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Journal of Chromatography A, 1038 (2004) 157–170
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Simultaneous extraction of tetracycline, macrolide and sulfonamide antibiotics from agricultural soils using pressurised liquid extraction, followed by solid-phase extraction and liquid chromatography–tandem mass spectrometry

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Received 3 November 2003; received in revised form 17 March 2004; accepted 17 March 2004

Available online 27 April 2004

Abstract

The veterinary antibacterial agents chlortetracycline (CTC), oxytetracycline (OTC), sulfadiazine (SDZ), erythromycin (ERY) and tylosin (TYL A, B, C and D) were extracted from soil using pressurized liquid extraction (PLE). Citric acid (pH 4.7) and methanol was used as extraction buffer, followed by tandem-solid-phase extraction (SPE) clean-up (SAX + HLB) for all compounds. For quantification two slightly different methods were employed using LC–MS–MS with MRM detection. The soil extraction method was validated using a loamy sand soil and a sandy soil, representing two typical Danish agricultural soils. Recoveries were 50–80% for the tetracyclines (CTC and OTC) and sulfadiazine (SDZ) and 60–100% for the macrolides (TYL and ERY). Limits of detection for the soil extraction method (LOD_{soil}) were 0.6–5.6 $\mu g\,kg^{-1}$ soil for CTC and OTC, 0.9–2.9 $\mu g\,kg^{-1}$ soil for SDZ and 2.4–5.5 $\mu g\,kg^{-1}$ soil for TYL A and ERY. Furthermore, the method was applied to field samples taken from two agricultural fields fertilised with liquid manure containing CTC and TYL A. These results showed a decline in the content of antibacterial agents throughout the sampling period of 155 days from 10 to 15 μg CTC kg^{-1} soil and 20–55 μg TYL A kg^{-1} soil to below or near the LOD_{soil} listed above. Finally, the method was applied to barley grains harvested from the fields. None of the antibacterial agents were measured in grain samples, but recoveries for spiked grain samples were similar to soil recoveries.

Keywords: Soil; Extraction methods; Environmental analysis; Antibiotics; Tetracyclines; Macrolides; Sulfonamides

1. Introduction

Tetracyclines [chlortetracycline (CTC) and oxytetracycline (OTC)], sulfonamides [sulfadiazine (SDZ)] and macrolides [tylosin A (TYL A) and erythromycin (ERY)], represent three groups of antibacterial agents that are widely used in Denmark in concentrated feeding of pigs, calves, poultry etc. for treatment of infectious diseases [1]. Table 1 shows the chemical structures and lists selected physico-chemical properties of the compounds. Several antibacterial agents typically used for various treatments of animals during a period of manure storage, can enter the environment simultaneously when liquid manure is applied to agricultural soils. In order to assess the fate of antibacterial agents in the environment, analytical methods that enable the simultaneous analysis of these compounds in manure or soil samples are needed.

The requirements for analytical methods are mainly dictated by the environmentally relevant concentrations. The concentration level of antibacterial agents in organic waste from animal production depends on a number of factors such as agricultural practise, animal species, number of treatment incidences, dose and the choice of antibacterial agent. Tetracyclines have been measured in concentrations ranging from 25–1000 $\mu g\,l^{-1}$ swine manure [2] and up to 20 $mg\,kg^{-1}$ in

