Journal of Chromatography A, 1509 (2017) 60–68

Contents lists available at ScienceDirect

Journal of Chromatography A

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

Full length article

Comprehensive determination of macrolide antibiotics, their synthesis intermediates and transformation products in wastewater effluents and ambient waters by liquid chromatography–tandem mass spectrometry

Ivan Senta, Ivona Krizman-Matasic, Senka Terzic*, Marijan Ahel

Division for Marine and Environmental Research, Rudjer Boskovic Institute, Bijenicka c. 54, 10000 Zagreb, Croatia

ARTICLE INFO

Article history:
Received 15 February 2017
Received in revised form 31 May 2017
Accepted 1 June 2017
Available online 3 June 2017

Keywords:
Antibiotics
Azithromycin
Erythromycin
Clarithromycin
Transformation products
Liquid chromatography–tandem mass spectrometry
Wastewater
Surface water

ABSTRACT

Macrolide antibiotics are a prominent group of emerging contaminants frequently found in wastewater effluents and wastewater-impacted aquatic environments. In this work, a novel analytical method for simultaneous determination of parent macrolide antibiotics (azithromycin, erythromycin, clarithromycin and roxithromycin), along with their synthesis intermediates, byproducts, metabolites and transformation products in wastewater and surface water was developed and validated. Samples were enriched using solid-phase extraction on Oasis HLB cartridges and analyzed by reversed-phase liquid chromatography coupled to electrospray ionization tandem mass spectrometry. The target macrolide compounds were separated on an ACE C18 PFP column and detected using multiple reaction monitoring in positive ionization polarity. The optimized method, which included an additional extract clean-up on strong anion-exchange cartridges (SAX), resulted in high recoveries and accuracies, low matrix effects and improved chromatographic separation of the target compounds, even in highly complex matrices, such as raw wastewater. The developed method was applied to the analysis of macrolide compounds in wastewater and river water samples from Croatia. In addition to parent antibiotics, several previously unreported macrolide transformation products and/or synthesis intermediates were detected in municipal wastewater, some of them reaching μg/L levels. Moreover, extremely high concentrations of macrolides up to mg/L level were found in pharmaceutical industry effluents, indicating possible importance of this source to the total loads into ambient waters. The results revealed a significant contribution of synthesis intermediates and transformation products to the overall mass balance of macrolides in the aquatic environment.

1. Introduction

In the last 20 years, a large number of studies [1–5] reported on widespread occurrence of antimicrobial compounds in wastewater and in the aquatic environment. Since the spread of antibiotic resistance is considered to be one of the most serious global threats to human health in the upcoming decades [6], the ubiquitous presence of antibiotic residues in the environment raised a great concern about possible contribution of the aquatic route to proliferation of the resistant bacterial strains [7]. In order to meet the needs of reliable and sensitive assessment of antibiotic exposure concentrations in different matrices, a number of analytical methods for the determination of antimicrobials in environmental samples have been described in the literature over the past decade. Many of the published methods are multiresidual and multiple-class methods, which typically include a smaller number of selected representatives from each antimicrobial class [8,9]. Moreover, the majority of the published methods have focused exclusively on the parent compounds [5], or, at best, include some major metabolites [10,11]. As a consequence, the contribution of transformation products (TPs), as well as synthesis intermediates and byproducts, to the total mass loads of antimicrobial-related compounds in the environment is much less understood.

Macrolide antibiotics represent one of the most prominent classes of antimicrobial agents, with widespread usage in both human and veterinary medicine. In the global antibiotic consumption in 2010, macrolides were ranked third [12], and their usage in human medicine in Croatia and other European countries [13] is in accordance with the global figures. Moreover, together with

* Corresponding author.
E-mail address: terzic@irb.hr (S. Terzic).

http://dx.doi.org/10.1016/j.chroma.2017.06.005
0021-9673/

penicilins, macrolides have reached the highest global sales among veterinary antimicrobials ($600 million in 2009) [14].

After therapeutic use, the macrolide antibiotics are released from the human body via urine and feces as a mixture of unchanged parent compound and various metabolites [15]. In addition, several literature reports [16–21] indicated that macrolides can be transformed in the environment either via microbial or photochemical degradation, yielding a number of TPs, mainly by *N*-demethylation, *O*-demethylation and hydrolytic cleavage of sugar residues. Nevertheless, most of the existing exposure data refer only to the parent macrolides, while the information on possible synthesis intermediates and/or TPs in the aquatic environment are still very scarce. In fact, to the best of our knowledge, none of the published methods for the quantitative determination of macrolide compounds in environmental samples tackled the problem of synthesis intermediates and byproducts. Our previous study, which focused on comprehensive analysis of freshwater sediments chronically polluted by wastewaters from the pharmaceutical industry [22], indicated that azithromycin synthesis intermediates can be found in significant concentrations along with high concentrations of the parent compound. Moreover, TPs of azithromycin and roxithromycin, formed by microbial phosphorylation, were identified in wastewater effluent of the membrane bioreactor [23]. However, due to the lack of methods for quantitative determination of macrolide synthesis intermediates and TPs, their contribution to the overall macrolide loads in wastewater effluents and in the aquatic environment could not be properly assessed.

The aim of this work was therefore to develop and validate a novel analytical method for comprehensive analysis of macrolide compounds in aqueous samples, with a special emphasis on azithromycin- and erythromycin-derived compounds. The relevance of this analytical approach will be demonstrated by the analysis of municipal wastewater, pharmaceutical industry effluent and receiving river water. Thus, the method is expected to provide a necessary tool for a more detailed assessment of the occurrence and fate of macrolide compounds in wastewater treatment and in the freshwater environment.

2. Experimental

2.1. Chemicals and materials

All analytes and internal standards, together with their abbreviations, are listed in Table 1, while their structures and properties can be found in Supplementary material (Table S1). AZI, EIE, EOX and CLA were kindly supplied by Pliva (Zagreb, Croatia). ROX was purchased from Sigma-Aldrich (Germany), while all other reference materials of macrolide compounds were supplied by Toronto Research Chemicals (Canada). AZI-PO_4 was prepared in our laboratory by microbial transformation of AZI (details can be found in Supplementary material with Fig. S1). The reference standard of CLA-OH was isolated from the urine of a patient treated with CLA (details can be found in Supplementary material with Fig. S2) and was used only as a qualitative standard.

Individual stock solutions of the target analytes, except AZI-PO_4, were prepared in methanol, at the concentration level of either 1 mg/mL or 2 mg/mL and stored at −20 °C. Working standard solutions, containing all analytes at 10, 1 and 0.1 μg/mL, were prepared by diluting the stock solutions with methanol and stored at 4 °C. The mixture of internal (surrogate) standards (AZI-d_3 and CLA-d_3) was also prepared in methanol at the concentration level of 5 μg/mL. HPLC grade methanol (MeOH) and acetonitrile (ACN) were delivered by BDH Prolabo (UK). Formic acid and ammonium formate were purchased from Sigma-Aldrich (Germany), while 25% ammonia solution in water was supplied by Merck (Darmstadt, Germany). Ultrapure water was produced using Elix-Milli-Q system (Millipore, Bedford, MA, USA).

