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Liquid chromatography–(tandem) mass spectrometry for the follow-up of the elimination of persistent pharmaceuticals during wastewater treatment applying biological wastewater treatment and advanced oxidation

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Abstract

The persistent and hardly eliminable pharmaceutical compounds carbamazepine, diazepam, diclofenac and clofibric acid were monitored in municipal wastewater by electrospray LC–MS and LC–MS-MS in positive and negative mode under high resolution and high mass accuracy conditions. While biological treatment by conventional and membrane bioreactors failed, the advanced oxidation methods using ozone (O_3), O_3/UV or hydrogen peroxide in combination with UV (H_2O_2/UV) successfully led to the complete elimination of these compounds. Target compounds could be confirmed as permanently present pollutants in Aachen-Soers wastewater in concentrations between 0.006 and 1.9 $\mu g\,l^{-1}$. Pharmaceuticals were determined after extraction using either C_{18} solid-phase extraction or by directly injecting them into the column without pre-concentration, achieving limits of quantification of 0.001 or 0.00001 $\mu g\,l^{-1}$, respectively.

Keywords: Advanced oxidation process (AOP); Biological wastewater treatment; Degradation products; Mass and tandem mass spectrometry; Polar pharmaceuticals

1. Introduction

At the end of the last decade pharmaceuticals emerged as a new class of organic pollutants of concern with a high potential to affect the environment and even human health [1]. Monitoring of wastewater [2] and surface waters [3] proved their presence after efficient analytical techniques, such as LC–MS and LC–MS-MS [4] came into routine use in order to determine these polar and persistent micropollutants [5]. Pharmaceuticals, once disposed off or excreted, either in their original form or as metabolites, often remain stable after conventional or even after advanced biological wastewater treatment processes. Therefore, wastewater discharges were identified as most important point sources of pharmaceuticals and their metabolites, which were found in the environment [1–3,5–10]. These compounds, often very polar and therefore, highly mobile in the aquatic environment, were observed in surface waters, groundwater or even drinking water. So their removal from drinking water as the most important food source for man is possible only by physico-chemical treatment, e.g., using membrane technologies like reverse osmosis (RO) or nano-filtration (NF) [11,12] or by means of activated carbon adsorption [13]. These advanced treatment steps are very effective yet quite energy- and material-intensive [14]. On one hand, the concentrates obtained from RO or NF treatment must be treated and discarded without emissions into the environment, while on the other hand the recycling and regeneration of the activated carbon as not only an effective but, moreover, an expensive adsorbent, becomes essential.

The elimination efficiency of conventional wastewater treatment does not provide a significant reduction of the concentration of pharmaceuticals and their metabolites before they are to re-enter the environment as literature data prove. With the knowledge that wastewater treatment plants discharge pharmaceuticals into surface waters like rivers and lakes [1,15] as well as into the sea [16,17], strategies for an advanced elimination of these pollutants became overdue. Highly efficient advanced biochemical [11,18–20], chemical or physico-chemical types of treatment [11,12,21–23] already were under research during the last decade. Membrane bioreactor treatment (MBR) alone, as an advanced combination of biological and physico-chemical processes, as well as activated sludge treatment and membrane filtration for biomass retention [11,18–20], all failed. With the application of ozone (O_3) and advanced oxidation processes (AOPs) for elimination, as reviewed by Kasprzyk-Hordern et

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al. [24], an improvement could be made, although elimination efficiency of the oxidation reagents was diminished by suspended, particulate organic matter found in conventionally treated wastewater [25,26]. That was the reason why our research focussed the on more promising advanced hybrid techniques, which are to eliminate compounds of high concern from wastewater more effectively, i.e., without generating highly loaded biological or activated carbon sludge or NF/RO concentrates. The hybrid process of O_3 or AOP application after a biological microfiltration process (MBR) seemed to be tailored to obtain elimination below the limits of detection by oxidation or mineralisation [27,28]. With the aim to determine elimination efficiency, we conducted experiments, which involved several of those representative drugs that literature reported as inherent in the effluents due to their persistence during biological treatment [1,6,8]. The target compounds, the three drugs carbamazepine, diazepam and diclofenac, as well as clofibric acid, which represents the metabolite of the lipid-reducing fibrates, could also be observed in feed and effluent of Aachen-Soers sewage treatment plant during conventional biological treatment.

Our goal was a far-reaching elimination of the persistent compounds under research by the application of a hybrid treatment using MBR treatment prior to oxidation by O_3 or AOPs. The pathways and whereabouts of the selected trace compounds and their biological and/or chemical degradation products through the treatment stages should be outlined qualitatively and quantitatively, as they can be determined by LC–MS and LC–MS^n.

2. Experimental

2.1. Materials

Ultra-pure water used in AOP treatment was prepared by a Milli-Q system (Millipore, Milford, MA, USA). Methanol used for desorption of pharmaceuticals and potential degradation products from the solid-phase materials, as well as acetone and methanol for SPE-conditioning purposes were Nanograde solvents purchased from LGC Promochem (Wesel, Germany). Acetonitrile and methanol used as mobile phases were of HPLC grade (LGC Promochem) and were used in gradient elution in combination with Milli-Q-purified water (Millipore).

Hydrogen peroxide solution 30% (H_2O_2) applied in AOP examinations was of "medical extra pure" grade (Merck, Darmstadt, Germany). Nitrogen gas for drying of solid-phase cartridges was of 99.999% purity. Oxygen for ozone generation was of "medical grade". All gases were products of Linde (Germany).

Carbamazepine, diclofenac sodium and clofibric acid were analytical grade compounds purchased from Sigma–Aldrich, Taufkirchen, Germany, while diazepam of pharmaceutical purity (Ph. Eur.) was purchased from Fagron, Barsbüttel, Germany. Stable isotopic labelled compounds for internal standard use were not commercially available.

