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Ultrasonic extraction of veterinary antibiotics from soils and pig slurry with SPE clean-up and LC–UV and fluorescence detection

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Abstract

A simple and rapid analytical method is presented in which the three veterinary antibiotics oxytetracycline (OTC), sulfachloropyridazine (SCP) and tylosin (TYL) are simultaneously extracted and determined in four different soils. Extractions were carried out by a combination of ultrasonic agitation and vortex mixing using a mixture of methanol, EDTA and McIlvaine buffer at pH 7 as the extractant solution. The extracts were then cleaned-up by a tandem solid phase extraction (SPE) method using an Isolute SAX anion exchange cartridge to remove natural organic matter and an Oasis HLB polymeric cartridge to retain the study compounds. Analysis was by HPLC–UV with additional fluorescence detection for SCP. Recoveries were in the range 68–85% for SCP in all soil types, 58–75% for OTC in sandy soils, 27–51% for OTC in clay containing soils, 74–105% for TYL and 47–61% in a clay soil. OTC and SCP were also extracted from liquid pig manure using a mixture of EDTA and McIlvaine buffer at pH 7 with ultrasonic agitation and vortex mixing with SPE clean-up and HPLC–UV analysis. Recoveries were greater than 77% and 58% for OTC and SCP, respectively. Limits of detection were 18 $\mu g\,kg^{-1}$ for OTC and SCP and 40 $\mu g\,kg^{-1}$ for TYL in soils and 70 $\mu g\,L^{-1}$ for OTC and 140 $\mu g\,L^{-1}$ for SCP in pig slurry.

Keywords: Veterinary antibiotics; Soils; Pig slurry; Ultrasonic extraction

1. Introduction

Antibiotics are used in large quantities in agriculture and can be excreted by animals as parent compounds or metabolites and may thus enter the environment via the application of manure to land as organic fertiliser or on pasture. Given the increasing concern over the potential presence of antibiotics in soil systems and the relatively few, although growing, number of papers that have been published in this area [1–3] there is a continuing need to develop analytical methods to allow both environmental monitoring studies and systematic fate and behaviour studies at laboratory and field scales to be conducted. The few soil analytical methods that have been published, include methods using radio labelled compounds [4,5], or where samples have been fortified to high concentrations [6,7] and so these methods are not as relevant to environmental monitoring studies.

Two key papers have been published recently which are more environmentally relevant. The tetracyclines (OTC), tetracycline (TC) and chlortetracycline (CTC) and their epimers and tylosin have been simultaneously determined in a manure amended sandy soil [2]. Soil samples were extracted by vortexing the soil with a citrate buffer followed by liquid/liquid extractions (LLE) using ethyl acetate (EtOAc) and then evaporation of the solvent and reconstitution in acetonitrile/ammonium acetate prior to LC–MS–MS analysis. Average recoveries of 67–86% for OTC, 33–47% for TC, 57–76% for CTC and 60–67% for TYL were achieved with R.S.D. varying from 4 to 29%. The fluoroquinolones

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ciprofloxacin and norfloxacin have been simultaneously determined in two sewage sludge amended soils; a clay and a sandy clay loam [3]. Soil samples were extracted by accelerated solvent extraction (ASE) using a phosphoric acid/acetonitrile buffer, with the extractant solution being cleaned-up by mixed phase cation exchange disks prior to LC–fluorescence analysis. Recoveries of better than 80% were achieved with precision better than 11%.

A number of papers have been published recently which describe the analysis of sulphonamide or tetracycline antibiotics in manure. CTC has been determined in pig slurry using bacterial whole-cell biosensors [8] and TC and its epimer 4-epi-tetracycline have been determined in pig slurry with recoveries of 94% and 97%, respectively after dilution with citric acid buffer, centrifugation and filtration with direct analysis by HPLC–UV [9]. Four sulfonamides, one acetyl metabolite and trimethoprim have been simultaneously extracted from pig slurry with recoveries ranging 77–91%. McIlvaine buffer at pH 5.5 was added to the manure, followed by a two stage LLE using *n*-hexane and EtOAc, centrifugation, drying overnight using sodium sulphate, filtration, rotary evaporation then solid phase extraction (SPE) clean-up prior to LC–MS–MS analysis [10]. Six sulfonamides, one acetyl metabolite and trimethoprim have been simultaneously extracted from pig slurry and cow manure with recoveries ranging 51–89%. The manure was adjusted to pH 9 using KOH, 1 g of NaCl added then vortexed, mixed with EtOAc, vortexed again and sonicated. The organic phase was then removed, evaporated under nitrogen, reconstituted with mobile phase and sonicated again and filtered prior to HPLC–MS analysis [11].

