	NT	Sed	The aquatic sediment was influenced by pharmaceutical industry	[111]
Díaz (2012)	UHPLC	Pharmaceuticals, illicit drugs, TPs and other organic pollutants (231 T + 1100 S)	T, S, NT	WW	Screening and identification	[16]
Eichhorn (2012)	UHPLC	Sildenafil (Viagra) and its N-demethylated metabolite	D	HPLC,A,SW	Elucidation of PhS TPs (8 from sildenafil +6 from N-demethylsildenafil) by LC-ESI-TOF MS+LC-APCI-QqQ MS+H/D exchange	[90]
Ferrer (2012)	HPLC	Pharmaceuticals and degradation	T	DW,GW,SW	Screening, quantification and confirmation	[36]

Author (year)	LC	Compound (no.) [a]	T/S/NT/D[a]	Matrix[b]	Observations[c]	Ref.
		products (100)		WW		
González-Mariño (2012)	HPLC	Illicit drugs and metabolites (24 T + 130 S)	T, S	WW	Screening, quantification and identification	[35]
Leknes (2012)	UHPLC[d]	Oseltamivir and its metabolite oseltamivir carboxylate	T	WW	Screening, quantification and confirmation	[66]
Martínez- Bueno (2012)	HPLC	Pharmaceuticals and illicit drugs (10 T, 1200 S)	T, S, NT	SW	Screening, quantification and identification	[116]
Nurmi (2012)	UHPLC[d]	Pharmaceuticals and pesticides (88 T + 201 S)	T, S, NT	WW	Evaluation of T,S,NT screening with spiked samples. Application of T,S to real WW. Theoretical library (6)	[117]
Bijlsma (2013)	UHPLC	Cocaine and its metabolite benzoylecgonine	D	SW	Elucidation of Hy, Cl and PhS TPs (16 from cocaine+10 from benzoylecgonine). 7 TPs detected in SW and WW samples by UHPLC-QqQ MS	[30]
Boix (2013)	UHPLC	Omeprazole	D	SW	Elucidation of TPs after Hy, Cl, PhS (17). 4 TPs detected in SW and WW samples by UHPLC-TOF MS and UHPLC-QqQ MS	[22]
Díaz (2013)	UHPLC	Pharmaceuticals, illicit drugs and TPs (150 organic pollutants)	T	SW, GW, WW	Qualitative validation	[121]
Masiá (2013)	UHPLC	Pharmaceuticals, illicit drugs and other emerging contaminants (250T+1100S)	T, S	WW, SW	Quantification (42 pesticides by QqQ), confirmation (QqQ and QTOF) and identification (QTOF)	[75]
Vergeynst (2013)	UHPLC	Pharmaceuticals (69)	T	SW	Screening, quantification and confirmation	[70]
Boix (2014a)	UHPLC	Omeprazole metabolites (24)	T	WW, SW	Retrospective screening of metabolites previously identified in metabolism study. 9 metabolites detected in samples	[23]
Boix (2014b)	UHPLC	THC-COOH (Cannabis metabolite)	D	SW	Elucidation of Hy, Cl and PhS TPs (19). 8 TPs detected in SW and WW samples by UHPLC-QqQ MS	[29]
Rodríguez-Álvarez (2014)	HPLC	Ethyl Sulfate (biomarker for ethanol tracing)	T	WW	Quantification and confirmation (QqQ vs QToF)	[68]

[a] T:Target, S: Suspect, NT: Non-Target; D: Degradation study

[b] HPLC: HPLC, distilled, ultrapure or demineralized water; SW: Surface Water; WW: Wastewater; GW: Ground Water; DW: Drinking Water; SS: Sewage Sludge; A: any artificial water matrix; MW: marine environment; Sed: Sediment; n.d.: not clearly defined

[c] PhN: Photolysis with natural sunlight; PhS: Photolysis with simulated sunlight; Bd: Biodegradation; Hy:Hydrolysis; Pc:Photocatalysis; Cl: Chlorination; Oz: Ozonation