The wastewater for our examinations originated from the Aachen-Soers municipal wastewater treatment plant (WWTP). The process performed was a conventional biological multistage treatment process. Before the wastewater was treated biologically, particles and sand were removed from wastewater by sieving or in the aerated grit removal tank, while the suspended solid were removed in the pre-settling tank. In the next step, biodegradable carbon compounds were eliminated through biological treatment. Nutrients like nitrogen and phosphorous were eliminated from wastewater either by nitrification, followed by a denitrification step or in a simultaneous precipitation after the addition of Fe^{2+} salts during aeration in the carbon-removal step. The sewage sludge was separated from the wastewater/bacteria suspension in the final clarification tank. The sludge was partly recycled while excess sludge was separated and treated anaerobically. Before the wastewater was discharged into the receiving water, the Wurm river, the effluent of final clarification was passed through a filter to remove traces of nitrate and phosphate. Permeate of the MBR was obtained with membrane modules and operated under semi-crossflow conditions (Kubota, Japan; pore size: 0.4 μm). Operational data for MBR are presented in literature [27].

From this wastewater treatment system feed of conventional and MBR, effluent of final clarification of conventional and permeate of MBR was taken as 3 h composite and time-correspondent samples over a period of 3 weeks and were used for analyses, spiking, O_3 and AOP treatment purpose. Sampling was only performed under dry-weather-flow conditions. The detention time of the conventional treatment plant then was 20 h, while the MBR detention time was calculated to 6 h.

The samples containing the pharmaceuticals were either stored at +4 °C and analysed as non-SPE-concentrated originals within 10 h after sampling or they were SPE-concentrated and also stored at +4 °C before they were desorbed and analysed. Results determined in the wastewater of Aachen-Soers and MBR treatment plant are shown in Table 2 (cf. Section 3).

2.2. Standards

For spiking experiments, O_3 and AOP treatment and quantification purposes, stock solutions of each pharmaceutical compound dissolved in acetonitrile or acetonitrile/water were prepared, containing 1 g l^{-1} of each compound. To reduce the tendency of hydrolysis, the stock solutions were kept at −18 °C. All working solutions were prepared freshly by diluting the stock solutions with Milli-Q water.

Calibration standards were obtained by diluting these stock solutions first to a drug concentration of 1 g l^{-1} each, before working solutions with concentrations of 0.1, 0.5, 1, 5, 10, 50 and 100 ng ml^{-1} for LC–MS were prepared by means of serial dilution. These were stored in glass bottles. Each concentration of pharmaceutical standards was applied to establish calibration curves and analysed in triplicate by MS in selected ion monitoring (SIM) mode after LC-separation on a reversed-phase (RP) C_{18} column, applying gradient elution. This confirmation step was performed prior to the entire procedure of extraction, and determination was validated three-fold by LC–SIM-MS. These standards were also used for spiking in the recovery experiments and prior to O_3 or AOP treatment. Product-ion spectra were obtained in LC–MS-MS or LC–MS^n mode.

2.3. Sample preparation

Commercially available solid-phase extraction (SPE) cartridges filled with C_{18} material from Baker (Deventer, The Netherlands) were used for concentration of the pharmaceuticals and their AOP degradation products. Prior to use, the cartridges were conditioned as prescribed by the manufacturer. All eluates from the solid-phase extraction were evaporated to dryness in a gentle stream of nitrogen at 30 °C. The residues then were taken up in 1 ml of methanol. Concentrates and depleted samples after SPE were used for injection in LC–MS analysis to confirm complete extraction of wastewater samples by SPE.

Depending on the degree of pollution determined as dissolved organic carbon (DOC), different amounts of centrifuged (feed) and membrane filtered (0.4 μm) (feed, permeate and treated permeate) wastewater were used in SPE procedure. The quantity of sample solution used for extraction was chosen in such a way that a maximum load of 7 mg DOC per 100 mg of SPE C_{18}-material was not exceeded. After samples for LC–MS analysis had been forced through the SPE cartridges, the columns were rinsed with one volume of purified water before they were dried in a gentle stream of nitrogen and stored at +4 °C. To ensure complete concentration of dissolved carbon on the SPE material, random distributed sample extracts, which were obtained from solutions after passage of the SPE cartridges were submitted to MS detection after a preceding lyophilisation procedure.

After the optimisation of SP-extraction recovery using spiked Milli-Q water, similar experiments were applied to real wastewater samples. For this purpose, 0.4 μm membrane filtered feed, MBR permeate and effluent of conventional treatment plant were spiked to reach concentrations of 100 ng ml^{-1}. As stable isotopic-labelled drugs were not available, standard solutions prepared from commercially available drugs were used for spiking.

All O_3 or AOP treatment steps (O_3/UV or H_2O_2/UV) were performed in a three-necked cylindrical glass reactor with a capacity of 2000 ml [29]. A 15 W medium pressure mercury lamp (Heraeus, Hanau, Germany), surrounded by a quartz thimble, was utilised during UV radiation. The flask and the quartz thimble were double-walled and water was used in a cooling circuit during reaction to maintain a temperature between 10 and 15 °C.

To perform oxidation either in pure water or wastewater after conventional or MBR treatment, 2 ml of the working solutions with concentrations of 1 mg ml^{-1} of selected drugs were spiked into 2 l of Milli-Q water, effluent and permeate, respectively, contained in the reaction flask. So the concentration of each pharmaceutical in the different types of wastewater was adjusted by spiking to exceed the native concentration by 1000 μg l^{-1} prior to oxidative treatment.

During ozonation experiments, O_3 was produced continuously from oxygen in an ozone generator (Sander, Uetze-Eltze, Germany). With the fixed gas flow and adjusted voltage, 2.6 g O_3 h^{-1} were generated. The gas, consisting of O_2 and O_3, was fed for 1 or 3 min into the wastewater, spiked Milli-Q water and wastewater using a sintered glass plug located at the bottom of the reactor while the aqueous solution of the drugs were thoroughly mixed by stirring.

The applications of O_3/UV or H_2O_2 in combination with UV (H_2O_2/UV) were carried out as mentioned before using O_3 or with the application of 0.5 ml of H_2O_2 l^{-1} aqueous sample at the physiological pH of the wastewater treatment process (about pH 6–8) with a simultaneous UV-radiation of 1 or 3 min, respectively.

In the course of the application of the oxidation reagents O_3, O_3/UV and H_2O_2/UV, 20 ml samples were taken after 0.0 and 3.0 min from the reaction flask to monitor the concentration of precursor drugs and their degradation products, respectively. Water samples from degradation pre-screening and treated wastewater samples (effluent and permeate) could be applied to LC–MS analyses with and without any pre-concentration step.