Solid-phase extraction (SPE) cartridges Oasis HLB (200 mg/6 mL) and Oasis MCX (150 mg/6 mL) were supplied by Waters (Milford, MA, USA), while SPE cartridges for an additional extract clean-up, Strata SAX (100 mg/3 mL), were purchased from Phenomenex (Torrance, CA, USA). Glass-fiber filters (GF/D) were delivered by Whatman (UK).

2.2. Sample collection and preparation

All raw wastewater (RW) and secondary effluent (SE) samples for the method development and validation were collected at the central wastewater treatment plant (WWTP) of the city of Zagreb. Industrial effluent was collected at the discharge point of the pharmaceutical industry into the Sava River, near the city of Zapresic. River water sample for the method validation was collected from the Sava River upstream from the discharge point, while the samples for the preliminary survey were collected at two additional locations downstream of the discharge point. All samples were processed within 24 h after collection. Typical sample volumes were 100 mL, 200 mL, 250 mL and 1 mL for RW, SE, river water and industrial effluent samples, respectively. After the filtration through glass-fiber filters, the mixture of surrogate standards (100 ng of each) was added and pH was adjusted with formic acid if necessary. In the preliminary experiments, two types of SPE cartridges were tested: Oasis HLB and Oasis MCX. In the final procedure, samples were extracted at the original pH (7–7.5) using HLB cartridges, previously conditioned with 6 mL of methanol, 6 mL of ultrapure water and 6 mL of spring water, at the flow rate of approximately 5 mL/min. Cartridges were then washed with 6 mL of ultrapure water and 2 mL of H_2O/MeOH (80/20, *v*/*v*), dried under the nitrogen stream and eluted with 4 mL of methanol at the flow rate of ≤1 mL/min. If the extracts were not analyzed on the same day, they were stored at −20 °C. The SPE procedures tested in the preliminary experiments, using either HLB cartridges or MCX cartridges, are described in Supplementary material.

Before the instrumental analysis, extracts were additionally cleaned up using strong anion-exchange cartridges Strata SAX. Extracts were loaded onto the cartridges previously conditioned with 3 mL of MeOH and eluates were collected in the glass tubes. The cartridges were additionally eluted with 4 mL of MeOH. The eluates were combined and evaporated to dryness under N_2 using a TurboVap evaporator (Caliper Life Sciences, Hopkinton, MA, USA). The residue was re-dissolved in 0.5 mL of 100 mM ammonium formate/MeOH (1/1, *v*/*v*) for instrumental analysis.

2.3. Instrumental analysis

Samples were analyzed using liquid chromatography–tandem mass spectrometry (LC–MS/MS) on a Thermo Electron TSQ AM (San Jose, CA, USA) LC–MS/MS system, which consisted of HPLC pump and autosampler (Surveyor) interfaced to a triple quadrupole mass spectrometer equipped with an electrospray ionization source.

Four HPLC columns were tested for the separation of target compounds during the method development: YMC Pro C18 (150 mm × 2,1 mm; 3 μm), Gemini C18 (150 × 3 mm; 3 μm), Synergi Hydro RP 80 Å (150 × 3 mm; 4 μm) and ACE C18 PFP (150 × 3 mm; 3 μm). Optimal separation was achieved on ACE C18 PFP column, by using gradient elution with 0.1% formic acid in water (*v*/*v*, eluent A) and acetonitrile (eluent B) at a flow rate of 0.4 mL/min. The gradient was as follows: 0–10 min from 15% to 30% B; 10–30 min from 30% to 45% B; 30–31 min from 45% to 95% B; 31–34 min 95% B (isocratic hold), 34–35 min from 95% B to 15% B; 35–45 min from 95% to 15% B (reconditioning to initial conditions). The injection volume was 20 μL.

Table 1
List of target macrolides and MS/MS parameters used for the MRM aquisition mode.

Compound	Abbreviation	RT/min	Precursor Ion (*m/z*)	Product Ion 1 (*m/z*)	CE 1/V	Product Ion 2 (*m/z*)	CE 2/V
Descladinosyl Azithromycin	DCA	4.5	296.2	116.0	15	158.0	12
Phosphorylated Azithromycin	AZI-PO_4	9.9	829.5	671.4	30	573.4	35
N-Desmethyl Azithromycin	N-DMA	10.8	368.3	158.0	19	420.1	15
N'-Desmethyl Azithromycin	N'-DMA	10.8	368.3	434.1	16	144.0	17
Azithromycin	AZI	11.0	375.2	591.3	16	83.1	31
Azithromycin *N*- Oxide	AOX	12.8	383.3	304.0	11	83.0	14
Descladinosyl Clarithromycin	DCC	13.8	590.4	158.0	18	116.0	31
14-hydroxy Clarithromycin	CLA-OH	14.5	764.5	606.2	23	158.0	33
Erythromycin Imino Ether	EIE	14.6	366.2	573.3	8	83.0	11
N-Demethyl Erythromycin	DME	15.5	720.5	144.0	24	562.2	15
Erythromycin	ERY	16.2	734.5	158.0	25	576.3	15
Erythromycin Oxime	EOX	16.8	749.5	591.3	16	158.0	29
Anhydro *N*-demethyl Erythromycin	DME-H_2O	18.4	702.5	544.2	10	508.2	17
Anhydro erythromycin	ERY-H_2O	19.6	716.5	558.2	19	158.1	41
N'-Demethyl Clarithromycin	DMC	21.8	734.5	144.0	23	576.2	12
N-Demethyl Erythromycin Enol Ether	DME-EE	22.2	702.5	544.2	10	508.2	17
Clarithromycin	CLA	22.9	748.5	158.0	34	590.3	26
Roxithromycin	ROX	23.1	837.5	158.0	42	679.3	27
Erythromycin Enol Ether	ERY-EE	23.6	716.5	558.2	19	158.1	41
Azithromycin $-d_3$[a]	AZI-d_3	11.0	376.8	594.3	16	83.0	28
Clarithromycin $-d_3$[a]	CLA-d_3	22.9	731.5	593.3	17	161.0	24

RT – retention time; CE – collision energy.
[a] Internal standard.

The target macrolides were analyzed in positive ionization polarity. Capillary voltage and temperature were 3500 V and 350 °C, respectively. Nitrogen was used as desolvation and auxiliary gas (40 and 10 arbitrary units, respectively). Detection and quantification was performed by using multiple reaction monitoring (MRM) mode, applying argon as a collision gas. Two characteristic transitions were selected for each target compound. Precursor ion was either protonated molecular ion, $[M+H]^+$, or doubly charged protonated molecular ion, $[M+2H]^{2+}$. First transition was used for quantification, while second transition, together with the ratio of the two transitions, was used for confirmation purposes. Collision energy and tube lens offset were optimized individually for each analyte by infusing their standard solutions at the concentration level of 10 μg/mL. The selected transitions, as well as the optimized collision energies, are listed in Table 1.