These previously published methods for soil and slurry have dealt with compounds from up to two different classes of antibiotics and with different media (e.g. tetracyclines and tylosin in a manure amended sandy soil and sulfonamides in sewage sludge amended clay and sandy soils) but have used extraction methods which either require relatively large volumes of solvent and buffer and can be laborious or require specialist or expensive instrumentation. Also, the use of expensive instrumentation, such as LC–MS–MS, with more sensitive and selective detection means a less rigorous sample clean-up is required. A simple and rapid method capable of simultaneously extracting compounds from two or more different classes of antibiotics from environmental matrices would, therefore, be highly advantageous. However, the different physico-chemical properties of antibiotics classes does make this problematical.

The aim of this study was, therefore, to develop a rapid and simple method to simultaneously extract compounds from three different classes of antibiotics from a range of soils for use in future laboratory fate, behaviour studies and environmental monitoring programmes. Subsequently, the method was also used as a basis for extracting two of these compounds from pig manure. Three of the most widely used classes of antibiotics are tetracyclines, sulfonamides and macrolides which may be used in conjunction to treat animals giving their different modes of action. These classes have recently been identified as having high potential to reach the environment in the UK [12] and have a wide range of physico-chemical properties. Three model antibiotics, one from each of these classes, respectively, were selected for investigation, namely, oxytetracycline (OTC), sulfachloropyridazine (SCP) and tylosin (TYL). These cover a range of physico-chemical properties, such as pK_a and octanol–water partitioning coefficients (K_{ow}) (Table 1). Chemical structures are given in Fig. 1.

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

Compound	CAS number	MW	pK_a	log K_{ow}
OTC	79-57-2	460.44	3.27, 7.32, 9.11[a]	−0.9[b]
SCP	80-32-0	284.7	1.76, 5.71[c]	−0.52[c]
TYL	1401-69-0	916.1	7.1[d]	3.5[d]

[a] Stephens et al. [13].
[b] Schumacher and Linn [14].
[c] Novartis [15].
[d] Tolls [16].

2. Experimental

2.1. Chemicals, reagents and standards

Methanol (MeOH), tetrahydrofuran (THF), acetonitrile (MeCN), water, trifluoroacetic acid (TFA), orthophosphoric acid (H_3PO_4) and citric acid were all HiPerSolv for HPLC^{TM} grade from BDH (Poole, Dorset, UK). EDTA disodium salt, disodium hydrogen orthophosphate anhydrous (Na_2HPO_4) and sodium acetate 3-hydrate (NaOAc) were all AnalaR® grade from BDH (Poole, Dorset, UK). Sulfachloropyridazine was Vetranal® grade from Riedel-de Haën (Gillingham, Dorset, UK), oxytetracycline, tylosin tartrate and fluorescamine were BioChemika grade from Fluka (Gillingham, Dorset, UK). Sulfachloropyridazine sodium salt was obtained from Novartis Animal Health

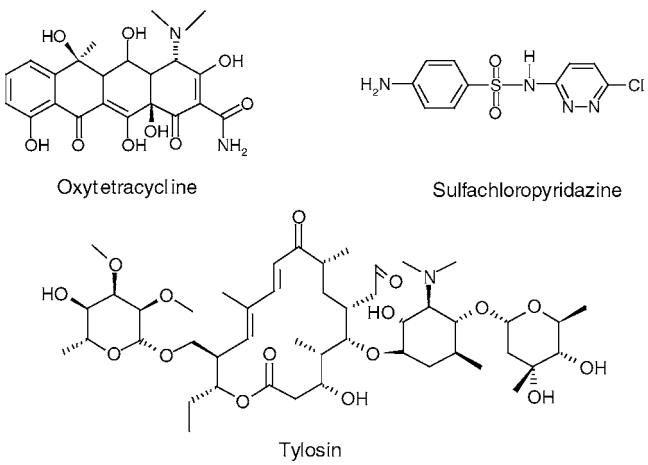

Fig. 1. Chemical structures of OTC, SCP and TYL.