2.4. Methods

2.4.1. Quantification procedure

Quantification of drugs in feed, conventional effluent and permeates as well as for degradation monitoring of AOP treated pharmaceuticals was performed by means of calibration curves. These were reconstructed by the results obtained in LC–MS-MS detection mode applying electrospray ionisation in positive or negative mode (ESI(±)) with an ESI interface from Thermo Electron (San Jose, CA, USA). Selected ions of pharmaceuticals as enlisted in Table 1 were quantified.

The analysis of drugs to examine the wastewater load of Aachen-Soers treatment plant was performed using a minimum of five different composite sample series from all treatment stages. Each sample was determined at least in triplicate. For O_3 and AOP treatment to follow up pharmaceuticals under oxidation the last of these samples series was selected for spiking. After the addition of the drugs, the resulting concentration was determined and was set to 100%. Elimination rates were calculated from quantification obtained by LC–MS-MS in ESI(±) mode summing up peak areas of the drugs in selected product-ion traces.

Table 1
Mass and tandem mass spectrometric data of selected pharmaceuticals

Pharmaceutical compound	Interface/ionisation mode	Precursor ion $m/z_{calculated}$	Precursor ion $m/z_{observed}$	Product-ion m/z	Normalised collision energy (%)
Carbamazepine	ESI(+)	237.1022 ($[M+H]^+$)	237.1013 ($[M+H]^+$)	194.0955	19
Diazepam	ESI(+)	285.0789 ($[M+H]^+$)	285.0778 ($[M+H]^+$)	257.0829	21
Clofibric acid	ESI(−)	213.0324 ($[M-H]^-$)	213.0318 ($[M-H]^-$)	126.9969	50
Diclofenac	ESI(−)	294.0094 ($[M-H]^-$)	294.0085 ($[M-H]^-$)	250.0217	23

Calibration curves for these compounds were obtained after separation on RP-C_{18} phase prior to MS in SIM mode and were also linear over a concentration range of 0.1–100 ng ml^{-1} (LC–MS: $r^2 \geq 0.995$).

The limits of quantification (LOQs) in LC–MS-MS mode were calculated by a signal-to-noise ratio of 3 (*S/N* 3:1), taking into account the amount of sample extracted, the volume of the extract analysed and the absolute spiking quantities of pharmaceuticals. For direct injection onto the analytical column, the volume of water applied to on-column-injection was determining the LOD as well as LOQ.

2.4.2. Flow injection analysis

Working in FIA–MS (flow injection analysis–mass spectrometric detection) mode, ESI interface from Thermo Electron (San Jose, CA, USA) was used to determine the oxidative degradation products of the pharmaceuticals. By-passing the analytical column on the LTQ Orbitrap (Thermo Electron, Bremen, Germany) the following conditions were applied in FIA–MS: injection volume: 10 μl; mobile phase methanol–water (30:70) containing 0.1% formic acid. The flow rate was 0.5 ml min^{-1}. The same ESI interface parameters used for HPLC were applied here for FIA.

2.4.3. Liquid chromatographic conditions

LC-separations were carried out with a MultoHigh C 18 column (RP-C_{18}, 5 μm, spherical; 125 mm × 2 mm, I.D.) from CS Chromatographie Service (Langerwehe, Germany). Gradient elution by means of (A) methanol/acetonirile 50:50 (v:v) in combination with (B) Milli-Q-purified water was applied both containing 0.1% formic acid. The gradient was programmed as follows: starting with (35% A)/(65% B) the concentration was increased linearly to (90% A)/(10% B) within 8 min. Up to 20 min, the composition was kept constant. The overall flow rate was 0.2 ml min^{-1}.

For LC-separations prior to the LTQ Orbitrap MS detector (Thermo Electron, Bremen, Germany), a Surveyor MS Plus pump (Thermo Electron, San Jose, CA, USA) was applied coupled with a HTC-PAL LC autosampler system (CTC Analytics, Zwingen, Switzerland).

2.4.4. MS and MS-MS systems and analysis

A LTQ Orbitrap hybride mass spectrometer (Thermo Electron) was used for research work in ESI-MS(±) mode. Instrument control, data acquisition and data processing were performed using Xcalibur software (Thermo Electron, San Jose, CA, USA).

For HPLC–MS-MS analysis, the ESI interface was operated using a sheath gas flow rate of 55 units, the auxiliary gas was set to 5 arbitrary units and the sweep gas flow was set to 5 units applying a mobile phase flow rate of 0.2 ml min^{-1}. The spray voltage was set to 3.0 kV. The temperature of the transfer capillary was 200 °C. The LTQ Ion Trap instrument was operated in normal scan mode with a resolution of 300 mmu at "Full Width at Half Mass" (FWHM).

Applying MS-MS, the LTQ Orbitrap was operated in CID mode, acquiring the high resolution high mass accuracy full scan MS^2 mass spectra produced by the LTQ linear trap working in SRM mode. The Orbitrap mass resolution was set to 60,000 at *m/z* 400 with a scan range dependent on the *m/z* of the precursor ions. No internal mass calibration was used. The LTQ isolation width was set to 1000 mmu, activation *Q* value was 0.25, activation time was 30 ms and the maximum fill time was 100 ms. The normalised collision energy was optimised on the most abundant product-ion.

Identification of oxidation products by MS^n was performed using the methanolic SPE-C_{18} eluates of AOP-treated pharmaceuticals. Fragmentation behaviour of precursors and degradation products in ultra-pure water were monitored by MS-MS.

Drugs and their oxidation products in wastewater were then identified by using their characteristic product-ions obtained from compounds concentrated after degradation experiments in Milli-Q water applying SRM in MS^n mode.