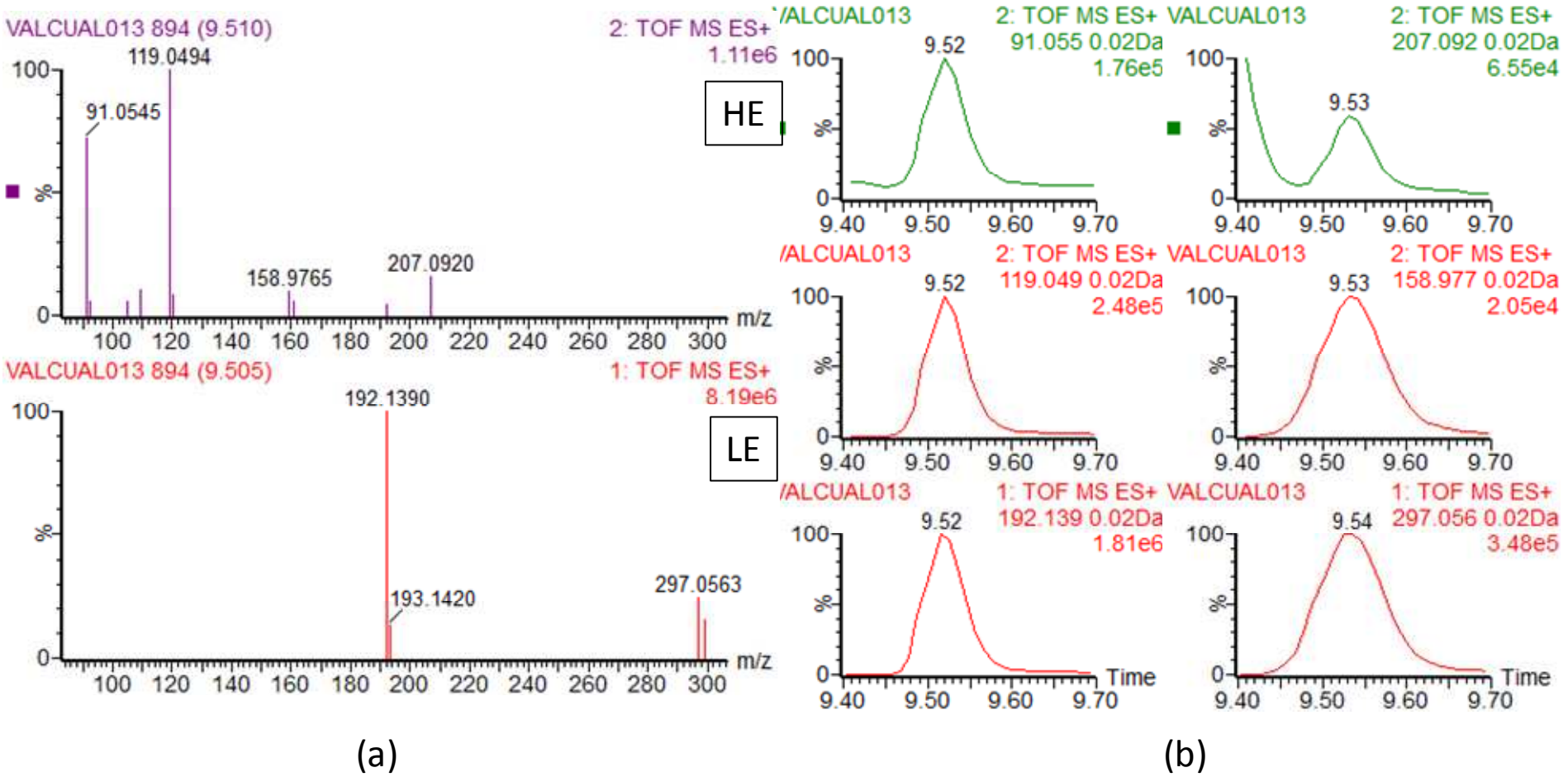
[d] Only TOF instrument used

Table 2. Literature overview on LC-LTQ Orbitrap MS applications.