Table 2
Characteristics of the soils used in this study

Property	Loamy sand	Sandy clay loam	Sandy loam	Clay loam
Sand (63 μm–2mm) (%)	85.3	52.3	69.2	42.6
Silt (2–63 μm) (%)	6.2	23.4	20.5	32.3
Clay (<2 μm) (%)	8.5	24.3	10.3	25.1
pH (1:2.5) in 0.01 M $CaCl_2$	4.8	6.2	6.6	6.8
Cation exchange capacity (mEq $100\,g^{-1}$)	14.8	22.9	11.4	22.4
Organic carbon (%)	1.3	3.5	1.3	2.2
Maximum water holding capacity (%)	35.4	55.9	40.2	48.0

(Basel, Switzerland) and oxytetracycline hydrochloride was obtained from Vericore Limited (Dundee, UK). McIlvaine buffers at pH 3, 4 and 7 were prepared by mixing 0.1 M citric acid and 0.2 M Na_2HPO_4. The soil extraction buffer was a mixture of methanol, 0.1 M EDTA and McIlvaine buffer (50:25:25). The SPE conditioning/washing buffer was prepared by diluting 15 ml of the soil extraction buffer to 400 ml total volume with distilled water and acidifying to pH 2.9 by adding 200 μl of H_3PO_4.

2.2. Apparatus

Extractions were carried out using a Yellowline TTS 2 vortex mixer, Ultrawave U300 ultrasonic bath and MSE Centaur 2 centrifuge. Solid phase extractions were carried out using Waters (Watford, UK) Oasis hydrophilic–liphophilic balance (HLB) polymer cartridges and International Sorbent Technology (Hengoed, Mid Glamorgan, UK) strong anion exchanger (SAX) cartridges. HPLC analysis was performed using a Dionex (Sunnyvale, CA, USA) Summit HPLC system controlled by Chromeleon software. Separations were performed on a 150 mm × 4.6 mm 4 μm, GENESIS C_{18} column from Jones Chromatography (Hengoed, Mid Glamorgan, UK).

2.3. Soils and pig slurry

Four different UK soils were investigated in this study: a loamy sand; a sandy clay loam; a sandy loam and a clay loam according to standard UK soil type definitions [17]. Properties of the soils are given in Table 2. For method development, the soils were air dried and sieved to <5.6 mm to ensure consistent moisture content and texture prior to fortification with mixed solutions of OTC, SCP and TYL at the given spiking levels. Pig slurry was collected from slurry pits under fattening sheds at a pig farm in Leicestershire, UK. The pigs had been continuously treated with TYL at the rate of 100 g per tonne of feed but were treated with neither sulfonamide nor with tetracycline antibiotics. The slurry was stirred prior to sample collection.

2.4. Soil extraction procedure

Approximately 4 g of soil was accurately weighed into a 15 ml centrifuge tube and 5 ml of extraction buffer (MeOH:0.1 M EDTA:McIlvaine buffer, 50:25:25) added. The tubes were vortex mixed for 30 s and were then placed into an ultrasonic bath for 10 min before being centrifuged at approximately 1200 g for 15 min. The supernatant was then decanted into a 500 ml glass bottle and the soil residue was extracted twice more and the (approximately) 15 ml total of supernatant was then combined and diluted to approximately 400 ml with distilled water to reduce the methanol content below 2% and then 200 μl of H_3PO_4 was added to reduce the pH to approximately 2.9.

2.5. Slurry extraction procedure

Exactly 2 ml of slurry was accurately pipetted into a 15 ml centrifuge tube immediately after homogenisation and 8 ml of extraction buffer (0.1 M EDTA:pH 7 McIlvaine buffer, 50:50) added. The tubes were vortex mixed for 30 s and then placed into an ultrasonic bath for 10 min before being centrifuged at approximately 1200 g for 15 min. Exactly 5 ml of the supernatant was then pipetted into a 15-ml centrifuge tube and 50 μl of H_3PO_4 and 50 μl of MeCN were added to adjust the sample pH and to help in precipitating proteins in the extract.

2.6. SPE clean-up of the soil and slurry extracts

SAX–HLB SPE cartridges were set up in tandem, pre-conditioned with methanol and SPE conditioning buffer and then the diluted soil extract or slurry extract was passed through the cartridges at $10\,ml\,min^{-1}$. The SAX cartridges were then removed and the HLB cartridge washed sequentially with: SPE washing buffer; 0.1 M NaOAc; distilled water; and 20% MeOH. The HLB cartridge was then air dried for 10 min and then eluted with 2 ml × 1 ml methanol to produce the extract for analysis.

