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Fast and robust simultaneous determination of three veterinary antibiotics in groundwater and surface water using a tandem solid-phase extraction with high-performance liquid chromatography–UV detection

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Abstract

A simple and robust analytical method is presented in which the three veterinary antibiotics oxytetracycline (OTC), sulfachloropyridazine (SCP) and tylosin (TYL) were simultaneously determined in surface water and groundwater. The three compounds were simultaneously extracted from the water samples using a mixture of methanol, EDTA and McIlvaine buffer (citric acid and sodium orthophosphate) and then cleaned-up and pre-concentrated by solid-phase extraction using sacrificial Isolute strong anion-exchange cartridges, to remove interfering organic material, and Waters Oasis hydrophilic–liphophilic balance polymer cartridges, to retain the compounds, in tandem. Analysis was performed using liquid chromatography with ultraviolet detection. Recoveries for river water samples spiked at 10 and 1 $\mu g\,l^{-1}$ were respectively 99.6 ± 4.6 and $99.4 \pm 8.4\%$ for OTC; 99.9 ± 2.2 and $105.0 \pm 5.7\%$ for SCP; and 94.9 ± 2.4 and $71.6 \pm 8.2\%$ for TYL. Overall limits of detection based on pre-concentrating 400 ml of sample were 0.35 $\mu g\,l^{-1}$ for OTC and TYL and 0.25 $\mu g\,l^{-1}$ for SCP.

Keywords: Water analysis; Environmental analysis; Antibiotics; Oxytetracycline; Sulfachloropyridazine; Tylosin

1. Introduction

Antibiotics used for veterinary purposes can be excreted (as the parent compound and/or metabolites) and therefore can subsequently enter the environment via manure spread directly onto farmland as fertiliser. There is increasing awareness and concern over the fate and environmental effects of antibiotics in the environment as witnessed by a growing number of recent review papers [1–4]. Possible impacts of antibiotics in the environment include both chronic and acute effects, e.g. toxicity [5–9] and the emergence of antibiotic resistance [10,11].

A number of papers describing analytical methodologies for the determination of antibiotics in waters have been published recently [12–16]. The majority of these studies have used solid-phase extraction (SPE) as a clean-up and pre-concentration step and then a chromatographic separation such as high-performance liquid chromatography (HPLC), capillary electrophoresis (CE) or gas chromatography (GC) with either fluorescence or mass spectrometric (MS) detection. Both fluorescence and MS detection are more sensitive and selective compared with ultraviolet detection which is advantageous in the case of environmental samples which generally contain low concentrations of antibiotics (in comparison with concentrations of antibiotic residues in food samples for which there are also many more published analytical methods than for environmental samples). These methods however, have largely focussed on only one, or at most two, classes of compound. Moreover, this study was performed as part of a much larger study into the environmental fate of veterinary antibiotics which

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Table 1
Physico-chemical properties of OTC, SCP and TYL

Compound	CAS no.	M_r	pK_a	log K_{ow}
Oxytetracycline	79-57-2	460.44	3.27, 7.32, 9.11 [20]	−0.9 [21]
Sulfachloropyridazine	80-32-0	284.7	1.76, 5.71 [22]	−0.52 [22]
Tylosin	1401-69-0	916.1	7.1 [23]	3.5 [23]

K_{ow}: octanol–water partition coefficient.

generated large numbers of samples [17–19] Therefore, it was necessary to develop a robust, simple, realistic and practical method capable of simultaneously extracting and analysing the study compounds, hence HPLC–UV was the analytical technique chosen.

The antibiotics selected for investigation were oxytetracycline (OTC), sulfachloropyridazine (SCP) and tylosin (TYL) which are members of the tetracycline, sulfonamide and macrolide groups of antibiotics, respectively. The selection of these compounds from three of the most widely used chemical classes of antibiotics with a range of physico-chemical properties (Table 1, Fig. 1) ensured that studies were relevant. Compounds from these antibiotic groups have differing modes of action and may be used in conjunction hence the relevance of a method able to analyse these compounds in environmental samples simultaneously. These groups of compounds have also recently been identified as having high potential to reach the environment in the UK [24].