3. Results and discussion

The persistent and hardly eliminable pharmaceutical compounds carbamazepine, diazepam, diclofenac, as well as the metabolite of the lipid reducing fibrates, clofibric acid, had been selected as target compounds for our examinations in order to monitor elimination during wastewater treatment. To reach one of the basic objectives, the determination of selected compounds in the complex matrix "wastewater", we first validated the analytical techniques for extraction, recovery and LC–MS and MS-MS analyses applying standards or wastewater samples spiked with these standards. After optimisation of SP-extraction, the recovery experiments led to satisfactory recovery rates of ≥96% using artificially generated wastewater samples. These were obtained from lowly contaminated wastewater spiked with pharmaceutical standards in the known quantities (Table 2) after the native drug load of the wastewater had been determined in standard addition mode.

The LOQs for the drugs were calculated by a signal-to-noise ratio of 3 (*S/N* 3:1) and enlisted in Table 2. For LOD- and LOQ-calculations after SPE, the amount of sample extracted, the volume of the extract analysed and the absolute quantities spiked were taken into account. For direct injection onto the analytical column, the LOD was determined by the quantity of water applied. Under direct injection conditions onto the column, LODs of 0.0002 or 0.0001 μg l^{-1} were observed for diclofenac and diazepam or carbamazepine and clofibric acid, respectively. The LOQs under the conditions applied were about five times higher (0.001 or 0.0005 μg l^{-1}; cf. Table 2, cf. Fig. 1). After C_{18}-SPE, using a concentration factor of 100 LODs and LOQs, the targets could be improved to the same extent, i.e., LODs and LOQs ≤ 10 ng l^{-1} in wastewater samples could be obtained.

After analytical requirements had thus been met, we focussed the goal to follow up and determine the elimination efficiency of the drugs and the drug metabolite during activated sludge treatment process or in a hybrid process. This process was a combination of advanced biological (MBR) and different chemical

Table 2
Load of pharmaceuticals in the different stages of Aachen wastewater treatment plant, limits of detection (LOD) and quantification (LOQ) in [$\mu g\,l^{-1}$] determined from selected product-ions recorded in LC–MS-MS mode

Pharmaceutical compound	Feed[a] wastewater ($\mu g\,l^{-1}$)		Recovery[b] (%)	Effluent[a] of final settling tank ($\mu g\,l^{-1}$)		Recovery[b] (%)	MBR-permeate[a] ($\mu g\,l^{-1}$)		Recovery[b] (%)	Original[a], without concentration ($\mu g\,l^{-1}$)	
	Original	C_{18}-SPE	C_{18}-SPE	Original	C_{18}-SPE	C_{18}-SPE	Original	C_{18}-SPE	C_{18}-SPE	LOD[c]	LOQ[c]
Diclofenac	1.9	1.5	97 ± 4	1.4	1.2	99 ± 2	1.4	1.2	98 ± 2	0.0002	0.001
Clofibric acid	–	0.019	95 ± 5	–	0.014	96 ± 2	0.007	0.006	100 ± 2	0.0001	0.0005
Carbamazepine	1.4	1.3	96 ± 3	1.4	1.0	101 ± 3	1.1	1.1	101 ± 2	0.0001	0.0005
Diazepam	–	0.08	101 ± 4	–	0.11	99 ± 3	0.09	0.07	99 ± 2	0.0002	0.001

Concentrations were determined without pre-concentration ("original") by on-column injection or after C_{18}-SPE followed by a methanol desorption.
[a] Data enlisted were determined as triplicates from a minimum of five wastewater samples (feed, effluent and permeate) which had been taken as time-correspondent samples from conventional and MBR treatment plant (sampling period: 3 weeks).
[b] Triplicate determination.
[c] In parallel with the SPE concentration factors of 100 the LODs and LOQs of the targets could be improved to the same extent.

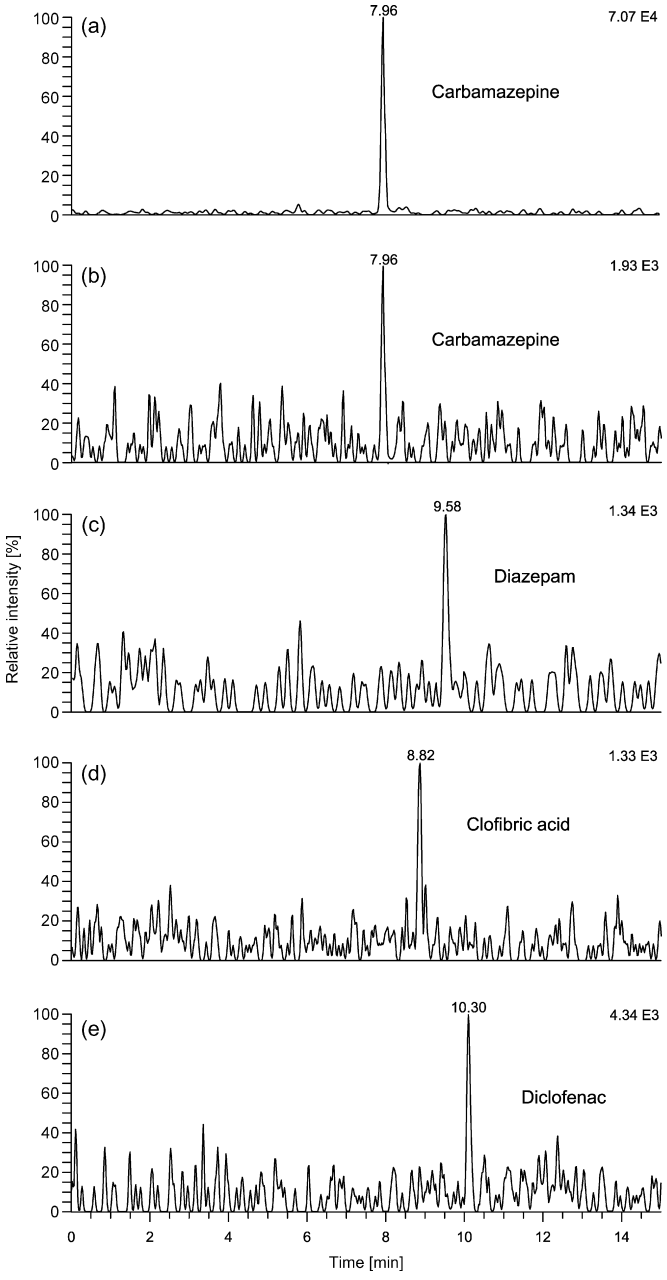

Fig. 1. LC–ESI(±)-MS-MS product-ion mass traces used for the LOQ determinations (b–e) of drugs which were spiked into wastewater and pre-concentrated by SPE: carbamazepine, t_R: 7.96 min, (a) concentration: 30 ng l^{-1}, *S/N* 20:1; (b) concentration: 5 ng l^{-1}, *S/N* 3:1; (c) diazepam, t_R: 9.58 min, concentration: 10 ng l^{-1}, *S/N* 3:1; (d) clofibric acid, t_R: 8.82 min, concentration: 5 ng l^{-1}, *S/N* 3:1 (e) diclofenac, t_R: 10.30 min, concentration: 10 ng l^{-1}, *S/N* 3:1. Separation by gradient elution performed on RP-C18 column. Precursor and product-ions are enlisted in Table 1. For LC–MS and MS-MS conditions, see Section 2.