2.7. Derivatisation of SCP

SCP was additionally determined in some samples using fluorescence detection following derivatisation with fluorescamine. 500 μl of the sample extract in methanol, 200 μl of McIlvaine buffer at pH 3 and 100 μl of a $1\,mg\,ml^{-1}$ fluorescamine solution in MeCN were vortex mixed in an HPLC sample vial and were then allowed to react for at least 8 h but not more than 32 h prior to analysis by HPLC.

2.8. HPLC analysis

The HPLC method for soil and slurry analysis was a gradient elution carried out with THF (solvent A), MeCN (solvent B) and 0.05% TFA 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 and an integral degasser was also present in the pump. Simultaneous detection was performed at 285 nm for SCP and TYL and at 355 nm for OTC. The HPLC method used for SCP analysis of the derivatised soil extracts was also a gradient method with the mobile phase composition (THF: A; MeCN: B; 0.05% TFA: C, as before) programmed as follows: A:B:C::5:15:80 from 0 to 1 min rising linearly to 5:85:10 from 1 to 6 min and then returning linearly to 5:15:80 from 6 to 7 min. Equilibration was then performed from 7 to 10 min at 5:15:80. The flow rate was 1 ml min^{-1} throughout with excitation and emission wavelengths of 400 and 495 nm, respectively.

2.9. Method performance

Four soils, described above, were used in the performance assessment. Each soil was fortified with mixed solutions of SCP, OTC and TYL so that all three study compounds were present in the soil. Three fortification levels were used, namely 0.2, 1.0 and 5.0 mg Kg^{-1}. Extractions were performed in triplicate and each extract was analysed in duplicate to allow calculation of method uncertainty. The slurry was fortified with mixed solutions of SCP and OTC at four levels, namely 1, 5, 10 and 20 mg/l^{-1}, with extractions carried out in triplicate. Unfortified soil and slurry blanks were also analysed. Extractions and analyses were conducted in a number of batches to ensure the overall precision covered day-to-day variability of the total analytical process, including extraction, SPE and HPLC analysis. The fortification levels chosen were based on predicated environmental concentrations using a commonly used exposure assessment model [18]. The data produced were used to calculate recoveries, precision and limits of detection and to confirm the method was suitable over the required range which ensured the method was validated to 'ad-hoc' criteria [19].

3. Results and discussion

3.1. Soil extraction method development

A number of different techniques are employed in the extraction of organic chemicals such as PAHs, PCBs and pesticides from soil. Traditional non-instrumental techniques, such as Soxhlet extraction and shake flask extraction are well established but can be labour intensive, time consuming and use relatively large volumes of organic solvents. Instrumental techniques such as supercritical fluid extraction (SFE), subcritical water extraction (SWE), pressurised fluid extraction (PFE), accelerated solvent extraction and microwave assisted extraction (MAE) are increasingly being used. These have the advantage of being automated and use smaller volumes of solvents and/or less toxic extractants, however the instruments themselves may be quite expensive. A number of recent papers have compared the relative merits of various extraction techniques [20–23]. An alternative technique, which is rapid and does not require large volumes of solvent or expensive instrumentation, is ultrasonic extraction and this method has been successfully applied to PAHs and pesticides [24,25] and was,therefore, used in this study.

Table 3
Sorption data for selected antibiotics in soils and pig manure

Compound	Matrix	K_{oc} (L kg^{-1})	K_d (L kg^{-1})
Tetracyclines			
TC	Clay loam soil[a]	40000	>400
OTC	Sandy/loamy soils[b]	27800–93300	420–1030
	Pig manure[c]	195	63–96
Sulfonamides			
SCP	Clay loam[d]		1.8
	Sandy loam[d]		0.9
Sulfathiazole	Loamy sand soil[a]	200	4.9
Sulfamethazine	Sandy/silty loam soils[a]	82–208	1.0–3.1
	Clay loam soil[a]	60	0.6
Macrolides			
TYL	Sandy/loamy soils[b]	550–7990	8.3–128
	Pig manure[c]	110	36–295

[a] Tolls [16].
[b] Rabølle and Spliid [27].
[c] Loke et al. [28].
[d] Boxall et al. [12].