2.4. Quantification and method validation

Quantitative determination was performed using appropriate internal (surrogate) standards. AZI-d_3 was used as a surrogate standard for AZI-derived compounds, while CLA-d_3 was used as a surrogate for other macrolide compounds.

The method validation included linearity, instrumental detection limit (IDL), method quantification limit (MQL), extraction recovery, accuracy, precision, matrix effect, as well as sample and extract stability. All validation parameters, except linearity, IDL and stability, were determined separately for each matrix (RW, SE, river water).

Linearity range was determined from 10-point internal standard calibration curves obtained by injecting standard solutions containing analytes in the concentration range from 1 to 1000 ng/mL and internal standards at the fixed concentration of 100 ng/mL. Linearity was verified by *F*-test, at 95% confidence level, according to IUPAC recommendations [24,25]. GraphPad Prism (version 6) was used for the statistical analysis.

IDL was determined from a four-point low-level calibration graph (0, 1, 2, 5 ng/mL), constructed with duplicates, at 95% confidence level, according to IUPAC recommendations [25]. MQL was calculated from IDL, taking into account typical concentration factor for each matrix.

Extraction recovery, accuracy, precision and matrix effect were all determined in the model experiments, performed in quadruplicate, with real water samples, which were spiked with the target compounds at the concentration level of 1 μg/L (RW and SE) or 200 ng/L (river water).

Extraction recovery was determined from the model experiments with samples spiked either before or after extraction, taking into account analytes already present in the original (non-spiked) sample. The extraction recovery was then calculated from the following equation:

$$\text{Extractionrecovery}(\%) = (A_{be} - A_{orig})/(A_{ae} - A_{orig}) \times 100$$

where A_{be} is the average response of analyte spiked to the sample before extraction, A_{ae} is the average response of analyte spiked to the extract after extraction and A_{orig} is the average response of analyte in the original sample.

The method accuracy was determined from the model experiments in which water samples were spiked with both analytes and surrogate standards before the extraction. Accuracy was calculated from the following equation:

$$\text{Accuracy}(\%) = (c_2 - c_1)/c_{0\,\times}\,100$$

where c_0, c_1 and c_2 represent the nominal spiked concentration, average concentration measured in the original sample and average concentration measured in the spiked sample, respectively. Method precision (repeatability), determined in the same experiment, was calculated as the relative standard deviation (RSD) of the analysis of spiked samples.

Matrix effect (signal suppression or enhancement) was calculated by comparing the average response of target compound spiked into the final extracts (A_{fe}) with the average response in matrix-free standard solution of the same concentration (A_{std}), taking into account analytes already present in the original sample (A_{orig}). Matrix effect was then calculated using the following equation:

$$\text{Matrixeffect}(\%) = (A_{fe} - A_{orig} - A_{std})/A_{std} \times 100$$

Sample and extract stability was assessed from the model experiments with RW. In these experiments, performed in triplicate, filtered RW samples were spiked with the target analytes at 1 μg/L. To assess the stability of target compounds in RW samples at −20 °C, spiked RW sample was divided into three sub-groups: one was processed on the same day, while the remaining two were

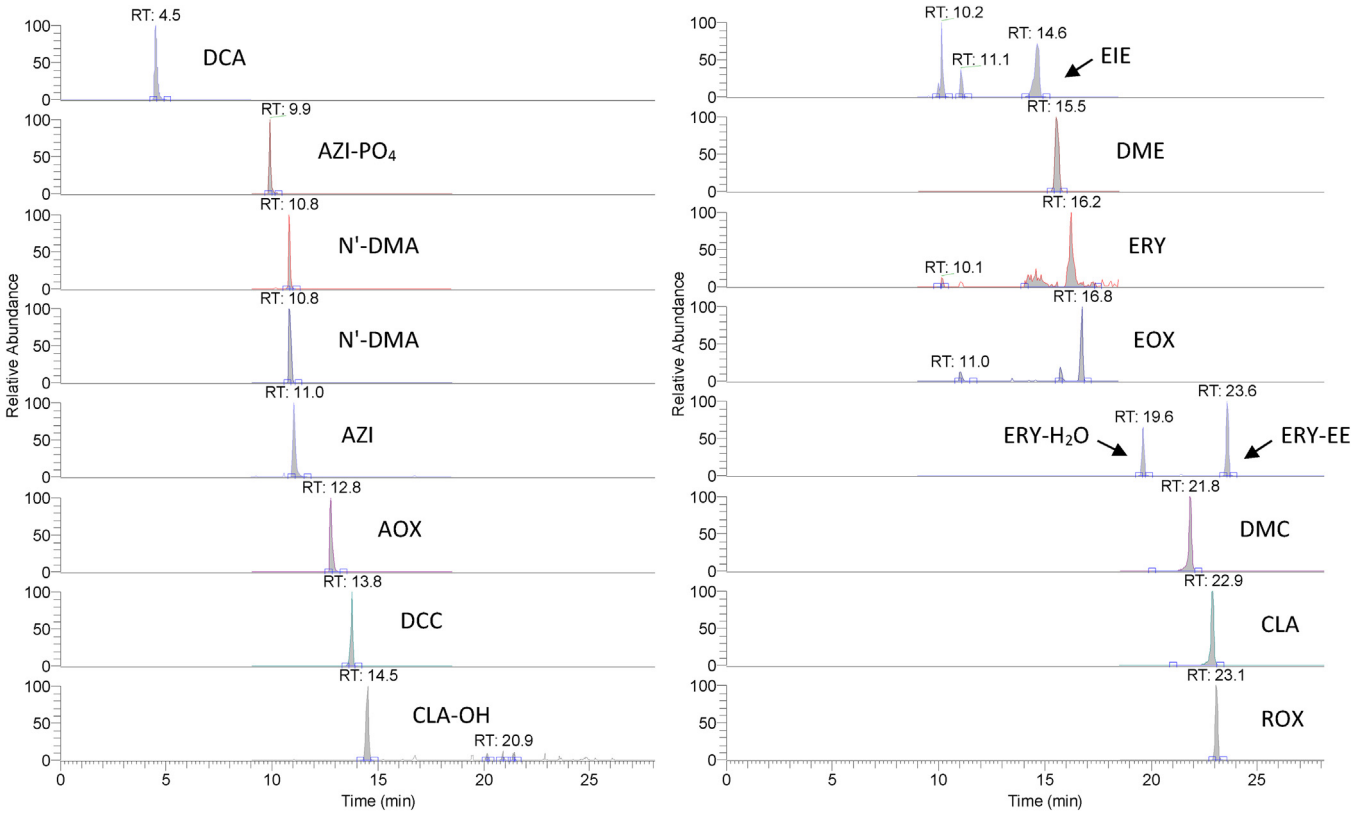

Fig. 1. MRM chromatogram of standard mixture of macrolide compounds (100 ng/mL) obtained on column ACE C18 PFP (150 × 3 mm; 3 μm).

processed after being stored at −20 °C for 7 and 35 days, respectively. The procedure to assess stability of target compounds in SPE extracts was similar to the protocol described above, but in this case all spiked sub-samples were extracted on the same day. The extracts were then analyzed on the same day and after storage at −20 °C for 7 and 35 days, respectively.

