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Rapid analysis of trace levels of antibiotic polyether ionophores in surface water by solid-phase extraction and liquid chromatography with ion trap tandem mass spectrometric detection

J.M. Cha, 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

The occurrence of antibiotics in surface and ground water is an emerging area of interest due to the potential impacts of these compounds on the environment. This paper details a rapid, sensitive and reliable analytical method for the determination of monensin A and B, salinomycin and narasin A in surface water using solid-phase extraction (SPE) and liquid chromatography–ion trap tandem mass spectrometry (LC–MS–MS) with selected reaction monitoring (SRM). Several product ions as sodiated sodium salts for MS–MS detection have been identified and documented with their proposed fragmentation pathways. Statistical analysis for determination of the method detection limit (MDL), accuracy and precision of the method is described. The average recovery of ionophore antibiotics in pristine and wastewater-influenced water was $96.0 \pm 8.3\%$ and $93.8 \pm 9.1\%$, respectively. No matrix effect was seen with the surface water. MDL was between 0.03 and 0.05 μg/L for these antibiotic compounds in the surface water. The accuracy and day-to-day variation of method fell within acceptable ranges. The method is applied to evaluate to the occurrence of these compounds in a small watershed in Northern Colorado. The method verified the presence of trace levels of these antibiotics in urban and agricultural land use dominated sections of the river.

Keywords: Antibiotics; Polyether ionophores; River; Agricultural

1. Introduction

The ionophore antibiotics (e.g. monensin (MON), salinomycin (SAL), narasin (NAR), etc.) produced by various strains of *Streptomyces* have microbiological activities against Gram-positive bacteria, fungi and protozoa. These antibiotics are used in veterinary applications as feed additives (coccidiostats) for poultry and livestock and as growth promoters for ruminants [1,2]. Their basic structure consists of multiple cyclic ethers, a free carboxylic acid group at one end of the molecule and a terminal alcohol group at the other, such that they are described as polyether antibiotics (Fig. 1). These ionophores readily form electrically neutral pseudo-macrocyclic complexes with polar mono and divalent cations, i.e. Na^+, K^+, Li^+, Cs^+, NH_4^+, Ca^{2+}, Mg^{2+} and Cu^{2+} in solution and are lipid soluble [2–4]. The transport of cations across the cell membrane by the ionophores is the main feature of their pharmacological activity.

When applied in veterinary medicine, a fraction of these drugs are metabolized to inactive compounds, but a significant amount is excreted as active metabolites unchanged via urine or feces. A variety of residual antibiotics have been found in WWTP effluents with concentrations as high as 6 μg/L [5]. U.S. Geological Survey reported that 95 organic wastewater contaminants (OWCs) including antibiotics were found in 80% of the 139 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 [6]. Donoho [7] revealed the presence of MON in cattle feces and urine, and Catherman et al. [8] found NAR (1.0–725.0 μg/kg) in poultry feces and manure.

* Corresponding author. Tel.: +1 970 491 8336; fax: +1 970 491 7727.
E-mail address: kcarlson@engr.colostate.edu (K.H. Carlson).

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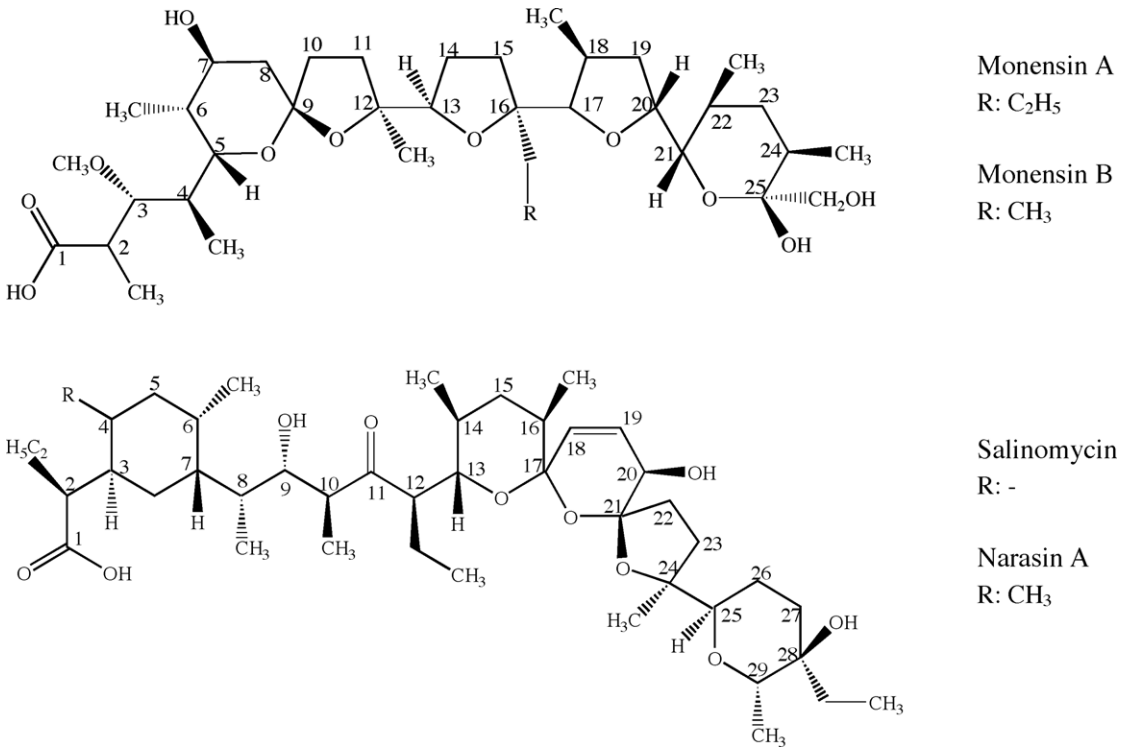

Fig. 1. Chemical structures of carboxylic polyether ionophores.

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 [9].

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 [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 polyether antibiotics (PEs) in both natural and wastewater environments.

Numerous methods for analytical determination of one or more of these ionophores in biological matrices (e.g. feeds, eggs, liver, human plasma, poultry tissue) have been reported in the literature [2,3,12,13]. Because ionophore antibiotics do not exhibit any significant UV absorbance [12], derivatization to form a UV-absorbing compound is generally required for their analysis by high-performance liquid chromatography (HPLC). Liquid chromatography–mass spectrometry (LC–MS) [3,13–15] or LC–MS–MS [2,16] has been used in the analysis of ionophore antibiotics because of its high sensitivity and ability to provide compound confirmation. Researchers have varied methodological approaches for a variety of biological matrices and site-specific equipment. The majority of LC–MS or LC–MS–MS methods for PEs in biological matrices are for single or triple quadrupole mass spectrometers [2,3,13–15] and ion trap tandem mass spectrometers [3]. However, Kiehl et al. [3] did not fully report electrospray ionization (ESI) data and procedures for the investigated MON using an ion trap tandem mass spectrometer. In water matrices, no study of the analytical method for the determination of MON A and B, SAL and NAR A has been conducted with single, triple quadrupole or ion trap mass spectrometers.