In attempt to extract the three study compounds simultaneously, it was important to consider the likely degree of binding of the compounds to soils and organic matter. Partition coefficient data for soil/water systems have been reviewed [16] and indicated that tetracyclines are very strongly sorbed, TYL is moderately strongly sorbed and sulfonamides are weakly sorbed to soils and that generally, these compounds are less strongly sorbed to pig manure than soils. Soil and organic carbon partitioning coefficients (K_{oc} and K_d) are summarised in Table 3. Specific data were also available for SCP which indicated that SCP did not strongly bind to two of the soils used for method development [26]. A logical starting point for the method development, therefore, was to select a buffer suitable for OTC extraction as this was the most strongly sorbed. OTC is known to form chelate complexes with metal ions and therefore, the use of chelating agents, such as EDTA and McIlvaine buffer, a mixture of citric acid and Na_2HPO_4, to extract OTC from food is commonplace [29]. McIlvaine buffer has also been used in the extraction

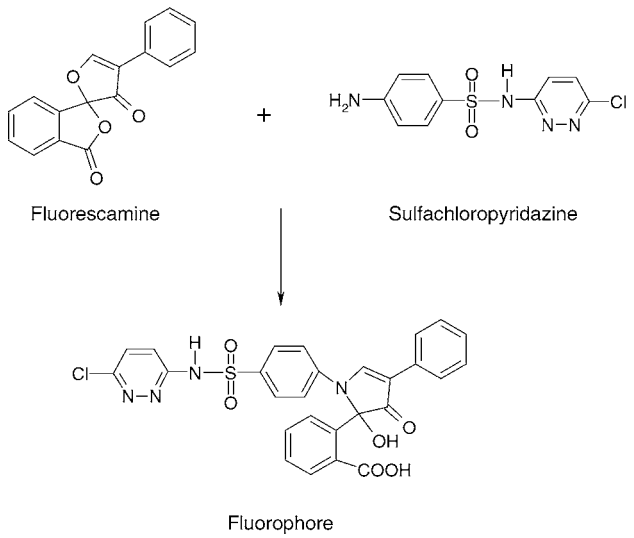

Fig. 2. Reaction of SCP with fluorescamine.

of sulfonamides from manure [10]. Therefore, these extractants were chosen for this study.

A number of experiments were then conducted to optimise the extraction recoveries with the procedure described in the extraction section ultimately being chosen. Initial experiments with two extractions gave poor recoveries for OTC, so a third extraction was added to the procedure. Initial extractions were also carried out using pH 4 McIlvaine buffer, which is most commonly used for extraction of tetracyclines from food but recoveries generally improved for all three compounds by using pH 7 buffer for all three extractions. A vortexing stage was also added to the procedure to help re-suspend the soil after centrifugation. The supernatant liquids produced by the extraction process were highly coloured with natural organic materials (NOM), which are likely to be mainly humic and fulvic materials. A tandem solid phase extraction procedure was used to enable the soil extracts to be cleaned-up. Diluting the soil extracts to a total volume of approximately 400 ml lowered the MeOH content to under 2% to ensure that the compounds were retained on the HLB cartridge during loading. Lowering the pH of the diluted extract to approximately 2.9 by the addition of 200 μl of H_3PO_4 ensured that the study compounds were either neutral or positively charged and therefore, were not retained on the sacrificial SAX anion exchange cartridge which removed a large proportion of the humic material with further humic material being removed during washing of the HLB cartridge.

3.2. Optimisation of the SCP derivatisation procedure

For some of the blank soil extracts, a small peak was observed at 285 nm at the same retention time as the SCP peak. In order to achieve selective and sensitive detection at lower concentrations, the use of fluorescence detection for SCP was investigated. The synthetic reagent fluorescamine is a non-fluorescent compound which rapidly reacts with primary aliphatic and aromatic amines to form a stable fluorescent compound (Fig. 2) and has been used by a number of workers to analyse sulfonamides in food [30,31]. A number of experiments were conducted to identify the optimum reaction conditions in which buffer pH, sample to buffer ratio and reaction time were investigated. McIlvaine buffer was used as the derivatisation reaction buffer as the stock solutions were already available having been prepared for the extraction stage. Generally, reaction kinetics were faster and fluorescence intensity of the derivatised fluorophore was greater at higher pH and with a greater buffer to sample

