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Review

Accurate mass screening and identification of emerging contaminants in environmental samples by liquid chromatography–hybrid linear ion trap Orbitrap mass spectrometry

A.C. Hogenboom [a,*], J.A. van Leerdam [a], P. de Voogt [a,b]

[a] *Kiwa Water Research, Chemical Water Quality and Health, P.O. Box 1072, Nieuwegein 3430 BB, The Netherlands*
[b] *Institute of Biodiversity and Ecosystem Diversity, University of Amsterdam, Nieuwe Achtergracht 166, Amsterdam 1018 WV, The Netherlands*

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ABSTRACT

The European Reach legislation will possibly drive producers to develop newly designed chemicals that will be less persistent, bioaccumulative or toxic. If this innovation leads to an increased use of more hydrophilic chemicals it may result in higher mobilities of chemicals in the aqueous environment. As a result, the drinking water companies may face stronger demands on removal processes as the hydrophilic compounds inherently are more difficult to remove. Monitoring efforts will also experience a shift in focus to more water-soluble compounds. Screening source waters on the presence of (emerging) contaminants is an essential step in the control of the water cycle from source to tap water. In this article, some of our experiences are presented with the hybrid linear ion trap (LTQ) FT Orbitrap mass spectrometer, in the area of chemical water analysis. A two-pronged strategy in mass spectrometric research was employed: (i) exploring effluent, surface, ground- and drinking-water samples searching for accurate masses corresponding to target compounds (and their product ions) known from, *e.g.* priority lists or the scientific literature and (ii) full-scan screening of water samples in search of 'unknown' or unexpected masses, followed by MS^n experiments to elucidate the structure of the unknowns. Applications of both approaches to emerging water contaminants are presented and discussed. Results are presented for target analysis search for pharmaceuticals, benzotriazoles, illicit drugs and for the identification of unknown compounds in a groundwater sample and in a polar extract of a landfill soil sample (a toxicity identification evaluation bioassay sample). The applications of accurate mass screening and identification described in this article demonstrate that the LC–LTQ FT Orbitrap MS is well equipped to meet the challenges posed by newly emerging polar contaminants.

Contents

* Corresponding author. Tel.: +31 306069668.
E-mail address: ariadne.hogenboom@kiwa.nl (A.C. Hogenboom).

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1. Introduction

Today, still 90% of the 100,000 chemicals in daily use have either no or insufficient health, safety or environmental impact data. The European Reach legislation seeks to provide a legal framework for dealing with chemicals ensuring a high level of health and environmental protection with the goal of achieving a sustainable development.

One of the major changes encompassed in the Reach legislation is that responsibility for chemical safety is shifted from government to industry. The safe use of chemicals has to be defined, and data on chemicals have to be provided by their manufacturers and down stream users. If no data are available on a chemical, then it can neither be registered nor marketed. The registration dossier includes: properties, intended uses, exposures assessment, production/import quantities, proposal for classification and labelling, risk assessment and proposed risk management measures, and testing proposal (protocols). The downstream uses should comply with supplier dossiers: (Material) Safety Data Sheets.

The information requested in the registration dossiers is production volume dependent (the higher the production volume, the sooner and the more data are required). Dossiers include information about (i) physicochemical properties; (ii) health (toxicological) data; (iii) environmental (ecotoxicological-fate-behaviour) data; (iv) compositional data; (v) chemical identity; (v) volume of production; (vi) uses and (vii) exposure data.

The registration dossier consists of a Chemical Safety Report (CSR) that includes

- summary of risk management measures,
- declaration that risk management measures are implemented and communicated,
- identification of the substance and physical and chemical properties,
- classification and labelling,
- environmental fate properties, and
- summary of chemical safety assessment (CSA).

The CSA involves the assessment of human health hazards, physicochemical and environmental hazards, persistence, bioaccumulation and toxicity (PBT) and very persistent very bioaccumulative (vPvB) properties and exposure as well as a risk characterization [1].

In the evaluation of the registration data, three major criteria play a pivotal role: persistence, bioaccumulation, and toxicity. Inevitably some existing substances will be banned or severely restricted due to their PBT scores. The legislation will force some producers to rationalize portfolios. One can therefore expect the Reach framework to promote innovation. A possible general development will be that newly designed chemicals will be less persistent, bioaccumulative or toxic. Most of the chemicals that bioaccumulate can be characterized as hydrophobic compounds. If innovation leads to an increased use of more hydrophilic chemicals, this may result in higher mobilities of the chemicals in aqueous media. As a result, the drinking water companies may face stronger demands on removal processes as the hydrophilic compounds inherently are more difficult to remove. Monitoring efforts will also experience a shift in focus to more water-soluble compounds, a tendency that has already become apparent during the last decade or so [2–5]. The shift towards the use of more hydrophilic compounds in industrial and consumer applications requires the development of appropriate analytical methods that can deal with specific properties and a multitude of different chemicals.

The applicability of nontarget analysis, *i.e.* full-scan screening, depends on current technical developments. Gas chromatography–mass spectrometry methods have greatly contributed to the characterization of (semi)volatile and thermostable contaminants in water whereas liquid chromatography–mass spectrometry (LC–MS) methods have been utilized to extend the investigation of water contaminants to non-volatile, (highly) polar, and thermally labile compounds, such as for example pharmaceuticals, pesticides, endocrine disrupting compounds and personal care products [6]. Nowadays, the major challenge is the analysis of highly polar compounds at trace concentration levels in aqueous environmental samples.

Our research is aimed to increase understanding of the sources, occurrence, behaviour and effects of chemicals in aquatic systems, and their consequences for (drinking) water quality and human health. Chemical (and biological) contaminants that end up in tap water may pose a potential threat to human health. Screening source waters (groundwater and surface water) on the presence of contaminants is an essential step in the control of the water cycle from source to tap water. The objective of this paper is to present some experiences with LC–high resolution MS–MS instrumentation, using the hybrid linear ion trap (LTQ) FT Orbitrap mass spectrometer, in the area of chemical water analysis. The Orbitrap is a new type of mass analyser, operating by radially trapping ions about a central spindle electrode. An outer barrel-like electrode is coaxial with the inner spindle-like electrode and mass/charge values are measured from the frequency of harmonic ion oscillations, along the axis of the electric field, undergone by the orbitally trapped ions. This axial frequency is independent of the energy and spatial spread of the ions. Ion frequencies are measured non-destructively by acquisition of time-domain image current transients, with subsequent fast Fourier Transforms (FFTs) being used to obtain the mass spectra. [7].

The use of the LTQ FT Orbitrap MS–MS combines the tandem mass spectrometry capability of the linear ion trap (LTQ) with the high resolution and mass accuracy capability of the FT Orbitrap. This combination allows high-quality accurate mass MS^n spectra to be acquired. Fourier transformation of the acquired transient allows wide mass range detection with high resolving power, mass accuracy, and dynamic range. High-accuracy mass measurements are within 2 parts per million (ppm) using internal standard and within 5 ppm with external calibration [8].