Analysis of the ionophore antibiotics presents substantial problems. The polyether compound exhibits lower limited solubility in water due to the formation of lipid-soluble cyclic complexes with alkali metal cations. The compounds with hemiactetal or ketal structures tend to be acid labile. While their lipophilic property facilitates their extraction from aqueous matrices, the equal lipophilicity of their salts is sensitive to the type of acid–base extraction. The appearance of multiple charge adducts for a single analyte of polyether ionophores, which readily form cyclic complexes with polar cations is an issue that some analytical techniques face.

This paper details a rapid, sensitive and reliable analytical method for the determination of MON A and B, SAL and NAR A in surface water using solid-phase extraction (SPE) and ion trap LC–MS–MS with ESI (+) and SRM. This study discusses product ions by fragmentation of ionophore–sodium ion complexes in an ion trap mass spectrometer. Several product ions as sodiated sodium salts for MS–MS detection have been identified and documented.

Statistical analysis for determination of the method detection limit (MDL), accuracy and precision of the method is described. The method is applied to evaluate the occurrence of these compounds in a small watershed in Northern Colorado that is influenced by WWTP effluents and agricultural landscapes.

2. Experimental

2.1. Materials and reagents

Monensin sodium salt (purity, 90–95%), salinomycin (purity, 96%), narasin (purity, 97%) 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 Inc. (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 monthly. Working solutions (10, 1 and 0.1 mg/L) were prepared fresh weekly by diluting the stock solution with deionized water and stored at 4 °C in the dark. Internal standard working solutions (1.0 mg/L) were prepared by diluting the standard solution (1000 mg/L in methanol) with deionized water, stored at 4 °C, and replaced with a fresh solution each week.

2.2. Description of the investigated area

A watershed-scale field 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 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.

2.3. Sample collection and preparation

Approximately 45 samples were collected from five locations on the Cache la Poudre (Poudre) River in northern Colorado, USA (Fig. 2) over a period of six months from January 1, 2004 to June 30, 2004. The samples were collected in triplicate in the center of the stream as a depth composite using a water grab sampler.

All water samples were filtered through 0.4-μm glass fiber filters (Millipore, MA, USA) and stored at 4 °C in refrigerators until they were extracted, typically within one week. SPE and measurement were performed on the same day since the solubility of the extracted PEs during freezing and thawing was variable.

2.4. Preparation of ionophore–sodium salt

To convert the investigated ionophores into a single sodium adduct species, appropriate amounts (0.005–1%, w/v) of sodium chloride as a surplus of sodium were dissolved in water samples and then the samples were left to stand for 30 min prior to SPE and LC–MS–MS analyses. The added amount of sodium chloride depends on the sum of cation concentrations (e.g. Na^+, K^+, Li^+, Cs^+, NH_4^+, Ca^{2+}, Mg^{2+} and Cu^{2+}) in water samples.

2.5. Solid-phase extraction

Because the ionophores form complexes with alkali metal cations, all glassware used was heated for 1 h at 450 °C,

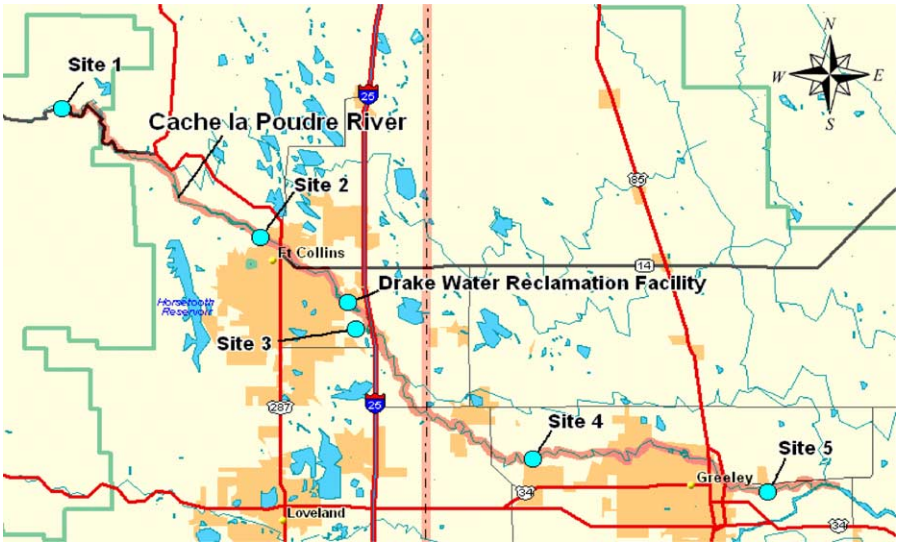

Fig. 2. Samples sites along the Cache la Poudre River in Northern Colorado.

cooled, rinsed with 5 mL of 5.0% Na_2EDTA and then 10 mL of HPLC grade water three times, and air-dried prior to SPE. SPE experiments were conducted using 60 mg/3 mL Oasis HLB cartridges (Waters, Millford, MA, USA). Cartridges were preconditioned with 3 mL of MeOH, 3 mL of 0.5 M HCl and 3 mL of deionized water at 8 in Hg on a vacuum manifold (PrepSep 12 port, Fisher Scientific, PA, USA). One-hundred and twenty millilitres of aqueous samples added at 0.005–1.0% (w/v) of sodium chloride were prepared for extraction. For controls and calibration curves, appropriate amounts of working solution containing each of the analytes was added. Because the investigated ionophores are acid and/or base labile, extraction using the HLB cartridges was performed with the neutral sample pH adjusted by 0.01 M NaOH to 7.5 immediately prior to extraction. To test the behavior of PEs spiked into a natural water matrix, a water sample was collected from the Poudre River Site 1. This water was used as a matrix because it has been shown in other studies by the authors [17,18] that this water was devoid of pharmaceutical compounds since it is essentially snow runoff. Before it was used as a matrix, the water was again analyzed using the developed method in this study and no PEs was 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 12 μL of the internal standard, 1.0 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 evaporation system (N-Evap, Organermation Associates Inc., 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 PEs.