3. Results and discussion

3.1. Selection of the target compounds

The method to be developed included four parent macrolides with the high usage in Europe (ERY, AZI, CLA and ROX), along with their known synthesis intermediates, human metabolites and TPs. The full list of the target compounds with their structural formula can be found in Table S1 (Supplementary materials).

ERY is a naturally occurring macrolide antibiotic, which has been used for more than 50 years. It is also used as a precursor in the synthesis of AZI [26]. At low pH, ERY converts rapidly to two inactive metabolites: ERY-H_2O and ERY-EE [27]. Due to the strongly acidic conditions in the human stomach, the conversion of ERY in treated patients is very efficient, with less than 5% of the oral dose being eliminated unchanged [15]. Therefore, most of the methods for the assessment of ERY in environmental samples are based on the determination of ERY-H_2O rather than ERY [1,10,28,29]. However, recent study by Kwon [19] showed that, in addition to ERY-H_2O, significant amount of ERY-EE and pseudoerythromycin enol ether could be formed during biodegradation under both aerobic and anaerobic conditions. ERY is mainly metabolized by demethylation in the liver [15] and DME could also be expected to occur in municipal wastewater as a result of human excretion. Due to the high structural similarity with ERY, its further transformation to dehydrated products is highly probable and has to be taken into account.

AZI has been one of the most prominent antibiotics in the last few decades. For example, it was the mostly prescribed outpatient antibiotic in the U.S. in 2010 [30]. AZI was discovered by the Croatian pharmaceutical company Pliva and it has been continuously produced in large quantities in the facilities situated near the city of Zagreb for almost 30 years. AZI is a semi-synthetic antibiotic made from ERY as a precursor, while EOX, EIE and N-DMA are all intermediates in this process [26]. All these compounds were therefore taken into account in our method. Beside the parent compound and the key synthesis intermediates, our method also included some additional metabolites and/or TPs, such as N'-DMA, DCA, AZI-PO_4 and AOX.

CLA is another semi-synthetic macrolide antibiotic derived from ERY, which has become its more advanced replacement by offering an improved acid stability. According to the literature [15,21,31], beside the parent compound, occurrence of 3 different metabolic/TPs could be expected in wastewater: DMC, DCC and CLA-OH.

Due to the lack of reference materials, only parent ROX was included in our method. However, this is not considered as the major drawback, since ROX is rarely used in Croatia and was almost never detected in samples collected in Croatia in significant concentrations [29,32].

3.2. LC–MS/MS method optimization

During the method development, four HPLC columns were tested for the separation of target macrolides. In the preliminary phase, 0.1% HCOOH was used as aqueous eluent, while MeOH, ACN and 0.1% HCOOH in MeOH were tested as organic eluents. Synergi Hydro column was found to be unsuitable, due to the pronounced

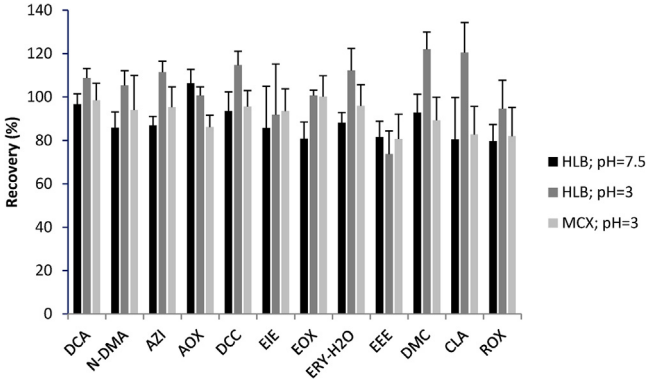

Fig. 2. The total extraction recoveries of target macrolides in spring water (spiking level: 1 μg/L; $n = 4$). For abbreviations see Table 1.

peak broadening of AZI-related compounds. Peak broadening was also observed on column YMC Pro C18. On the other hand, peak shapes were better on other two columns, Gemini C18 and ACE PFP. The separation on these columns was fairly similar and PFP column was selected for the further method development.

The selection of organic eluent proved to be an important factor for the successful separation of target macrolides, because peak shapes considerably improved when ACN was employed instead of MeOH. Although different gradient programs were tested, full separation could not be accomplished in two cases: N-DMA and N′-DMA co-eluted and could not be fully separated from AZI, while CLA and ROX also partially co-eluted (Fig. 1). Nevertheless, high selectivity of the method was assured by highly specific MRM transitions (Table 1). An excellent example is the differentiation between N-DMA and N′-DMA. These two compounds are structural isomers, with the only difference being the position of the methyl group. As a consequence, the most abundant product ions (577.3 and 83.0) are common for both compounds. In order to distinguish these two co-eluting compounds, the less abundant, but specific, transitions were finally selected (Table 1). MS/MS spectra of these two isomers, obtained on UPLC-QTOF/MS instrument (Waters Corp., Milford, MA, USA), can be found in Supplementary material (Fig. S3). On the other hand, two other structural isomers, ERY-H_2O and ERY-EE, shared all major product ions (Table 1), however their reliable determination was assured by the full chromatographic separation (Fig. 1).

3.3. Optimization of extraction procedure and extract work-up

For the optimization of extraction procedure, spring water, spiked with the target compounds, was used as a matrix. Two types of SPE cartridges were used in the preliminary experiments: Oasis HLB and Oasis MCX. The Oasis HLB cartridges were selected since they have been successfully applied for the extraction of parent macrolide antibiotics from different environmental water samples [10,28,29], while the selection of MCX phase was justified by its increased affinity for organic cations, which may provide a basis for additional selectivity. The HLB cartridges were tested at both original pH (approximately 7.5) and pH 3, while the MCX cartridges were tested only at pH 3 in order to protonate basic macrolide compounds, thus increasing their interaction with this mixed-mode cation exchange sorbent. In the preliminary experiments, these three different SPE protocols were tested by applying sequential elution with 6 mL of MeOH (fraction 1) and 6 mL of 1% ammonia solution in MeOH (fraction 2). The eluates were processed and analyzed separately. The total recoveries are presented in Fig. 2, while the combined recoveries for each fraction are presented in Fig. S4. As can be seen from Fig. 2, the total recoveries exceeded 75% for all investigated compounds and were fairly similar for all of the three protocols applied. However, significant differences were observed regarding the distribution of target compounds between the two eluates (fractions). Using HLB cartridges, all macrolides eluted almost completely in MeOH fraction (fraction 1). The additional recovery in fraction 2 contributed with only 2–7% and 1–13% to the total extraction recovery at the original pH and pH 3, respectively. In contrast, a more complex situation was observed with MCX cartridges. None of the target macrolides could be quantitatively eluted with MeOH from MCX sorbent. A relatively high recovery (61–78%) in fraction 1 was generally obtained for the compounds having only one nitrogen (ERY-H_2O, DCC, DMC, and CLA). The remaining macrolides, mostly azithromycin-like compounds, which contain an additional nitrogen in the lactone ring, were more strongly retained on the MCX sorbent, probably due to the stronger ionic interactions with the cation exchanger, and their recoveries in fraction 1 were negligible (0–7%). For the quantitative elution of macrolides from MCX cartridges it was necessary to apply basified MeOH as the eluting solvent, which suggests the importance of ionic interactions for the MCX sorbent.