The LTQ FT Orbitrap MS has several advantages compared to triple–quadrupole MS that are important for amongst others the screening for and identification of unknown compounds: (i) the sensitivity in full-scan is higher, (ii) accurate mass are used to calculate the most favourable elemental composition

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and (iii) the accurate mass of the product ions (in MS^n) can be determined. Several advantages of the LTQ FT Orbitrap MS over quadrupole/time-of-flight MS are: (i) high ion transmission resulting in higher MS^n sensitivity and detection limits and (ii) a higher intensity range over which accurate mass data can be acquired.

In this paper we demonstrate the versatility of the LTQ FT Orbitrap MS using exemplary studies conducted at the Kiwa Water Research (KWR) Institute. KWR's mass spectrometric research employs a two-pronged strategy: (i) exploring surface, ground- and drinking-water samples looking for accurate masses corresponding to target compounds (and their product ions) known from, *e.g.* priority lists or the scientific literature, (ii) full-scan screening of water samples in search of 'unknown' or unexpected masses, followed by MS^n experiments to elucidate the structure of the unknowns. Applications of both approaches to emerging water contaminants are presented below.

2. Analytical techniques and approaches used

2.1. General sample treatment approach

Preconcentration of water samples was carried out on Oasis-HLB 200 mg solid-phase material in a glass cartridge with a volume of 5 mL (Waters, Milford, MA, USA). Oasis-HLB is a porous co-polymer [poly(divinyl–benzene–co-*N*-vinylpyrrolidone)] with an adsorption capacity for both lipophilic and hydrophilic compounds [9]. For the analysis of pharmaceuticals, preconcentration of the samples was carried out on the Oasis MCX 500 mg solid-phase material in a glass cartridge with a volume of 6 mL. Oasis-MCX is a strong mixed-mode cation exchanger, water wettable, polymeric sorbent which provides dual modes of retention – ion exchange and reversed phase – on a single, high surface area, organic polymer that is stable from pH 0 to 14 [9].

One liter of the water sample spiked with two SPE internal standard compounds fenuron and chloroxuron in order to check the SPE procedure and enable the calculation of the SPE recoveries. In addition, the sample pH was adjusted to 2. Both the Oasis-HLB and Oasis MCX cartridges were conditioned before use by washing with acetonitrile, methanol and water acidified to pH 2. Samples were then passed through the cartridges under vacuum. The Oasis-HLB and Oasis-MCX cartridges were washed with water acidified to pH 2 and dried under vacuum. The Oasis-HLB cartridges were eluted with 3× 2.5 mL of acetonitrile. The Oasis-MCX cartridges were eluted with 2.5 mL acetonitrile and 2× 2.5 mL 5% NH_4OH solution. Cations cannot be enriched by the Oasis-MCX procedure and the NH_4OH solution is used for elution of acids from the ion exchange material.

The eluates were evaporated to 250 μL under a gentle flow of nitrogen at 45 °C. Two hundred and fifty microliter of a solution of the internal standards 1H-benzotriazole-d_4, atrazine-d_5 and bentazone-d_6 with a concentration of 2 mg/L in water was added to the extracts. The final concentration of the internal standard fenuron, chloroxuron, 1H-benzotriazole-d_4, atrazine-d_5 and bentazone-d_6 is 1 mg/L.

2.2. Liquid chromatography-LTQ FT Orbitrap mass spectrometry (LC–LTQ FT Orbitrap MS)

The LC–DAD-LTQ-FT Orbitrap MS system consisted of a Surveyor autosampler model Plus, a Surveyor quaternair gradient LC-pump, a Surveyor Photo Diode Array (DAD) model Plus detector and an LTQ-FT Orbitrap mass spectrometer (Thermo Electron GmbH, Bremen, Germany). The linear ion trap (LTQ) part of the hybrid MS system was equipped with an Ion Max Electrospray Ionization (ESI) probe and operated in the positive- and negative-ion mode. Full-scan accurate mass spectra (mass range from 50 to 1300 Da) were obtained at high resolution (100,000 FWHM) and processed using Xcalibur v.2.0 software. The electrospray source conditions were: capillary voltage 3.6 kV (positive-ion measurements), 2.5 kV (negative-ion measurements), heated capillary temperature 275 °C, capillary voltage 30 V and tube lens 70 V.

Table 1
The scan type settings of the linear ion trap (LTQ) part and the Orbitrap part of the LTQ FT Orbitrap MS

Scan event no.	Measurement in	Scan type	
1	Orbitrap part	Full-scan accurate mass	
2	LTQ part	DDA MS^2	Most abundant ion of scan event no. 1
3	LTQ part	DDA MS^3	First most abundant ion of scan event no. 2
4		DDA MS^3	Second most abundant ion of scan event no. 2
5		DDA MS^3	Third most abundant ion of scan event no. 2
6	LTQ part	DDA MS^4	First most abundant ion of scan event no. 3
7		DDA MS^4	Second most abundant ion of scan event no. 3
8		DDA MS^4	First most abundant ion of scan event no. 4
9		DDA MS^4	Second most abundant ion of scan event no. 4
10		DDA MS^4	First most abundant ion of scan event no. 5
11		DDA MS^4	Second most abundant ion of scan event no. 5

The mass spectrometer was operated in a data-dependent-acquisition (DDA) mode in which both MS and MS^n spectra were acquired without the need to specify parent masses. In this mode, the acquisition software probed the MS spectra in real-time on a scan-by-scan basis to select the most intense parent ions for MS^n analysis. This technique is capable of finding true unknowns since the method does not require any pre-selection of masses. The instrument is initially set to operate in full-scan ('survey') mode until an ion exceeds a preset threshold at which point the instrument switches into the product-ion mode (MS^n). The mass resolution was set at 100,000 FWHM for both screening and quantitative analysis. The products ions were generated in the LTQ trap at a normalized collision energy setting of 35% and using an isolation width of 2 Da. The scan type settings are presented in Table 1. No exclusion list was used. The total cycle time depends upon the resolution; at a resolution of 100,000 FWHM the total cycle time is about 1.8 s. The results were used to create a (full-scan) accurate mass MS and an MS^n database to enable identification of compound in future screening.

The DAD spectral range was set from 200 to 350 nm. Acquisition and data processing were performed with Xcalibur v.2.0 software.

Five microliters of the final extract was injected into the LC system consisting of a 100 mm × 2.0 mm i.d. column packed with 3-μm Omnisphere C_{18} material (Varian-Chrompack, Middelburg, the Netherlands). The guard column was 4.0 mm × 2.0 mm i.d. packed with pellicular C_{18} material, 25–35 μm (Varian-Chrompack). The analytical column and the guard column were maintained at a temperature of 21 °C in a column thermostat.