Author (year)	LC	Compound (no.)[a]	T/S/NT/D[a]	Matrix[b]	Observations[c]	Ref.
Hogenboom (2009)	HPLC	Pharmaceuticals, illicit drugs and other emerging contaminants (3000 T)	T, NT	SW,WW,GW,DW	Screening, quantification and identification	[118]
Hollender (2009)	HPLC	Emerging contaminants (220 T, including 77 pharmaceuticals); known oxidation TPs of 5 pharmaceuticals (S)	T,S	WW	Screening and removal efficiency for 220 pollutants after Oz degradation. Screening of known oxidation products of 5 pharmaceuticals	[85]
Kern (2009)	HPLC	Plausible TPs assembled using computer-aided prediction (UM-PPS) of pesticides (24) +biocides (7)+pharmaceuticals(21). TPs reported in literature of pharmaceuticals (21) and pesticides (31). Total: 1794 proposed TPs (890 from pharmaceuticals)	S	SW	19 TPs identified in SW (7 from pharmaceuticals) → 12 confirmed with ref st	[28]
Helbling (2010)	HPLC	Plausible TPs assembled using computer-aided prediction (UM-PPS) of pharmaceuticals (6) + pesticides (6). Degradation study	S, D	WW	26 TPs identified after Bd (13 from pharmaceuticals). UM-PPS predicted the structures of 21 TPs. Elucidation resulted in 26 TPs	[84]
Kern (2010)	HPLC	Plausible TPs assembled using computer-aided prediction (UM-PPS) + TPs reported in literature (7)	S	WW	12 TPs identified after Bd. Application to real samples (quantification by QqQ)	[27]
Bagnati (2011)	HPLC	Cocaine and benzoylecgonine	T	WW, SW	Evaluation of HRMS capabilities: screening, quantification and confirmation	[122]
Pinhancos (2011)	UHPLC	Pharmaceuticals (8)+metabolite of caffeine	T	DW	Screening, quantification and confirmation	[48]
Wille (2011)	UHPLC[d]	Pharmaceuticals (16) and pesticides (13)	T	MW	Screening, quantification and confirmation	[49]
Bijlsma (2012)	HPLC	Illicit drugs and metabolites (24)	T	WW	Quantification, confirmation and removal efficiency	[77]
Cahill (2012)	HPLC	Pharmaceuticals (5) and pesticides (4)	T	WW	Screening, quantification and confirmation	[78]
Calza (2012a)	HPLC	Clarithromycin and Carbamazepine	D	HPLC	Elucidation of Pc TPs (28 from carbamazepine and 29 for clarithromycin)	[31]
Calza (2012b)	HPLC	Lincomycin	D	HPLC	Elucidation of Pc TPs(21)	[32]
de Jongh (2012)	HPLC	Pharmaceuticals and TPs (26)	T	SW, DW, GW	Screening and quantification	[79]
ter Laak (2012)	HPLC	Pharmaceuticals, illicit drugs, personal care products and other organic pollutants (635)	T	GW	Screening and confirmation	[82]

Author (year)	LC	Compound (no.)[a]	T/S/NT/D[a]	Matrix[b]	Observations[c]	Ref.
Bijlsma (2013)	HPLC	Illicit drugs and metabolites (24 T + 2 S)	T, S	WW	Quantification and confirmation.	[20]
Calza (2013)	HPLC	Lincomycin, Clarithromycin and Carbamazepine	D	SW	Elucidation of PhS TPs (19 from lincomycin, 21 from carbamazepine and 6 from clarithromycin).13 TPs detected in samples	[99]
Chiaia-Hernandez (2013)	HPLC	Pharmaceuticals, personal care products and other organic pollutants (> 180 T+ 80 S)	T, S	Sed	Screening and identification (APPI and ESI)	[81]
van der Aa (2013)	HPLC	Illicit drugs and metabolites (34)	T	DW, SW, WW	Screening and quantification (incl. QqQ, Orbitrap) from different research groups	[123]
Rodayan (2014)	UHPLC	Illicit drugs and TPs (7)	D	WW	Elucidation of Oz TPs (10). Removal efficiency	[50]
Emke (2014)	HPLC	Amphetamine and MDMA	T	WW	Enantiomeric profiling. Quantification and confirmation	[83]
Hug (2014)	HPLC	Novel micropollutants (98 T+ 2160 S)	T, S, NT	WW	Screening and identification	[26]
Kosma (2014)	HPLC	Pharmaceuticals and personal care products (18)	T,S	WW	Confirmation of positives detected by LC-MS. Removal efficiency by LC-MS. Identification of trimethoprim TPs (2).	[80]
Schymanski (2014)	HPLC[e]	Polar organic contaminants (364 T + approx. 180 S	T, S, NT	WW	Screening and identification	[119]
Zonja (2014)	HPLC	Zanamivir	D	A, SW	Identification of TPs after PhN and PhS (4). 1 TP confirmed with ref st	[51]