Although all of the three investigated extraction protocols provided high recoveries of the target compounds, the protocol using HLB cartridges at the original sample pH was selected for further method development, because it allowed good recovery of all target compounds using a simple one-step elution with pure methanol. The main reason for selecting original pH instead of pH 3 was the simplicity and better compatibility with further extract work-up.

Since the final extract volume affects evaporation time, additional experiment was performed in order to optimize the solvent volume used for the elution of macrolides from HLB cartridges. For that purpose, spring water spiked with the target compounds was extracted using the selected SPE procedure. After sample percolation, the cartridges were sequentially eluted with three 2 mL-portions of MeOH. The subsequent analysis of sub-eluates showed that all target macrolides can be quantitatively eluted with 4 mL of MeOH, (less than 2% of the total recovery was found in the third fraction; Fig. S5). The protocol using MCX cartridges requires additional elution with basified MeOH, which increases the evaporation time and the amount of co-extracted interfering compounds that could enhance the matrix effect.

When the selected extraction procedure was applied to RW, as the most complex of all investigated matrices, it was observed that heavily-loaded RW extracts caused a significant peak deterioration and shift in retention times of several macrolides (Fig. 3A). This effect was more pronounced for the analytes with shorter retention times, especially for DCA, resulting in two completely distorted peaks. This problem of chromatographic interferences was fully resolved by an additional extract clean-up on strong anion-exchange sorbent (Fig. 3B), which probably removed some interfering acidic matrix components. It should be noted that all target macrolides could be quantitatively eluted from SAX cartridges in a single (MeOH) fraction.

3.4. Method validation

The method validation parameters, obtained for three different aqueous matrices, are presented in Table 2. As can be seen, linear range for most of the target compounds was between 1 and 500 ng/mL, with few exceptions for which linear range was up to 200 ng/mL. IDLs were in the low pg-range (injected on column). MQLs were generally in the low ng/L range.

Recoveries were high for all the selected compounds in all investigated matrices. In fact, the recoveries of all the analytes were higher than 90% in RW and river water. On the other hand, slightly lower recoveries were obtained for most of the compounds in SE, but they were typically higher than 70% and within the acceptable criteria [33]. It should be stressed that ERY was omitted from the

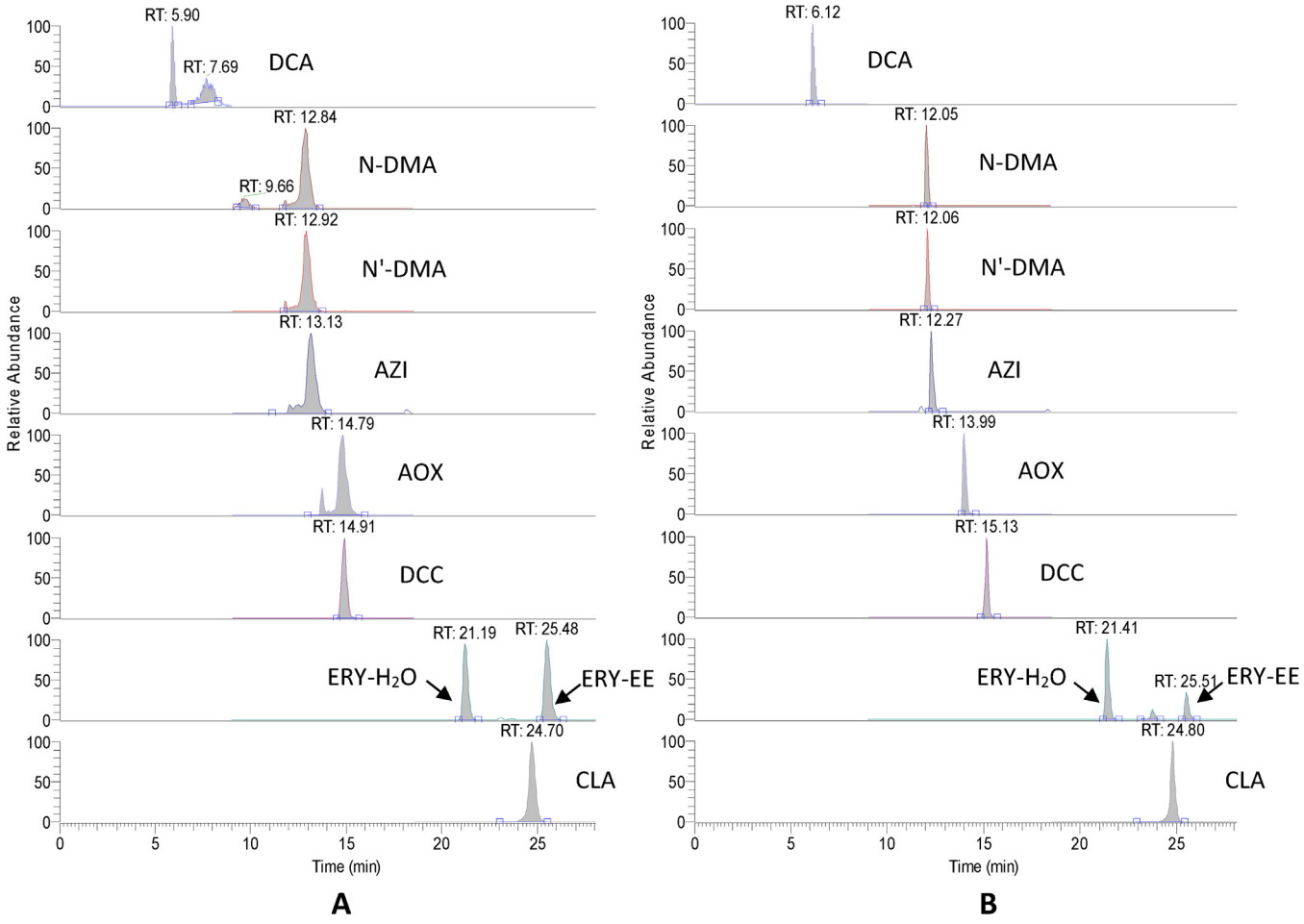

Fig. 3. MRM chromatogram of raw wastewater extract spiked with target macrolides (1 μg/L): A) without extract clean-up; B) after extract clean-up on Strata SAX cartridge. For abbreviations see Table 1.

macrolide model mixture used for the method validation in order to avoid its possible tampering with quantitation of ERY-EE and ERY-H_2O. A separate recovery experiment using the same procedure, performed with ERY and DME, indicated their complete transformation into the corresponding dehydrated TPs during the sample work-up.