For positive-ion measurements, a linear gradient of acetonitrile (5–100%) and water with 0.05% formic acid was used in 40 min and held at this composition for an additional 10 min. For negative-ion measurements, a linear gradient of acetonitrile (5–100%) and water with 0.05% ammonium formate was used with the same gradient profile as for positive-ion measurements. The analytical column

Table 2
Presence of pharmaceuticals quantified in treated sewage effluents (STP), in μg/L (samples taken in Summer 2007)

Compound	Elemental composition	STP 1	STP 2	STP 3	STP 4	STP 5
Atenolol	$C_{14}H_{22}N_2O_3$	0.2	0.5	1.5	0.3	0.3
Bezafibrate	$C_{19}H_{20}ClNO_4$	<	<	<	<	<
Carbamazepine	$C_{15}H_{12}N_2O$	0.4	0.5	0.5	0.6	0.4
5-Chloramphenicol[a]	$C_{11}H_{12}Cl_2N_2O_5$	–	–	–	–	–
Clindamycine	$C_{18}H_{33}ClN_2O_5S$	<	<	<	<	<
Clofibric acid[a]	$C_{10}H_{11}ClO_3$	–	–	–	–	–
Cyclofosfamide	$C_7H_{15}Cl_2N_2O_2P$	–	–	–	–	–
Diclofenac	$C_{14}H_{11}Cl_2NO_2$	0.2	0.2	0.1	0.2	0.2
Fluoxetine	$C_{17}H_{18}F_3NO$	–	–	–	–	–
Furosemide	$C_{12}H_{11}ClN_2O_5S$	–	0.2	0.2	<	0.2
Ibuprofen[a]	$C_{13}H_{18}O_2$	–	–	–	–	–
Ifosfamide	$C_7H_{15}Cl_2N_2O_2P$	–	<	<	–	–
Lidocaïne	$C_{14}H_{22}N_2O$	0.1	0.1	0.1	0.1	<
Lincomycine	$C_{18}H_{34}N_2O_6S$	–	–	–	–	<
Metoprolol	$C_{15}H_{25}NO_3$	1.8	2.0	2.6	1.8	2.7
Metronidazol	$C_6H_9N_3O_3$	–	–	–	–	–
Naproxen[a]	$C_{14}H_{14}O_3$	–	–	0.6	–	–
Nitrofurantoin	$C_8H_6N_4O_5$	–	–	–	–	–
Paroxetine	$C_{19}H_{20}FNO_3$	<	<	<	<	<
Phenazone	$C_{11}H_{12}N_2O$	<	<	<	<	<
Propyphenazone	$C_{14}H_{18}N_2O$	<	<	<	<	<
Salbutamol	$C_{13}H_{21}NO_3$	–	–	–	–	–
Sotalol	$C_{12}H_{20}N_2O_3S$	1.6	1.5	1.9	0.7	2.6
Sulfadiazine	$C_{10}H_{10}N_4O_2S$	–	–	–	–	–
Sulfamethoxazole	$C_{10}H_{11}N_3O_3S$	0.4	0.7	0.6	1.1	0.4
Tramadol	$C_{16}H_{25}NO_2$	0.2	0.3	0.3	0.3	0.2
Trimethoprim	$C_{14}H_{18}N_4O_3$	<	<	<	<	<

'–' not detected; '<' detected, but below quantification limit of compound.
[a] Analysed in negative-ion mode; the others in positive-ion mode.

was re-equilibrated for 15 min between consecutive runs. The flow rate of the mobile phase was 0.3 mL/min. The LC column effluent was split via a post-column splitter from 300 to 125 μL/min which was introduced into the source of the mass spectrometer. In order to obtain maximum structural information, the samples were analysed in both full-scan mode with accurate mass measurements and in the product-ion scan mode in the ion trap part with nominal mass measurements in a single analysis.

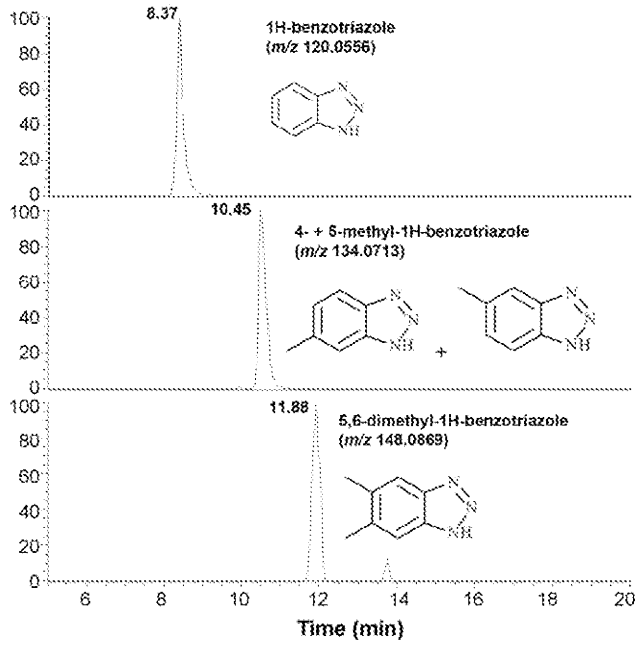

Fig. 1. LC–MS (positive ion-mode) extracted-ion chromatograms of benzotriazoles in a drinking water sample based on accurate mass with a mass window of 5 ppm.

2.3. Data processing

The undeniable appeal of using LTQ FT Orbitrap MS for target compound analysis is related to its high mass resolution potential, corresponding to a mass assignment accurate to four decimal places. The ability to determine the m/z of an ion to within 5 ppm allows the determination of a unique elemental composition based on the mass defect of the constituent atoms. The ability to closely match the expected/theoretical mass with the observed mass greatly increases the reliability of identification. To find the exact masses of the ions of interest, the deconvolution software program Formulator was used.

The total list of accurate masses detected in a sample was corrected manually for masses also found in ultra-pure water. Based on the accurate mass, the (possible) elemental composition of the peaks of interest was calculated using the elemental composition tool within the Xcalibur software. Parameter settings were: C 0–40, H 0–100, N 0–10, O 0–15, P 0–5, and F 0–5; even electron ions for the precursor ions; odd and even electron ions for the product ions. The appropriate numbers of the elements Cl, Br, S and Si were determined from the specific isotope pattern and added if required. Isotope ratio $^{13}C/^{12}C$ was checked in order to assign the number of C. The double-bond equivalent (DBE) parameter was used as an indicator of the stability (degree of π-electron conjugation) of the calculated elemental composition and was set dependent to the existence of an UV signal.

In this study, the calculated elemental compositions with a maximum deviation of 5 ppm from the measured exact mass were considered. To ensure correct mass calibration, the exact masses of the MS internal standard compounds were checked regularly.