wastewater treatment methods (O_3 and AOPs): MS- and MS-MS-techniques were performed for analyses.

FIA–MS, as having bypassed the analytical column as a useful tool for surfactant analysis [30,31] in sewage water in the past, was applicable neither for qualitative nor quantitative drug analysis. Reliable detection and identification of pharmaceutical target compounds selected and present as trace and ultra-trace compounds could not be achieved but for the application of MS- and MS-MS-detection in high-resolution mode (HR).

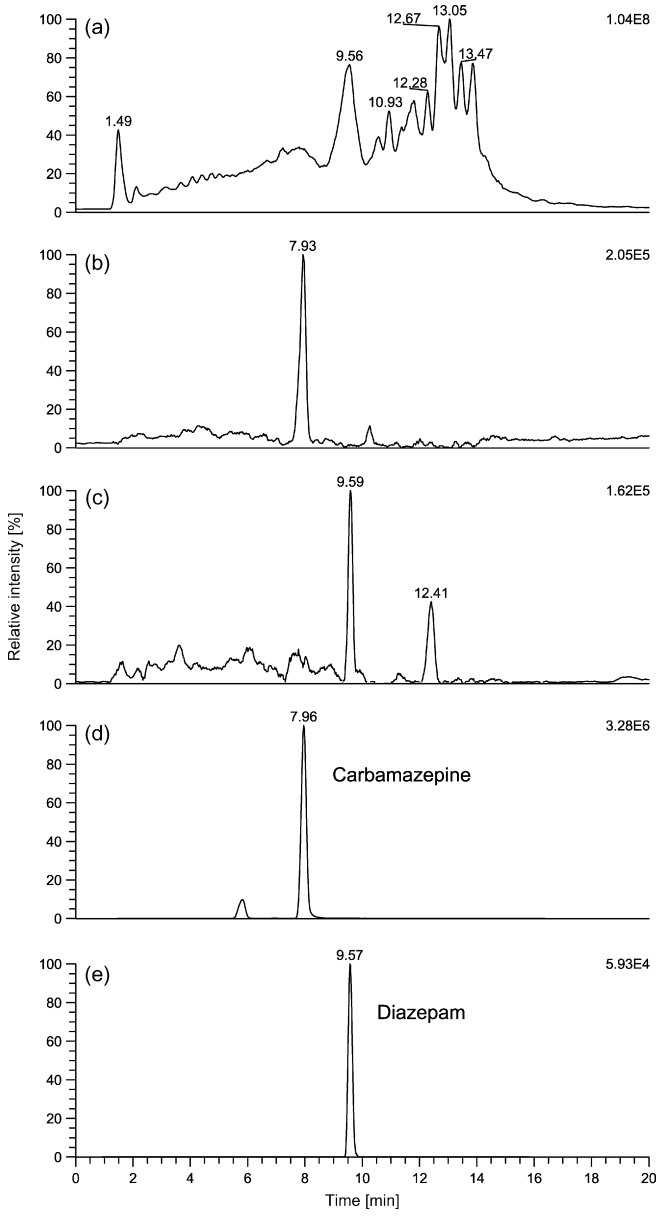

Fig. 2. (a) LC–ESI(+)-MS total-ion current tracing for the C_{18}-SPE-concentrated feed extract of Aachen-Soers treatment plant, displayed in normal scan mode with a resolution (FWHM) of 1000 mmu. Extracted mass trace of (b) carbamazepine (t_R 7.93 min) and (c) diazepam (t_R 9.59 min), extracted with a resolution of 1 mmu. (d) LC–ESI(+)-MS-MS product-ion mass traces of carbamazepine (t_R: 7.96 min; carbamazepine concentration: 1.3 ng ml^{-1}) and (e) diazepam (t_R: 9.57 min; diazepam concentration: 0.08 ng ml^{-1}), displayed with a resolution of 1 mmu. Separation by gradient elution as in Fig. 1. For LC–MS and MS-MS conditions, see Section 2.

