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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

S. Yang, K.H. Carlson*

Department of Civil and Environmental Engineering, Colorado State University, Fort Collins, CO 80523-1372, USA

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Abstract

A method using solid-phase extraction (SPE) combined with high-performance liquid chromatography–ion trap tandem mass spectrometry (LC–MS–MS) has been developed for determination of trace concentrations of erythromycin-H_2O (ETM-H_2O), roxithromycin (RTM) and tylosin (TLS) in natural and waste water matrices. These macrolides (MLs) were extracted from water samples using Oasis HLB cartridges, and the average recovery was 93.6 ± 8.6, 92.1 ± 10.0, and 94.3 ± 8.9% for ETM-H_2O, RTM and TLS in surface water, respectively. For water from the influent of a wastewater treatment plant (WWTP), the average recovery was 84.8 ± 14.0, 83.2 ± 13.1, and 86.1 ± 13.4% for ETM-H_2O, RTM and TLS, respectively. Method detection limits in a natural water matrices were 0.07, 0.03, and 0.05 μg/l for ETM-H_2O, RTM, and TLS, respectively. Fragment or product ions from MS spectra using in-source collision-induced dissociation and MS–MS spectra have been identified. The accuracy and day-to-day variation of the method fell within acceptable ranges. The method was evaluated by studying the occurrence of the three macrolides on a river and a WWTP in northern Colorado. None of the antibiotics were detected in the stream except immediately downstream of a WWTP, a result consistent with their presence in the influent and effluent of the treatment facility.

Keywords: Water analysis; Environmental analysis; Antibiotics; Macrolides

1. Introduction

Macrolide antibiotics including erythromycin (ETM), roxithromycin (RTM), and tylosin (TLS) are an important group of pharmaceuticals in today's human and veterinary medicine practice. These antibiotics are basic and lipophilic molecules that consist of a macrocyclic lacton ring containing 14, 15, or 16 atoms with sugars linked via glycosidic bonds (Fig. 1) [1].

These macrolide antibiotics exhibit similar antibacterial properties and are active against Gram-positive and some Gram-negative bacteria, and are particularly useful in the treatment of Mycoplasmas, *Haemophilus influenzae*, Chlamydia species and Rickettsia [2]. Antibiotics in this class are an important alternative for patients exhibiting penicillin sensitivity and allergy [3]. In addition, this class of antibiotic compounds has been widely used both for the prevention and treatment of disease and as feed additives to promote growth in concentrated animal feeding operations (CAFOs) [4].

After application, a fraction of the drugs are metabolized to inactive compounds, but a significant amount is excreted as active metabolites. A variety of residual antibiotics have been found in wastewater treatment plant (WWTP) effluents with concentrations as high as 6 μg/l [5]. The US Geological Survey measured concentrations of 95 organic wastewater contaminants (OWCs) containing antibiotics in water samples from a network of 139 streams across 30 states during 1999 and 2000. This reconnaissance study indicated that OWCs were found in 80% of the streams sampled [6]. Antibiotic concentrations as high as 1.9 μg/l were found and only 10 of 24 compounds measured were not detected in any of the streams. For the 84–104 streams that were sampled (it varied by compound), the frequency of detection of at least one antibiotic was 22%.

* Corresponding author. Tel.: +1-970-4918336; fax: +1-970-4917727.
E-mail address: kcarlson@engr.colostate.edu (K.H. Carlson).

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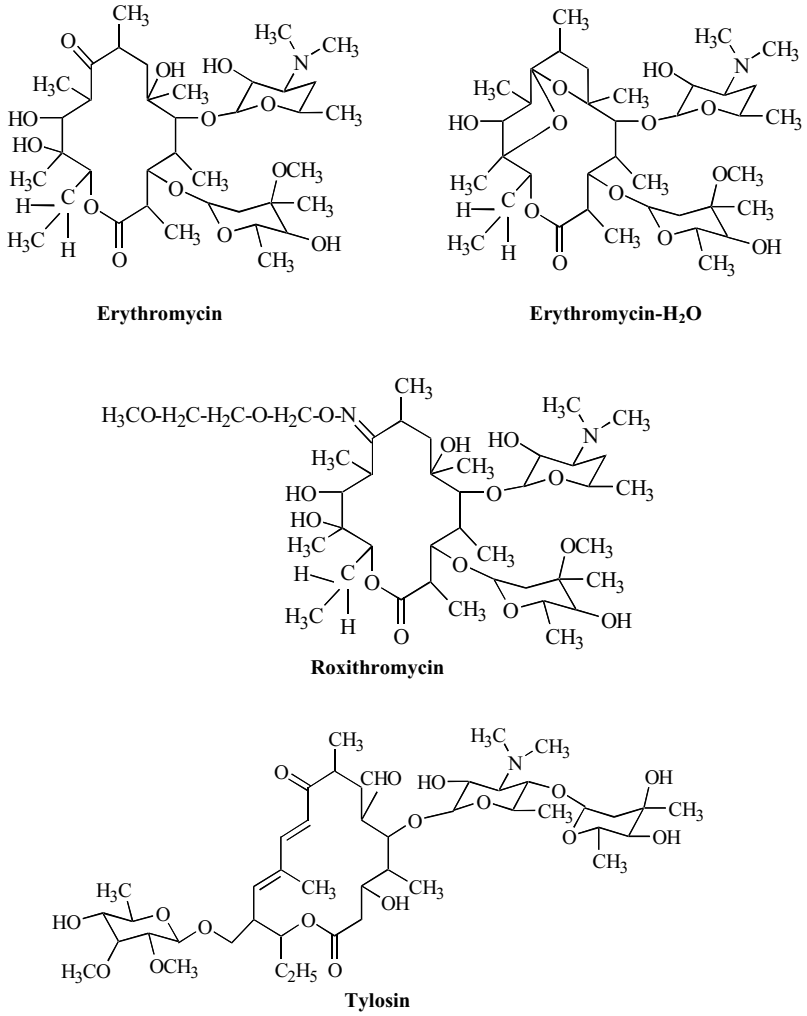

Fig. 1. Chemical structures of erythromycin, erythromycin-H_2O, roxithromycin and tylosin.

The presence of antibiotics in the aquatic environment has created two concerns. The immediate concern is the potential toxicity of these compounds to aquatic organisms and humans through drinking water. In addition, there is growing concern that release of antibiotics to the environment contributes to the emergence of strains of disease-causing bacteria that are resistant to even high doses of these drugs [7,8].

The origin of antibiotic contamination in surface and ground waters is considered to be point and non-point source discharges of municipal and agricultural wastewater [9,10]. Since few studies have been conducted on the occurrence, fate and transport of antibiotics in the environment [11] there are several questions that need to be answered on local and watershed levels. Thus, there is a need for sensitive and reliable analytical methods to measure concentrations of macrolides (MLs) such as ETM, RTM and TLS in both natural and wastwater environments.

LC–MS or LC–MS–MS methods [5,6,12,13] have been used in the analysis of antibiotics because of their high sensitivity and ability to provide compound confirmation. There are few studies of chromatographic methods for the determination of MLs in water matrices. Kolpin et al. [6] confirmed the analytical methods for ETM-H_2O, RTM and TLS in streams using LC–MS. Hirsch et al. [5,12] measured ETM-H_2O, RTM and clarithromycin in surface water, groundwater and STP effluents using LC–MS–MS. In addition, Hamscher et al. [13] and Sacher et al. [14] reported the analytical methods for TLS in water, soil and liquid manure, and for six macrolides including ETM, RTM and TLS in groundwater using LC–MS–MS, respectively. These studies did not describe total ion and mass chromatograms (TICs), mass spectra, fragment ions and/or product ions for the investigated macrolides in environmental matrices in detail. For example, Hirsch et al. [5] and Sacher et al. [14] did not report mass spectra for the investigated MLs. Hamscher et al. [13] did not show total ion and mass chromatograms for TLS. Without this information, it can be difficult to vary methodological approaches for different water matrices and site-specific equipment.