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 520 nm for PEs. The mass spectrometry was a Finnigan LCQ Duo ion trap (ThermoQuest, CA, USA) equipped with a heated capillary interface, and an electrospray ionization source. ThermoQuest Xcalibur software was employed to control the mass spectrometric conditions. PEs were separated using a short Xterra MS C_{18} column (2.1 mm × 50 mm) with a 2.5 μm pore size (Waters, Millford, MA, USA) in combination with a guard column of the same type (2.1 mm × 4 mm) from Phenomenex Inc. (Torrance, CA, USA). An injection volume 40 μL and a ternary gradient with a flow rate of 0.30 mL/min were used. The LC column temperature was kept at 25 °C. Mobile phase A was water with 0.1% formic acid, mobile phase B was methanol and mobile phase C was acetonitrile. Separations of PEs were achieved with the following mobile phase gradient program: at 0 min A/B/C = 18:74:8, 9 min A/B/C = 13:1:86, 10 min A/B/C = 18:74:8. The investigated PEs eluted in only 5.84 min. A 10-minute post-time allowed re-equilibration of the column.

2.7. Ion trap tandem mass spectrometry

Full scan mode was used to acquire mass spectra, precursor ions as protonated sodium salts and product ions as sodiated sodium salts from standard PE solutions. Mass spectral data shown in this study were acquired on a LCQ Duo ion trap tandem 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 (7 mg/L) coming from an integrated syringe pump at a flow rate of 5 μL/min was mixed with mobile phases A/B/C at a 15:32:53 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 for PEs, 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 38 and 25 V. These 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 and auxiliary gas flow rate each was set at 50 units (a scale of arbitrary units in the 0–100 range defined in the LCQ system) and capillary temperature was 175 °C. MS–MS parameters for PEs including their proposed structures, and collision energy and isolation width (*m*/*z*) are summarized in Table 1.

2.8. Quantitation

The product ion, $[M + Na - H_2O]^+$ as a sodiated sodium salt 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 SIM. For SRM the product ion of the highest intensity for the investigated MON A and B, SAL and NAR A was reported in boldface in Table 1. Quantitation was based on a detector response defined as the ratio of the base peak ion (the specific product ion of interest) to the base peak ion of the internal standard. Calibration curves constructed for PEs spiked into water samples before extraction ranged from 0.05 to 5 μg/L in deionized water and surface water (Poudre River Sites 1 and 3).

2.9. Statistical analysis

The method detection limit (MDL) was determined using the recommended US EPA method for MDL determination [19] on the basis of the variability of multiple analyses of

Table 1
MS–MS parameters for analysis of ionophore antibiotics

Formula of MON A	Product ions (*m*/*z*)	Formula of MON B	Product ions (*m*/*z*)
Nominal molecular mass (Da)	670.5	Nominal molecular mass (Da)	656.4
Isolation width (*m*/*z*)	3.3	Isolation width (*m*/*z*)	3.2
Normalized collision energy (%)	33	Normalized collision energy (%)	30
Precursor ion		Precursor ion	
Protonated sodium salt		Protonated sodium salt	
$[M + Na]^+$, $C_{36}H_{62}O_{11}Na^+$	693.5	$[M + Na]^+$, $C_{35}H_{60}O_{11}Na^+$	679.4
Product ions		Product ions	
Sodiated sodium salts		Sodiated sodium salts	
$[M + Na - H_2O]^+$, $C_{36}H_{60}O_{10}Na^+$	**675.5**[a]	$[M + Na - H_2O]^+$, $C_{35}H_{58}O_{10}Na^+$	**661.5**[b]
$[M + Na - 2H_2O]^+$, $C_{36}H_{58}O_9Na^+$	657.5	$[M + Na - 2H_2O]^+$, $C_{35}H_{56}O_9Na^+$	643.4
$C_{34}H_{58}O_8Na^+$	617.5	$C_{24}H_{42}O_7Na^+$	465.3
$C_{34}H_{56}O_7Na^+$	599.4	$C_{24}H_{40}O_6Na^+$	447.2
$C_{25}H_{44}O_7Na^+$	479.3		
$C_{25}H_{42}O_6Na^+$	461.3		

Formula of SAL	Product ions (*m*/*z*)	Formula of NAR A	Product ions (*m*/*z*)
Nominal molecular mass (Da)	750.5	Nominal molecular mass (Da)	764.5
Isolation width (*m*/*z*)	3.0	Isolation width (*m*/*z*)	3.2
Normalized collision energy (%)	35	Normalized collision energy (%)	35
Precursor ion		Precursor ion	
Protonated sodium salt		Protonated sodium salt	
$[M + Na]^+$, $C_{42}H_{70}O_{11}Na^+$	773.5	$[M + Na]^+$, $C_{43}H_{72}O_{11}Na^+$	787.5
Product ions		Product ions	
Sodiated sodium salts		Sodiated sodium salts	
$[M + Na - H_2O]^+$, $C_{42}H_{68}O_{10}Na^+$	**755.5**[c]	$[M + Na - H_2O]^+$, $C_{42}H_{66}O_9Na^+$	**769.5**[d]
$[M + Na - 2H_2O]^+$, $C_{42}H_{66}O_9Na^+$	737.4	$C_{30}H_{50}O_7Na^+$	545.3
$C_{29}H_{48}O_7Na+$	531.4	$C_{29}H_{48}O_7Na^+$	531.4
$C_{29}H_{46}O_6Na^+$	513.3	$C_{29}H_{46}O_6Na^+$	513.3
$C_{23}H_{36}O_6Na^+$	431.3	$C_{23}H_{36}O_6Na^+$	431.3
$C_{23}H_{34}O_5Na^+$	413.2	$C_{23}H_{34}O_5Na^+$	413.2
$C_{19}H_{34}O_5Na^+$	365.1	$C_{20}H_{36}O_5Na^+$	379.1
$C_{13}H_{22}O_4Na^+$	265.1	$C_{14}H_{24}O_4Na^+$	279.1

[a,b,c,d] Product ions (*m*/*z*) of the highest intensity for SRM and quantitation are reported in bold face.

seven surface water (Poudre River Sites 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 were carried out with six surface water (Poudre River Sites 1 and 3) extracts spiked with 0.1, 1.0 or 2.0 μg/L of PEs for three days. Each day, six extracts spiked at three different concentrations in the two water matrices were analyzed.

3. Results and discussion

3.1. Liquid chromatography

The LC method employing a ternary gradient sequence combined with ESI (+)–MS–MS allowed the rapid, sensitive, selective and reliable determination of the investigated PEs in water matrices. The mass peaks corresponding to PEs appeared on the total-ion chromatograms (TICs) monitored at the selected product ion. The data were processed by creating reconstructed total-ion chromatograms (RTICs) for each analyte as shown in Fig. 3. These results indicate that efficient separation of PEs was achieved in only 5.84 min by the short C_{18} column using a column temperature (25 °C), a volumetric flow rate of 0.30 mL/min and mobile phases in a ternary solvent system, meaning fast analysis of the investigated ionophore antibiotics.