2. Experimental

2.1. Equipment

Analysis for this study was performed using a Dionex (Sunnyvale, CA, USA) Summit HPLC system comprising a GINA 50 autosampler and a P580 quaternary gradient pump with a UVD 170S UV–visible spectrophotometric detector which allowed four wavelengths to be simultaneously monitored. Separations were performed on a Genesis C_{18} 150 cm × 4.6 mm internal diameter column packed with 4 μm ODS from Jones Chromatography (Hengoed, Mid Glamorgan, UK). The system was controlled by Chromeleon software. Oasis hydrophilic–liphophilic balance (HLB) polymer solid-phase extraction cartridges were purchased from Waters (Watford, UK) and Isolute polymer (ENV+), strong anion-exchange (SAX) and C_{18} solid-phase extraction cartridges were purchased from International Sorbent Technology (Hengoed, Mid Glamorgan, UK).

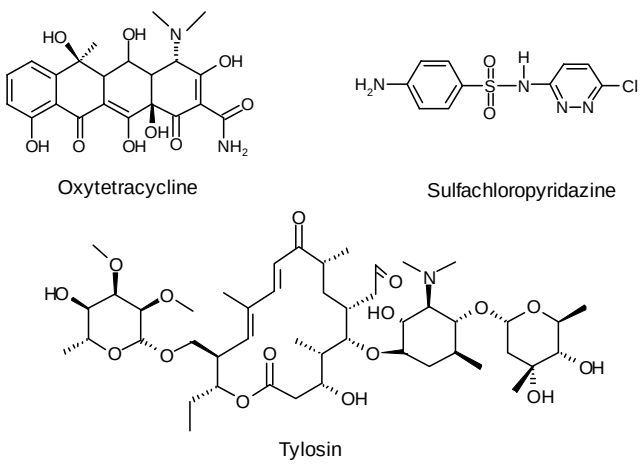

Fig. 1. Chemical structures of OTC, SCP and TYL.

2.2. Chemicals, reagents and standards

Methanol, tetrahydrofuran, acetonitrile, water, trifluoroacetic acid, orthophosphoric acid and citric acid were all HiPerSolv for HPLC grade from BDH (Poole, UK). EDTA disodium salt, disodium hydrogenorthophosphate anhydrous, sodium acetate 3-hydrate and sodium sulphite were all AnalaR grade from BDH. Sulfachloropyridazine was Vetranal grade from Riedel–de Haën (Gillingham, UK), oxytetracycline and tylosin tartrate were BioChemika grade from Fluka (Gillingham, UK). Sulfachloropyridazine sodium salt was obtained from Novartis Animal Health (Basel, Switzerland) and oxytetracycline hydrochloride was obtained from Vericore Limited (Dundee, UK).

0.1 M Na_2EDTA solution was prepared by dissolving 18.6 g in 500 ml of distilled water, 0.2 M citric acid was prepared by dissolving 21.0 g in 500 ml distilled water and 0.4 M Na_2HPO_4 was prepared by dissolving 28.4 g in 500 ml of distilled water. Extraction/adjustment buffer was EDTA–McIlvaine buffer (50:50) prepared by mixing 150 ml of 0.1 M EDTA, 90 ml 0.2 M citric acid, 60 ml 0.4 M Na_2HPO_4 and 3 ml H_3PO_4. Conditioning/washing buffer for SPE was prepared by diluting the extraction buffer 20-fold to match the matrix after sample preparation. Single compound stock solutions of 1 mg ml^{-1} were prepared by dissolving the Vetranal and BioChemika antibiotic solids in methanol. Mixed calibration standards were prepared in methanol at 0.0, 0.1, 0.5, 1.0, 2.0, 5.0, 10.0, 25.0 and 50.0 μg ml^{-1} from the stock solutions. A mixed QC standard at 5.0 μg ml^{-1} was prepared in the same manner but from stocks prepared from the SCP–Na and OTC–HCl.

2.3. Chromatographic conditions

A gradient elution was carried out with tetrahydrofuran (solvent A), acetonitrile (solvent B) and 0.05% trifluroacetic

acid in water (solvent C). The flow rate was 1 ml min^{-1} throughout. The mobile phase composition was programmed as follows: A–B–C (5:2.5:92.5) from 0 to 4 min rising linearly to (5:75:20) from 4 to 18 min and then returning linearly to (5:2.5:92.5) from 18 to 20 min. Equilibration was then performed from 20 to 25 min at (5:2.5:92.5). All eluents were degassed in an ultrasonic bath under vacuum prior to analysis in addition to their being an integral degasser fitted in the pump. The injection volume was 20 μl. Simultaneous detection was performed at 285 nm for SCP and TYL and at 355 nm for OTC, with additional simultaneous monitoring at 260 nm for SCP and TYL and 370 nm for OTC to allow selectivity checks to be made.