The majority of LC–MS or LC–MS–MS methods for macrolide antibiotics in biological and environmental matrices that have been reported are for single or triple quadrupole mass spectromers [5,12,14–16]. There is a need to document analytical methods for macrolides in water matrices using ion trap mass spectromers [13,17]. Ion trap LC–MS, LC–MS–MS and/or LC–multiple MS (MS^n) are important exploratory instruments in environmental chemistry and can if used properly, also be good quantitative instruments for environmental studies of trace levels of antibiotics. The ability to perform multiple stages of MS, its performance in providing high quality full scan MS–MS data, and its very low price point as compared to a triple quadrupole mass spectrometer make it a very compelling instrument to use for this application. Because of this, it is important to have a variety of approaches for analyzing potential important antibiotics in a variety of matrices so that they can be compared for their robustness.

MLs show poor UV absorbance, indicating that specific, selective and sensitive UV detection of these compounds is difficult. ETM and RTM, which are made up of three elements, the erythronolide aglycone and two sugar moieties (desosamine and cladinose) (Fig. 1) show no measurable UV absorbance [2]. To overcome this problem, low UV wavelengths, where substantial UV absorption occurs, have been used. Horie et al. [18] analyzed five MLs containing TLS with a UV detector with wavelength programming (no fixed wavelength). Other researchers have described electrochemical detection due to the poor UV detection of MLs and the need for derivatization prior to LC [19].

Electrospray ionization (ESI) as a soft ionization technique producing an abundance of $M \pm H$ ions and/or adduct ions is preferred due to its higher sensitivity, better reproducibility, and commercial availability [5,6,12,13,20]. Many antibiotic compounds are non-volatile with high molecular weights and they respond well to positive electrospray ionization, ESI (+), which makes LC–MS or LC–MS–MS the choice for separation and analysis.

This paper details a sensitive and reliable analytical method for the simultaneous determination of ETM-H_2O, RTM, and TLS in water using SPE, and LCQ Duo ion trap LC–MS–MS with positive ion electrospray ionization, ESI (+) and selected reaction monitoring (SRM). In addition, several fragment or product ions for both MS using in-source collision-induced dissociation (CID) and MS–MS detection have been identified and documented. This study also discusses different product ions in an ion trap and a triple quadrupole LC–MS–MS system, indicating that the ion trap LC–MS–MS is also a good quantitative instrument for environmental studies of trace level antibiotics in water matrices. Statistical analysis for determination of the method detection limit (MDL), accuracy and precision of the method is described. The method is evaluated by studying the occurrence of these compounds in a small watershed in Northern Colorado.

2. Experimental section

2.1. Materials and reagents

Erythromycin and tylosin tartrate salt were purchased from ICN Biomedicals (Aurora, OH, USA). Roxithromycin and Na_2EDTA (purity, 99%) were obtained from Sigma–Aldrich (St. Louis, MO, USA). Simatone, the internal standard (1000 mg/l in methanol) was purchased from Absolute Standards (Hamden, CT, USA). Stock solutions of the standards were prepared by dissolving each compound in methanol at a concentration of 100 mg/l and stored at −20 °C in the dark. Fresh stock solution was prepared every month. Working solutions (50, 10, 5, 1, 0.5, and 0.1 mg/l) were prepared fresh daily by diluting the stock solution with deionized water, and intermediate storage at 4 °C in the dark. Internal standard working solutions (0.5 mg/l) were prepared by diluting the standard solution (1000 mg/l in methanol) with deionized water, stored at 4 °C, and replaced by a fresh solution every week.

2.2. Description of the investigated area

To evaluate the utility of the method, a watershed-scale field screening study was conducted on the Cache la Poudre (Poudre) River in northern Colorado, USA (Fig. 2). The Poudre River originates near the continental divide in Rocky Mountain National Park flowing through steep mountainous terrain for approximately 43 miles (ca. 65 μm) before entering the Front Range city of Fort Collins. After traveling through Fort Collins, the river moves through approximately 45 miles of mostly agricultural landscape before joining the South Platte River in Greeley, CO, USA. Due partly to the semi-arid nature of the Front Range of Colorado, there are no significant tributaries to the Poudre River and therefore the inputs to the river are predominantly point sources in the urban landscape of Fort Collins and non-point sources in the agriculture areas outside of the city. These factors coupled with the source being snowmelt with minimal anthropogenic influences make this a good watershed to study the occurrence evolution of antibiotics through pristine, urban and agricultural landscapes.

2.3. Sampling and collection points

A minimum of 16 samples were collected from each of five locations on the Cache la Poudre River (Table 1) over a period of 16 months from 1 July 2002 to 31 October 2003. The samples of duplicates were collected in the center of the stream as a depth composite using a water grab sampler. Also, we collected 24 h composite samples from the influent and effluent of the Fort Collins Drake Water Reclamation Facility (DWRF) every 2 months over a period of 16 months.

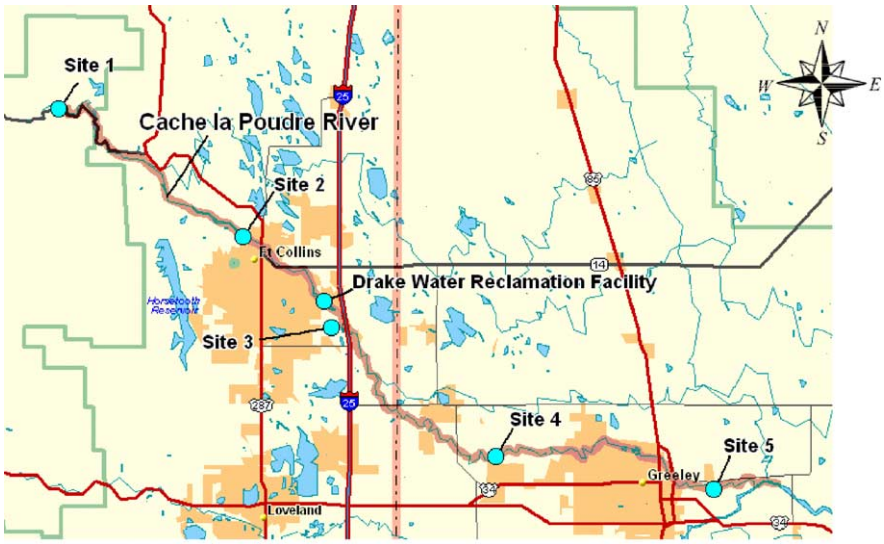

Fig. 2. Samples sites along the Cache la Poudre River in Northern Colorado.

2.4. Sample preparation

All of the water and wastewater samples were filtered through 0.2 μm glass fiber filters (Millipore, MA, USA) and stored at 4 °C in refrigerators until they were extracted, typically within 1 week. WWTP influents were centrifuged at 3000 rpm for 40 min at 4 °C in a centrifuge (IEC Centra CL 3R, MA, USA) with a cooling system and then pre-filtered through a paper filter under vacuum before being filtered through a glass fiber filter. Solid-phase extraction and measurement were performed on the same day since the solubility of the extracted MLs during freezing and thawing was variable.

2.5. Solid-phase extraction

Solid-phase extraction (SPE) experiments were conducted using 60 mg/3 ml Oasis HLB cartridges (Waters, Millford, MA, USA). Cartridges were preconditioned with 3 ml of MeOH and 3 ml of deionized water at 8 in.Hg on a vacuum manifold (PrepSep 12 port, Fisher scientific, PA, USA; 1 in.Hg = 338.638 Pa). Aqueous samples were prepared for extraction by adding 0.5 ml of 5% Na_2EDTA to a flask containing 120 ml of water. For controls and calibration curves, appropriate amounts of working solution containing each of analytes was added. Extraction using the HLB cartridges was performed with the sample pH adjusted to ~5.0 by addition of 40% H_2SO_4 immediately prior to extraction. Additionally, extraction was performed with the sample adjusted to acidic, neutral, and basic pH in order to determine which form of ETM is present in water samples. To test the behavior of MLs spiked into a natural water matrix, a water sample was collected from the Poudre River site 1 (Fig. 2). This water was used as a matrix because it has been shown in other studies [21,22] by the author that this water was devoid of pharmaceutical compounds since it is essentially snow runoff. Before it was used as a matrix, the water was analyzed using the developed method and no MLs were detected.