To obtain an elemental composition, first a self-created (accurate mass) MS and MS^n database containing about 3000 water pollutants was searched. The self-created database includes amongst others the measured mass, theoretical mass, retention time, KRetI (retention time relative to two internal standards, see for formula Tables 4 and 5), sample type, product ions, (most

Table 3
Compounds identified in a screening study for illicit drugs and their metabolites in sewage treatment plant (STP) effluent, surface water (SW) and drinking water (DW)

Compound	Sample type/location/year								
	DW/1/2007	SW/1/2007	SW/2/2007	SW/3/2007	SW/4/2007	SW/5/2007	STP/1/2007	DW/4/2006	SW/4/2006
Cocaine + metabolite									
Cocaine	–	–	–	–	–	–	–	–	–
Benzoylecgonine	–	+	+	+	+	–	–	–	+
Methadone	–	–	–	–	–	–	+	–	–
Benzodiazepines + metabolite									
Diazepam	–	–	–	–	–	–	–	–	–
Nordazepam	–	–	–	–	–	–	+	–	–
Oxazepam	–	+	–	–	+	–	+	–	+
Desalkylflurazepam	–	+	+	+	+	+	+	–	–
δ-9-Tetrahydrocannabinol (δ-9-THC) + metabolites									
δ-9-THC	–	+	–	–	–	+	–	–	–
11-Hydroxy-δ-9-THC	–	–	–	–	–	–	–	–	–
9-Carbonic acid-δ-9-THC	–	+	+	+	+	+	–	–	–

'–' not detected; '+' detected.

probable) elemental composition, code of mass. Second, the NIST library was searched and last Internet sites such as Chemfinder (www.chemfinder.com) and Chemspider (www.chemspider.com) were searched. The structures found in the libraries were evaluated based on the fragmentation patterns observed in the simultaneously acquired product-ion spectra.

2.4. Strategies of accurate mass screening for (non)target compounds and unknowns

Full-scan accurate mass measurements were compared with theoretical exact masses of known environmental microcontaminants and/or with our self-created accurate mass database. For an accurate mass that could not be found in our accurate mass database, an elemental composition was proposed. The elemental composition could be used to search electronic databases (other than spectral databases) to find out whether the unknown was ever patented, studied or commercialized.

MS^n measurements were performed to obtain information of fragment ions generated in the linear ion trap (nominal mass of product ions) within the same analysis. In addition, the accurate masses of these product ions could be obtained in a second analysis. The masses of these fragment ions were linked with precursor compound masses and were ordered in a so-called 'fragmentation tree'.

The final identification of the compound was based on the above steps.

2.5. Confirmation and quantification

Confirmation of the identity was done by comparing the retention time and fragmentation pattern of the compound to that of a synthesized reference standard. If no information on the reference standard was available, the theoretical log K_{ow} was calculated based on the proposed structure and compared to the retention time of the compound detected. It is known both from theory and practice (own experience) that a relationship exists between the log K_{ow} and the retention time of a compound [10,11].

For quantification purposes, the standard addition method was used. Calibration curves were constructed by plotting the ratio of the analyte quantifying ion response to the response of ion for the internal standard against the fortified concentrations in the range of 0.05–10 μg/L. The concentration of the analyte in each non-fortified sample was obtained by extrapolation of the calibration curve.

In addition, the (SPE) internal standards chloroxuron and fenuron were used to monitor the sample preparation process. The (MS) internal standards 1H-benzotriazole-d_4, atrazine-d_5 and bentazone-d_6 were used to compensate for ionization suppression.

3. Accurate mass screening and identification of emerging contaminants in environmental samples: some examples

3.1. Pharmaceuticals

Pharmaceuticals have become important emerging contaminants. Due to their wide spread presence in the (aquatic) environmental there are concerns about possible effects, both on wildlife and humans. A major concern for pharmaceuticals also includes the development of bacterial resistance to antibiotics. It is estimated that approximately 3000 different substances are used as pharmaceutical ingredients worldwide today, including analgesics, antibiotics, antidiabetics, β-blockers, contraceptives, lipid regulators,

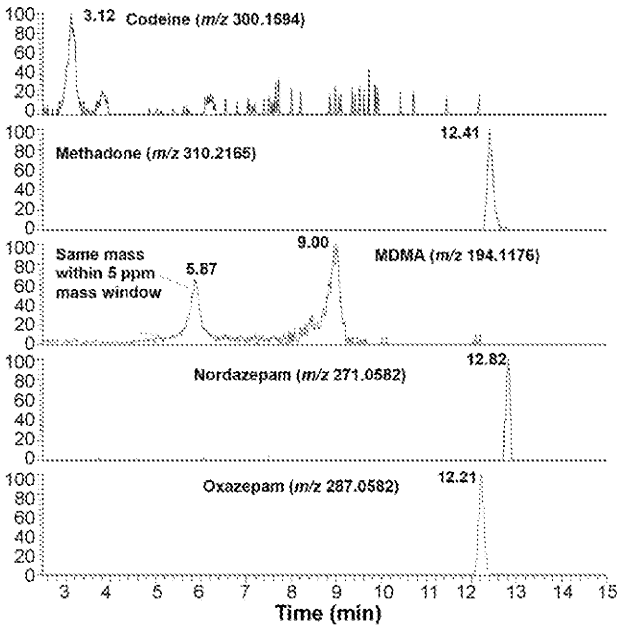

Fig. 2. LC–MS (positive-ion mode) extracted-ion chromatograms of illicit drugs in a Dutch STP effluent water sample based on accurate mass with a mass window of 5 ppm.

Table 4
Results of screening for polar organic microcontaminants in groundwater with off-line SPE and LC–LTQ FT Orbitrap MS^n analysis

Code positive-ion measurement	Accurate mass of $[M+H]^+$ (Da)	MW (Da)	KRetI (min)	DBE	Most probable elemental composition (M)	Relative mass deviation (ppm)
07-006-pos	248.12775	247	25.27	7.0	$C_{14}H_{17}NO_3$	−1.5
07-007-pos	266.13839	265	28.45	6.0	$C_{14}H_{19}NO_4$	−1.1
07-008-pos	248.12786	247	29.05	7.0	$C_{14}H_{17}NO_3$	−1.0
07-009-pos	220.13280	219	32.49	6.0	$C_{13}H_{17}NO_2$	−1.8
Code negative-ion measurement	**Accurate mass of $[M-H]^-$ (Da)**	**MW (Da)**	**KRetI (min)**	**DBE**	**Most probable elemental composition (M)**	**Relative mass deviation (ppm)**
07-003-neg	264.12383	265	21.22	6.0	$C_{14}H_{19}NO_4$	−1.1
07-004-neg	294.13422	295	22.45	6.0	$C_{15}H_{21}NO_5$	−1.6
07-006-neg	278.13961	279	23.36	6.0	$C_{15}H_{21}NO_4$	−0.6