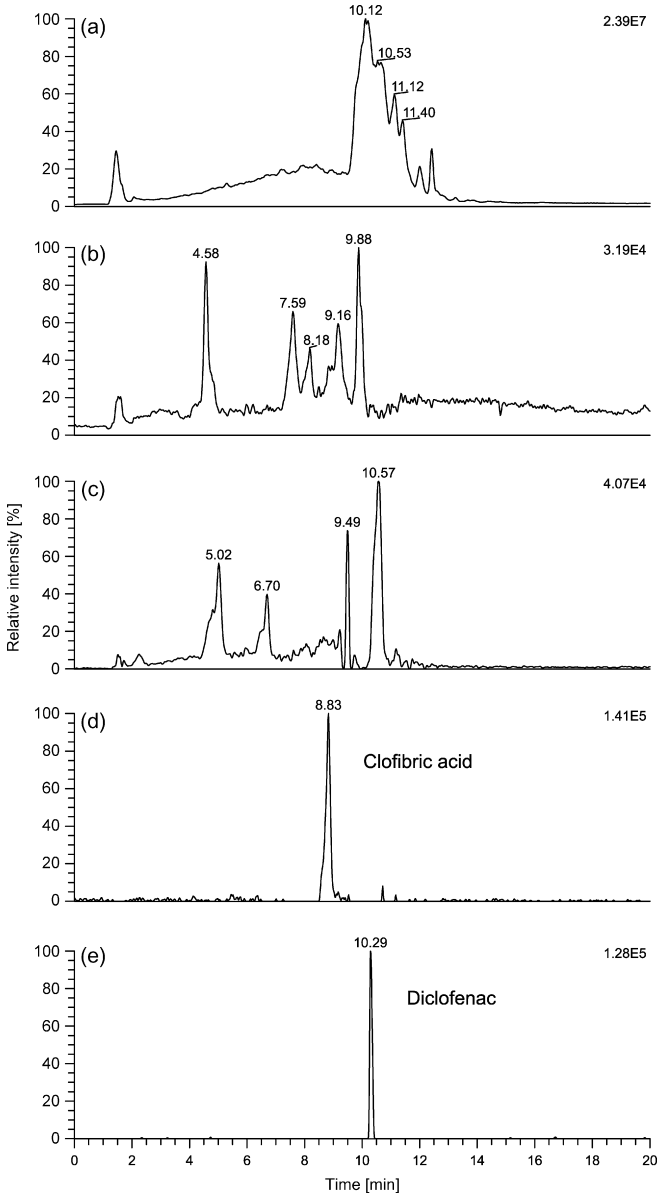

Fig. 3. (a) LC–ESI(−)-MS total-ion current tracing for the C_{18}-SPE-concentrated feed extract of Aachen-Soers treatment plant as in Fig. 1a. Extracted mass trace of (b) clofibric acid and (c) diclofenac, recorded and displayed as in Fig. 2b and c. (d) LC–ESI(−)-MS-MS product-ion mass traces of clofibric acid (t_R: 8.83 min; clofibric acid concentration: 0.02 ng ml^{-1}) and (e) diclofenac (t_R: 10.29 min; diclofenac concentration: 1.5 ng ml^{-1}) as in Fig. 2d and e. Separation by gradient elution as in Fig. 1. For LC–MS and MS-MS conditions, see Section 2.

3.1. Wastewater analysis by LC–(HR)MS

The reason for this failure was that municipal wastewater, as it has been used predominantly for our examinations, consists of surfactants – non-ionic and anionic surfactants – and personal care products (POPs) present in high concentrations. In Figs. 2a and 3a, the total ion current traces (TIC) of C_{18}-SPE-concentrated feed samples in the positive or negative mode when using a linear ion trap for detection are presented as consisting of the signals of LC-separated surfactants mentioned before—non-ionic surfactants of alkylpolyethyleneglycolether type (AEO) and anionic surfactants of linear alkylbenzenesulfonic type (LAS).

The extracted mass traces of the respective target compounds enlisted in Table 1 (cf. Section 2), and here recorded in normal scan mode with a resolution (FWHM) of 1 mmu, are presented in Fig. 2b (carbamazepine) and c (diazepam) or Fig. 3b (clofibric acid) and c (diclofenac), respectively. The intensity of the TIC differs from the selected ion current intensities that were extracted as high resolved ion mass traces in Fig. 2b and c by

a factor of 10^3. So carbamazepine and diazepam can be quite easily recognised in these positively ionised mass traces. In the negatively ionised mass traces (cf. Fig. 3b and c), also extracted under high-resolution conditions, though, the targets diclofenac and clofibric acid cannot be identified without any knowledge about their retention behaviour (t_R).

3.2. Wastewater analysis by LC–(HR)MS-MS

The options of the hybrid mass spectrometer – high resolution combined with high mass accuracy when recording spectra on the orbitrap – were used to identify unambiguously both the positively and negatively ionised targets carbamazepine, diazepam, diclofenac and clofibric acid. Contrary to the use of C_{18}-SPE-concentrated samples, wastewater without any pre-concentration was directly injected onto the column, then separated and analysed in CID mode on the orbitrap. The positively or negatively recorded mass traces of characteristic product-ions (cf. Table 1) in MS-MS mode, as shown in Fig. 2d and e or Fig. 3d and e, respectively, had been recorded with a signal selection of 1 mmu.

These signals obtained in product-ion mode were used to quantify the wastewater load from direct injection before the same quantification was performed and confirmed by the use of C_{18}-SPE extracts. The results in Table 2, showing the load of the target pharmaceuticals, were mean values obtained by the analyses of a minimum of five different composite samples (cf. Section 2). Contrary to the determination in LC–MS mode using the linear trap, the targets now could be quantified easily in high resolution and high mass accuracy with excellent *S/N* ratios by means of the orbitrap and by recording selected product-ions in MS-MS mode (cf. Table 1).

The results we obtained prove that the target pharmaceuticals were present in all municipal wastewater samples in concentrations above the LOQs (cf. Table 2). As these samples had been taken over a period of 3 weeks and, as time-correspondent samples, targeted pharmaceuticals and the metabolite clofibric acid could be confirmed as micropollutants, permanently present in untreated and treated wastewater of Aachen-Soers treatment plant. In the feed effluent of conventional treatment and permeate of MBR treatment, the micropollutants were observed in low microgram per litre or nanogram per litre ranges, respectively.

The results we obtained without concentration or after C_{18}-SPE, were comparable, though, in that a slight tendency to higher concentrations became apparent when we had used the original wastewater for a direct on-column injection without any pre-concentration step. The analysis of all drugs without pre-concentration was possible only in MBR permeate. Yet the determination of diazepam and clofibric acid as present in low concentrations both in feed and final effluent was disturbed by matrix compounds, if no C_{18}-SPE was performed prior to the analyses.

The comparison of the observed concentrations of all pharmaceuticals under research during the different treatment stages, i.e., in effluents of pre-settling tank (feed) and final clarifier of conventional treatment and permeate of MBR, proves that concentration changes that can be determined for all compounds in the time-correspondent samples lie within the analytical variance of the determination method. So the objective to eliminate the polar compounds by either biochemical degradation or adsorption onto the sewage sludge could be reached neither by conventional biological treatment nor by MBR treatment.