Water samples were passed through the cartridges at 5 ml/min and then, rinsed with 3 ml of deionized water. The analytes were eluted with 5 ml of MeOH into a test tube containing 24 μl of the internal standard, 0.5 mg/l simatone. Simatone was chosen as an internal standard because it eluted within the same chromatographic time frame as the analytes, responded well in ESI (+) mode, and had no noticeable matrix effects. The extracts were concentrated under a flow of N_2 gas to about 50 μl using a nitrogen

Table 1
Study sampling sites

Sample	Sampling sites
Cache La Poudre River, Site 1	Greyrock National Recreation Trail, Fort Collins, CO
Cache La Poudre River, Site 2	Shields Street Bridge, Fort Collins, CO
Cache La Poudre River, Site 3	Drake Waste Water Treatment Plant, Fort Collins, CO
Cache La Poudre River, Site 4	Hwy 392 Bridge, Windsor, CO
Cache La Poudre River, Site 5	Weld Country Municipal Airport, Greeley, CO
WWTP influent	Drake Water Reclamation Facility, Fort Collins, CO
WWTP effluent	Drake Water Reclamation Facility, Fort Collins, CO
NIC effluent	Northwest Intermediate Clarifier, Fort Collins, CO

evaporation system (N-Evap, Organermation Associates, MA, USA). To this, 70 μl of mobile phase A was added. The resulting solutions were transferred to 0.5 ml amber autosampler vials to prevent photodegradation of MLs.

2.6. Liquid chromatography

The LC system was a HP 1100 LC (Agilent, Palo Alto, CA, USA) with a variable wavelength UV detector. The UV monitoring wavelength was 215 nm for ETM-H_2O, 205 nm for RTM and 287 for TLS. The mass spectrometry was a Finnigan LCQ Duo ion trap (ThermoQuest, CA, USA) equipped with a heated capillary interface, and an ESI source. ThermoQuest Xcalibur software was employed to control the mass spectrometric conditions.

MLs were separated using a 50 mm × 2.1 mm Xterra MS C_{18} column with a 2.5 μm pore size (Waters, Millford, MA) in combination with a guard column of the same type (4 mm × 2.1 mm) from Phenomenex (Torrance, CA, USA). An injection volume 20 μl and a binary gradient with a flow rate of 0.32 ml/min were used. The column temperature was kept at 45 °C. Mobile phase A was water with 0.1% formic acid and mobile phase B was acetonitrile with 0.1% formic acid. Separations of MLs were achieved with the following mobile phase gradient program: at 0 min A–B (80:20), 12 min A–B (65:35), 13 min A–B (80:20). The investigated MLs eluted within 13 min. A 10 min post time allowed re-equilibration of column.

2.7. Ion trap mass spectrometry

Full scan mode was used to acquire mass spectra, protonated molecular ions (or precursor ions), fragment ions and/or product ions from standard solutions of MLs. Mass spectral data shown in this study were acquired on a LCQ Duo ion trap mass spectrometer equipped with an ESI source operated in positive ion mode.

Infusion into the ion trap tandem mass spectrometer was performed as follows: the flow of standard compounds (10 mg/l) coming from an integrated syringe pump at a flow rate of 5 μl/min was mixed with mobile phases A–B at a 80:20 ratio through a T-piece for tuning the mass spectrometer and optimizing the ESI source. The ESI source and MS–MS parameters were automatically optimized and saved in a tune file. Spray needle voltage was set at 4.5 kV, automatic gain control (AGC) was on, maximum isolation time was 300 ms, and three microscans per scan were acquired. Voltages on capillary and tube lens were 46 and 20 V, respectively which were set by automatic optimization using the LCQ autotune program on the mass spectrometer instrument. Nitrogen was used as a sheath and auxiliary gas. Helium was used as the collision gas in the ion trap. The optimized tune conditions were as follows: sheath gas flow rate was set at 40 units (a scale of arbitrary units in the 0–100 range defined in the LCQ system), the auxiliary gas was turned off, and capillary temperature was 165 °C. MS–MS parameters including their proposed structures, and collision energy and isolation width (m/z) are summarized in Table 2.

In addition, fragmentation was produced via in-source CID in the LCQ ion trap mass spectrometer. Nitrogen was used as nebulizer and drying gases at flow rates of 40 and 8 units, respectively. The first ion optic octapole and lens voltage were −3.5 and −32 V, respectively. The heated capillary temperature was set to 165 °C. In-source CID on the ion trap mass spectrometer was done by increasing the dc voltage difference between the first ion optic octapole and skimmer to produce enough energy which induces decomposition of protonated molecular ions upon collision with residual nitrogen gas molecule from the nebulizing source. The ion collisions cause weaker ions to break apart so that a full MS scan in the ion trap can actually produce fragment ions. The fragment ions for each analyte obtained by in-source CID and their proposed structures are summarized in Table 2.

2.8. Quantitation

The product ion producing the highest intensity was used for SRM and quantitation to increase analytical sensitivity and selectivity in LC–MS–MS mode. For the internal standard, the protonated molecular ion, $[M + H]^+$ was chosen for the selected ion monitoring (SIM). For SRM the product ion of the highest intensity at m/z = 558.2, 679.3, and 772.3 was used for ETM-H_2O, RTM and TLS, respectively (Table 2). Quantitation was based on the ratio of the base peak ion (the above mentioned product ion) to the base peak ion of the internal standard. Calibration curves constructed for MLs spiked before extraction into water samples ranged from 0.05 to 5 μg/l in deionized water, surface water (the Poudre River 1) and WWTP influent, respectively and were linear, with correlation coefficients R^2 >0.99 for the MS–MS procedure. Because Poudre River 3 and WWTP influent already contained ETM-H_2O, RTM and/or TLS, a calibration curve for ETM-H_2O, RTM and/or TLS in these matrices was constructed by subtracting the level concentration from the spiked concentration, respectively. Concentrations for the MLs were calculated reproducibly by using the standard calibration curves that were based on the internal standard, simatone.

2.9. Statistical analysis

The method detection limit (MDL) was determined using two methods: (1) a signal-to-noise ratio (S/N), which can be measured directly using the instrument software, and (2) the US Environmental Protection Agency (EPA) recommended method for MDL determination [23] based on the variability of multiple analyses of seven surface water (Poudre Rivers 1 and 3) extracts spiked at a concentration of 0.2 μg/l. To assess the accuracy and day-to-day variation of the LC–MS–MS method, repeatability experiments

Table 2
MS and MS–MS parameters

	Macrolides			
	ETM-H_2O	RTM	TLS	Simatone[a]
Nominal molecular mass (Da)	715.4	836.1	915.5	197.2
LC–ESI–MS				
Protonated molecular ions (*m/z*)	716.3[M + H–H_2O]$^+$	837.3[M + H]$^+$	916.4[M + H]$^+$	**198.2**[b] [M + H]$^+$
Fragment ions (*m/z*)	576.3[M + H–desosamine]$^+$ 558.2[M + H–desosamine–H_2O]$^+$ 540.2[M + H–desosamine–2H_2O]$^+$ 522.2[M + H–desosamine–3H_2O]$^+$ 158.0[desosamine + H]$^+$	679.5[M + H–desosamine]$^+$ 158.1[desosamine + H]$^+$ 116.5[cladinose + H–OCH_3]$^+$	772.3[M + H–$C_7H_{12}O_3$]$^+$	
LC–ESI–MS–MS				
Isolation width (*m/z*)	3	3	3	
Normalized collision energy (%)	22	23	33	
Precursor ions (*m/z*)	716.4[M + H–H_2O]$^+$	837.5[M + H]$^+$	916.6[M + H]$^+$	
Product ions (*m/z*)	698.3[M + H–2H_2O]$^+$ 684.1 658.3 640.2 **558.2**[c][M + H–desosamine–H_2O]$^+$ 540.2[M + H–desosamine–2H_2O]$^+$ 522.2[M + H–desosamine–3H_2O]$^+$	716.3[M + H–$C_4H_9NO_2$–H_2O]$^+$ **679.3**[c][M + H–desosamine]$^+$ 558.3[M + H–desosamine–$C_4H_9NO_2$–H_2O]$^+$ 522.3[M + H–desosamine–$C_4H_9NO_2$–3H_2O]$^+$	**772.3**[c][M + H–$C_7H_{12}O_3$]$^+$	

[a] Simatone: internal standard.
[b] Protonated molecular ions (*m/z*) of internal standard in SIM.
[c] Product ions (*m/z*) of the highest intensity for SRM and quantitation are reported in boldface.

were carried out with six surface water (Poudre River 3) and WWTP influent extracts spiked with 0.1 μg/l, 1.0 μg/l, 2.0 μg/l or 5.0 μg/l of three macrolide antibiotics for 3 days, respectively. Each day, six extracts spiked at three different concentrations in the two water matrices were analyzed.