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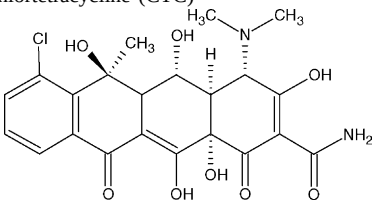
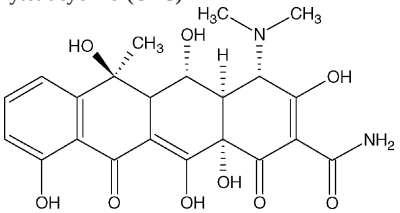
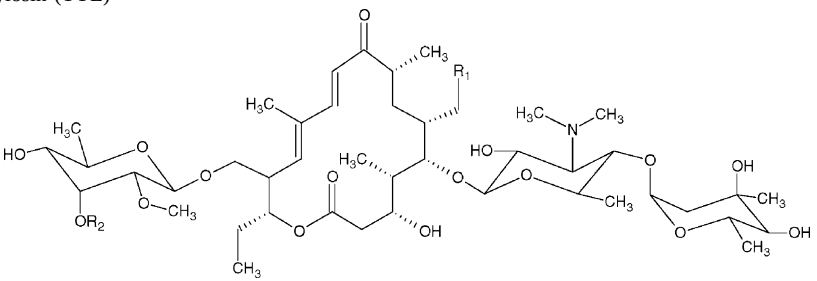
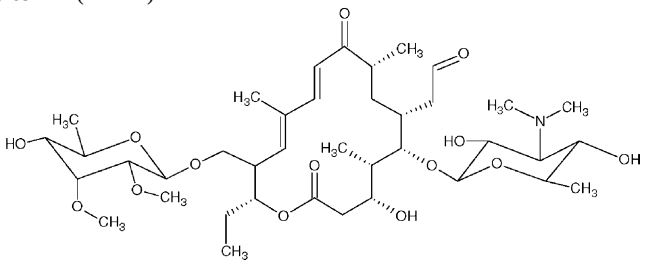
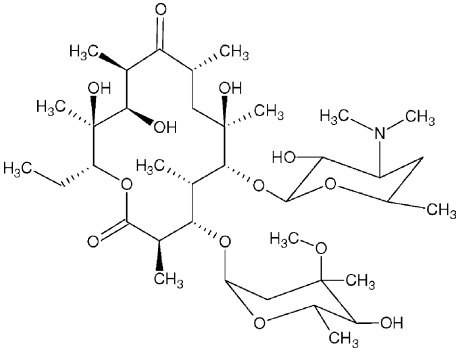
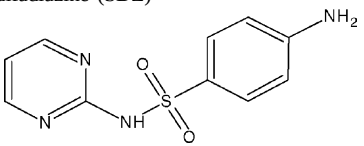
doi:10.1016/j.chroma.2004.03.034

Table 1
Chemical structure and selected physico-chemical properties for the antibacterial agents

Compound structure	M_w (g/mol)	pK_a	log K_{ow}[a]	K_d (l kg^{-1})[b]
Chlortetracycline (CTC) 	478.9	3.30, 7.44, 9.27 [29]	−0.36 [30]	–
Oxytetracycline (OTC) 	460.4	3.27, 7.32, 9.11 [29]	−0.89 [30]	680 ± 69 (Askov) 670 ± 149 (Lundgaard) [14]
Tylosin (TYL) 				
Tylosin A (TYL A) R_1=CHO R_2=CH_3	916.1	7.73 [31]	1.63 [31] 2.50 ± 0.84 [32]	128 ± 20 (Askov) 10.8 ± 0.7 (Lundgaard) [14]
Tylosin C (TYL C) R_1=CHO R_2=H	902.1	–	2.20 ± 0.84 [32]	–
Tylosin D (TYL D) R_1=CH_2OH R_2=CH_3	918.2	–	2.17 ± 0.83 [32]	–

Tylosin B (TYL B) 	771.9	–	1.66 ± 0.75 [32]	–
Erythromycin (ERY) 	747.9	8.88 [31]	3.06 [31]	–
Sulfadiazine (SDZ) 	250.3	6.15 [28]	−0.092 [28]	2.0 [33]

(–) No data available.

[a] $\log K_{ow}$: logarithm of the octanol/water distribution coefficient.

[b] K_d: soil/water distribution coefficient.

manure and bedding [3]. Sulfonamide content in manure has been measured up to 20 mg kg^{-1} [4] while the content of macrolides are generally lower, e.g. <110 μg TYL kg^{-1} manure [3] and 2.5 μg ERY l^{-1} swine manure [2]. When liquid manure is amended to agricultural soils a considerable dilution of the antibacterial agents is anticipated and the resulting soil concentrations has been estimated to range from 10 μg kg^{-1} soil [3] to 450–900 μg kg^{-1} soil for tetracyclines [5], but most environmental samples show lower concentrations, e.g. 4–7 μg CTC kg^{-1} soil [6] and 6–7 μg OTC kg^{-1} soil [3]. The objective of the method development was quantification of selected antibacterial agents in concentrations down to the low μg kg^{-1} soil level.