3.3. Oxidative treatment for the elimination of drugs in native wastewater

This failure of the biological degradation or adsorptive elimination of the targets led to an additional application of other physico-chemical treatment steps to these samples using O_3 alone, O_3 or H_2O_2, both in combination with UV radiation. This sequential treatment using O_3 or AOP after the biological MBR treatment can be regarded as a hybrid process that combines the far-reaching elimination of bio-degradable wastewater content by MBR and oxidative degradation of persistent drugs. MBR treatment eliminates all particles and easily biodegradable organic compounds, while O_3 or AOP treatment degrades or mineralises persistent pollutants, e.g., pharmaceuticals. All target compounds present in the feed and effluent or in the permeate of treated wastewater were reduced in concentration below their LOQs, i.e., $\geq$97.5%, though no additional adjustment of pH was performed during ozone and AOP treatment in laboratory scale [29] in order to improve treatment conditions.

3.4. Degradation and follow-up of pharmaceuticals in spiked Milli-Q water

With these results, our objectives to follow up the biochemical as well as chemical degradation by LC–MS was not met, since the desired metabolites or chemical degradation products could not be observed under these conditions. Our decision therefore was to spike the wastewater prior to the application of O_3 or AOP reagents. However, before real wastewater samples were spiked, preliminary examinations were performed using Milli-Q water, which had been spiked with concentrations of 1 mg l^{-1}, far above the real concentration level of these pharmaceuticals in the environment. The examinations in ultra-pure water in combination with FIA–MS(±) detection were aiming at collecting information about the chemical degradability of the target compounds and their oxidation products formed during O_3 or AOP treatment. By performing FIA–MS prior to LC–MS we expected an increase of probability to recognise degradation products, among precursor compounds in spiked Milli-Q water, generated by oxidation. The reason for conducting this procedure was the fact that LC-separation behaviour of chemical degradation products was unknown. These oxidation products should be more polar than precursor drugs. LC–MS may therefore lead to a poor separation result or even no separation at all.

Though no complete elimination of selected pharmaceuticals by this treatment could be reached (cf. Table 3), only the chemical degradation products of carbamazepine (CBZ), as already reported by McDowell et al. [22], could be recognised by FIA–MS before the degradation products were isolated and examined by LC–MS and LC–MS-MS. In addition, a new compound with an *m/z* ratio of 226.08626 was observed.

Table 3
Elimination efficiency in (%) of different physico-chemical treatment methods (O_3 and AOP) applied to ultra-pure water and wastewater (feed, conventional effluent and MBR-permeate) spiked with carbamazepine, diazepam, diclofenac and clofibric acid to exceed the native concentration by 1000 $\mu g\,l^{-1}$

Pharmaceutical compound	Elimination[a]	Treatment											
		O_3				O_3/UV				H_2O_2/UV			
		Milli-Q	Feed	Effluent	MBR	Milli-Q	Feed	Effluent	MBR	Milli-Q	Feed	Effluent	MBR
Diclofenac	(%)	93 ± 3	100 ± 5	100 ± 4	100 ± 2	95 ± 3	100 ± 3	100 ± 3	100 ± 2	92 ± 4	98 ± 4	100 ± 2	99 ± 3
Clofibric acid	(%)	65 ± 4	88 ± 4	89 ± 2	90 ± 2	72 ± 4	99 ± 4	97 ± 4	98 ± 2	70 ± 2	92 ± 5	98 ± 1	97 ± 6
Carbamazepine	(%)	60 ± 2	99 ± 5	96 ± 4	99 ± 1	81 ± 2	95 ± 3	92 ± 2	99 ± 3	73 ± 2	95 ± 5	92 ± 3	96 ± 3
Diazepam	(%)	20 ± 3	95 ± 4	53 ± 3	65 ± 3	27 ± 4	90 ± 5	77 ± 2	88 ± 5	10 ± 3	95 ± 5	61 ± 4	73 ± 5

Concentrations were determined in in ESI-LC–MS-MS mode without pre-concentration by on-column injection.
[a] Data were determined as triplicates from three samples (Milli-Q water, feed, effluent and permeate) each treated as enlisted.

Together with the product-ion spectra and the structures of the known degradation compounds (cf. Fig. 4a–c), the product-ion spectrum of this first-observed unknown degradation product obtained after O_3/UV and confirmed by LC–MS-MS is presented in Fig. 4d. The fragmentation pattern of the $[M+H]^+$-ion of the oxidation product with a *m/z* ratio of 226.08626 looks similar to the BQM product-ion spectrum in Fig. 4a, which contains also the same fragment ions at *m/z* 180.08 and 208.07. The calculation of the sum formula using high-resolution data led to the elemental composition of $C_{14}H_{12}O_2N_1$ for the $[M+H]^+$-ion at *m/z* 226.08626. The proposed structure of this new oxidation product is shown in Fig. 4d I. Alternatively the structure of the di-aldehyde (cf. Fig. 4d II) would also fit into the elemental composition, but a loss of *m/z* 18 (H_2O) seems to be only possible for an ion with structure I as also observed for aromatic and aliphatic carboxylic acids in ESI(+) mode [34]. The loss of CO (*m/z* 28) resulting in the fragment at *m/z* 198 is supporting the proposed structure I though the structure of this ion is not favoured energetically. The definite structure of the ion *m/z* 226 could not yet be determined and confirmed without the application of NMR [34].

The other target pharmaceuticals treated in Milli-Q water were more or less eliminated, but no oxidation products could be observed by MS.

3.5. Degradation and follow-up of pharmaceuticals in spiked wastewater

With this information about the degradation of the targeted pharmaceuticals in ultra-pure water, the feed, the effluent of the final clarifier and the permeate of the MBR were spiked in order to increase the concentration of the compounds under examination moderately. The elevated concentrations should help to follow up compounds during AOP degradation processes. The same reagents as used before were then applied to these spiked real environmental samples. Contrary to our examinations in Milli-Q water, O_3 and AOP treatment of spiked wastewater resulted in very high elimination rates (≥88%) for all compounds in the feed. Diazepam elimination observed in effluent and permeates, however, was reduced to ≥53%, while diazepam concentration in spiked Milli-Q water could only be diminished to ≤27% (cf. Table 3). For all targets including CBZ, no chemical degradation products as found for CBZ in spiked Milli-Q