3. Results and discussion

3.1. Degradation of erythromycin (ETM)

Experiments for the analysis of ETM and related degradation products in this study exhibited strong pH sensitivity for ETM, indicating that ETM is not detected in its original form but as a degradation product (ETM-H_2O) with an apparent loss of one molecule of water (Fig. 1). Surface water extracts adjusted to acidic, neutral, and basic pH during the SPE procedures were analyzed as both ETM and ETM-H_2O in the acidic mobile phase gradient. Only ETM-H_2O was detectable at pH <7 on the basis of the protonated molecular ion (*m/z*), 716.3 or product ions (*m/z*), 558.2 of highest intensity for ETM-H_2O in LC–MS or LC–MS–MS (Table 2). This result agrees with the finding of Hirsh et al. [13] that only ETM-H_2O exists at pH <7. Kolpin et al. [6] also measured ETM as the degraded product, ETM-H_2O. These results imply that this loss of one molecule of water already occurred in aquatic solutions, indicating that ETM-H_2O is already present in the aquatic environment. ETM was also measured as ETM-H_2O in this study, assuming that ETM was totally converted into ETM-H_2O in both of SPE procedures with the sample pH adjusted to ∼5.0 and acidic mobile phase gradient since no ETM was detected. Additionally, since the orally applied ETM has to pass through strongly acidic conditions in the stomach, the degraded product ETM-H_2O, does not exhibit the original antibiotic properties [24].

3.2. Liquid chromatography and ion trap mass spectrometry

LC employing a simple gradient system combined with ESI (+)–MS–MS allowed the rapid, sensitive, selective and reliable determination of the investigated MLs in water matrices. The three MLs investigated were difficult to detect at the same fixed wavelength due to the generally poor absorbance of these compounds. Therefore, the extracts were analyzed by a UV detector with the following wavelength programming. During the first 8 min of the analysis, the wavelength for TLS was 287 nm. After 8 and 9.5 min of analysis, the wavelength for ETM-H_2O was set at 215 nm. Then after 9.5 and 12 min of analysis, the wavelength for RTM was 205 nm (Fig. 3).

The mass peaks corresponding to MLs appeared on the total ion and mass chromatograms monitored at the selected product ion. The data were processed by creating reconstructed ion chromatograms (RICs) for each analyte as shown in Fig. 3. These results indicate that efficient separation of the three MLs was achieved by the short C_{18} column using an elevated column temperature (45 °C) and mobile phases in a binary solvent system. The elevated temperature (45 °C) demonstrated that resolution increased with an increase in temperature due to a significant decrease in the height equivalent to a theoretical plate (HETP) of some of compounds of interest. Acidic mobile phases, which were more suitable for reversed-phase chromatography would not dissociate any residual silanols to weakly anionic species that could strongly retain the basic MLs [18]. As shown in Fig. 4, providing the elevated temperature optimized the protonation of TLS resulting in better mass peak symmetry of this compound compared to 15 and 25 °C. The degradation product, ETM-H_2O, was separated with good peak symmetry using the acidic mobile phases. In addition, because MLs may not be stable in acidic solutions, we investigated the stability of concentrated extracts of each ML (10 mg/l) at 45 °C for 30 min. No effect on the ML detection or quantitation was observed using these acidic mobile phase conditions with this method.

Figs. 5 and 6 show full scan MS spectra using in-source CID and full scan MS–MS spectra for a standard solution of 10 mg/l ETM, RTM and TLS with the ESI (+) source, respectively. Molecular mass, protonated molecular ions and fragment ions at *m/z* for LC–MS, and precursor ions and product ions at *m/z* for LC–MS–MS including their proposed structures, and collision energy and isolation width (*m/z*) are listed in Table 2.

For ion trap mass spectrometry (MS) using in-source CID, ETM-H_2O, RTM and TLS exhibited characteristic fragmentation and the predominant ion for three MLs under the acidic mobile phase conditions was $[M + H–H_2O]^+$ for ETM-H_2O and $[M + H]^+$ for RTM and TLS, respectively. As can be seen in Table 2 and Fig. 5, ETM exhibited the apparent loss of H_2O, indicating that the degradation product, ETM-H_2O became a protonated molecular ion, $[M + H–H_2O]^+$. ETM-H_2O and RTM exhibited various fragment ions that related to losses of their two characteristic sugars (desosamine and cladinose) and H_2O. These mass spectra and fragment ions agree with the findings of Hirsch et al. by LC–MS–MS in water using a triple quadrupole mass spectrometer with ESI (+) [5] and those of Delepine et al. by a particle beam (PB) LC–MS with positive chemical ionization (PCI) in bovine muscle [25]. Fragmentation of TLS produced only a single fragment ion, *m/z* 772.3 corresponding to $[M + H–C_7H_{12}O_3]^+$ in two mass ranges *m/z* 150–1000 and 770–920. Fig. 5 shows the mass spectrum for TLS in the mass range *m/z* 770–920, indicating that the mass spectrum and the 772.3 ion agree with those of MS–MS for the mass range *m/z* 700–920 in water, soil and liquid manure reported by Hamscher et al. [13] and Delepine et al. [25].

For the ion trap tandem mass spectrometer (MS–MS), mass spectra and product ions were clearly observed in full scan mode for ETM-H_2O, RTM, and TLS (Fig. 6). For

