Journal of Chromatography A, 1426 (2015) 77–90

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Development and validation of a generic nontarget method based on liquid chromatography – high resolution mass spectrometry analysis for the evaluation of different wastewater treatment options

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ARTICLE INFO

Article history:
Received 14 August 2015
Received in revised form 4 November 2015
Accepted 5 November 2015
Available online 10 November 2015

Keywords:
QTOF
Micropollutants
Process evaluation
Nontarget analysis
LC conditions
Wastewater
Direct injection

ABSTRACT

A comprehensive workflow for using nontarget approaches as process evaluation tools was implemented, including data acquisition based on a LC–HRMS (QTOF) system using direct injection and data post-processing for the peak recognition in "full scan" data. Both parts of the approach were not only developed and validated in a conventional way using the suspected analysis of a set of spiked known micropollutants but also the nontarget analysis of a wastewater treatment plant (WWTP) effluent itself was utilized to consider a more environmental relevant range of analytes. Hereby, special focus was laid on the minimization of false positive results (FPs) during the peak recognition. The optimized data post-processing procedure reduced the percentage of FPs from 42% to 10–15%. Furthermore, the choice of a suitable chromatography for biological treated wastewater systems was also discussed during the method development. The workflow paid also attention to differences in the performance levels of the LC–HRMS system by implementation of an adaption system for intensity variations comparing different measurements dates or different instruments. The application of this workflow on wastewater samples from a municipal WWTP revealed that more than 91% compounds were eliminated by the biological treatment step and that the received effluent contained 55% newly formed potential transformation products.

1. Introduction

The water quality of the urban water cycle is directly affected by the discharge of treated effluents from WWTPs [1]. The main objectives of WWTPs are the removal of organic carbon and of nutrients (e.g. nitrogen and phosphorous) [2] from the wastewater and the formation of a barrier for fecal bacteria and pathogens [1]. Therefore, the performance of WWTPs and the received effluent quality are characterized by physicochemical parameters (e.g. pH and temperature), chemical parameters (e.g. biological (BOD) and carbon oxygen demand (COD), amount of ammonia and phosphorus) and operational parameters (e.g. total suspended solids (TSS), hydraulic (HRT) and solids retention time (SRT)) [2–4]. The implementation of these objectives is mainly realized by the biological treatment step, which often bases on conventional activated sludge. Fixed-bed and membrane bioreactors are further examples [5,6]. In addition to standard operational parameters, the occurrence and behavior of micropollutants (MPs, e.g. pharmaceuticals and personal care products) has become an additional important factor determining the efficiency of the treatment. In conventional WWTPs, MPs are often only partially removed [7–9]. Therefore, the establishment of further advanced treatments steps and their impact on an improved removal efficiency came in a stronger focus [10]. Examples of these advanced post-treatments are the utilization of activated carbon, ozonation/sand filtration combinations or advanced oxidation procedures [3,11,12]. Usually a selected set of known MPs is quantified before and after the treatment step using *target analysis* for the evaluation of its removal efficiency [13–15]. This method, for small organic MPs, is usually based on LC–MS/MS measurements (liquid chromatography tandem mass spectrometry). The implementation of high resolution mass spectrometry coupled to liquid chromatography (LC–HRMS) for screening of organic MPs in aqueous systems using full scan mode opens new possibilities [7,16]. One of them is the extension of the restricted number of considered analytes of target analysis by a post subsequent extraction of XICs (extracted ion chromatograms) for a certain set of known analytes from the full scan data. This more comprehensive screening of MPs is often referred to as *suspected analysis* [17–20]. Another advantage of the high resolution data is that the accurate mass in combination with fragmentation information of a compound is frequently sufficient

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http://dx.doi.org/10.1016/j.chroma.2015.11.014

for the assignment of a measured signal to a certain compound and authentic standards are not always required [21]. Fragmentation experiments are often supplementary obtained during a LC–HRMS measurement. Additionally, the post-selection of analytes can be modified as often as required for a data set and new identified analytes can be taken into account during every new sample processing [7,22]. However, in both target and suspected analysis only known MPs are considered and valuable information about unknown MPs or transformation products is excluded from the evaluation. The *nontarget analysis* is one potential option to cover this knowledge gap. Beside known MPs, also "unknown compounds" are detected within the same chromatographic run. "Unknowns" not only mean really new compounds (e.g. transformation products), but analytes whose identity for this case is not expected. Similar to the suspected analysis, full scan data are extracted and a list of features and their corresponding XICs is achieved. The term *feature* in this work is defined as the result of the extraction procedure containing an exact *m*/*z*-ratio (mass-to-charge-ratio, mass) at a certain retention time (RT) combined with the knowledge of the intensity for each sample. In contrast to suspected analysis, the features are not directly associated with certain MPs, but rather are found by automated peak finding software. In most recent studies, the main objective of the nontarget analysis is to lay the groundwork for a subsequent identification procedure [23–28].