2.4. Sample collection

Approximately 20 l of river-water was collected from the River Trent at Shardlow, Derbyshire, UK, to provide real environmental matrix water for method development and validation. Drain flow samples (400 ml maximum) were automatically collected during flow events in a 'v-notch' weir-box from the drain outflow pipe of a tile drained clay loam field in Osgathorpe, Leicestershire, UK where a field scale study to investigate environmental fate was undertaken. Liquid pig manure, sourced from a farm where the animals were treated continuously with TYL at 100 g t^{-1} of feed, was fortified with OTC and SCP before application to the field. The field was 1.55 ha in area and the slurry application rate was 45,000 l ha^{-1} (in line with UK agricultural practice). The slurry was fortified such that the application rates of OTC and SCP were 0.9 and 1.2 kg ha^{-1}, respectively (predicted environmental concentrations calculated using published methodologies [25]). Surface water samples were collected from a variety of drainage ditches in the River Soar catchment area in north west Leicestershire from farms in the area surrounding the pig farm which use the pig slurry as organic fertiliser.

2.5. Sample preparation

The Trent river water was filtered through Whatman GMF-150 2.0 μm glass fibre filters prior to fortification and extraction. The clay site drainflow samples were centrifuged at approximately 1200 g for 10 min, and then filtered to 0.8 μm through Whatman nylon membrane filters under vacuum. The samples had 5 ml of extraction buffer and 2 ml methanol added per 100 ml of sample and then were thoroughly mixed prior to SPE. SAX and HLB SPE cartridges were set up in tandem, conditioned with 5 ml methanol then 5 ml conditioning/washing buffer and then the samples were loaded at 10 ml min^{-1}. After loading, the SAX cartridges were removed and the HLB cartridges were washed sequentially with 5 ml conditioning/washing buffer, 2.5 ml 0.1 M NaOAc, 5 ml distilled water and 2 ml 20% methanol. The cartridges were then air dried for 10 min and the compounds were eluted with 2 ml × 1 ml aliquots of methanol.

2.6. Method validation

Method validation was carried out using an approach based upon existing recommendations [39]. Three batches of single compound stock solutions (1 mg ml^{-1}) of SCP, OTC and TYL in methanol were prepared separately (i.e. nine solutions in total). From these stock solutions three batches of mixed spiking solutions in methanol (at 0.0, 0.5 and 5.0 μg ml^{-1}, nine solutions in total) and three batches of mixed calibration solutions in methanol (at 0.0, 0.1, 0.5, 1.0, 2.0, 5.0, 10.0, 25.0 and 50.0 μg ml^{-1}, 27 solutions in total) were prepared. 500 ml samples of river water were fortified with 1 ml of spiking solution to give either 0 μg l^{-1} (blank), 1 μg l^{-1} (low spike) or 10 μg l^{-1} (high spike). Spiking was carried out in duplicate therefore 18 extractions were performed in total as three separate sets of six. Each separate extraction set was carried out on a different day and contained two of the blanks, two low spikes and two high spikes, such that all three separately prepared batches of spiking solutions were represented and the two spikes at each level were from different batches. The 18 extraction solutions and 27 calibration solutions were all analysed in triplicate in random order over a 5-day period with freshly prepared mobile phase used for each run and with the UV lamp turned off and on between each run. Thus, the procedure should provide highly robust validation data covering inter- and intra-run variability.

3. Results and discussion

3.1. Method development

3.1.1. Cartridge selection

Simple initial experiments to determine SPE cartridge suitability were conducted using 500 ml of 40 μg l^{-1} mixed aqueous solutions of OTC, SCP and TYL and using methanol as an eluent. Initially, a silica based C_{18} cartridge was used and the pH of the sample was adjusted with phosphoric acid or potassium hydroxide. The C_{18} cartridges proved poor at simultaneously retaining all three compounds at acidic, neutral and basic pH probably because C_{18} cartridges are poor at retaining charged molecules (there is no pH at which all three study compounds are neutral, see Table 1) and because of interactions between the OTC and silanol groups in the SPE cartridge.