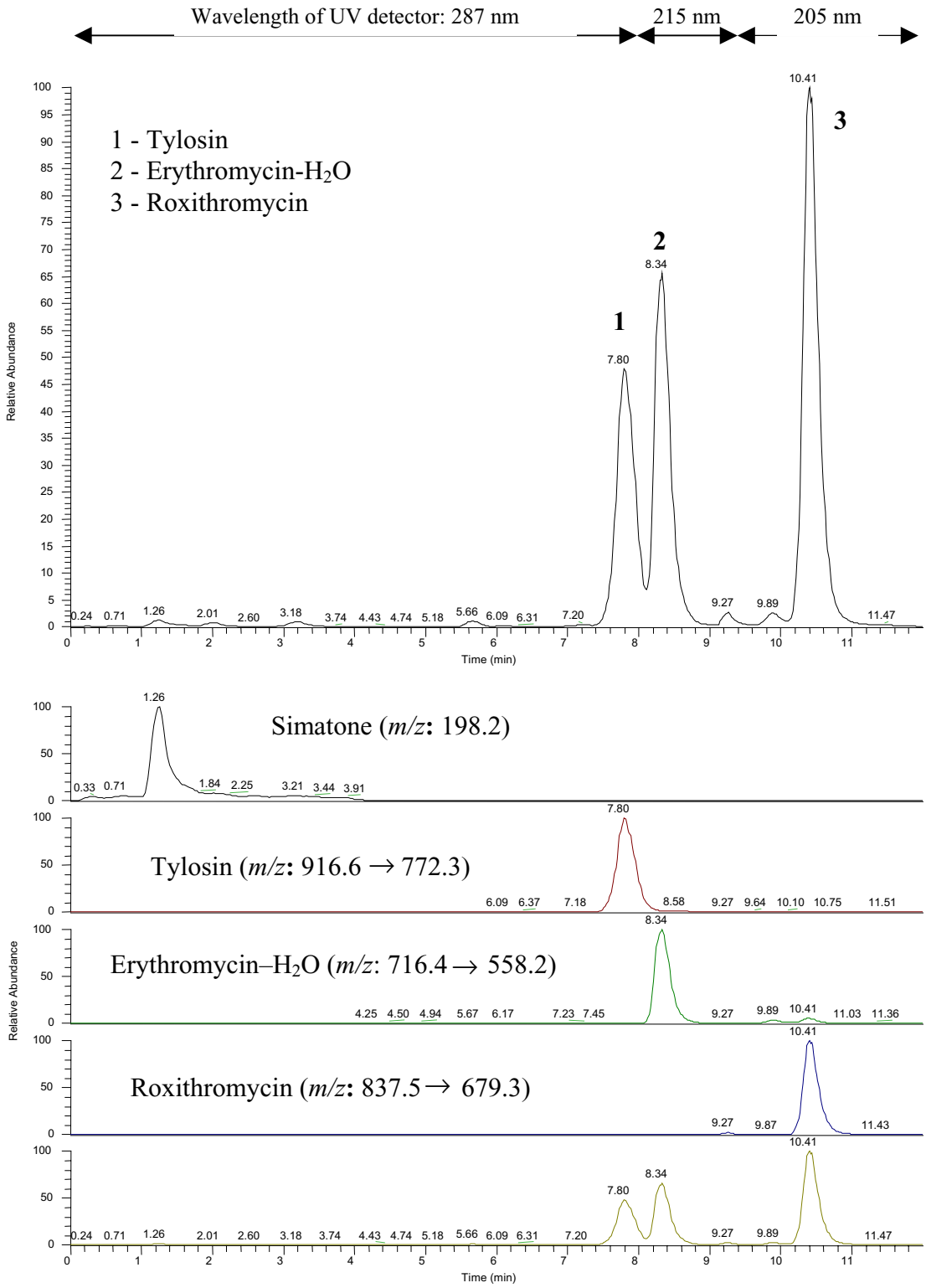

Fig. 3. Total ion and mass chromatograms and the applied UV wavelength programming of erythromycin-H_2O, roxithromycin, and tylosin spiked at 2 μg/l for the extracted Poudre River 1 as a surface water matrix using SRM LC–MS–MS. Masses (*m/z*) indicate precursor → product ions used for quantitation.

ETM-H_2O the product ion (*m/z*), 558.2 for ETM-H_2O exhibited the highest intensity under the acidic mobile phase conditions in MS–MS due to conversion of ETM to ETM-H_2O. Fragmentation produced the same product ions that were seen in the MS, but the mass spectra did not exhibit the 158.0 ion corresponding to [desosamine + H]$^+$ over the mass range *m/z* 300–1000 selected in the ion trap MS–MS (Table 2 and Fig. 6). The mass spectrum for RTM exhibited

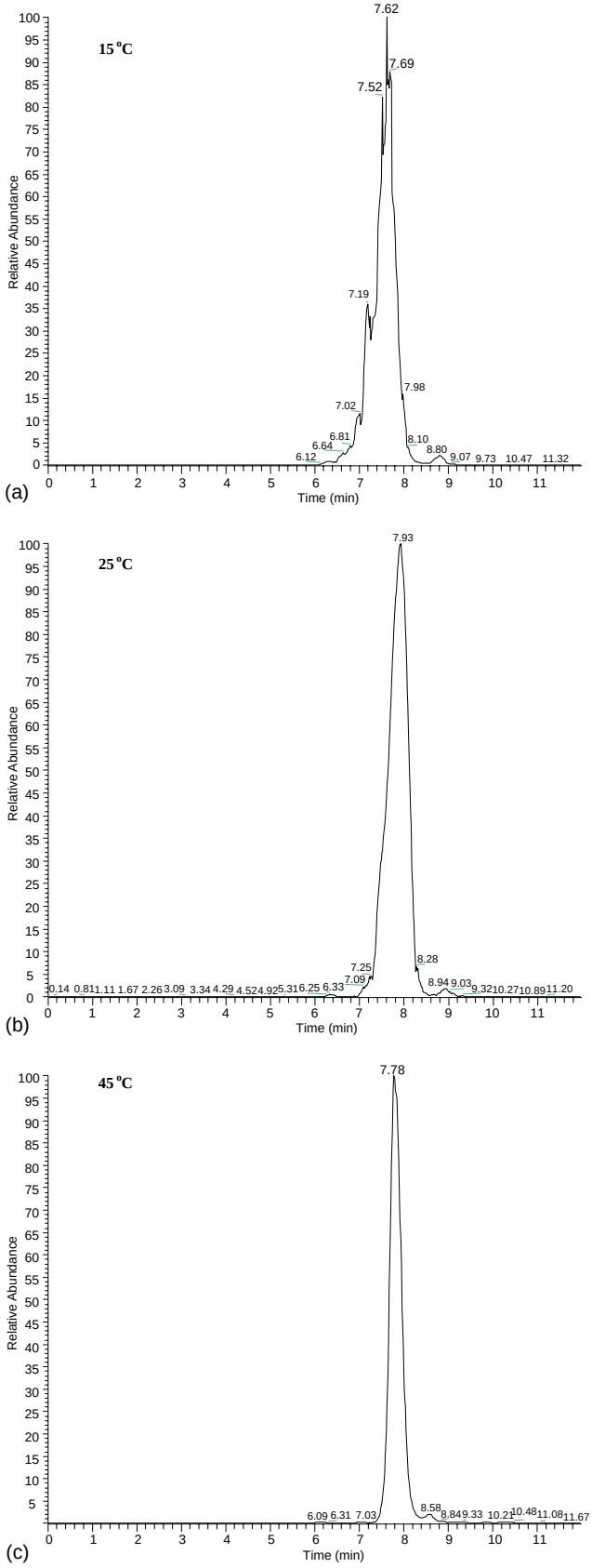

Fig. 4. Mass chromatograms of tylosin (*m/z*: 916.6 → 772.3) spiked at 2 μg/L for the extracted surface water (the Poudre River 1) at column temperatures of 15, 25, and 45 °C using SRM LC–MS–MS.

three different product ions that were related to the loss of $C_4H_9NO_2$ (Fig. 1). These product ions were not observed in this study using MS or Hirsch et al. using MS–MS [5]. For TLS only the 772.3 ion, $[M + H–C_7H_{12}O_3]^+$, was exhibited in the mass spectrum in the MS–MS mode, a result consistent with the mass spectra reported by Hamscher et al. [13] and Delepine et al. [25], and MS using in-source CID in this study. However, other studies [14–17] reported the 174.1 ion, $[C_8H_{15}NO_3 + H]^+$, the 100.9 and/or 101.2 ion as well as the 772.3 ion as product ions in triple quadrupole MS–MS. It is not surprising that a triple quadrupole and an ion trap system, both operating in MS–MS mode, may exhibit different product ions for the specific application.

3.3. Deviations in product ions in an ion trap tandem and a triple quadrupole mass spectrometers

In the case of the TLS mass spectrum described in this paper, it is possible that one of the fundamental operating conditions of the ion trap MS–MS system prevented us from seeing the ion at *m/z* 174.1, 101.2 or 100.9; alternatively, it is also possible that the formation of the ion at *m/z* 174.1 or 101.2 takes place after multiple fragmentation steps (e.g., MS^n).

When an ion trap system performs MS–MS, it does so utilizing a set of physical equations that define the range of product ions that can be stored following excitation and fragmentation, given a particular precursor ion. Unless specifically changed, the '*q*' value that is used for MS–MS excitation by default on the LCQ Duo system limits the range of product ions that can be stored following MS–MS fragmentation to approximately one third of the mass of the precursor ion. That is, with isolation of the TLS ion at *m/z* 916.6 in this study, the default conditions for MS–MS would likely only show product ions down to a range of *m/z* 250 or so. Thus, even if the ion at *m/z* 174.1, 101.2, or 100.9 was present in the fragmentation process, it may not have been stored in the ion trap for later detection during mass analysis. However, a triple quadrupole system does not operate with an identical set of physical principles to the ion trap system, and does not have this mass range issue in the product ion mass analysis.

Another reason that the ion trap might not be able to see an ion that is observed on a triple quadrupole system is that the ion may be formed not by a single fragmentation step, but by multiple fragmentation steps. When an ion is selected for fragmentation in an ion trap, it is just that ion that is then collisionally activated and fragmented. Because this is done with a resonant frequency that is specific for the isolated ion, any fragment ions formed are unable to absorb any further energy (since their *m/z* value and thus their frequency are now different) and fragment further. The MS–MS process on an ion trap is selective in precursor ion isolation and in precursor ion activation.

In a triple quadrupole, an isolated precursor ion is directed into a collision cell and accelerated through a cloud of inert gas (typically argon, although nitrogen is sometimes used).