Code, MS database entry code; MW, molecular weight; KRetI, Kiwa Retention Index:

$$Rty_a = Rt_{Fn} + \frac{Rt_{Cx} - Rt_{Fn}}{Rt'_{Cx} - Rt'_{Fn}}(Rtx_a - Rt_{Fn})$$

with Rty_a = corrected retention time of compound a relative to internal standards fenuron and chloroxuron; Rtx_a = measured retention time of compound a; Rt_{Cx} and Rt_{Fn} = set retention times of the internal standards; Rt'_{Cx} and Rt'_{Fn} = measured retention times of the internal standards; DBE, double bond equivalents.

antidepressants and impotence drugs. However, only a small subset of these compounds (~150) has been investigated in environmental studies [12]. Pharmaceuticals represent a wide variety of compound(s) (classes), *i.e.* a wide variety of compound structures and functional groups, and have a broad spectrum of physicochemical characteristics. Most of these compounds are water-soluble for which analyses by LC–MS–MS is the obvious technique of choice. Different analytical methods for the determination of various pharmaceuticals in different types of water were developed [13–15].

To screen for the presence of pharmaceuticals in water, samples were taken from different sewage treatment plants (STP) effluents. Sample preparation and analysis of the extracts by LC–LTQ FT Orbitrap MS (in both positive- and negative-ionization mode) were performed as described in Section 2.

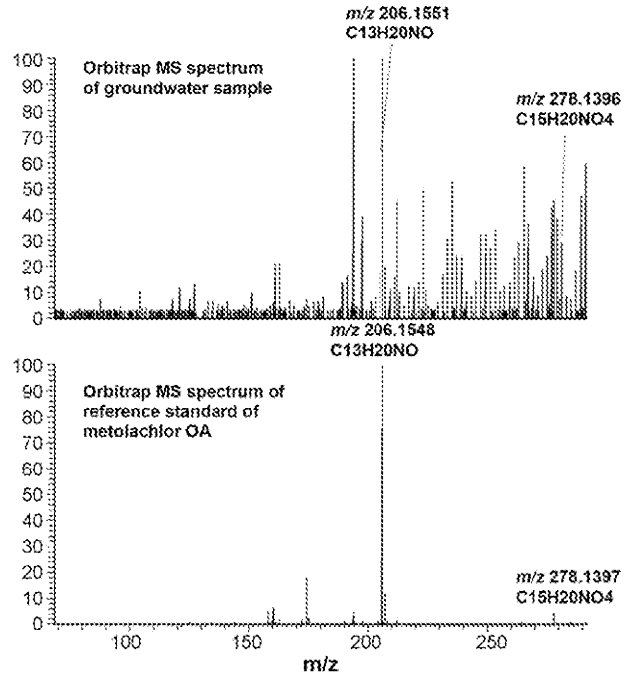

Fig. 3. Full-scan accurate mass spectrum (negative-ion mode) of metolachlor oxalinic acid (metolachlor OA) detected in a groundwater sample (top) and a standard solution (bottom). Elemental composition: $C_{15}H_{21}NO_4$, theoretical mass of $[M-H]^-$ *m/z* 278.1398. Some background ions were observed in the sample.

Quite a number of pharmaceuticals were identified (based on accurate mass and MS^n fragmentation pattern), such as the β-blockers atenolol, furosemide, metoprolol, solatol and trimethoprim, the anti-epileptic carbamazepine, the antibiotics clindamycine and sulphametoxazole, the analgesics diclofenac, naproxen and tramadol and the anaestatic lidocaïne to name a few. However, one has to keep in mind that the true confirmation of the compound's identity can only be done by analysis of and comparison with a synthesized reference standard. To confirm the presence of the compounds detected in the water samples and calculate the concentration levels, a quantitative method was developed (for selected pharmaceuticals, see Table 2). In short, for quantification the standard addition method was used. Hereto, an effluent sample was spiked with selected pharmaceuticals at concentration levels of 1.0 and 5.0 μg/L. The concentration of the pharmaceutical detected in the effluent samples was based on the response in the spiked effluent sample. Limits of quantification varied between 0.1 and 1.0 μg/L in effluent water. Subsequent analyses of the extracts confirmed the presence of certain pharmaceuticals in effluents at concentration levels between 0.1 and 2.7 μg/L (Table 2).

3.2. Benzotriazoles

1H-benzotriazoles are complexing agents that are used as anti-corrosives, *e.g.* in engine coolants, aircaft deicers, or anti-freezing liquids, and for silver protection in dish washing liquids. They are soluble in water, resistant to biodegradation, and are only partially removed in wastewater treatment [16].

Sample preparation and analysis of the extracts by LC–LTQ FT Orbitrap MS were performed as described in Section 2.

Screening for benzotriazoles in different types of water revealed the presence of some benzotriazoles, such as 1H-benzotriazole, and 4-methyl and 5-methyl-1H-benzotriazole in surface and drinking water (based on accurate mass measurements comparison).

To confirm the presence of these 1H-benzotriazoles in the water samples and calculate the concentration levels, a quantitative LC–MS (in positive-ion mode) method was developed. In short, 5 benzotriazoles, 1H-benzotriazole, 4-methyl-1H-benzotriazole, 5-methyl-1H-benzotriazole, 5,6-dimethyl-1H-benzotriazole and 5-chloro-1H-benzotriazole and 3 benzothiazoles, benzothiazole, 2-hydroxybenzothiazole, 2-(methylthio)benzothiazole could be analysed within one analysis. All five benzotriazoles and three benzothiazoles showed a linear response between 0.01 and 1.0 μg/L. For the majority of the compounds, recoveries were >70% in drinking, ground and surface water. The quantification limits

Table 5
Results of LTQ FT Orbitrap MS^n screening of a polar extract of a landfill soil sample

Code positive-ion measurement	Accurate mass of $[M+H]^+$ (Da)	MW (Da)	KRetI (min)	DBE	Most probable elemental composition (M)	Relative mass deviation (ppm)
07-pos-028	194.09587	193	21.7	10.0	$C_{14}H_{11}N$	−2.8
07-pos-029	295.12207	294	28.6	16.0	$C_{21}H_{14}N_2$	−3.1
07-pos-030	204.08029	203	35.3	12.0	$C_{15}H_9N$	−2.4
07-pos-031	223.14749	222	44.2	9.0	$C_{17}H_{18}$	−3.1
Code negative-ion measurement	Accurate mass of $[M-H]^-$ (Da)	MW (Da)	KRetI (min)	DBE	Most probable elemental composition (M)	Relative mass deviation (ppm)
07-neg-013	293.10736	294	40.5	16.0	$C_{21}H_{14}N_2$	−3.6
07-neg-014	281.15387	282	42.0	9.0	$C_{19}H_{22}O_2$	−2.9