Careful optimization of the extraction procedure, which included an additional extract clean-up on anion-exchange cartridges, resulted in relatively low matrix effects for most of the analytes. Even in the heavily-loaded RW extracts, the signal suppression was typically lower than 30%. Generally, matrix effects of river water extracts were the lowest. The only exception was DMC, for which signal suppression up to 46% was observed.

Owing to the high recoveries and relatively low matrix effects, the method accuracy was very good in most of the cases, although only 2 internal standards were used for the quantification. There were only few exceptions, such as DMC in river water (56%), which is mostly the consequence of higher matrix effect for this compound. It should also be noted that accuracy of AZI-PO_4 in RW was assessed using external calibration, because the assessment based on internal standard calibration (153%) suggested that AZI-d_3 was not a fully suitable surrogate standard for this analyte.

Method precision was excellent and comfortably within the acceptable criteria [33], with RSD values lower than 5% for all the compounds in all investigated matrices.

Regarding sample stability, all of the selected compounds were found to be stable in both filtered RW and RW extracts if stored at −20 °C up to 7 days (Fig. S6). Some degradation was observed after 35 days for DCA in filtered RW and EIE in RW extract, respectively. The concentration of ERY-H_2O decreased both in filtered RW and RW extract after 35 days. This decrease, which was more pronounced for RW extract than for filtered RW, was accompanied with the comparable increase of ERY-EE concentration. This suggested that transformation between the two dehydrated degradation products of ERY can occur during the prolonged storage. Taken together, the stability experiments suggest that, if the collected samples cannot be analyzed the same day, they can be either kept frozen at −20 °C (after filtration) or extracted and stored at the same temperature for at least 5 weeks.

3.5. Application to real water samples

The developed method was successfully applied for the analysis of selected macrolides in real wastewater and river water samples from Croatia. The results are presented in Table 3. It should be noticed that all concentrations are in μg/L. As can be seen, only EIE, DME-EE and ROX were below the detection limits in all samples. All other macrolide compounds were detectable in the analyzed samples, many of them at high frequencies and in relatively high concentrations. As expected, significant differences were observed between municipal wastewater and industrial effluents. The concentrations of parent macrolide antibiotics in municipal wastewater (RW and SE) of the city of Zagreb (0.77–9.7 μg/L AZI and 0.16–0.47 μg/L CLA) were generally in line with the previous reports on their occurrence in samples collected at WWTP of the city of Zagreb [32], as well as in wastewaters in other countries [2,5].

Table 2
Method validation parameters for determination of macrolides in water samples (spiking level: 1 μg/L (RW and SE), 200 ng/L (river water); $n = 4$).

	DCA	AZI-PO_4	N-DMA	N'-DMA	AZI	AOX	DCC	EIE	EOX	ERY-H_2O	ERY-EE	DMC	CLA	ROX
Linearity range (ng/mL)	1–500	1–200	1–500	2–200	1–200	1–500	1–500	1–200	1–200	1–500	1–500	1–500	1–500	2–200
r^2	0.9977	0.9954	0.9979	0.9919	0.9971	0.9967	0.9985	0.9977	0.9975	0.9965	0.9977	0.9965	0.9981	0.9959
F_{exp} (Critical F)	2.2 (3.0)	1.9 (3.2)	2.1 (3.0)	3.3 (3.6)	2.5 (3.2)	2.0 (3.0)	2.1 (3.0)	1.5 (3.2)	1.3 (3.2)	1.9 (3.0)	1.5 (3.0)	1.5 (3.0)	2.4 (3.0)	3.4 (3.6)
IDL (pg)	42	15	39	30	34	8	6	30	14	34	5	30	8	11
Raw wastewater (RW)														
Recovery (%)	95	117	92	93	106	94	93	106	94	94	92	93	92	93
Accuracy (%)	101	104[a]	95	107	94	104	101	96	99	112	103	75	96	107
Repeatability (%)	1	1[a]	2	2	2	2	4	1	3	2	3	1	1	1
Matrix effect (%)	−22	20	−24	−17	−18	−22	−9	−14	−10	6	−2	−31	−10	2
MQL (ng/L)	32	11	30	22	26	6	4	23	10	26	4	23	6	8
Secondary effluent (SE)														
Recovery (%)	81	125	67	88	71	104	89	82	76	78	72	110	79	76
Accuracy (%)	108	121	101	109	111	110	88	78	86	91	75	91	87	83
Repeatability (%)	3	2	2	3	5	1	1	2	1	1	3	3	1	4
Matrix effect (%)	−10	−11	−5	−21	−32	−32	−9	−8	3	−1	1	−43	−2	−1
MQL (ng/L)	16	6	15	11	13	3	2	12	5	13	2	11	3	4
River water														
Recovery (%)	94	102	102	103	98	106	93	100	108	91	107	105	97	96
Accuracy (%)	117	98	95	101	99	106	113	104	96	128	63	56	95	94
Repeatability (%)	2	3	2	3	1	3	2	2	2	2	4	2	2	3
Matrix effect (%)	−4	0	−9	−1	0	−2	−3	2	−6	−4	−2	−46	−9	−1
MQL (ng/L)	13	4	12	9	10	3	2	9	4	10	2	9	2	3

For abbreviations see Table 1. IDL – instrumental detection limit; MQL – method quantification limit.
[a] Determined using external calibration.