Polymer based cartridges were then investigated as these are much better able to simultaneously retain a wide range of charged and neutral polar and non-polar compounds and thus they are increasingly being used in preference to C_{18} for multi-analyte environmental and pesticide analysis [26–28]. Two cartridges were tested: Isolute ENV+ and Oasis HLB. The HLB cartridge gave very good (>90%) recoveries for all three compounds simultaneously, however for the ENV+ cartridge, whilst a recovery of 88.5 ± 3.3% for SCP was achieved, neither OTC nor TYL were recov-

ered. This is in agreement with other work [16] in which no tetracyclines were recoverable from ENV+ cartridges whereas HLB cartridges gave good recoveries for a range of sulfonamides and tetracyclines. Other studies [27] with pesticides have also found the HLB cartridge to be preferred for multi-compound extractions. Therefore, the HLB cartridge was selected for the method development.

Having achieved good recoveries for all three compounds simultaneously in spiked distilled water using HLB cartridges, spiking tests were carried out using natural waters. The HLB cartridges proved to be efficient at also retaining the dissolved natural organic matter (NOM) in the samples and consequently the methanol extract produced was very coloured and the subsequent chromatograms had a significant interfering 'hump'. This caused problems when quantifying the compounds and recoveries were significantly reduced, especially for OTC. The next step therefore was to determine a suitable extractant buffer to use for real samples and to try to reduce the amount of NOM retained on the HLB cartridge.

3.1.2. Extraction buffer selection

Tetracyclines are known to form chelate complexes with a range of metal ions and organic molecules and recoveries can be affected in biomatrices [29]. The addition of EDTA as a chelating agent to water samples and pH adjustment with HCl has been used recently for water samples with relatively low concentrations of NOM [16]. The use of McIlvaine buffer, a mixture of citric acid and sodium phosphate usually at pH 4, and EDTA is common in extracting tetracyclines from milk, tissue and other biomatrices [29]. Therefore, a mixture of McIlvaine buffer and EDTA was chosen as a suitable extractant to use.

3.1.3. Sample clean-up

A number of different methods have been used recently to reduce the amount of NOM, which is predominantly composed of humic and fulvic acids [30], in extracts of natural waters. These include: ultrafiltration [31]; the use of acetone as SPE eluent as humic acids have much lower solubility in this solvent relative to methanol or acetonitrile [32]; sample adjustment to pH 7 rather than acidifying to pH 2 or 3, to reduce the degree to which humic and fulvic acids are retained on polymer cartridges [33]; chemical methods such as oxidation using hydrogen peroxide [34] or reduction using sodium sulphite [35]; and adsorption and ion exchange [36]. A number of experiments using some of these approaches were performed, with the use of anion exchange to remove the NOM showing most promise. The model structure of humic acid [30] indicates that the predominant functional groups are carboxyl and phenol with the carboxyl groups ionising to give a negative charge in aqueous solution depending on pH. Experiments were conducted in which the extraction was simplified by passing the samples through an SAX cartridge first in tandem with an HLB cartridge. The final sample pH was adjusted to ca. 2.9 by addition of H_3PO_4 to the McIlvaine–EDTA extraction buffer, which ensured that OTC, SCP and TYL were either positively charged or neutral and would pass through the SAX cartridge but that carboxyl functional groups of the humic acids would remain negatively charged and be retained by the SAX which could then be discarded. This pH of 2.9, selected because of the pK_a values of the compounds, should also aid the retention of the compounds on the HLB cartridge as a previous study found that optimum recovery for tetracyclines and sulfonamides was achieved by lowering the sample pH to <3.0 [20]. Although this approach required the use of two SPE cartridges it was successful in removing the majority of the humic material without significantly reducing analyte recovery.

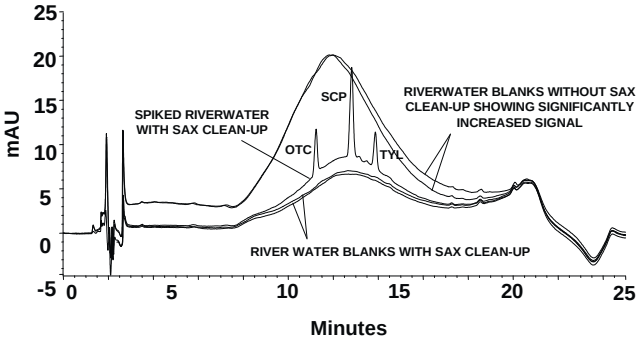

Fig. 2. Chromatograms of blank river-water with and without the inclusion of the SAX clean-up step and spiked river-water with clean up (UV detection at 285 nm).