Code, MS database entry code; MW, molecular weight; KRetI, Kiwa Retention Index:

$$Rty_a = Rt_{Fn} + \frac{Rt_{Cx} - Rt_{Fn}}{Rt'_{Cx} Rt'_{Fn}} (Rtx_a - Rt'_{Fn})$$

with Rty_a = corrected retention time of compound a relative to internal standards fenuron and chloroxuron; Rtx_a = measured retention time of compound a, Rt_{Cx} and Rt_{Fn} = set retention times of the internal standards, Rt'_{Cx} and Rt'_{Fn} = measured retention times of the internal standards; DBE, double bond equivalents.

were between 0.01 and 0.03 μg/L. More details on the method development of benzotriazoles and benzothiazoles in water are described in Ref. [17]. Subsequent analyses of the extracts confirmed the presence of 1H-benzotriazole, methyl-1H-benzotriazole and dimethyl-1H-benzotriazole in drinking water. Fig. 1 shows the LC–MS (positive-ion mode) extracted-ion chromatograms of 1H-benzotriazoles: 1H-benzotriazole, methyl-1H-benzotriazole and dimethyl-1H-benzotriazole in a drinking water sample. The concentration levels were 0.1 μg/L for both 1H-benzotriazole and methyl-1H-benzotriazole and 0.01 μg/L for dimethyl-1H-benzotriazole. Concentration levels observed in surface water were between 0.1 and 1.0 μg/L for 1H-benzotriazole, methyl-1H-benzotriazole and dimethyl-1H-benzotriazole.

3.3. Illicit drugs and metabolites

The use of illicit drugs is increasing worldwide, and millions of individuals are reported to be current users of cocaine, heroin, amphetamine-like stimulants and other drugs. Zuccato et al. [18] published in 2005 a first study in which concentration levels of cocaine and its metabolite benzoylecgonine in surface and wastewater were used to calculate environmental loads and to estimate cocaine consumption in different communities. As a consequence of the worldwide interest in this approach, determination of illicit drugs (and their metabolites) has become an important issue not only for forensic sciences, but also in environmental studies. Few studies deal with controlled drugs in environmental samples; the majority of these studies focus on wastewater effluents and surface water [19,20]. Currently, the impact and incidence of most of these compounds in the aquatic environment, as well as the effectiveness of the water treatment processes in their elimination, are unknown.

The screening approach was applied to explore the occurrence of these substances in different types of water in the Netherlands. Sample preparation and analysis of the extracts by LC–LTQ FT Orbitrap MS (in both positive and negative-ion mode) were performed as described in Section 2. Several water samples from different origin, *i.e.* treated sewage (effluent) water, surface water, groundwater and drinking water were analysed.

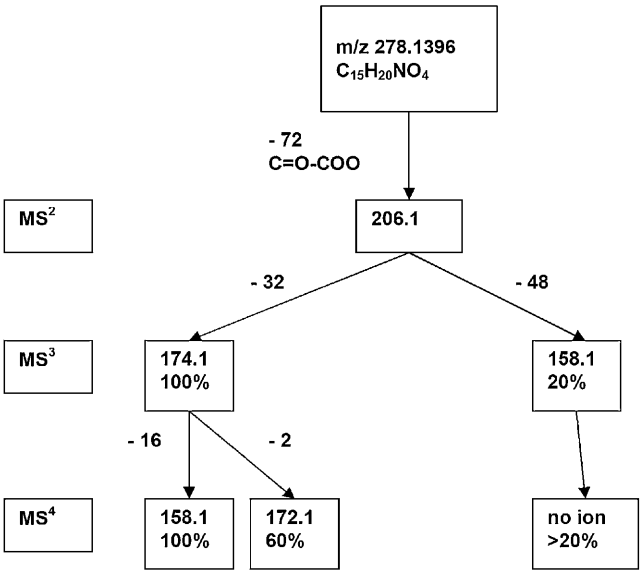

Fig. 4. Fragmentation pathway of metolachlor oxalinic acid (negative ionization). The deprotonated ion is measured with accurate mass, the product ions are measured data dependent in the LTQ with nominal masses. Only ions with a relative abundance >20% are shown. The product ion with mass 206.1 and the ion 206.1551 in the full scan spectrum are the same. Degradation of the oxanilic acid part of the molecule [M−H−72] is observed.

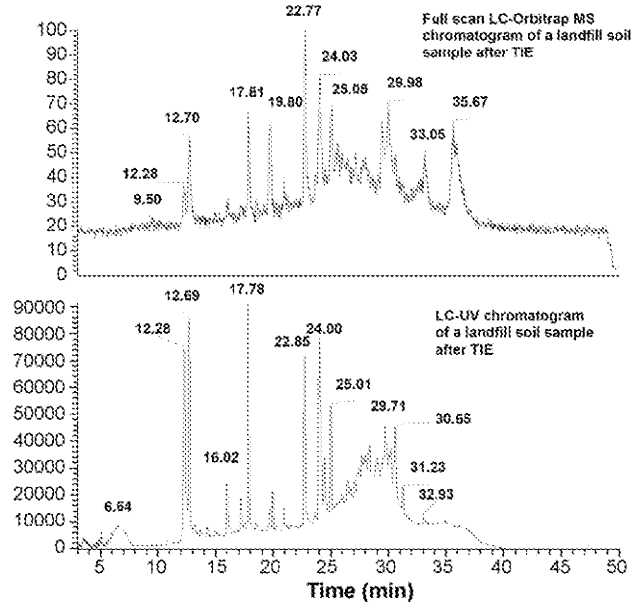

Fig. 5. LC–MS chromatogram (positive-ion mode; top) and LC–UV chromatogram (maximum spectrum plot; bottom) of the polar soil extract after TIE.

Table 3 presents the results of screening for illicit drugs and metabolites in different types of water (effluent, surface and drinking water) and at different locations. A positive identification (based on accurate mass and retention time) of an illicit drug is assigned as '+' in the table. Those compounds assigned as '–' were not detected. In addition to the results presented in Table 3, the opiates codeine, heroine, morphine, 6-monoacetylmorphine (6-MAM) and the amphetamine methylenedioxymethamphetamine (MDMA) were identified in one surface water sample and in one STP effluent water sample. The presence of these compounds could not be confirmed nor quantified in our laboratory, because at that moment in time no formal license for storage of reference standards was available. Fig. 2 shows LC–MS (positive-ion mode) extracted-ion chromatograms for the identification of some illicit drugs and metabolites in a STP effluent water sample. The presence of codeine, MDMA, nordazepam and oxazepam in this STP effluent water was confirmed by an independent laboratory (with a license for storage and analysis of drug reference standards).