[a] T:Target, S: Suspect, NT: Non-Target; D: Degradation study
[b] HPLC: HPLC, distilled, ultrapure or demineralized water; SW: Surface Water; WW: Wastewater; GW: Ground Water; DW: Drinking Water; SS: Sewage Sludge; A: any artificial water matrix; MW: marine environment; Sed: Sediment
[c] PhN: Photolysis with natural sunlight; PhS: Photolysis with simulated sunlight; Bd: Biodegradation; Hy:Hydrolysis; Pc:Photocatalysis; Cl: Chlorination; Oz: Ozonation
[d] Exactive instrument used
[e] Q Exactive instrument used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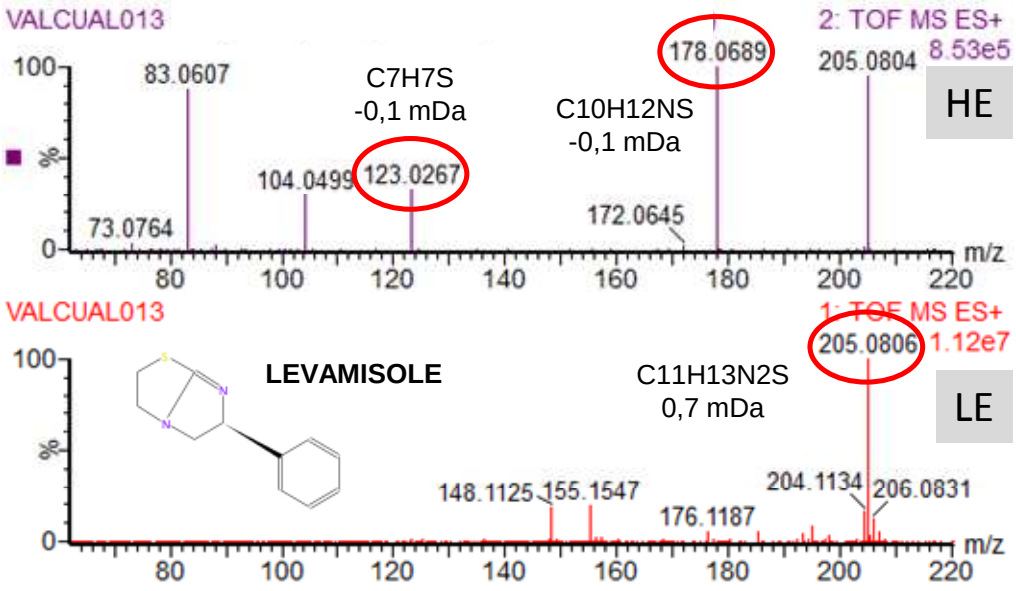
Time
(a)
(b)

Fig 1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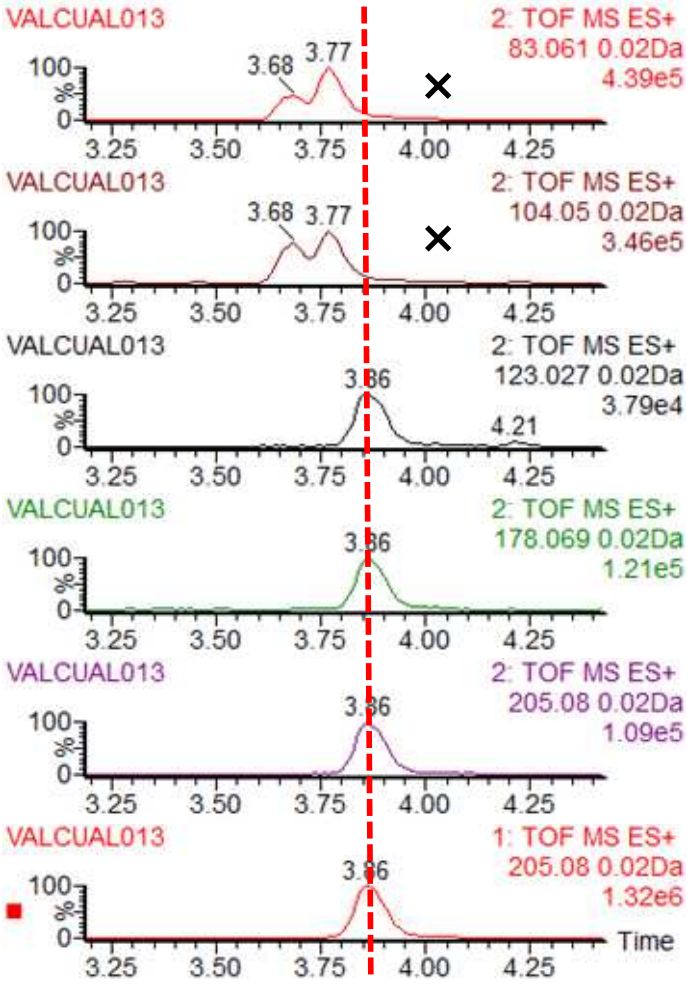
m/z