3.2. Determination of ionophore components in standard materials

Interestingly, the ionophore antibiotic standard materials are known to contain their derivatives as impurities. To determine components of derivatives in the ionophore standards (e.g. monensin sodium salt, purity = 90–95%; salinomycin, purity = 96%; narasin, purity = 97%) used in this study, 2 mg/L of each standard solution was detected in the LC–MS–MS method with SRM developed in this study, followed by the comparison of mass peak areas. Standard monensin (MON) comprised MON A and B (Fig. 1). MON B (an analogue of MON A that has a methyl instead of an ethyl group) was detected with a 0.91 min shorter retention time as compared to that of MON A (Fig. 3). The average

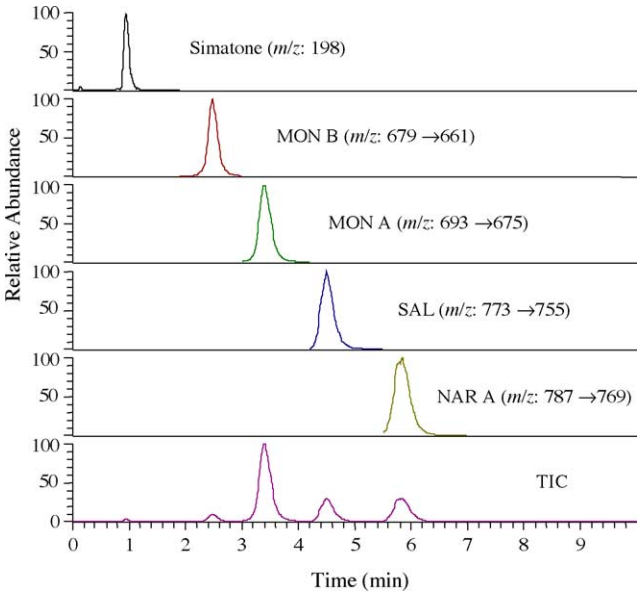

Fig. 3. Reconstructed total-ion chromatograms of ionophore antibiotics spiked at a concentration of 2 μg/L before extraction for 120 mL of Poudre River Site 1 as the surface water matrix using LC–MS–MS in SRM. *m/z* indicates precursor ion → product ion used for quantitation.

composition of MON A and B in the MON standard material used was 87.7 and 12.3% with a 1.2% standard deviation, respectively.

Derivatives of standard SAL were not detected in even full scan LC–MS mode. Standard NAR mostly comprised NAR A as the major component (Fig. 1). These results for SAL and NAR were consistent with those of Harris et al. [20], reporting the derivatives of less than 5% relative to the main components. Thus, MON A and B, SAL and NAR A were quantitated in LC–MS–MS with SRM in this study.

3.3. Electrospray ionization characteristics of ionophore antibiotics

The high affinity of ionophore antibiotics for alkali metal cations as various impurities present in sample matrices and mobile phases has been described in the literature on electrospray ionization [3,16,21,22]. Analysts have tried to force all of the analyte to a particular charge state in a similar manner such as the addition of a particular modifier in the sample matrix and/or mobile phase. Several studies on electrospray ionization showed the predominance of protonated sodium salt, $[M + Na]^+$ and the lack of protonated molecular ion, $[M + H]^+$ [2,3,12,16]. Blanchflower and Kennedy [14,15] added 0.8% NaOH solution to egg and poultry tissue extract to quantitate MON, SAL and NAR using the $[M + Na]^+$ ion, followed by the addition of 15 g of sodium sulfate in the 5 g portion of animal tissues by Matabudul et al. [2] and the conversion of ionophores into a single adduct species using dichloromethane (DCM) and alkali chlorides by Volmer and Lock [16]. For ionophores, basic modifiers such as ammonium and sodium acetate were added to the mobile phase in order to ensure sodium adduct formation [3,13,15]. Because the modifier and mobile phase are in great excess of the analyte, this can be effective, as the ion formation reaction can be driven far to the right in terms of its equilibrium. These results indicate that it is desirable to quantitate a sodium adduct ion for the ionophores in LC–MS and/or LC–MS–MS. To develop sensitive, specific and reproducible LC–MS and/or LC–MS–MS methods for ionophore antibiotics, it is desirable to convert the ionophores into a single sodium adduct species in the sample matrix and then to enhance the ionization process in the mobile phase containing sodium acetate to ensure a surplus of sodium in order to ensure sodium adduct formation.

To improve sensitivity, selectivity and specificity for the chosen sodium ion of analyte in this study, appropriate amounts (0.005–1%, w/v) of sodium chloride were dissolved in water samples prior to SPE and LC–MS–MS analyses to convert the investigated ionophores into a single sodium adduct species. The added amount of sodium chloride depends on the sum of cation concentrations (e.g. Na^+, K^+, Li^+, Cs^+, NH_4^+, Ca^{2+}, Mg^{2+} and Cu^{2+}) in water samples from the investigated Poudre River Sites 1–5. The sum of cation concentrations in the investigated surface water ranged from 20 mg/L (Poudre River Site 1) to 500 mg/L (Poudre River Site 5). Sodium acetate as a basic modifier in the mobile phase may be advantageous to maximize sensitivity and specificity for the sodium adduct ions of PEs in MS–MS analyses but we did not use the basic modifier as a non-volatile compound in the mobile phase due to the problems with clogging of the interface of the mass spectrometer without a Z-spray.

3.4. Fragmentation of ionophores in the electrospray ion trap tandem mass spectrometer

This study reports the investigation of protonated sodium adduct ion ($[M + Na]^+$) fragmentation in a positive electrospray ion trap tandem mass spectrometer. Mass spectra and product ions as sodiated sodium salts for PEs were clearly observed in the full MS–MS scan mode of the ion trap tandem mass spectrometer. Fig. 4 shows full scan MS–MS spectra of precursor ion, $[M + Na]^+$ for a standard solution of 7 mg/L MON A and B, SAL and NAR A with ESI (+) source, and their proposed fragmentation pathways. Molecular mass (Da), precursor ions as protonated sodium salts and product ions as sodiated sodium salts at *m/z* for LC–MS–MS including their proposed structures, and collision energy and isolation width (*m/z*) are listed in Table 1. Each of the PEs exhibited characteristic fragmentation with the ESI (+) source and the precursor ion observed for all analytes was $[M + Na]^+$.