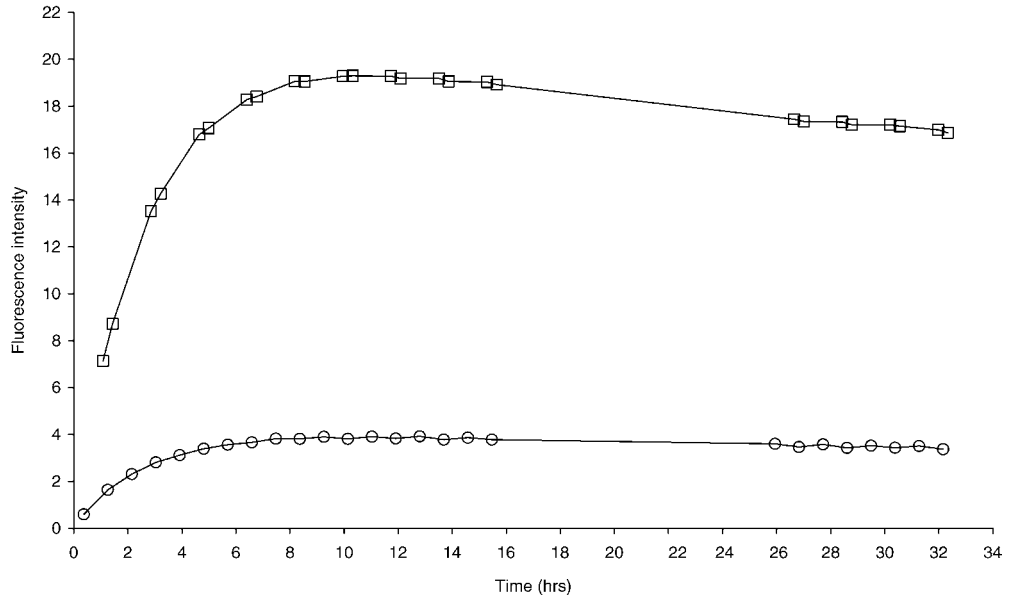

Fig. 3. Fluorescence intensity vs. reaction time for SCP standards in methanol (□) 0.5 μg ml^{-1}; (○) 0.1 μg ml^{-1}.

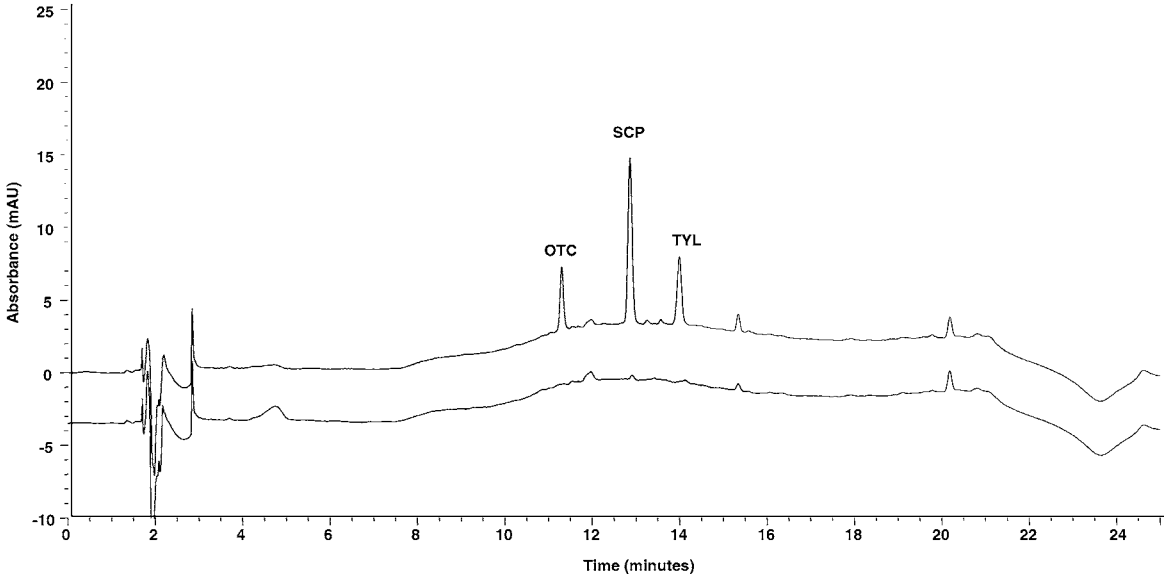

Fig. 4. Chromatogram of sandy loam soil blank and spiked with 1 mg kg^{-1} of the study compounds.

ratio, but the fluorophore was relatively unstable and a loss of fluorescence intensity occurred over a period of a very few hours which was not convenient for reproducible analysis. In the optimum reaction conditions chosen, a fluorescence intensity maximum was achieved after 8-h reaction time, and the fluorescence signal remained stable for at least 24 h more (Fig. 3).