(a)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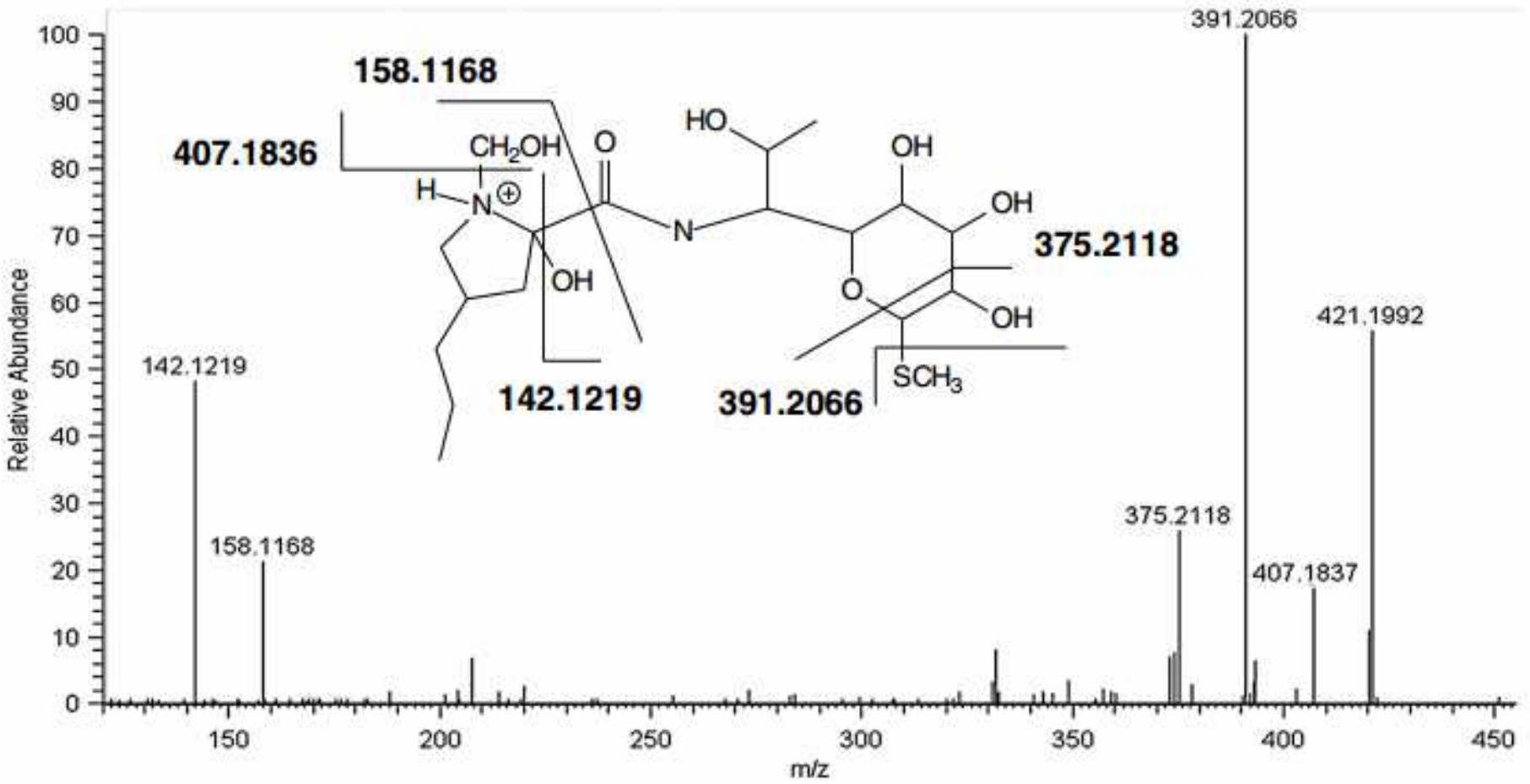
(b)