For MON A and B (Table 1 and Fig. 4A), fragmentations of MON A and B were the result of openings of the cyclic ether rings as illustrated in Fig. 4A. Fragmentation of MON A with ion trap MS–MS in this study produced neutral losses of 18 and 36 Da corresponding to the sequent losses of H_2O, $[M + Na - H_2O]^+$ and $[M + Na - 2H_2O]^+$ from the precursor ion, $[M + Na]^+$. According to the nomenclature for ionophore

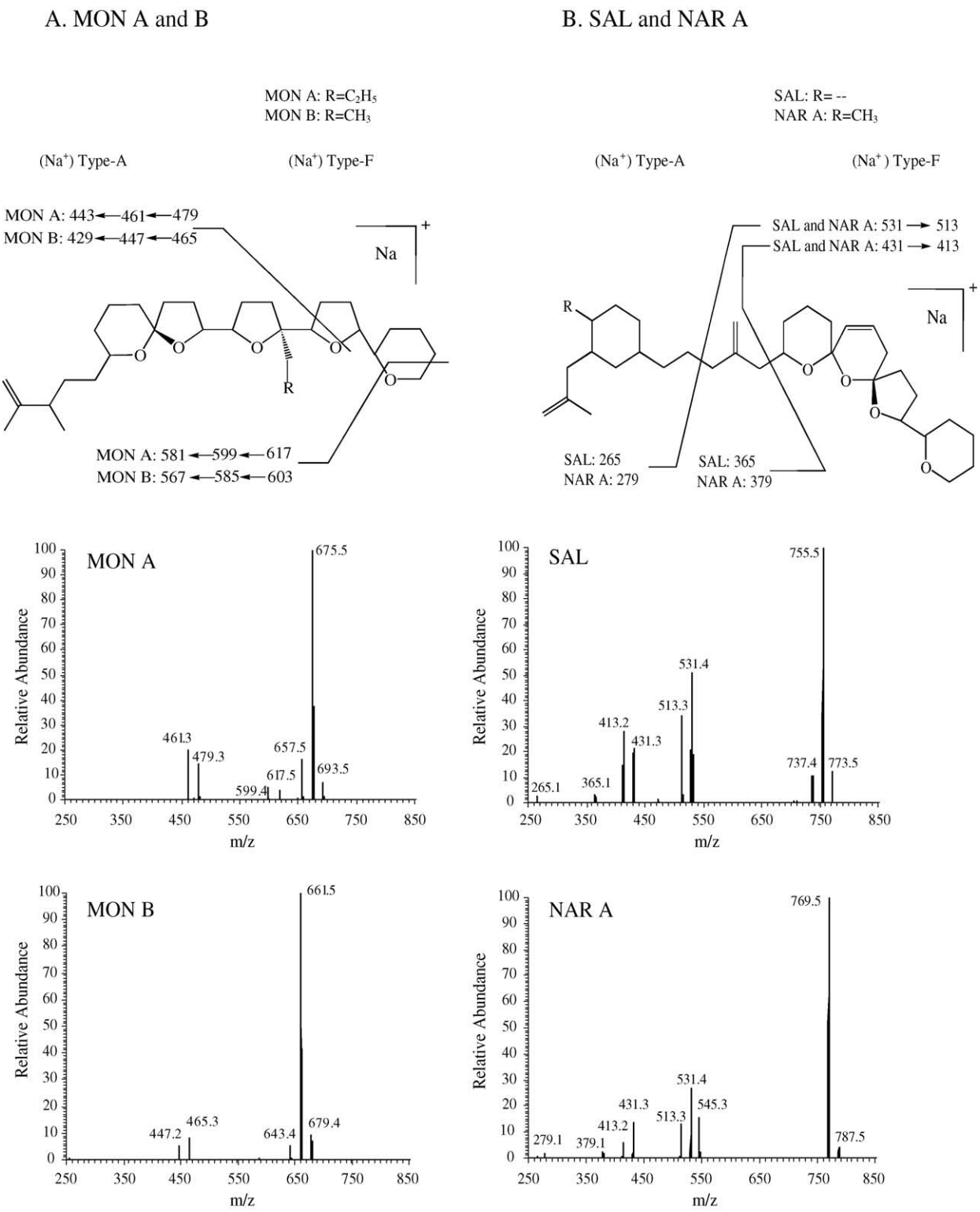

Fig. 4. Proposed MS–MS fragmentation pathways of solidated sodium adduct ions and full scan MS–MS spectra of (A) MON A and B, (B) SAL and NAR A.

antibiotics suggested by Kim et al. [22], fragmentations containing the terminal carboxyl group of the molecule or the terminal hydroxyl group were named as Type A or Type F ions, respectively. It means that these complementary ions correspond to charge retention via the sodium ion on each side of the broken bond for the left hand and the right hand of the molecule. MON A exhibited Type-A product ions (e.g. *m/z* 617, 599, 479, 461 due to losses of H_2O from the 617 or 479 ion) and no Type-F product ions were observed in this ion trap MS–MS mode. MON B exhibited the product ions

(*m*/*z* 465 and 447) at 14 Da lower than their counterparts in MON A spectra due to the different alkyl substituents at the C(16) (Fig. 4A). However, no *m*/*z* 603, 585 and 567 in MON B relative to their counterparts (*m*/*z* 617, 599, 581) in MON A were observed in the ion trap MS–MS of this study.

For SAL and NAR A (Table 1, Fig. 4B), fragmentation of SAL and NAR A with ion trap MS–MS in this study produced neutral losses of 18 Da and/or 36 Da corresponding to the subsequent losses of H_2O, $[M + Na - H_2O]^+$ and/or $[M + Na - 2H_2O]^+$ from the precursor ion, $[M + Na]^+$ as observed in the fragmentation of MON A and B. Two major fragmentation pathways (Fig. 4B) were observed in the ion trap tandem mass spectrometer. The major fragmentation pathways involved β-cleavage of the oxygen-activated C–C bonds on each side of the C(11) carboxyl function, as illustrated in Fig. 4 (B). In the first fragmentation pathway of SAL, β-cleavage and subsequent hydrogen migration occurred at C(9)–C(10), resulting in two production ions, *m*/*z* 531 and 265. The 531 ion was then followed by loss of H_2O, resulting in *m*/*z* 513. The second fragmentation pathway of SAL was due to bond dissociation at C(12)–C(13) to give *m*/*z* 431 and 365, followed by *m*/*z* 413 due to loss of H_2O from the 431 ion. Thus, SAL exhibited both of the Type-A product ions (e.g. *m*/*z* 265, 365) and Type-F product ions (e.g. *m*/*z* 531, 513, 431, 413). NAR A exhibited the same fragmentation pathways as compared to those of SAL, indicating that the 531, 513, 431 and 413 ions (Type F product ions) are identical to those in SAL. For Type-A product ions, NAR A exhibited *m*/*z* 279 and 379 instead of *m*/*z* 265 and 365 for SAL due to the differences in substitution between the two ionophores at C(4).