Additional experiments were then carried out to attempt to further clean up the extract. The addition of 10% methanol to samples has aided in the extraction of more non-polar compounds by helping to prevent adsorption [37] although other work has indicated that analyte recoveries can be adversely affected by the addition of methanol to samples [38]. It was found however that addition of 2% methanol to the samples did not affect recoveries and therefore this step was included in the SPE procedure.

Additional washing stages were also tested to try to remove interferences from the HLB that had not been removed on the SAX cartridge. Various buffers, usually matched to the concentration of buffer in the samples, and dilute solvents are routinely used in SPE to wash cartridges after loading and prior to elution. After a series of experiments sequential washes with diluted McIlvaine–EDTA extraction buffer, 0.1 M NaOAc, distilled water and 20% methanol were found to remove further interferences (cartridge effluent was coloured) without affecting analyte recovery. The final SPE procedure is described in sample preparation. The necessity of the clean up stage is illustrated in Fig. 2, in which river waters were extracted with and without the inclusion of an SAX cartridge.

Table 2
Average recoveries and limits of detection (LODs) in river water samples

Compound	Spike level ($\mu g\,l^{-1}$)	Recovery ($\mu g\,l^{-1}$)	Recovery (%)	Uncertainty (%)	R.S.D. (%)	LOD ($\mu g\,l^{-1}$)
Oxytetracycline	1	0.99	99.4	8.4	8.5	0.35
	10	9.96	99.6	4.6	4.6	
Sulfachloropyridazine	1	1.05	105	5.7	5.4	0.25
	10	9.99	99.9	2.2	2.2	
Tylosin	1	0.72	71.6	8.2	11.5	0.35
	10	9.49	94.9	2.4	2.5	

3.2. Method validation

Method validation data have been summarised in Tables 2 and 3.

3.2.1. Calibration linearity and range

Fitting a linear least squares regression through the calibration gave acceptable values for r^2 in all cases, but when the regression equation was used to re-calculate the concentrations from the peak areas of the standards, large errors were observed for the lower concentrations with the nominal $0.10\,\mu g\,ml^{-1}$ standards recalculated as $0.50\,\mu g\,ml^{-1}$ (500% error), $0.31\,\mu g\,ml^{-1}$ (310% error) and $0.33\,\mu g\,ml^{-1}$ (330% error) for OTC, SCP and TYL, respectively. Plotting residuals indicated that the regression lines were biased by the more concentrated standards and were not passing close to the origin or the lower concentration standards, and also indicated that a second order curve would be more appropriate for the OTC calibration. Data have been presented which suggest that the reliance on r^2 values as an acceptance criterion is inadequate [40] and the data generated during this validation study bear this out. Acceptable regression curves were generated by: (i) inclusion of the origin in the regression (in the software set-up a zero peak area for the blank excludes (0.0) as a calibration point); (ii) limiting the regression to the range $0.1–10\,\mu g\,ml^{-1}$; (iii) using a second order curve for the OTC calibration.

3.2.2. Recoveries, accuracy and precision

Very good recoveries were observed for both OTC and SCP at both spiking levels and although the recovery of TYL was poorer it was still greater than 70% at $1\,\mu g\,l^{-1}$ and approached 95% at $10\,\mu g\,l^{-1}$. The precision was slightly poorer for all three compounds at the lower spiking level but overall was comparable to the inherent uncertainty of the analytical stage, as calculated from the calibration accuracy and precision, indicting that the presence of NOM in the extracts did not cause significant deterioration of data quality.

3.2.3. Selectivity and detection limits

To ensure the method selectivity, SCP and TYL peaks were also determined at 260 nm and OTC peaks were also determined at 370 nm. Peak area ratios between the two different wavelengths (i.e. 260 and 285 nm for SCP and TYL; 355 and 370 nm for OTC) were calculated both for the standards dissolved in MeOH (matrix free) and the sample extracts to ensure that the method did not suffer from interferences at the selected wavelengths and retention times. Peak area ratios for the sample extracts were not significantly different from the ratios obtained for the standards indicating that the method was selective at the chosen analytical wavelengths. Analytical limits of detection (for matrix free samples without a pre-concentration factor) were calculated based on the variability of the $0.10\,\mu g\,ml^{-1}$ standard in methanol and were $40\,\mu g\,l^{-1}$ for OTC, $30\,\mu g\,l^{-1}$ for SCP and $70\,\mu g\,l^{-1}$ for TYL. Overall methodological limits of detection have been calculated based on the variability of the low spike samples. These are greater than simply applying an extraction pre-concentration factor to the analytical limits of detection as would be expected for real samples compared to matrix free samples. The use of a standard, rather than a blank, to calculate limits of detection is acceptable provided that the concentration of the standard proves to be sufficiently close to the limit of detection [41].