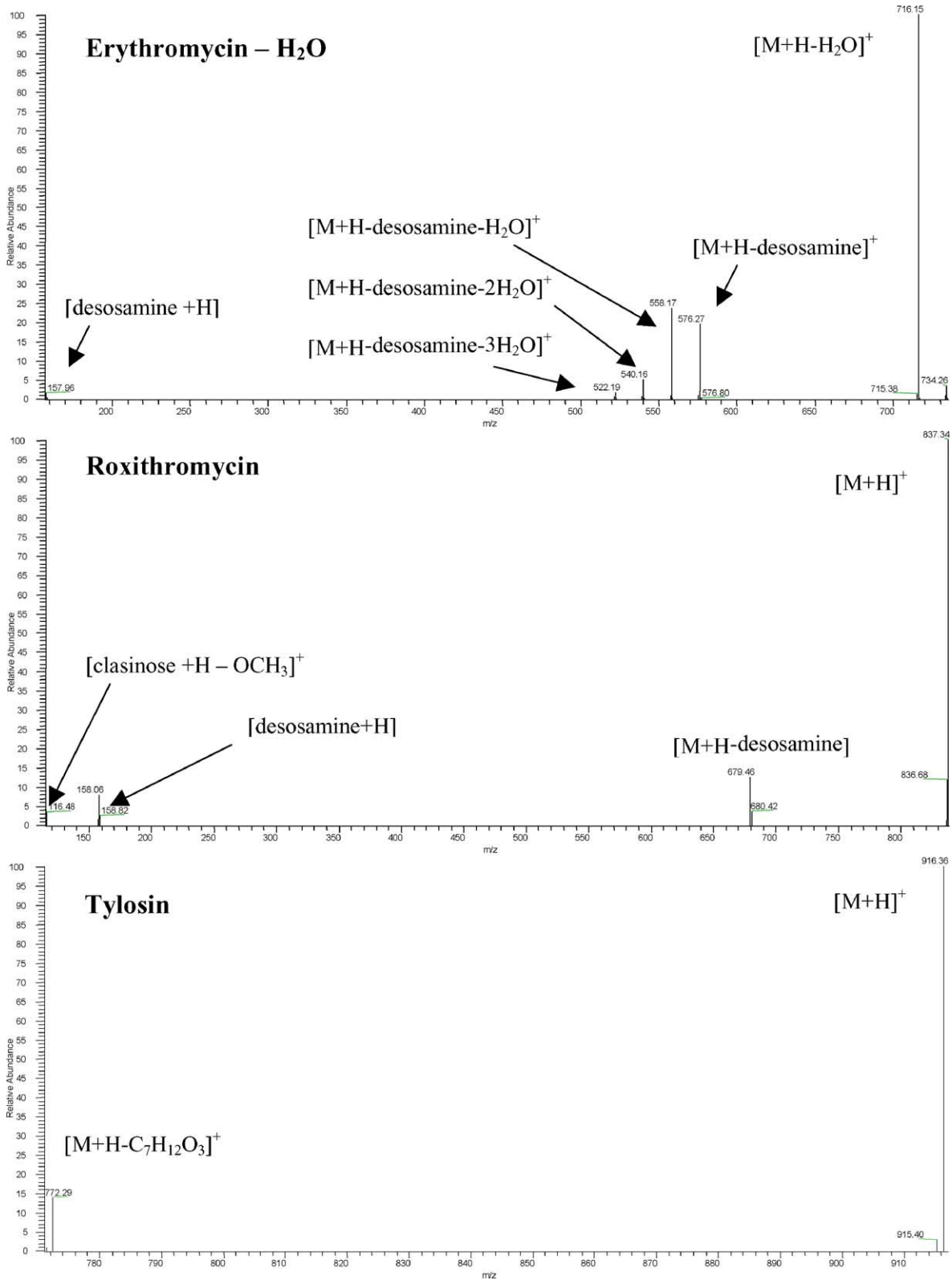

Fig. 5. Full scan MS spectra using in-source CID of erythromycin-H_2O, roxithromycin, and tylosin.

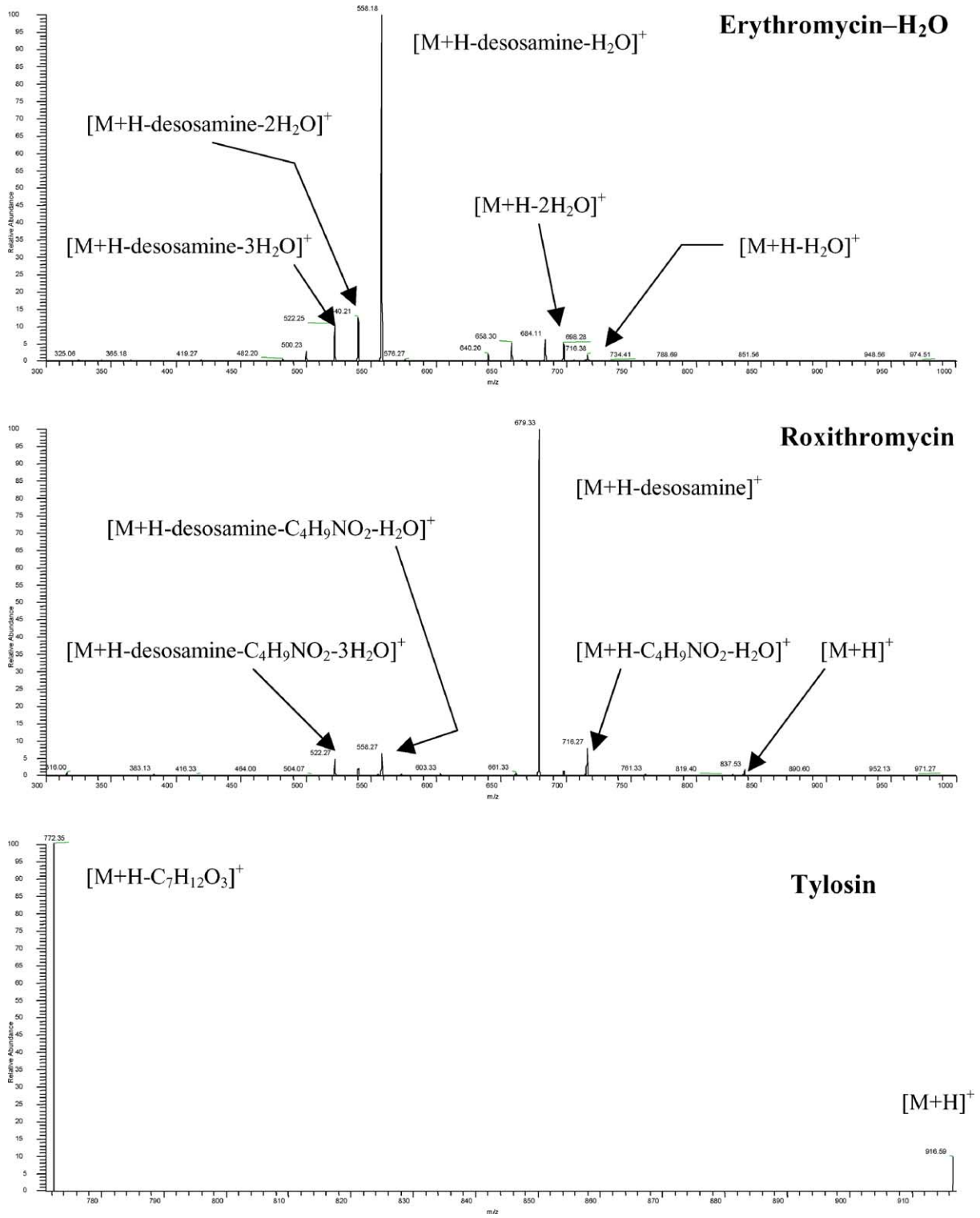

Fig. 6. Full scan MS–MS spectra of erythromycin-H_2O, roxithromycin and tylosin.

This acceleration is done via a dc voltage offset, meaning that any ion that either enters the collision cell (i.e. the chosen precursor ion) or is formed in the collision cell (i.e. a product ion) can be fragmented. The MS–MS process on a triple quadrupole system is thus selective in precursor ion isolation but not selective in precursor ion activation, indicating that any ion formed in the collision cell can also be fragmented.