In this paper, a simple and robust method is proposed for the simultaneous extraction of CTC, OTC, SDZ, TYL A and ERY, together with the degradation products (and impurities) TYL B, TYL C and TYL D, from soil. As shown in Table 1, the three groups of antibacterial agents represent a wide range of different physico-chemical properties and a method capable of simultaneous analysis was a compromise to accommodate different properties. During the method development focus was concentrated on the tetracyclines, as strong sorption of these compounds to soil particles (see Table 1) causes difficulties when extracting the antibacterial agents from soil and manure [3,6,7]. Extraction methods for antibacterial agents have been developed for several matrices, e.g. animal food products [8–10] and environmental water samples [11–13], while only few methods have been developed for extraction of the antibacterial agents from soil and manure [3,6,7,14]. These extraction methods utilise a range of different extraction solvents and are generally based on mechanical shaking, ultrasonication or vortex mixing. Pressurized liquid extraction (PLE; Dionex trade name ASE for accelerated solvent extraction) is an alternative to these methods, that allows soil extractions to be performed under high pressure (500–3000 psi; 1 psi = 6894.76 Pa) at elevated temperatures (50–200 °C) and several cycles of fresh solvent can be applied [15]. PLE has various advantages over other methods such as better reproducibility, reduced use of extraction solvent and reduced time for sample preparation. The most important parameters to optimise when using PLE are extraction time, pressure, temperature and number of solvent cycles, as well as water content of the matrix [15]. Besides the extraction technique, the choice of solvent is a critical parameter in optimising the effectiveness of the method, e.g. polarity of the solvent and pH.

For the simultaneous extraction of OTC, CTC, TYL, ERY and SDZ from soil, we applied PLE using a Dionex ASE 200 system. Optimum conditions with regard to extraction solvent and number of extraction cycles were investigated. Soil extraction was followed by pre-concentration and clean-up of the PLE extracts using a solid-phase extraction (SPE) method with strong anion exchange (SAX) and hydrophilic lipophilic balance (HLB) cartridges, placed in tandem. For chemical analysis and quantification two different reversed-phase HPLC methods coupled with electrospray ionisation (ESI) MS–MS analysis with MRM (multiple reaction monitoring) detection were employed, based on a method developed by Loke et al. [16].

The soil extraction method was validated by measuring recoveries, linearity, day-to-day variation and limits of detection and quantification, for two different soils representing typical Danish agricultural fields, i.e. a loamy sand soil (Askov) and a sandy soil (Lundgaard). The extraction method was applied to samples taken from the two fields on several occasions during a 155-day period after treatment with antibiotic containing pig manure and to barley plants grown on the two fields.

2. Experimental

2.1. Antibacterial agents and chemicals

The antibacterial agents used in this study were purchased from the following companies: OTC hydrochloride (95.7%) from Unikem, Copenhagen, Denmark; chlortetracycline hydrochloride (79%), tylosin tartrate (89.8%), erythromycin (>99%) and sulfadiazine sodium salt (>99.0%) all from Sigma Aldrich, Germany. Professor Hoogmartens (Facultait Farmaceutische Wetenschappen, Leuven, Belgium) kindly donated tylosin A (TYL A), tylosin B (TYL B), tylosin C (TYL C) and tylosin D (TYL D). 4-Epi-chlortetracycline hydrochloride (ECTC) (97%) from Acros Organics, Geel, Belgium, was used for identification in chromatograms.

The following chemicals were used for the extraction buffer, SPE and HPLC: formic acid (GR for analysis, 98–100%), citric acid monohydrate, sodium acetate anhydrous (GR for analysis) and sodium hydroxide pellets (GR for analysis), all from Merck, Darmstadt, Germany. Methanol of HPLC grade was obtained from KEBO Lab. (Albertslund, Denmark).