Table 3
Summary HPLC validation data

Standard ($\mu g\,ml^{-1}$)	Oxytetracycline			Sulfachloropyridazine			Tylosin		
	Calculated ($\mu g\,ml^{-1}$)	Accuracy (%)	R.S.D. (%)	Calculated ($\mu g\,ml^{-1}$)	Accuracy (%)	R.S.D. (%)	Calculated ($\mu g\,ml^{-1}$)	Accuracy (%)	R.S.D. (%)
0.10	0.08	75.3	17.2	0.11	111	7.88	0.12	116	17.9
0.50	0.49	97.8	5.17	0.50	101	4.71	0.50	100	7.12
1.00	0.96	95.9	5.25	0.99	99.0	2.38	0.99	98.5	4.30
2.00	1.95	97.7	8.87	2.01	101	4.82	2.01	100	2.60
5.00	5.04	101	2.77	5.06	101	1.64	5.05	101	2.33
10.0	9.99	99.9	5.76	9.97	99.7	4.69	9.98	99.8	3.24

Table 4
Quality control data

Parameter	Oxytetracycline	Sulfachloropyridazine	Tylosin
Nominal concentration ($\mu g\,ml^{-1}$)	5.00	5.00	5.00
Average ($\mu g\,ml^{-1}$)	4.75	5.02	4.96
S.D. ($\mu g\,ml^{-1}$)	0.25	0.16	0.18
Precision (%)	5.26	3.16	3.57
Bias (%)	−4.99	0.41	−0.76
n	249	249	249

3.2.4. Quality control

Since validation in July 2001 following completion of the method development the method has been routinely used. For each analytical run an independently prepared mixed QC standard in methanol has been analysed at the start and end and after not more than every 10 samples during each run. The QC standard should therefore provide a long term indication of the applicability of the HPLC analytical method. QC data covering the period July 2001–January 2003 are summarised in Table 4 and indicate that the method shows good long-term accuracy and precision.

3.3. Sample results

The study compounds were not detected in the river water samples collected for method development. SCP and OTC were observed in the drainflow samples at peak concentrations of 613.2 and 36.1 $\mu g\,l^{-1}$, respectively, whereas TYL was not detected. The study compounds were not detected in the surface water samples. The lack of detections for TYL in the drainflow and surface water samples suggests that TYL was degraded in the slurry store prior to application. The presence of OTC and SCP in the drainflow samples is expected given that the slurry was fortified with these compounds. Results from the field study are summarised and the processes governing the fate of these compounds are discussed in greater detail elsewhere [17,19] and are beyond the scope of this method development paper.

3.4. Wider applicability of the method

Since validation for the compounds discussed in this paper, the extraction method has been tested on a range of other antibiotic compounds and initial results indicate that the method can be successfully applied to a much greater range of compounds, with recoveries in the range 68–105% having been achieved for other tetracyclines, sulfonamides and fluoroquinolones [42].

4. Conclusions

A robust and simple extraction and analytical method for the simultaneous determination of the veterinary antibiotics oxytetracycline, sulfachloropyridazine and tylosin in water samples has been developed which has allowed a number of experiments into the fate and behaviour of these compounds in water systems to be performed. The method was fully fit for purpose for these fate studies with suitable recoveries and limits of detection. The use of a tandem sacrificial anion exchange cartridge with a polymer cartridge for analyte retention ensured the method was effective at removing humic interferences from even highly contaminated water samples without significantly affecting the recovery of the study compounds and thus enabled HPLC–UV to be used for analysis of the extracts which significantly simplified the overall method. Preliminary data from additional method development studies indicate that the generic nature of the extraction method allows it to be used for a wider range of antibiotic compounds, both from the three classes of compound investigated and also for fluoroquinolones. The use of MS detection for analysis of these extracts has allowed the method to be used as the basis for environmental monitoring studies

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