3.4. Identification of an unknown

During a screening programme of groundwater quite a number of unknown (polar) organic compounds were detected. In Table 4 some results of a few compounds are presented, *i.e.* the accurate mass of their protonated molecules, the most probable elemental composition, the DBE and the relative mass error (between theoretical and measured accurate mass) in ppm. Since the mass accuracy error of the LTQ FT Orbitrap MS is relatively very small it was possible to assign an elemental composition in the cases presented in the table. The proposed elemental compositions of the compounds detected all contain C, H, N and O. Cl, Br and S were not observed. They are typified as aromatic-nitrogen containing compounds (DBE between 6.0 and 7.0) and some are isomers, such as for example compound with code 07-006-pos and 07-008-pos ($C_{14}H_{17}NO_3$). In addition, the 4 compounds differ only in 1 C (C_{13} or C_{14}), 1 or 2 O (O_2–O_4) and 2 H (H_{17} or H_{19}). Based on the fragmentation pattern observed the compound 07-009-pos was assigned as alachlor ($C_{14}H_{17}NO_3Cl$) minus CH_3Cl [21]. This compound was detected in photodegradation experiments of alachlor in water, but was not detected in the environment. The elemental composition of 07-007-pos ($C_{14}H_{19}NO_4$) and 07-008-pos ($C_{14}H_{17}NO_3$) differ only by H_2O. The compound 07-007-pos probably corresponds with the compound detected in negative-ion mode as 07-003-neg $C_{14}H_{19}NO_4$. With this in mind the elemental compositions of all chloroacetanilides and their metabolites were considered. Fig. 3 shows the full-scan accurate mass spectra (negative-ion) of the compound detected in a groundwater sample (top) and a reference standard solution of metolachlor oxanilic acid (OA) (bottom). Although the compound is present at a very low concentration level the MS database still is able to assign the compound. For confirmation purposes, the accurate mass and isotope pattern (ratio $^{13}C/^{12}C$) of the deprotonated ion of compound at *m/z* 279 detected in a groundwater sample and a standard reference solution of metolachlor oxanilic acid (OA) were compared. In addition, the accurate mass and isotope pattern (ratio $^{13}C/^{12}C$) of the most intense product ion of compound with *m/z* 279 detected in a groundwater sample and a standard reference solution of metolachlor OA were compared. In the same analytical run as the full-scan accurate mass measurements, MS^n mass experiments (see Table 1) were performed with the LTQ to construct a socalled 'fragmentation tree' with nominal masses. The deprotonated ion is measured with accurate mass, the product ions are measured data dependent (DDA) in the LTQ with nominal masses. Fig. 4 shows the fragmentation pathway of metolachlor OA (negative-ion mode). On the basis of the results presented above the compound *m/z* 279 (07-006-neg; $C_{15}H_{21}NO_4$) detected in a groundwater sample was confirmed to be metolachlor OA. In addition, the compound with code 07-006-pos and 07-008-pos appeared to be product ions of metolachlor in positive-ion mode. Metolachlor OA can be analysed in both positive and negative-ion mode.

The compound 07-007-pos and 07-003-neg and 07-004-neg are probably compounds related to chloroacetanilides and their metabolites, but have not been identified so far due to a lack of reference standards.

3.5. Toxicity identification evaluation in combination with chemical analysis

The toxicity identification evaluation (TIE) approach can also help in identifying emerging contaminants in the environment. In TIE bioassays, which measure biological effects in organisms or in-vitro systems, are used to direct fractionation of complex samples and subsequent chemical analysis, thereby ensuring that chemicals identified with the approach may actually cause effects. A contaminated soil sample from a former municipal landfill site was subjected to assay directed fractionation. Extracts corresponding to 10 g soil were prepared with pressurised liquid extraction and GPC, as described in [22] and fractionated in three consecutive series of fractionations using reversed-phase HPLC. Initially, five large fractions were collected and subjected to bioassay testing, an embryotoxicity assay employing zebrafish. Those yielding a response in the bioassay were further fractionated into 20 intermediate fractions. These were again tested separately. Six of these yielded a biological response, and these were further fractionated into three small fractions each, which were again tested. Details of the fractionation based on time of elution from the column and testing procedures can be found in Ref. [22]. The small fractions that yielded a response were analysed with GC–MS and the most polar fraction showing effects was also analysed using the LTQ FT Orbitrap MS instrumentation, as described in Section 2. The compounds that were observed either in positive- or negative-ion mode are shown in Table 5. The response of the most intensive peak in the mass spectrum was used to this end. The LC–LTQ FT Orbitrap MS chromatogram (positive-ion mode) of the polar extract is shown in Fig. 5. The most likely elemental compositions of the protonated or deprotonated molecules corresponding to the accurate masses observed are listed in Table 5.

The compounds with codes 7-pos-028, 07-pos-029 en 07-pos-030 are most likely aromatic structures containing a nitrogen atom. MS^2, MS^3 and MS^4 experiments did not show product ions, which is typical for polycyclic aromatic compounds (PAHs). Compound 07-pos-029 is probably the same compound as the one with code 07-neg-013.

In the polar fraction, both the GC and the LC screen tentatively identified the heterocyclic PAH methylacridine (*m/z* 193, $C_{14}H_{11}N$, several isomers possible). This compound is a metabolite of acridine, a member of the azaarene group of heterocyclic PAHs, which contain one nitrogen atom in one of the aromatic rings. The azaarenes are more water-soluble than the well-known homocyclic PAHs, and are known to cause genotoxicity [23,24]. Other candidates for the elemental composition $C_{14}H_{11}N$ could be methylphenanthridine and vinylcarbazole. The LC–MS screening of the polar fraction also exclusively revealed a compound with a molecular weight of 203.24, which could be represented by isomers of the nitrogen PAH azapyrene. Similar to methylacridine, azapyrene is a nitrogen-containing PAH which has been found in river sediment samples [25]. Standards of several of the candidates were tested. 9-Methylacridine and 4-azaapyrene were found to indeed yield responses in the bioassays similar to the small extracts, and were also found to end up in the small fraction when subjected

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Table 6
Proposal for additional LC/MS criteria to be implemented in 2002/657/EC taken from Nielen et al. [27]

Goal	Mass resolution (FWHM)	Mass accuracy (mDa)	Remarks
Screening	≥10,000	±50 (window)	Relative retention time ≤2.5%
Confirmation	≥10,000	≤5	1.5 identification points/ion or product ion; at least one ion ration; relative retention time ≤2.5%
HR confirmation	≥20,000	≤5	Two identification points/ion or product ion; at least one ion ratio; relative retention time ≤2.5%
MS/MS identification of unknowns	≥10,000	≤5	Confirmation postulated structure by NMR and/or confirm accurate masses at mass resolution ≥70,000 (FWHM)

to the RP-HPLC triple fractionation procedure. Three other peaks (with tentative elemental compositions $C_{21}H_{14}N_2$, $C_{19}H_{22}O_2$, and $C_{17}H_{18}$) have not been identified so far due to a lack of reference standards.

This study demonstrates the usefulness of the LTQ FT Orbitrap MS in a TIE approach to reveal toxic compounds of unknown identity in environmental samples.