Full scan MS–MS spectra for the investigated ionophore antibiotics (Fig. 4) clearly exhibited product ions as sodiated sodium salts in the ion trap tandem mass spectrometer. Under the given conditions, no protonated molecular ions, $[M + H]^+$ or multiple charge adducts (e.g. $[M + Cat]^+$, $[M + 2Cat - H]^+$, $Cat = Na^+$, K^+, etc. and $[M + NH_4]^+$) were observed (Fig. 4). This indicates that the investigated ionophores were totally converted into a single sodium adduct species by adding sodium chloride to water samples because no multiple charged adducts were observed in this assay. This implies that the compounds show a very high affinity for sodium and the large thermodynamic stability for larger sodium adduct ions versus a smaller proton. It is likely that there is enough 'residual' sodium from other sources (standard monensin sodium salt of the ionophore standards) such that we have no difficulty in generating sodium adduct ions. It is potentially the case that there is already enough sodium present from our current methodology (conversion of ionophores into a sodium adduct ion using an appropriate amount of sodium chloride and addition of sodium hydroxide to adjust sample pH to 7.5) to force the ionization reaction the direction we would like. In addition, formic acid added to the mobile phase in this study had the benefit of improving the spectral quality of mass spectra (Fig. 4) because this effect may be due to the protonation of the terminal carboxyl groups at low pH, thereby reducing and/or eliminating the possibility of $[M + 2Cat - H]^+$ formation. Also, protonation of the carboxyl function at low pH could lessen the extent of hydrogen bonding between the carboxyl function and the terminal hydroxyl group, thus making cyclic complex formation more difficult. These results indicate that the investigated PEs show considerable sensitivity, selectivity and specificity for the chosen sodium ion ($[M + Na]^+$) and the specific product ion ($[M + Na - H_2O]^+$) of the highest intensity for quantitation of analyte in LC–MS–MS analysis with SRM of this study (Table 1, Fig. 4).

3.5. Recovery comparison

Ionophore antibiotics show multiple charge adduct ions for a single analyte with the various ratio of the multiple charge species in different water matrices, resulting in various recoveries in SPE and then a loss of raw sensitivity and precision in LC–MS–MS analysis. To reduce recovery variability in SPE, the investigated ionophores were converted into a single sodium adduct species by adding sodium chloride to water samples before extraction to promote the formation of a single sodium adduct species.

The recoveries of PEs from the HLB cartridges were measured by extracting analytes from 120 mL of deionized water spiked at 0.07–50.0 μg/L and surface water spiked at 0.07–5.0 μg/L. Poudre River samples from Sites 1 and 3 were selected as reference matrices for surface water. Recoveries of PEs were determined using the ratio of the concentration of analyte for extract spiked before extraction to the concentration of analyte for extract spiked after extraction. The average recovery in deionized water (Table 2) was 99.9 ± 6.8% at the investigated concentration range, 90.1 ± 8.2% with a 0.07 μg/L spike, and 93.8 ± 6.9% with a 1.5 μg/L spike. No concentration dependence was observed. The average recovery of PEs in Poudre River Site 1 was 96.0 ± 8.3% at the investigated concentration range, 88.3 ± 9.6% with a 0.07 μg/L spike, and 94.0 ± 9.1% with a 1.5 μg/L spike, indicating the HLB cartridges also gave reproducible recoveries for PEs and were effective for the isolation of the PEs. Recoveries in Poudre River Site 1 water were similar to those in deionized water indicating that matrix effects in surface water were minimal.

To assess the matrix effects for PEs in more complex surface water, recovery was also determined with water from Poudre River Site 3, immediately downstream of the WWTP. Because MON A and NAR A were detected at the sample site using the developed method, recovery in this matrix over a period of six months was determined using a concentration calculated by subtracting the measured background concentration from the spiked concentration. For Poudre River Site 3 (Table 2) the average recovery of PEs was 93.8 ± 9.1% at the investigated concentration range, 88.2 ± 10.6% with a 0.07 μg/L spike, and 95.1 ± 10.3% with a 1.5 μg/L spike, indicating that matrix effects in the more complex surface water matrix were also minimal. In addition, the average

Table 2
Recoveries of ionophore antibiotics from 120 mL of water samples

Ionophores	Recovery, X ± SD (%)								
	Deionized water (μg/L)			Poudre River Site 1 (μg/L)			Poudre River Site 3 (μg/L)		
	0.07	1.5	0.07–50.0	0.07	1.5	0.07–5.0	0.07	1.5	0.07–5.0
MON A	85.6 ± 10.5	95.0 ± 8.3	96.9 ± 8.6	83.6 ± 12.2	92.4 ± 11.7	94.1 ± 11.5	86.1 ± 13.2	92.6 ± 11.8	93.7 ± 12.3
MON B	91.2 ± 6.4	92.7 ± 5.1	99.0 ± 4.9	89.2 ± 6.8	92.7 ± 5.3	94.8 ± 5.7	88.6 ± 7.4	93.4 ± 8.7	92.3 ± 6.9
SAL	90.5 ± 7.1	95.1 ± 7.4	102.2 ± 6.5	91.7 ± 10.1	96.5 ± 10.4	99.3 ± 8.9	89.5 ± 10.9	101.7 ± 11.2	93.9 ± 9.8
NAR A	93.1 ± 8.6	92.0 ± 6.8	101.6 ± 7.2	88.5 ± 9.4	93.2 ± 8.8	95.6 ± 7.2	88.7 ± 10.8	92.9 ± 9.6	95.4 ± 7.2
Average recovery	90.1 ± 8.2	93.8 ± 6.9	99.9 ± 6.8	88.3 ± 9.6	94.0 ± 9.1	96.0 ± 8.3	88.2 ± 10.6	95.1 ± 10.3	93.8 ± 9.1

recovery of lower concentrations of 0.07 μg/L for the investigated PEs in deionized water and surface water were lower (88.0–90.0%) as compared to more than 92.0% at higher concentrations but the difference was not statistically significant. Likewise, recoveries of MON A followed the lower and higher concentration trend observed with MON B, SAL and NAR A in deionized water and surface water.

3.6. Quantitation

It is desirable that both a compound as an internal standard and target compounds should have structural similarities such that it reflects the properties of the target compounds during the entire analytical procedure. This implies that it would be more ideal to have an internal standard (e.g. isotopically labeled compound, structurally similar compound) for each class of antibiotics. Unfortunately, this equates to the added cost and the difficulties in obtaining the ideal compound. Thus, simatone was chosen as an internal standard for PEs in this study and previous studies [17,18] because it eluted within the same chromatographic time frame as the analytes, responded well in ESI (+) mode and did not exhibit noticeable matrix effects. As shown in RTICs (Fig. 2) of this study, simatone (spike concentration of 0.1 μg/L) also eluted much earlier in the chromatogram and was therefore much less affected by latter eluting interferences. Lindsey et al. [23] and Kolpin et al. [6] reported that internal standard simatone did not exhibit matrix effects in analysis of tetracyclines and sulfonamides in surface water, ground water and U.S. streams. To evaluate matrix effects of internal standard, we compared the peak area of internal standard (0.1 μg/L) in extracts including internal standard and PEs for 120 mL water samples analyzed over a period of four months. The average peak area and standard deviation of the internal standard in deionized water and surface water (Poudre River Sites 1–5) was 64,529,774 ± 10.5% and 61,957,146 ± 13.3%, respectively. These values in surface water did not differ statistically from those in deionized water. Standard deviation (10.5% versus 13.3%) of peak areas for the internal standard in the two water matrices is within the recommended acceptable values difference of 15.0% [24]. This indicates that the internal standards do not exhibit matrix effects in surface water. Thus, concentrations for PEs were calculated reproducibly by using the standard calibration curves, which were constructed using a detector response defined as the ratio of the base peak ion (the specific product ion of highest intensity) to the base peak ion of the internal standard. PEs measured in this surface water were not corrected for the matrix effect of the internal standard.