Table 3
Occurrence of macrolide compounds in wastewater and river water samples from Croatia.

	c (μg/L)																
	AZI	EOX	EIE	N-DMA	N'-DMA	DCA	AOX	AZI-PO_4	ERY-H_2O	ERY-EE	DME-H_2O[a]	DME-EE[a]	CLA	DCC	DMC	CLA-OH[a]	ROX
Municipal wastewater																	
Zagreb RW 09/05/2016	1.01	<MQL	<MQL	<MQL	<MQL	0.15	0.008	0.12	<MQL	<MQL	<MQL	<MQL	0.30	0.05	<MQL	0.32	<MQL
Zagreb RW 11/05/2016	0.77	<MQL	<MQL	<MQL	<MQL	0.05	0.02	<MQL	<MQL	<MQL	<MQL	<MQL	0.20	0.02	0.05	0.39	<MQL
Zagreb RW 14/12/2016	9.70	<MQL	<MQL	0.07	0.09	3.60	0.02	0.79	0.04	0.01	<MQL	<MQL	0.47	0.14	0.10	0.97	<MQL
Zagreb SE 09/05/2016	1.06	<MQL	<MQL	<MQL	0.03	0.23	0.02	0.30	<MQL	<MQL	<MQL	<MQL	0.22	0.09	0.03	0.38	<MQL
Zagreb SE 11/05/2016	0.89	<MQL	<MQL	<MQL	0.03	0.19	0.02	0.31	<MQL	<MQL	<MQL	<MQL	0.16	0.05	<MQL	0.48	<MQL
Industrial effluent																	
06/02/2016	2137	3355	<MQL	2341	<MQL	1501	16	9.0	2009	22	69	<MQL	<MQL	<MQL	<MQL	<MQL	<MQL
28/05/2016	3776	936	<MQL	5660	<MQL	2182	34	90	1069	50	8	<MQL	<MQL	<MQL	<MQL	<MQL	<MQL
River water																	
Sava Zapresic 28/05/2016	<MQL	<MQL	<MQL	<MQL	<MQL	<MQL	<MQL	<MQL	<MQL	<MQL	<MQL	<MQL	<MQL	<MQL	<MQL	<MQL	<MQL
Sava– Krapina cofluence 28/05/2016	1.4	1.3	<MQL	5.5	<MQL	4.1	<MQL	0.86	2.1	<MQL	<MQL	<MQL	<MQL	<MQL	<MQL	<MQL	<MQL
Sava Podsused 28/05/2016	3.4	19	<MQL	8.6	<MQL	5.3	<MQL	<MQL	10	<MQL	<MQL	<MQL	<MQL	<MQL	2.0	<MQL	<MQL

RW – raw wastewater; SE – secondary effluent; MQL – method quantification limit.
[a] Semi-quantitative determination: concentrations of CLA-OH were calculated based on calibration curve for CLA; concentrations of DME-H_2O and DME-EE were calculated based on calibration curves for ERY-H_2O and ERY-EE.

The analyzed metabolites and TPs of AZI and CLA were also detected in similar concentrations, contributing significantly to the total load of macrolides in the investigated municipal wastewater. In the RW sample collected in December 2016, the concentration of parent AZI of 9.7 μg/L, was accompanied with the elevated concentrations of DCA and AZI-PO_4 as well (3.6 and 0.79 μg/L, respectively). Similarly, the estimated concentration of CLA-OH was generally higher than the concentration of CLA. While the CLA metabolites were previously reported in the literature [21], most of the detected AZI-derived compounds are reported for the first time in this study. Comparison of their concentrations in RW and SE indicated very low removal of TPs and even suggested their possible formation during biological treatment. However, a more systematic approach will be needed to support these initial observations.

The concentration of AZI-related compounds was especially high in the effluent of pharmaceutical industry, where concentrations of AZI, N-DMA, DCA, EOX and ERY-H_2O reached mg/L level. Most of these compounds are associated with AZI synthesis. ERY-H_2O was the predominant form of ERY in our samples, however it should be noted that some of the detected ERY-H_2O could have been formed during the analysis. Tran et al. [34] suggested that the environmental concentration of ERY-H_2O could be assessed by subtracting the ERY concentration from the apparent ERY-H_2O concentration measured by LC–MS/MS. However, considering the well-known instability of parent ERY, including its possible transformations during the sample storage and analysis, such assessment is prone to significant errors. Moreover, our data and the recent literature [19] suggest that the total concentration of ERY should be expressed as the sum of ERY, ERY-H_2O and ERY-EE. The concentration of the dehydrated TPs of ERY metabolite DME was much lower than that of the corresponding ERY TPs. Furthermore, N-DMA and EOX are also synthesis intermediates from the AZI synthesis, while DCA could be either a byproduct from the AZI synthesis or a TP formed in the wastewater effluent. All these AZI-related compounds were highly abundant with concentrations exceeding 5 mg/L. These typical AZI synthesis intermediates were also detected in the Sava River downstream of the pharmaceutical effluent discharge, reaching concentrations up to 19 μg/L. This is considerably higher than the usual concentrations of macrolide antibiotics in surface waters [2–4]. In contrast, none of the synthesis intermediates was detected in the sample collected at the location upstream of the discharge point. It is important to note the concentration of AZI-related compounds in the receiving water was even higher than the concentration of the parent compounds, which underlined the importance of our approach.

4. Conclusions

The developed method allows for the first time quantitative determination of an extended range of macrolide compounds, including parent macrolide antibiotics and their metabolites, synthesis intermediates, byproducts and TPs, in wastewater and in the wastewater-impacted aquatic environment. The preliminary analysis of real water samples from Croatia indicated municipal wastewaters and especially effluents from the pharmaceutical industry as important inputs of macrolide-derived compounds into the natural waters, with a significant contribution of so far neglected synthesis intermediates and TPs to the overall mass balance. This method is therefore an essential tool for a more comprehensive study of the occurrence and fate of macrolides in wastewater treatment and in the aquatic environment.

Acknowledgments

We are thankful to the staf of Central WWTP of the city of Zagreb for the cooperation in wastewater collection. We thank Giovanna Guarinoni Ardito, Marin Matosic and Nikolina Udikovic Kolic for their contribution in the preparation of phosphorylated azithromycin. Technical assistance of Nenad Muhin is also highly appreciated. The research was financially supported by Croatian Science Foundation (project COMPASS; grant number IP-2014-09-7031).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.chroma.2017.06.005