4. Identification power of accurate mass by LTQ-FT Orbitrap MS

4.1. Some experiences

The identification of the target compounds as described in the previous examples is based upon the retention time and the accurate mass in full-scan MS. In our experience the relative mass error of the LTQ FT Orbitrap MS is stable between 1.0 and 3.0 ppm for masses higher than 150 Da. For masses below 150 Da, a mass error of 5.0 ppm is observed. The mass error is stable in time, in other words hardly any drift of mass is observed. The maximum drift is 1.0 ppm in 3–4 days. In our experience the instruments is very robust. In addition, the instrument is very robust.

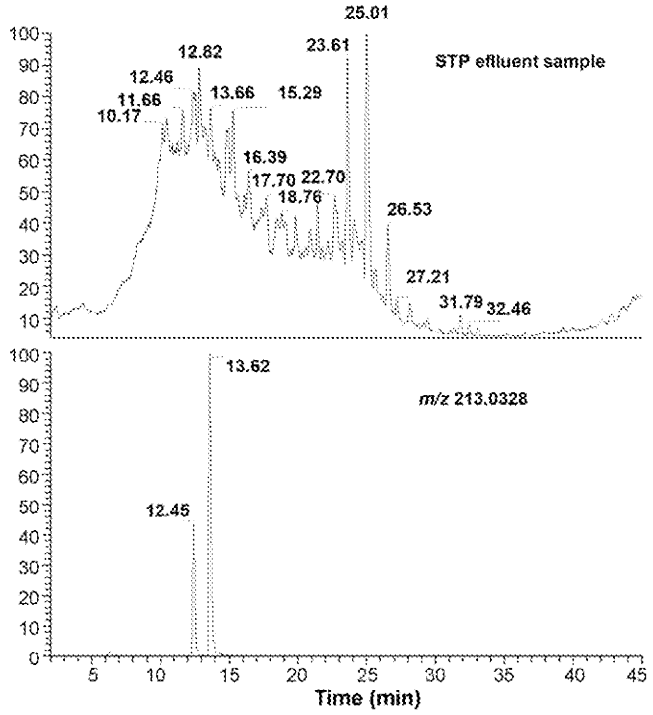

Fig. 6. LC–MS full scan (negative-ion mode) chromatogram (top) and extracted-ion chromatogram (bottom) of a STP effluent water sample spiked with pharmaceuticals at 1.0 μg/L level. Extracted-ion trace of clofibric acid ($C_{10}H_{11}ClO_3$, *m/z* 213.0328; bottom) with a mass window of 5 ppm.

For assigning the elemental composition a number of instrument characteristics are important: (i) mass error; (ii) low mass drift in time; (iii) high mass resolution, *i.e.* $^{13}C/^{12}C$ ratio is easily observed, which gives a good indication of the number of C atoms in the elemental composition.

The total MS cycle time is highly dependent on the mass resolution, *i.e.* the higher the mass resolution the higher the cycle time. For example, a mass resolution of 100,000 (FWHM) will result in a cycle time of about 2 s (1 'scan' = 2 s). To obtain enough datapoints over a chromatographic peak the cycle time versus mass resolution and LC peak shape/width need to be optimized. Similar high MS cycle times are observed when both accurate mass of precursor and product ions are obtained in a single analytical run. Cycle times can be increased when working at lower mass resolution; however, this may increase accurate mass error.

For this reason we chose to work with conventional LC in combination with the LTQ FT Orbitrap MS. Within a screening analysis, the following data are recorded: (i) LC retention time; (ii) MS accurate mass with a resolution of 100,000; (iii) MS^n product ions in the LTQ with nominal mass.

4.2. Identification criteria for confirmation of a screening result

For confirmation of a non-compliant screening result LC relative retention time criteria (<2.5%) and mass spectrometric identification criteria need to be fulfilled; the latter being based on the concept of identification points: an ion (or precursor ion) contributes 1 point and each multiple reaction monitoring (MRM) product ion 1.5 points (low mass resolution MS). Thus acquiring at least two MRM ion transitions yields the minimum requirement of four identification points and allow the calculations of at least one ratio of the product ions [26].

Highly sophisticated ion-trap based accurate mass Fourier Transform mass spectrometry, such as LTQ FT Orbitrap MS can be applied for identification of small organic molecules. As described above structural elucidation by accurate mass MS^n measurements and comparison with a synthesized reference compounds can disclose the identity of the unknown compound. However, criteria are missing: in 2002/657/EC high resolution mass spectrometry (HRMS) is defined as MS at a mass resolution of 10,000 according to the 10% valley definition [26]. For modern instruments such as TOF MS, FT ICR MS and LTQ FT Orbitrap MS the resolution is usually specified as full-width-at-half-maximum (FWHM), which

is approximately the double (requiring 20,000 FWHM). No criteria for mass accuracy are given, but two identification points per ion (or precursor ion), and 2.5 per MRM product ion are earned by HRMS, *i.e.* the requirements are less stringent than confirmation criteria for low resolution LC–MS–MS above.

Nielen et al. [27] proposed additional criteria for the use of accurate mass technologies, to be implemented in Commission Decision 2002/657/EC (see Table 6).

Based upon proposed criteria of Nielen et al. (see Table 6) the number of identification points obtained would be 2 points for the accurate mass precursor ion/product ion (in HRMS) and 1.5 points per nominal mass product ion. This would bring the total number of identification points to 3.5 points for the combination one accurate mass precursor ion and one nominal mass product ion and a total of 4.0 in case one accurate mass product ion is considered. In addition, one ion ratio should also be considered.

In our previous examples identification was based upon (i) LC retention time, (ii) accurate mass of the precursor ion (R = 100,000) and (iii) nominal masses of product ions which meets the proposed criteria.

The next practical example (also) illustrates that MS^n product ions are necessary for identification. During screening of treated sewage effluent water samples some pharmaceuticals were detected. Fig. 6 shows the LC–MS (negative-ion mode) chromatogram (top) of a STP effluent sample spiked with pharmaceuticals at 1.0 μg/L level. In the extracted-ion trace of clofibric acid ($C_{10}H_{11}ClO_3$, m/z 213.03279) (bottom) 2 closely eluting peaks were observed. MS^n experiments revealed that the compound with retention time 13.62 min had a product ion at m/z 126.9959 which was identified as clofibric acid. The compound with retention time 12.45 min has one product ion at m/z 141.0114. This compound was identified as mecoprop. In addition to the accurate mass of the precursor ion, specific product ions are necessary to identify a compound.

5. Conclusion

High-resolution mass spectrometry coupled to LC is a very powerful combination for screening and identification purposes. The applications of accurate mass screening and identification described in this article demonstrate that current day analytical instrumentation is well equipped to meet the challenges posed by newly emerging polar chemicals, for which Reach and other EU legislation, such as the Water Framework Directive and the Groundwater directive constitute the legal framework.

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