Calibration curves constructed for PEs spiked into water samples before extraction ranged from 0.05 to 5 μg/L in deionized water and surface water (Poudre River Sites 1 and 3). The calibration curves were linear with correlation coefficients (R^2) > 0.99 for the MS–MS procedure. Because Poudre River Site 3 already contained MON A and NAR A, a calibration curve for these ionophores in this matrix was constructed by subtracting the level concentration from the spiked concentration. The ionophore concentrations in Poudre River samples from Sites 1–5 were determined reproducibly by using the standard calibration curves for Poudre River Site 3 since these would include matrix effects that are present in surface water.

3.7. Method detection limit, accuracy and precision

The method detection limit (MDL) was determined using the US EPA recommended method for MDL determination [19]. Calculations of MDL using the US EPA method were based on the variability of multiple analyses of seven surface water (Poudre River Site 1 and 3 each) extracts spiked at a concentration of 0.2 μg/L for PEs. 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 MON A and B, SAL and NAR A extracted from 120 mL of surface water was 0.03, 0.05, 0.04 and 0.04 μg/L, respectively.

The accuracy and the variability of the method were determined using six Poudre River Site 1 and 3 extracts spiked with 0.1, 1.0 and 2.0 μg/L of PEs over a period of three days. Because Poudre River Site 3 (immediately downstream of the WWTP influent) is a more complex water matrix, samples from this site were used to assess the accuracy and precision of the method. The results are summarized in Table 3. The accuracy range was −7.5 to +9.8% in the two water matrices. This accuracy range is well within the recommended acceptable values of −30 to +20% [25]. The relative standard deviations (RSDs) calculated from these experiments ranged

Table 3
The accuracy and day-to-day variation of the LC–MS–MS method in surface water (the Poudre River Sites 1 and 3) extracts from 120 mL spiked with 0.1, 1.0 or 2.0 μg/L of ionophore antibiotics

Samples	*n*	Spike concentration (μg/L)	MON A			MON B			SAL			NAR A		
			Mean concentration (μg/L)	Accuracy (%)	RSD[a] (%)	Mean concentration (μg/L)	Accuracy (%)	RSD[a] (%)	Mean concentration (μg/L)	Accuracy (%)	RSD[a] (%)	Mean concentration (μg/L)	Accuracy (%)	RSD[a] (%)
Day 1														
Poudre River, Site 1	6	0.10	0.10	3.6	8.3	0.10	−1.3	7.4	0.11	6.4	6.4	0.11	8.7	8.6
	6	1.00	1.03	3.3	10.1	1.00	−0.1	5.0	1.01	0.6	5.5	1.09	8.6	7.6
	6	2.00	2.04	1.9	7.2	1.98	−0.8	6.5	1.96	−1.8	7.2	2.02	0.9	6.7
Poudre River, Site 3	6	0.10	0.11	9.4	13.9	0.09	−7.5	8.5	0.11	8.6	7.8	0.10	−1.8	9.2
	6	1.00	0.99	−1.4	11.4	1.04	3.8	6.6	1.10	9.6	8.6	1.07	7.3	11.9
	6	2.00	1.96	−1.8	8.0	1.96	−22	7.1	2.04	1.8	6.6	1.93	−3.3	7.0
Day 2														
Poudre River, Site 1	6	0.10	0.10	−2.5	10.6	0.10	−3.7	7.0	0.10	−1.4	7.0	0.11	5.4	8.8
	6	1.00	0.97	−3.5	9.6	1.02	1.8	7.5	1.04	3.7	7.3	0.96	−3.6	9.3
	6	2.00	2.02	0.8	7.3	2.01	0.7	6.2	2.10	4.9	6.6	2.00	0.1	7.1
Poudre River, Site 3	6	0.10	0.10	−2.9	12.3	0.11	5.1	8.9	0.10	−1.6	9.2	0.11	8.8	12.4
	6	1.00	1.05	5.3	8.6	1.10	9.8	8.4	0.96	−3.6	8.6	1.06	6.3	8.3
	6	2.00	2.02	1.2	10.1	1.86	−6.8	6.5	1.99	−0.6	6.0	1.97	−1.5	7.3
Day 3:														
Poudre River, Site 1	6	0.10	0.09	−5.2	10.4	0.10	−1.7	7.3	0.10	−0.2	7.1	0.09	−5.2	9.8
	6	1.00	1.06	5.6	9.1	1.10	9.7	6.8	0.98	−2.4	6.2	0.98	−2.2	6.1
	6	2.00	1.98	−1.1	7.8	1.99	−0.5	6.0	2.09	4.4	6.7	1.94	−3.2	8.5
Poudre River, Site 3	6	0.10	0.10	−0.8	12.4	0.10	−4.3	9.5	0.11	6.4	8.9	0.09	−6.5	11.7
	6	1.00	1.06	6.3	10.1	1.01	1.4	6.1	1.06	6.4	9.1	0.97	−3.1	8.5
	6	2.00	2.02	0.9	9.7	2.06	2.9	7.4	2.03	1.7	5.6	2.08	3.8	6.2

[a] RSD: relative standard deviation.

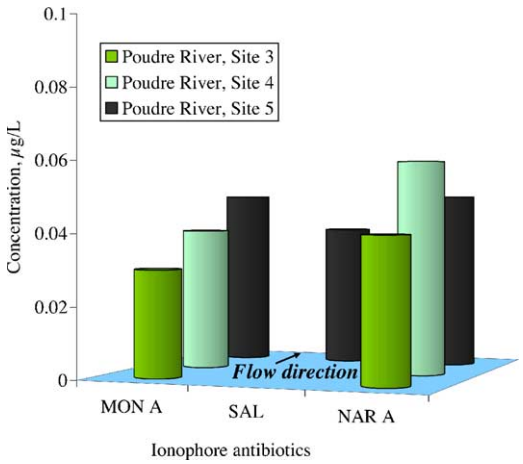

Fig. 5. Occurrence of ionophore antibiotics in the Poudre River. Concentrations shown are the triplicate average of three samples at each site over a period of six months. Number of samples at each site, N = triplicates × three samples × five frequencies = 45.

from 5.0 to 10.6% and 6.1 to 13.9% in the Poudre River Sites 1 and 3 samples, respectively. No concentration dependence was observed.