3.3. Slurry extraction method development

Initial experiments were conducted using only citric acid as an extractant as has been previously used for TC analysis in pig manure [9]. Recoveries of OTC were dependent on concentration, however, falling from 83% at 10 mg l^{-1} to 29% at 1 mg l^{-1}. Ultimately, a mixture of EDTA and McIlvaine buffer at pH 7 was selected as being suitable, but MeOH was not added to the extractant solution as this dissolved more sample matrix without improving recoveries and produced a very coloured sample. A single extraction stage proved adequate given the liquid nature of the slurry. Again, a tandem SAX–HLB SPE procedure was used to clean-up the extract for HPLC analysis. It was not possible to quantify TYL in the slurry extracts owing to an interference peak that modification of the HPLC conditions was not able to be separated from the TYL peak. Recoveries for the final extraction procedure are given in Table 5.

3.4. Recoveries and limits of detection

Generally recoveries for all three compounds were lower in the soils with higher clay and organic carbon, especially for OTC and TYL which have relatively high sorption coefficients in soils. This is likely to be explained by the greater clay content, i.e. greater proportion of smaller sized soil particles giving a greater surface area for sorption in the clay loam. Recoveries were reasonably consistent on a per soil basis for each compound and were considered to be fit for purpose. The recoveries in the soils and pig slurry are summarised in Tables 4 and 5. In soils, the limits of detection for OTC, SCP and TYL were approximately 18, 18 and

Table 4
Recoveries for the study compounds in different soils

Soil type	Spiking level (mg Kg^{-1})	n	Recovery (%, mean ± S.D.)		
			OTC	SCP	TYL
Loamy sand	0.2	6	58 ± 9	80 ± 2	105 ± 7
	1.0	6	63 ± 2	72 ± 4	85 ± 4
	5.0	6	62 ± 2	79 ± 3	79 ± 3
Sandy clay loam	0.2	6	29 ± 4	77 ± 5	81 ± 7
	1.0	6	27 ± 1	76 ± 3	79 ± 3
	5.0	6	31 ± 2	78 ± 3	79 ± 2
Clay loam	0.2	6	47 ± 6	73 ± 6	48 ± 9
	1.0	12	38 ± 4	68 ± 10	47 ± 4
	5.0	6	51 ± 4	73 ± 2	61 ± 3
Sandy loam	0.2	6	68 ± 12	76 ± 4	74 ± 5
	1.0	6	65 ± 7	80 ± 1	85 ± 2
	5.0	6	75 ± 3	85 ± 4	86 ± 4

Table 5
Recoveries for OTC and SCP in pig slurry

Spiking level (mg l^{-1})	n	Recovery (%, mean ± S.D.)	
		OTC	SCP
1	3	80 ± 5	70 ± 4
5	3	77 ± 2	58 ± 2
10	3	79 ± 1	58 ± 3
20	3	102 ± 5	89 ± 3

40 μg kg^{-1}, respectively, based on analysis of blanks. In the pig slurry, the limits of detection for OTC and SCP were approximately 70 and 140 μg kg^{-1}, respectively. An example chromatogram is given in Fig. 4

4. Conclusions

A simple, inexpensive and practical soil extraction method has been developed which required only small sample weights and extractant volumes and allowed rapid simultaneous extraction of antibiotics from different soil types at environmentally relevant concentrations with reasonable recoveries and precision. The relatively low recoveries for OTC and TYL in the soils containing clay were more than compensated for by the fact that separate extractions for each compound were not required. The use of a tandem SPE clean-up procedure, which proved successful in removing a great deal of NOM matrix from the extracts, allowed analysis to be performed using more widely available LC–UV and LC–fluorescence. Furthermore, the method was successfully applied to liquid manure samples for simultaneous OTC and SCP analysis with acceptable recoveries. Given the widely different physico-chemical properties of the study compounds, the method has shown the potential to be applicable to other major classes of antibiotics in a range of media types.

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