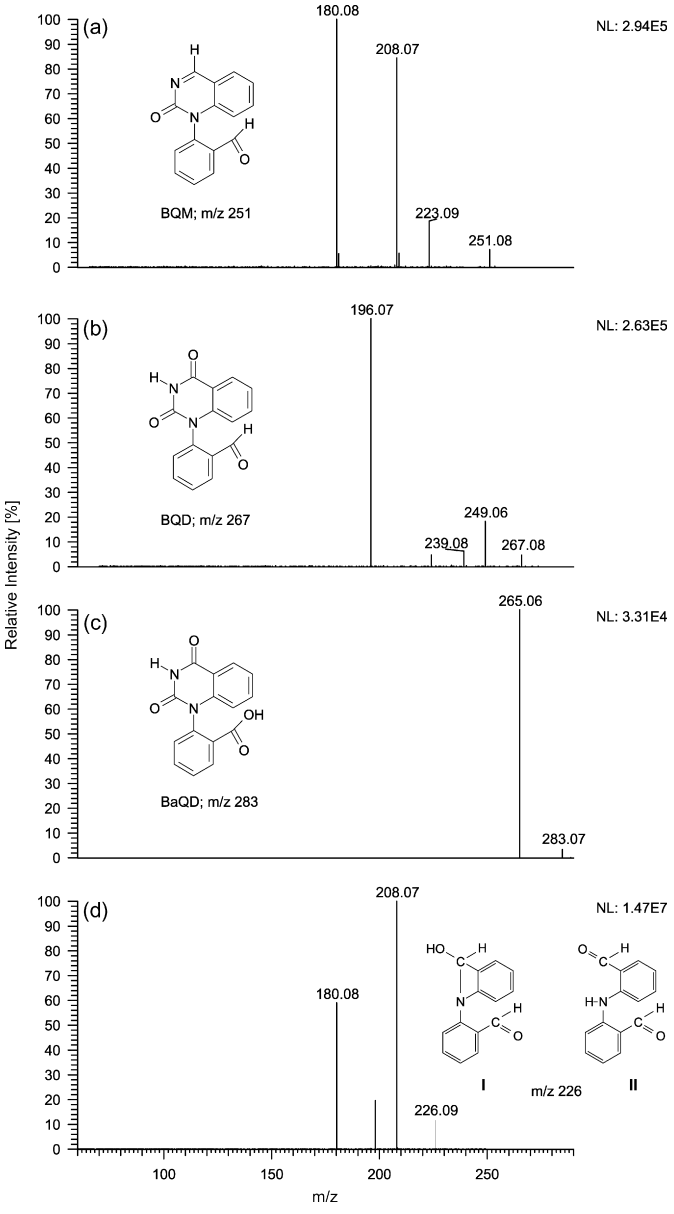

Fig. 4. LC–ESI(+)-MS-MS product-ion mass spectra and structures (a–c) of already known [22] degradation products of carbamazepine. (d) Fragmentation pattern and proposed structure of unknown degradation products of carbamazepine. AOP products were determined by LC–MS-MS after C_{18}-SPE and methanol desorption applying separation conditions as in Fig. 1. For MS-MS conditions, see Section 2.

water could be observed during the treatment of spiked wastewater. Though selected mass trace analyses of CBZ degradation products were performed, only the non-degradable residues of the target compounds were observed, yet no oxidation products. The reduced oxidation efficiency observed for all target compounds when oxidation was performed in Milli-Q water can be attributed to the complete absence of heavy metal ions, whereas in wastewater, a broad spectrum of heavy metal ions is present as catalysing oxidation. This absence of metal ions reduces the velocity of degradation or prevents mineralisation.

4. Conclusions

LC–MS- and LC–MS-MS-examinations performed on a hybrid mass spectrometer confirmed that the polar pharmaceutical compounds carbamazepine, diazepam, diclofenac and the metabolite of the lipid reducing fibrates, clofibrinic acid, were permanently present in municipal wastewater of Aachen-Soers treatment plant. Their concentrations were low but easily detectable in the nanogram per litre range, which is in accordance with applied quantities in human health care [2,7]. Their elimination by biological wastewater treatment – conventional or advanced MBR treatment – failed and therefore, the pharmaceuticals were, in undiminished concentrations, discharged with the wastewater into the environment. This discharge could be diminished with additional treatment steps by using O_3 or the AOP reagents O_3 and H_2O_2, both in combination with UV. The hybrid treatment process helped to solve problems by eliminating all target compounds contained in wastewater from Aachen-Soers conventional and MBR treatment plants below their LODs. Information about the fate of oxidation products generated in advanced wastewater treatment was not yet available. In our examination, oxidation products could only be detected in Milli-Q water but not in treated wastewater samples, i.e., O_3-degradation products of carbamazepine as found in ultra-pure water, could not be observed in both non-spiked and spiked wastewater samples under these conditions. Traces of heavy metals as present in wastewater, but not in ultra-pure water, served as catalysts, as confirmed by the addition of Fe^{2+}/Fe^{3+}-traces. Nevertheless, further examinations by applying O_3 or AOP treatment would become overdue because an increase of bio-toxicity in wastewater or surface water biocenosis cannot be excluded [32,33].

Despite the problem generated by a dominant wastewater matrix, all the above-mentioned results were obtained and could easily be confirmed by the follow-up of the target compounds in the biological and chemical treatment processes using ESI-LC–MS and ESI-LC–MS-MS. Product-ion spectra were recorded in positive and negative mode when applying high resolution in combination with high mass accuracy on an orbitrap mass spectrometer. This ensured efficient and selective quantitative determination of the polar, hardly eliminable pollutants by direct injection of wastewater samples onto the column without pre-concentration, reaching LOQs of 0.0005–0.001 $\mu g\,l^{-1}$ in positive and negative mode, respectively. A 100-fold lower value for both LODs and LOQs were obtained after the more time-consuming and cost-intensive C_{18}-SPE concentration step, which confirms the elimination of the pharmaceuticals below their LODs.

The procedure of improving LOQs by SPE-concentration led to slightly reduced, yet satisfactory recovery rates, while an overload of SPE phases could be excluded by following the prescription of the SPE manufacturer. The reduced recovery was possibly caused by interferences due to competitive adsorption of polar pharmaceuticals and the less polar wastewater matrix on the C_{18}-SPE phase.

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