Fig 2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Relative Abundance
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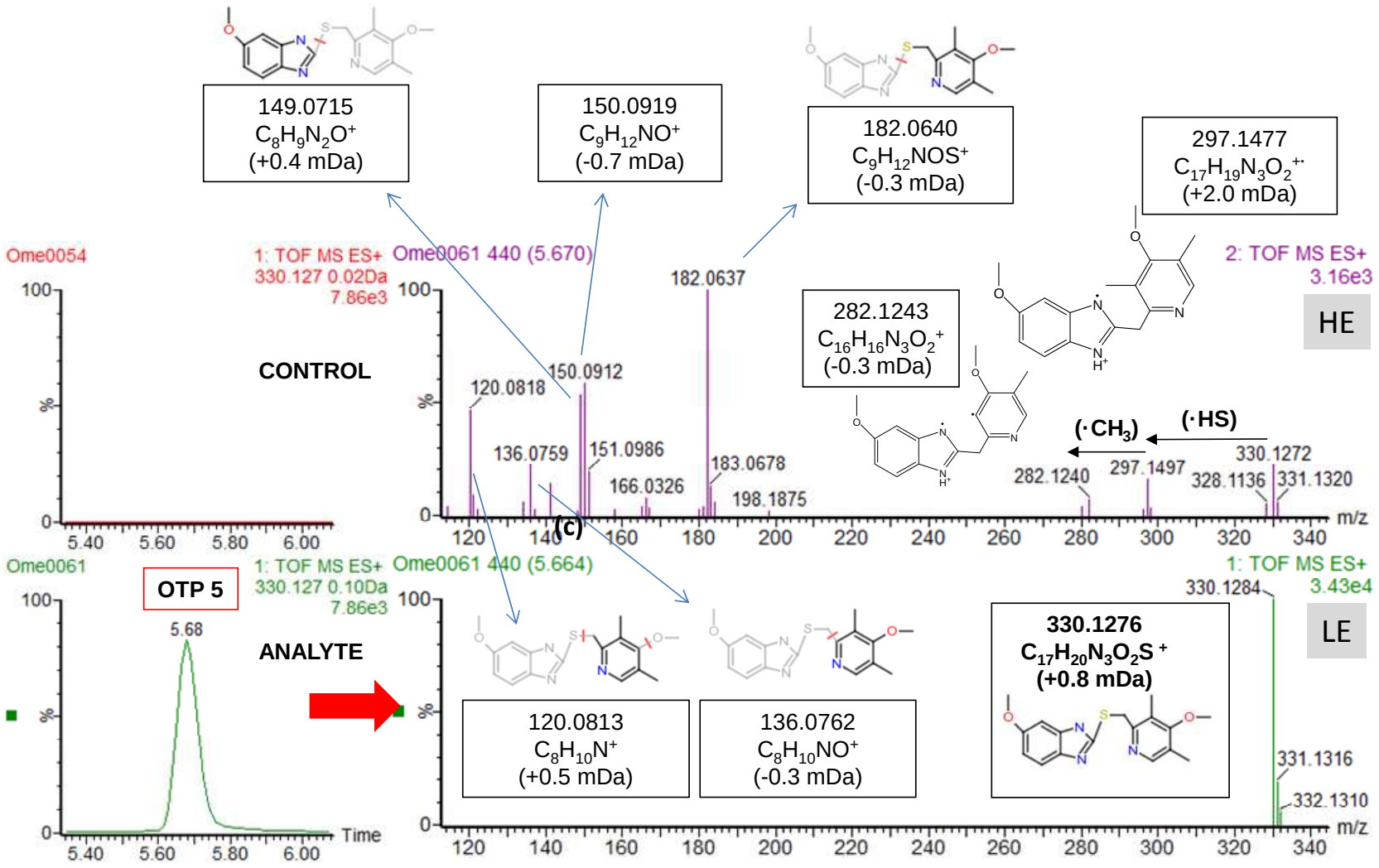
Fig 3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(·CH_3)
(·HS)
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328.1136
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331.1320
m/z
(c)
Ome0061
OTP 5
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ANALYTE
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Time
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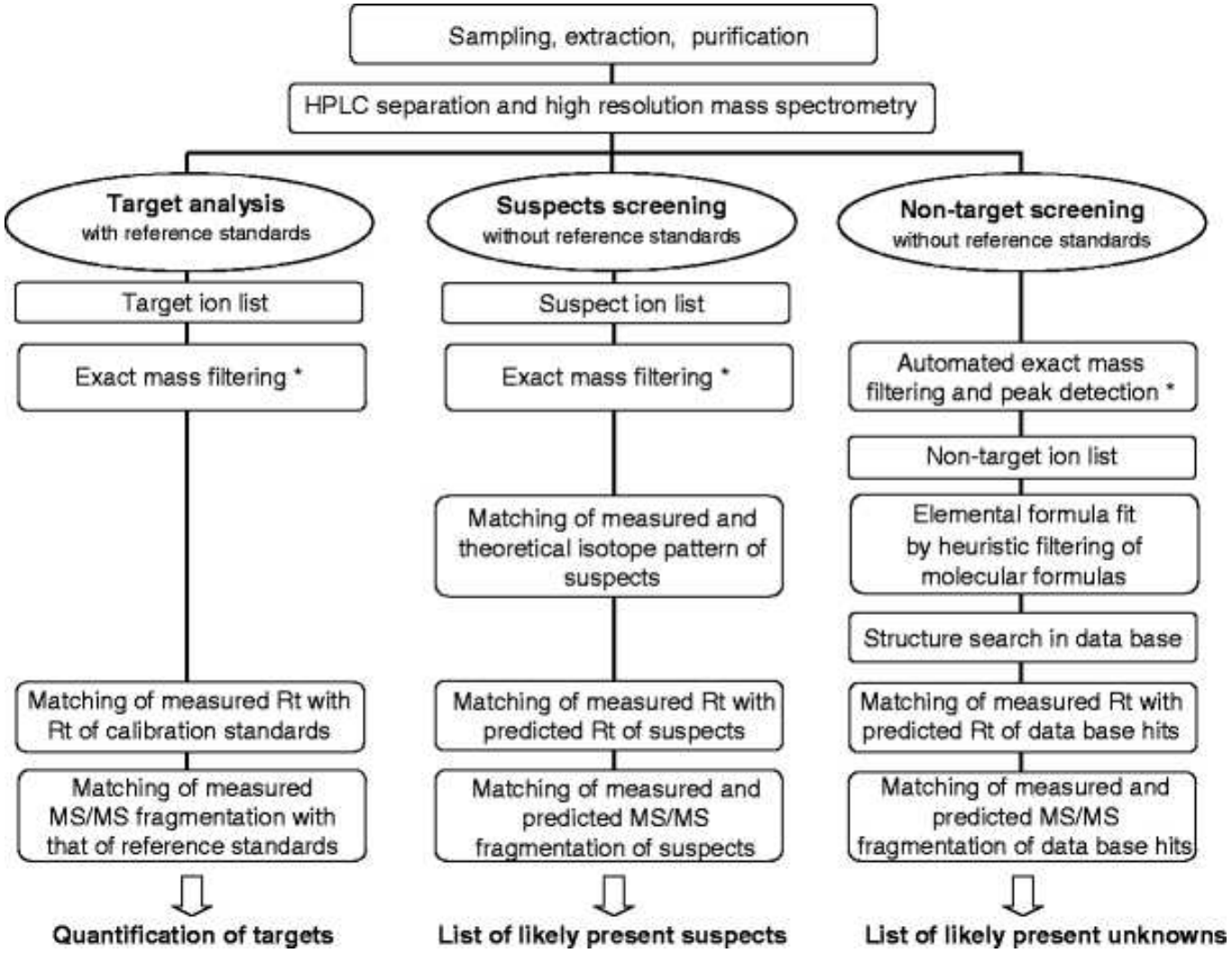
m/z

(a) (b)

Fig 4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Target ion list
Exact mass filtering *
Matching of measured Rt with Rt of calibration standards
Matching of measured MS/MS fragmentation with that of reference standards
Quantification of targets
(a)
Suspects screening
without reference standards
Suspect ion list
Exact mass filtering *
Matching of measured and theoretical isotope pattern of suspects
Matching of measured Rt with predicted Rt of suspects
Matching of measured and predicted MS/MS fragmentation of suspects
List of likely present suspects
(b)
Non-target screening
without reference standards
Automated exact mass filtering and peak detection *
Non-target ion list
Elemental formula fit by heuristic filtering of molecular formulas
Structure search in data base
Matching of measured Rt with predicted Rt of data base hits
Matching of measured and predicted MS/MS fragmentation of data base hits
List of likely present unknowns
(c)

Fig 5