In addition to that, the nontarget approach can also be applied at an earlier date, before the identification procedure is implemented. In this case the whole list of features can be used to display relationships or differences within a sample set without the knowledge of the corresponding MPs. For instance, Müller et al. used this technique to determine the influences of a landfill leachate toward the final effluent of a water purification process [29]. The main difference between these nontarget approaches – identification vs. process evaluation – is that the focus is shifted from compound identification toward peak recognition. Hence, primarily the lowest confidence level defined by Schymanski et al. [30] will be used for the process evaluation. Therefore, in contrast to most recent studies, the peak recognition itself and other data post-processing steps, which influence the resulting list of features, also need to be validated and not only the recovery of target analytes [20,21].

For the first time, this work presents the development and validation of a generic nontarget LC–HRMS method designed for the evaluation of biological wastewater treatment. First, as one of the important parts of the data acquisition, a suitable chromatography for the separation of a broad range of polarities (polar – media polar compounds) was chosen. The essential steps for the nontarget procedure from the sample to the final list of peaks were developed and discussed in detail separately to subsequently generate an overall workflow for the nontarget approach. This workflow was applied to a set of influent and effluent samples from a municipal WWTP to demonstrate the potential of the nontarget analysis for process evaluation.

2. Materials and methods

2.1. Chemicals

Acetonitrile (ACN) and methanol (MeOH) (both LiChrosolv® hypergrade for LC–MS) were purchased from Merck (Darmstadt, Germany). Formic acid (FA, LC–MS grade) was purchased from Sigma-Aldrich (Seelze, Germany) and ultra-pure water (UPW) was obtained from a Milli-Q system (Millipore, Billerica, MA, USA).

The list of target substances used for method validation (target analyte mixture) as well as respective isotopic labeled internal standards (IS) is described in Tables 1 and 2. The compounds were chosen as representatives for organic MPs covering a wide range of polarity ($\log K_{OW}$: −4.16 to 7.75) and a wide mass range (119–836 Da). Standards of these substances were prepared separately in MeOH at a concentration of 1 g/L and were stored at −25 °C.

2.2. Environmental samples

Freezed aliquots of a 24 h-composite sample of the effluent (September 2013) from the municipal WWTP Koblenz–Wallersheim (KO) were used as reference matrix for optimization and validation of the nontarget method. Prior to freezing, the effluent sample was filtered (0.45 μm, regenerated cellulose, Spartan, Whatman, USA). WWTP effluent and UPW were spiked with an isotopic labeled internal standard (IS) mixture to obtain a final concentration of 1 μg/L of each IS. This UPW sample (with IS) was used for blank correction. Additionally, target analytes were also spiked to both matrices at different concentration levels (0.01–2.0 μg/L) for the optimization and validation procedure. For the X-ray contrast media the ten-fold and for acesulfame the twenty-fold concentrations were used. The KO sample at a spike concentration of 1 μg/L was used for the optimization process of the peak extraction and the post-screening of LC columns and LC eluents.

Furthermore, a grab sample of the river Rhine taken at Koblenz (November 2014) and corresponding samples from the effluent of the primary clarifier and the treated effluent of the WWTP of Koblenz–Wallersheim (February 2015) were used to determine matrix effects. These samples were further prepared as described above. In addition, spiked influent and effluent samples were 1:1 (d1) and 1:3 diluted (d2) with UPW.

2.3. LC–ESI-QTOF MS measurement

Measurements were carried out by the injection of 100 μL of the sample into an Agilent 1260 Series LC system (Agilent Technologies, Waldbronn, Germany) consisting of a membrane degasser, a binary high-pressure gradient pump, a high performance autosampler and a column thermostat. The chromatographic separation was achieved on a Zorbax Eclipse Plus C18 column (2.1 mm × 150 mm, 3.5 μm, Agilent) equipped with a Security Guard (2.0 mm × 4 mm, AQ C18, Phenomenex, Aschaffenburg, Germany).

The gradient of the LC method was composed by the following steps within the total run time of 27 min. UPW (A) and ACN (B), each containing 0.1% FA, served as mobile phases. After an isocratic step for 1 min, a linear gradient was applied from 2% to 20% B within 1 min. Afterwards, a linear gradient with reduced slope was used from 20% to 98% B within 14.5 min. An isocratic step followed for 5.5 min, then within 0.1 min, the initial conditions were reached again and were kept for 5 min constant to re-equilibrate the column. The flow rate was 0.3 mL/min and the column temperature was 40 °C.

The LC system was coupled to a hybrid quadrupole time of flight mass spectrometer (QTOF) (SCIEX TripleTOF 5600, Darmstadt, Germany). The QTOF system was equipped with a DuoSpray ion source and a TurboIonSpray™ probe for ESI experiments. A post-column divert valve (Rheodyne, Darmstadt, Germany) was used to discard the first 2 min and the last 7 min of the LC eluent for protecting the HRMS from highly polar salts and very apolar compounds. An additional flow of 0.3 mL/min UPW/ACN (1:1, v/v) pumped by an Agilent G1311B quaternary LC pump (Agilent) compensated the missing flow from the LC during waste positing operation. Electro spray ionization (ESI) was used in positive and negative ion mode in separate runs. The parameters for positive