For the TLS results in this study, it could be that the first transition that occurs is the fragmentation of the TLS ion at *m*/*z* 916.6 to a fragment at *m*/*z* 722.3. At this point, on the ion trap system, the ion at *m*/*z* 722.3 receives no

further energy, and does not continue to fragment. However, on the triple quadrupole system, that ion at *m*/*z* 722.3 may continue to fragment, forming the ion at *m*/*z* 174.1, 101.2 or 100.9. The other operating conditions on the system that may have affected which product ions were observed may have included the original method of forming the precursor ion (ESI or atmosphere pressure chemical ionization (APCI)) and whether or not they were other adducts (i.e. sodium) used for mass analysis. Once the ion is formed, however, the differences in product ion mass spectra may be related more to the mass analyzer than any LC or other 'front end' conditions. In addition, for ETM-H_2O and RTM in the ion trap MS–MS of this study, the 158.0 ion for ETM-H_2O, and the 158.1 ion and the 116.5 ion for RTM were not detected due to the limited mass range in the ion trap.

3.4. Recovery comparison

MLs can potentially sorb to residual metals on SPE cartridges and glassware, resulting in irreversibly binding to the cartridge and lowering recovery. Na_2EDTA was utilized in this study to chelate metals that are sufficiently soluble in water and prevent interference with the extraction of MLs. The pre-elution of the HLB cartridges with MeOH led to very clean extracts for the final LC–MS–MS analysis.

The recoveries of MLs from the HLB cartridges were measured by extracting analytes from 120 ml of deionized water and surface water spiked at 0.05–5 μg/l for MLs. Cache la Poudre River site 1 was selected as a reference matrix for surface water since no MLs were detected with the developed method. The 60 mg HLB cartridges were selected for analysis of MLs in the water matrices because they do not contain silanols. Basic molecules containing amino sugar(s) like MLs are strongly affected by silanol groups [18]. Recoveries of MLs for SPE were determined in deionized water and surface water (Cache la Poudre River Site (1) using the following equation:

$$\text{Recovery (\%)} = \frac{\text{detector response for extracted analyte}}{\text{detector response for non-extracted analyte}} 100 \quad (1)$$

Where detector response is the area of the mass chromatogram for extracted or non-extracted analyte divided by the area of the mass chromatogram for the internal standard that was added.

The internal standard simatone did not exhibit noticeable matrix effects since the average recovery was 99.5 ± 4.1 and 98.7 ± 6.5% (calculated using peak areas for non-extract and extract) with a constant 0.1 μg/l spike in surface water and WWTP influent, respectively. Therefore, MLs measured in these matrices were not corrected for the recovery of the internal standard.

Recoveries of MLs are the average of duplicates of 0.05, 0.2, 0.5, 1, 2, and 5 μg/l of MLs spiked in deionized water and surface water (Poudre River 1). As shown in Table 3, the average recovery of MLs in deionized water was 95.9 ± 8.8% at the investigated concentration range, 95.5 ± 11.3 and 99.6 ± 8.8% at 0.05 and 2 μg/l spike, respectively and no concentration dependence was observed.

The average recovery of MLs in surface water was 93.3 ± 11.3% at the investigated concentration range, 91.9 ± 13.3% with a 0.05 μg/l spike, and 94.5 ± 10.4% with a 2 μg/l spike, indicating the HLB cartridges also gave reproducible recoveries for MLs and were effective for the isolation of the MLs. Recoveries of MLs in surface water did not differ statistically from those of MLs in deionized water (Table 3). Because of the reproducible recoveries of MLs, it was assumed that they did not exhibit matrix effects in surface water such as sulfonamide antibiotics as reported previously [21].

ML recoveries were more variable at lower concentration and with more complex water matrices such as WWTP influent. To assess the matrix effects for MLs in more complex surface waters and the WWTP influent, recovery was also determined from a calibration curve for the ML extracts spiked in deionized water, surface water and WWTP influent. Because all three macrolide antibiotics were found at Poudre River Site 3, this sample was chosen to confirm matrix effects in surface water. Recoveries of MLs in the Poudre River Site 3 and WWTP influent over a period of 16 months were determined using the calibration curves (Table 4). For surface water (Poudre River Site 3), the average recovery of ETM-H_2O, RTM and TLS was 93.6 ± 8.6, 92.1 ± 10.0, and 94.3 ± 8.9% at 0.1 and 1.0 μg/l spike concentrations, respectively. These results indicate that MLs did not exhibit matrix effects in the more complex surface water matrix (Table 4) and that no difference for recovery is observed between the two methods (Eq. (1), calibration curves).

Table 3
Recoveries of macrolides from 120 ml of water samples

ML	Recovery, X ± S.D. (%)					
	Deionized water (μg/l)			Surface water, Cache la Poudre River 1 (μg/l)		
	0.05–5	0.05	2	0.05–5	0.05	2
ETM-H_2O	95.9 ± 8.1	96.4 ± 10.6	98.6 ± 8.8	93.5 ± 11.8	91.2 ± 12.6	94.3 ± 11.3
RTM	96.2 ± 8.7	95.1 ± 11.8	99.5 ± 9.2	93.9 ± 10.5	93.5 ± 13.8	95.6 ± 9.9
TLS	95.7 ± 9.5	94.9 ± 11.5	100.8 ± 8.4	92.5 ± 11.7	90.9 ± 13.5	93.8 ± 10.1
Average recovery	95.9 ± 8.8	95.5 ± 11.3	99.6 ± 8.8	93.3 ± 11.3	91.9 ± 13.3	94.5 ± 10.4

Table 4
Macrolide recoveries for 120 ml of sixteen surface waters (Cache la Poudre River 3) and eight WWTP influents spiked at 0.1 μg/l, 1.0 μg/l and/or 3.0 μg/l concentration over a period of 16 months

ML	Recovery, $X \pm$ S.D. (%)					
	Surface water (μg/l), Cache la Poudre River 3			WWTP influent (μg/l)		
	0.1	1.0	Average recovery	0.1	3	Average recovery
ETM-H_2O	92.4 ± 8.3	94.7 ± 8.9	93.6 ± 8.6	84.1 ± 14.7	85.5 ± 13.2	84.8 ± 14.0
RTM	90.8 ± 9.5	93.4 ± 10.4	92.1 ± 10.0	82.5 ± 13.4	83.9 ± 12.8	83.2 ± 13.1
TLS	94.6 ± 10.1	94.0 ± 7.6	94.3 ± 8.9	85.9 ± 12.8	86.2 ± 13.9	86.1 ± 13.4

For the WWTP influent samples spiked with 0.1–5 μg/l MLs, recovery variability ranged from 5 to 20% from run to run. The calculated concentration variability was 0–3 μg/l on the basis of deionized water, indicating matrix interference. As shown in Table 4, average recovery of ETM-H_2O, RTM and TLS was 84.8±14.0, 83.2±13.1, and 86.1±13.4% with spiked concentrations of 0.1 and 3.0 μg/l in eight WWTP influent samples over a period of 16 months. The lower recoveries relative to deionized and surface waters are likely due to the presence of organic matter (OM) in the WWTP matrix. Recovery efficiency has been shown to correspond to the solubility of humic and fulvic acid (natural dissolved organic matter) in the solvent used to elute antibiotics in the SPE cartridges [9]. Most matrix effects resulted in an increase in signal intensity although some resulted in total suppression of the MS signal. It is hypothesized that the OM has surfactant properties that could enhance the MS signal intensity by promoting ionization in the positive electrospray.

3.5. Method detection limit

The method detection limit (MDL) was determined using two methods, a signal-to-noise ratio (S/N), which can be measured directly using the instrument software, and the US Environmental Protection Agency (EPA) recommended method for MDL determination [23]. The MDL based on a signal-to-noise ratio (S/N) greater than 6 was determined to be 0.05 μg/l for the three ML compounds extracted from 120 ml of water samples. Hirsch et al. [5,12] obtained a limit of quantitation of 0.02 μg/l for ETM-H_2O and RTM in water based on the second lowest calibration curve point of the linear correlation. Hamscher et al. [13] obtained the limit of quantitation of 0.1 μg/l (based on a S/N greater than 6) and a limit of detection of 0.05 μg/l (based on a S/N greater than 3) for TLS. The limit of quantitation or the limit of detection depend on the volume of sample extracted, the extent of complexity of water matrices as well as the S/N chosen (e.g., commonly 3–10).