References

[1] R. Hirsch, T. Ternes, K. Haberer, K.L. Kratz, Occurrence of antibiotics in the aquatic environment, Sci. Total Environ. 225 (1999) 109–118.
[2] K. Kümmerer, Antibiotics in the aquatic environment—a review –part I, Chemosphere 75 (2009) 417–434.
[3] P.A. Segura, M. François, C. Gagnon, S. Sauvé, Review of the occurrence of anti-infectives in contaminated wastewaters and natural and drinking waters, Environ. Health Perspect. 117 (2009) 675–684.
[4] A.J. Watkinson, E.J. Murby, D.W. Kolpin, S.D. Costanzo, The occurrence of antibiotics in an urban watershed: from wastewater to drinking water, Sci. Total Environ. 407 (2009) 2711–2723.
[5] T. Zhang, B. Li, Occurrence transformation, and fate of antibiotics in municipal wastewater treatment plants, Environ. Sci. Technol. 41 (2011) 951–998.
[6] Review on Antimicrobial Resistance, Tackling drug-resistant infections globally: Final report and recommendations, London, 2016. Available at: https://amr-review.org/sites/default/files/160518_Final%20paper_with%20cover.pdf (Accessed 10 February 2017).
[7] F. Baquero, J.L. Martínez, R. Cantón, Antibiotics and antibiotic resistance in water environments, Curr. Opin. Biotechnol. 19 (2008) 1260–1265.
[8] M. Seifrtová, L. Nováková, C. Lino, A. Pena, P. Solich, An overview of analytical methodologies for the determination of antibiotics in environmental waters, Anal. Chim. Acta 649 (2009) 158–179.
[9] L.J. Zhou, G.G. Ying, S. Liu, J.L. Zhao, B. Yang, Z.F. Chen, H.J. Lai, Occurrence and fate of eleven classes of antibiotics in two typical wastewater treatment plants in South China, Sci. Total Environ. 452–453 (2013) 365–376.
[10] A. Göbel, C.S. McArdell, M.J.F. Suter, W. Giger, Trace determination of macrolide and sulfonamide antimicrobials a human sulfonamide metabolite, and trimethoprim in wastewater using liquid chromatography coupled to electrospray tandem mass spectrometry, Anal. Chem. 76 (2004) 4756–4764.
[11] E. Gracia-Lor, M. Ibáñez, T. Zamora, J.V. Sancho, F. Hernández, Investigation of pharmaceutical metabolites in environmental waters by LC–MS/MS, Environ. Sci. Pollut. Res. 21 (2014) 5496–5510.
[12] T.P. Van Boeckel, S. Gandra, A. Ashok, Q. Caudron, B.T. Grenfell, S.A. Levin, R. Laxminarayan, Global antibiotic consumption 2000–2010: an analysis of national pharmaceutical sales data, Lancet Infect. Dis. 14 (2014) 742–750.
[13] European Centre for Disease Prevention and Control (ECDC), Summary of the Latest Data on Antibiotic Consumption in the European Union, ESAC-Net Surveillance Data, European Centre for Disease Prevention and Control, 2015, Stockholm. Available at: http://ecdc.europa.eu/hr/eaad/antibiotics-news/Documents/antimicrobial-consumption-ESAC-Net-summary-2015.pdf (Accessed 10 February 2017).
[14] Center for Disease Dynamics, Economics & Policy, State of the World's Antibiotics, CDDEP, Washington, D.C, 2015, Available at: https://cddep.org/sites/default/files/swa_2015_final.pdf (Accessed 10 February 2017).
[15] R.C. Baselt, Disposition of Toxic Drugs and Chemicals in Man, 3rd ed., Biomedical Publications, Foster City, California, USA, 2004.
[16] P. Calza, S. Marchisio, C. Medana, C. Baiocchi, Fate of antibacterial spiramycin in river waters, Anal. Bioanal. Chem. 396 (2010) 1539–1550.
[17] L. Tong, P. Eichorn, S. Pérez, Y.H. Wang, D. Barceló, Photodegradation of azithromycin in various aqueous systems under simulated and natural solar radiation: kinetics and identification of photoproducts, Chemosphere 83 (2011) 340–348.
[18] S.R. Batchu, V.R. Panditi, K.E. O'Shea, P.R. Gardinali, Photodegradation of antibiotics under simulated solar radiation: implications for their environmental fate, Sci. Total Environ. 470–471 (470) (2014) 299–-.
[19] J.W. Kwon, Environmental impact assessment of veterinary drug on fish aquaculture for food safety, Drug Test. Anal. 8 (2016) 556–564.
[20] E. Topp, J. Renaud, M. Sumarah, L. Sabourin, Reduced persistence of the macrolide antibiotics erythromycin, clarithromycin and azithromycin in agricultural soil following several years of exposure in the field, Sci. Total Environ. 562 (2016) 136–144.

[21] M. Ibáñez, V. Borova, C. Boix, R. Aalizadeh, R. Bade, N.S. Thomaidis, F. Hernández, UHPLC-QTOF MS screening of pharmaceuticals and their metabolites in treated wastewater samples from Athens, J. Hazard. Mater. 323 (2017) 26–35.
[22] S. Terzic, M. Ahel, Nontarget analysis of polar contaminants in freshwater sediments influenced by pharmaceutical industry using ultra-high-pressure liquid chromatography—quadrupole time-of-flight mass spectrometry, Environ. Pollut. 159 (2011) 557–566.
[23] S. Terzic, I. Senta, M. Matošić, M. Ahel, Identification of biotransformation products of antimicrobials in membrane bioreactor treatment by ultrahigh-performance liquid chromatography/quadrupole time-of-flight mass spectrometry, Anal. Bioanal. Chem. 40 (2011) 353–363.
[24] K. Danzer, L.A. Currie, Guidelines for calibration in analytical chemistry. Part 1. Fundamentals and single component calibration, Pure Appl. Chem. 70 (1998) 993–1014.
[25] A.C. Olivieri, Practical guidelines for reporting results in single- and multicomponent analytical calibration: a tutorial, Anal. Chim. Acta 868 (2015) 10–22.
[26] S. Schönfeld, Azithromycin and novel azalides, in: W. Schönfeld, H.A. Kirst (Eds.), Macrolide Antibiotics, Springer Basel AG, 2002 (pp. 73–96).
[27] A. Hassanzadeh, J. Barber, G.A. Morris, P.A. Gorry, Mechanism for the degradation of erythromycin A and erythromycin A 2¢-ethyl succinate in acidic aqueous solution, J. Phys. Chem. A 111 (2007) 10098–10104.
[28] S. Yang, K.H. Carlson, Solid-phase extraction-high-performance liquid chromatography-ion trap mass spectrometry for analysis of trace concentrations of macrolide antibiotics in natural and waste water matrices, J. Chromatogr. A 1038 (2004) 141–155.
[29] I. Senta, S. Terzic, M. Ahel, Simultaneous determination of sulfonamides, fluoroquinolones macrolides and trimethoprim in wastewater and river water by LC–tandem–MS, Chromatographia 68 (2008) 747–758.
[30] L.A. Hicks, T.H. Taylor, R.J.N. Hunkler, U.S. outpatient antibiotic prescribing, 2010, Engl. J. Med. 368 (2013) 1461–1462.
[31] A. Buchicchio, G. Bianco, A. Sofo, S. Masi, D. Caniani, Biodegradation of carbamazepine and clarithromycin by Trichoderma harzianum and Pleurotus ostreatus investigated by liquid chromatography—high-resolution tandem mass spectrometry (FTICR MS-IRMPD), Sci. Total Environ. 557 (2016) 733–739.
[32] I. Senta, S. Terzic, M. Ahel, Occurrence and fate of dissolved and particulate antimicrobials in municipal wastewater treatment, Water Res. 47 (2013) 705–714.
[33] A. Gustavo González, M. Ángeles Herrador, A practical guide to analytical method validation, including measurement uncertainty and accuracy profiles, TrAC-Trends Anal. Chem. 26 (2007) 227–238.
[34] N.H. Tran, H.J. Chen, T.V. Do, M. Reinhard, H.H. Ngo, Y.L. He, K.Y.H. Gin, Simultaneous analysis of multiple classes of antimicrobials in environmental water samples using SPE coupled with UHPLC-ESI–MS/MS and isotope dilution, Talanta 159 (2016) 163–173.