3.8. Occurrence, distribution, and fate of ionophore antibiotics

The average concentrations of four PE compounds measured along the Poudre River are shown in Fig. 5. As expected, no PE compounds were detected in the pristine river section (Site 1) since there are no urban or agricultural influences. No PE compounds were detected at the fringes of the urban area (Site 2) either indicating minimal urban or agricultural influences.

The first occurrence of MON A (0.03 μg/L) and NAR A (0.04 μg/L) is at Site 3, immediately downstream of the WWTP, around the lower density animal feed operations (AFOs) and concentrated animal feed operations (CAFOs) near to urban area. These trace concentrations are most likely due to the influence of agricultural since all four of these antibiotics have only veterinary applications.

As agricultural land use pre-dominates, i.e. Site 4 (around the greater density AFOs and CAFOs) and Site 5 (around the greatest density AFOs and CAFOs), a general increase in MON A and NAR A concentration is noted relative to Site 3. The results indicate that the two ionophore compounds are present at higher concentrations in the river with when the land use is dominated by agriculture activity. SAL (0.04 μg/L) was first detected along the river with the greatest influence of agriculture. No MON B compound were detected at any of the sites.

Four PE compounds found in this study were present at trace levels (0.03–0.06 μg/L) in the river water, although the compounds have been used as a growth promoter and/or coccidiostats in cattles, chickens and turkeys. This reflects that the PE compounds exhibit lower limited solubility in water matrices and form complexes with alkali metals cations in the sample matrices. This indicates that these compounds may be present at higher concentrations in sediments, manures and lagoon waters. Thus, it is desirable to evaluate the occurrence and fate of ionophore antibiotics in these matrices as well as surface water. The SPE–LC–MS–MS method in this study was first applied to evaluate the occurrence of four ionophore antibiotics in the Cache la Poudre River influenced by WWTP effluents and the surrounding agricultural activities, and therefore will be applied to develop analytical methods for these compounds in more complex environmental matrices of manure and lagoon water eventually.

4. Conclusions

A SPE–LC–MS–MS method with SRM has been shown to be accurate, reliable and robust for the determination of three ionophore veterinary antibiotics. The method was able to quantify two analogues of monensin (A and B) and the results showed a significant fraction of the total (approximately 12%) was monensin B.

Ionophores will readily form different complexes with alkali metal cations in different water matrices, resulting in various recoveries in SPE and then a loss of raw sensitivity and precision in LC–MS–MS analyses. Sensitivity, selectivity and specificity for the chosen protonated sodium ion and the specific product ion of analyte in LC–MS–MS analysis with SRM were improved by adding NaCl before extraction to promote the formation of a single sodium adduct species.

The average recovery of ionophore antibiotics in pristine and wastewater-influenced water was $96.0 \pm 8.3\%$ and $93.8 \pm 9.1\%$, respectively. No matrix effect was seen with surface water. The MDL was between 0.03 and 0.05 μg/L for MON A and B, NAR A, SAL with an accuracy of better than ±10%. The method verified the presence of trace levels (0.03–0.06 μg/L) of these antibiotics in urban and agricultural land use dominated sections of the river.

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References

[1] J.W. Westley, Polyether Antibiotics, Marcel-Dekker, New York, 1982 (chapter 6).
[2] D.K. Matabudul, B. Conway, I. Lumley, S. Sumar, Food Chem. 75 (2001) 345.
[3] D.E. Kiehl, R.K. Julian, J.A.S. Kennington, Rapid Commun. Mass Spectrom. 12 (1998) 903.

[4] E. Mercurio, M. Pellegrini, D.F. Mierke, Biopolymer 42 (1997) 759.
[5] R. Hirsch, T. Ternes, K. Haberer, A. Mehlich, F. Ballwanz, K. Kratz, J. Chromatogr. A 815 (1998) 213.
[6] D.W. Kolpin, E.T. Furlong, M.T. Meyer, E.M. Thurman, S.D. Zaugg, L.B. Barber, H.T. Buxton, Environ. Sci. Technol. 36 (2002) 1202.
[7] A.L. Donoho, J. Anim. Sci. 58 (1984) 1528.
[8] D.R. Catherman, J. Szabo, D.B. Batson, A.H. Cantor, R.E. Tucker, J.R. Mitchell, Poult. Sci. 70 (1991) 120.
[9] American Society of Microbiologists, The role of antibiotics in agriculture, Critical Issues Colloquia, American Academy of Microbiology, Washington, DC, 2002.
[10] B. Halling-Sorensen, S.N. Nielson, P.E. Lanzky, L.F. Ingerslev, Chemosphere 36 (2) (1998) 357.
[11] E.M. Thurman, M. Lindsey, National Ground Water Association Emerging Issues Conference Proceedings, Minneapolis, Minnesota, USA, 7–8 June 2000, National Ground Water Association, Westerville, OH, USA, 2000, p. 19.
[12] G. Dusi, V. Gamba, J. Chromatogr. A 835 (1999) 243.
[13] S.B. Turnipseed, J.E. Roybal, A.P. Pfenning, S.A. Gonzales, J.A. Hurlbut, M.R. Madson, J. AOAC Int. 84 (2001) 640.
[14] W.J. Blanchflower, D.G. Kennedy, Analyst 120 (1995) 1129.
[15] W.J. Blanchflower, D.G. Kennedy, J. Chromatogr. B 675 (1996) 225.
[16] D.A. Volmer, C.M. Lock, Rapid Commun. Mass Spectrom. 12 (1998) 157.
[17] S. Yang, K.H. Carlson, J. Chromatogr. A 1038 (2004) 141.
[18] S. Yang, J.M. Cha, K.H. Carlson, Rapid Commun. Mass Spectrom. 18 (2004) 2131.
[19] P.M. Berthouex, L.C. Brown, Statistics for Environmental Engineers, second ed., Lewis Publications, 2002, p. 119.
[20] J.A. Harris, C.A.L. Russell, J.P.G. Wilkins, Analyst 123 (1998) 2625.
[21] R.P. Schneider, M.J. Lynch, J.F. Ericson, H.G. Fouda, Anal. Chem. 63 (1991) 1789.
[22] Y.H. Kim, J.S. Yoo, C.H. Lee, Y.M. Goo, M.S. Kim, J. Mass Spectrom. 31 (1996) 855.
[23] M.L. Lindsey, M.T. Meyer, E.M. Thurman, Anal. Chem. 73 (2001) 4640.
[24] M.J. Avery, Rapid Commun. Mass Spectrom. 17 (2003) 1971.
[25] M. Cherlet, S. De Baere, S. Croubels, P. De Backer, Anal. Chim. Acta 473 (2002) 167.