Caculations of MDL using the EPA method [23] were based on the variability of multiple analyses of seven surface water (the Poudre Rivers 1 and 3 each) extracts spiked at a concentration of 0.2 μg/l for each of the three MLs. The MDL was determined by multiplying the sample standard deviation calculated from each group of the extracts spiked at the concentration by the Student's *t*-variate for a one-sided *t*-test at the 99% confidence level with $n - 1$ degrees of freedom. The MDL for ETM-H_2O, RTM and TLS extracted from 120 ml of surface water were 0.07, 0.03 and 0.05 μg/l, respectively.

3.6. Accuracy and precision

The accuracy and the variability of the method were determined using six surface water and WWTP influent extracts spiked with 0.1 μg/l, 1.0 μg/l, 2.0 μg/l or 5.0 μg/l of three macrolide antibiotics over a period of 3 days. Because Poudre River Site 3 (immediately downstream of the WWTP influent) and the WWTP influent sample are more complex water matrices, these were used to assess the accuracy and precision of the method. The results are summarized in Table 5. The accuracy range was −12.0 to +13.0% for the three ML antibiotics in two water matrices. This accuracy range is within the acceptable value of −30 to +20% recommended [15,26]. The relative standard deviations (R.S.D.s) calculated from these experiments ranged from 2.9 to 11.7 and 7.2 to 19.8% for three MLs in the Poudre River Site 3 and WWTP influent, respectively, and no concentration dependence was observed. However, the R.S.D.s for WWTP influent are greater than those of the Poudre River Site 3 matrix. These results indicate matrix effects may be important, the result being an increase or suppression of the MS signal intensity.

3.7. Occurrence, distribution, and fate of macrolides

The SPE-LC–MS–MS method was used to determine the occurrence of three macrolides in the Cache la Poudre River through pristine, urban and agricultural landscapes (Table 6). All three compounds were found in samples collected at site 3, immediately downstream of the WWTP. Although only ETM-H_2O and TLS were detected in the WWTP effluent, the concentration of RTM in the river was close to the MDL and therefore it is likely that the WWTP is the source. TLS was found at the highest concentrations in the WWTP influent but the activated sludge process appears to have removed 95% of the compound.

Several other observations can be made based on the occurrence data. Significant natural attenuation mechanisms (e.g. photolysis, biodegrdation) must be present in the river

Table 5
The accuracy and day-to-day variation of the LC–MS–MS method in surface water (the Poudre River 3) and WWTP influent extracts from 120 ml spiked with 0.1 μg/l, 1.0 μg/l, 2.0 μg/l or 5.0 μg/l of three macrolide antibiotics

Samples	*n*	Spike concentration (μg/l)	ETM-H_2O			RTM			TLS		
			Mean concentration (μg/l)	Accuracy (%)	R.S.D. (%)	Mean concentration (μg/l)	Accuracy (%)	R.S.D. (%)	Mean concentration (μg/l)	Accuracy (%)	R.S.D.[a] (%)
Day 1											
Poudre River 3	6	0.1	0.09 ± 0.01	−4.9	10.5	0.09 ± 0.01	−12.0	11.4	0.11 ± 0.01	13.0	8.8
	6	1.0	1.03 ± 0.05	3.5	4.8	1.10 ± 0.07	10.2	6.4	1.08 ± 0.08	8.2	7.4
	6	2.0	2.09 ± 0.06	4.8	2.9	2.01 ± 0.16	0.6	8.0	2.14 ± 0.12	7.3	5.6
WWTP influent	6	0.1	0.11 ± 0.02	12.1	17.8	0.09 ± 0.01	−6.0	10.6	0.11 ± 0.02	11.0	18.0
	6	2.0	2.25 ± 0.22	12.8	9.8	1.91 ± 0.17	−4.3	8.9	2.29 ± 0.36	10.8	16.3
	6	5.0	4.84 ± 0.56	−3.1	11.6	5.48 ± 0.65	9.7	11.9	5.26 ± 0.74	5.3	14.1
Day 2											
Poudre River 3	6	0.1	0.09 ± 0.01	−4.9	10.5	0.09 ± 0.01	−8.1	10.9	0.09 ± 0.01	−5.0	10.5
	6	1.0	1.12 ± 0.12	12.1	10.7	1.03 ± 0.11	3.5	10.6	1.05 ± 0.04	5.5	3.8
	6	2.0	2.17 ± 0.19	8.8	8.7	2.01 ± 0.07	0.7	3.5	1.88 ± 0.22	−5.8	11.7
WWTP influent	6	0.1	0.09 ± 0.01	−11.9	11.4	0.11 ± 0.02	11.9	17.9	0.09 ± 0.01	−12.0	11.4
	6	2.0	2.19 ± 0.17	9.8	7.7	1.90 ± 0.14	−2.6	7.2	2.11 ± 0.17	5.8	8.0
	6	5.0	5.56 ± 1.10	11.3	19.8	4.99 ± 0.97	−0.1	19.4	5.32 ± 0.78	6.5	14.6
Day 3											
Poudre River 3	6	0.1	0.11 ± 0.01	11.1	9.0	0.09 ± 0.01	−5.1	10.5	0.09 ± 0.01	−5.0	10.5
	6	1.0	0.88 ± 0.09	−11.5	10.2	1.11 ± 0.12	11.5	10.8	1.09 ± 0.11	9.1	10.1
	6	2.0	2.19 ± 0.09	9.8	4.1	2.05 ± 0.15	2.7	7.3	2.15 ± 0.07	7.7	3.2
WWTP influent	6	0.1	0.11 ± 0.02	13.0	17.7	0.11 ± 0.01	11.1	9.0	0.09 ± 0.01	−9.0	11.0
	6	2.0	2.25 ± 0.34	12.8	15.1	2.21 ± 0.29	10.7	13.1	1.93 ± 0.26	−3.3	13.4
	6	5.0	5.49 ± 0.72	9.9	13.1	5.62 ± 0.88	12.5	15.6	5.24 ± 0.45	4.9	8.6

[a] R.S.D.: relative standard deviation.

Table 6
Occurrence of macrolide antibiotics in the Poudre River

Sample location	μg/l, X ± S.D. (%)		
	Erythromycin-H_2O	Roxithromycin	Tylosin
Cache La Poudre River 1	Nd[a]	ND	ND
Cache La Poudre River 2	ND	0.04 ± 0.001	ND
Cache La Poudre River 3	0.17 ± 0.03	0.06 ± 0.002	0.13 ± 0.01
Cache La Poudre River 4	ND	ND	ND
Cache La Poudre River 5	ND	ND	ND
WWTP influent	0.20 ± 0.01	ND	1.15 ± 0.07
NIC effluent	0.14 ± 0.02	ND	1.11 ± 0.06
WWTP effluent	0.08 ± 0.005	ND	0.06 ± 0.004

MDL for ETM-H_2O, RTM and TLS: 0.07, 0.03, and 0.05 μg/l, respectively.
[a] ND: less than the level of MDL for each of macrolides.

environment since none of the three compounds is found at site 4, approximately 12 miles downstream. Also, it appears that the only source of these macrolide antibiotics in this watershed is from a point-source WWTP. Sites 4 and 5 are heavily influenced by the surrounding agricultural activities but none of the macrolide antibiotics were measured at either of these locations. Since a previous study by the authors showed a significant occurrence of four tetracyclines at these sites [21], the absence of macrolides in this study indicates that these compounds are not being used for growth promotion, the largest source of agricultural antibiotics to the environment.

4. Conclusions

A sensitive and reliable method for identificatioin and quantification of ETM-H_2O, RTM and TLS in water has been developed using SPE and LC–MS–MS with MDLs of 0.07, 0.03, and 0.05 μg/l for ETM-H_2O, RTM, and TLS, respectively. Oasis HLB sorbent showed good recoveries with the average of 93.6 ± 8.6, 92.1 ± 10.0, and 94.3 ± 8.9% for ETM-H_2O, RTM and TLS in surface water, respectively. For water from the WWTP influent, the average recovery was 84.8 ± 14.0, 83.2 ± 13.1, and 86.1 ± 13.4% for ETM-H_2O, RTM and TLS, respectively. Using both LC–MS

and LC–MS–MS, mass spectra and fragment or product ions were clearly observed for all three MLs in full scan mode. However, specific fragement or product ions for an analyte depended on the MS or MS–MS method that was used.

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