2.2. Soils

Samples of soil were collected from the Ap horizon (0–20 cm) from two fields at a Danish research station, with well-described soil properties [17]. The two soils are a sandy soil (Lundgaard) and a loamy sand soil (Askov), respectively, representing typical Danish agricultural soils. Important soil properties for the two soils are listed in Table 2. To achieve homogeneous soil samples, the soils were air dried to moisture content of approximately 5% water and sieved through a 2 mm sieve before further handling. Ottawa sand standard, general-purpose grade (S/0365/63), was purchased from Fisher Scientific UK (Leicestershire, UK).

2.3. Pressurized liquid extraction (PLE)

The extraction of antibacterial agents from soil was performed by PLE, using an ASE 200 system from Dionex (Sunnyvale, CA, USA). The system was operated with pressure resistant steel extraction cells with a volume of

Table 2
Selected soil properties for the loamy sand soil (Askov) and the sandy soil (Lundgaard) used for optimisation and validation of the soil extraction method

Soil	Soil depth (cm)	Texture (%)					pH ($CaCl_2$)	Cation-exchange capacity (meq./100 g)
		Clay (<2 μm)	Silt (2–20 μm)	Fine sand (20–200 μm)	Coarse sand (200–2000 μm)	Organic C		
Loamy sand soil	0–20	11.3	10.7	37.9	37.5	1.6	6.1	10.0
Sandy soil	0–20	5.2	4.8	24.4	63.2	1.4	5.6	6.7

33 ml and lined with glass-fibre filters from Dionex (part no. 049458, size 1.983 cm).

Approximately 10 g soil sample was mixed with 10 g Ottawa sand before added to the extraction cell. The extraction buffer consisted of a 1:1 (v/v) mixture of methanol and 0.2 M citric acid buffer with pH adjusted to 4.7 with NaOH. The automated PLE program was as follows: Extraction with approximately 30 ml extraction buffer at 1500 psi for 10 min, followed by flushing of the extract into a collection vial. Then static extraction with additionally 30 ml of extraction buffer for 3 min and flushing into the same collection vial. The total final volume of extract was approximately 60 ml (depending on soil moisture and mass). Extractions were performed at room temperature as the tetracyclines are converted to their epi- or anhydroform when heated [18,19]. Optimum conditions for the PLE method are summarised in Table 3.

Between each run the extraction cells were cleaned by ultra-sonication for 15 min in a mixture of Milli-Q water methanol (50:50, v/v), followed by 15 min ultra-sonication in Milli-Q water.

2.4. Solid-phase extraction (SPE)

Clean up and pre-concentration was performed using a combination of SAX cartridges (strong anion exhange, 500 mg sorbent, 6 ml cartridge) purchased from Isolute, IST, Mid Glamorgan, UK and Oasis HLB cartridges [poly(divinylbenzene–co-*N*-pyrrolidone), 200 mg sorbent, 6 ml cartridge] purchased from Waters, Milford, MA, USA. Cartridges were placed in tandem to simultaneously remove negatively charged humic material (SAX) and retain the antibacterial agents (HLB) [20]. The SAX cartridge was placed on top of the HLB cartridge and both columns were conditioned first with 2 ml methanol and then 2 ml 0.04 M citric acid buffer (pH 4.7). PLE extracts (60 ml) containing approximately 30 ml methanol were diluted with Milli-Q water to a methanol content below 10%. The diluted samples were passed through both SPE-columns at approximately 5 ml min^{-1} and after extraction the columns were washed with 2 ml 0.04 M citric acid buffer (pH 4.7) and 2 ml 0.1 M potassium acetate and dried under vacuum for 15 min. Then the SAX cartridge was removed and the antibacterial agents were eluted from the HLB-sorbent with 2 ml methanol.

For samples with expected low levels of antibacterial agents, method sensitivity was improved by combining three PLE extracts prior to pre-concentration and thereby achieving higher concentration in the final extracts.

Experiments were performed to determine recoveries of the antibacterial agents CTC, OTC, SDZ, TYL (A, B, C and D) and ERY for the tandem SPE (SAX+HLB) clean-up step only. Sample matrix was obtained by extracting non-spiked samples from the loamy sand soil (Askov) using the PLE method described in Section 2.3. These PLE extracts were fortified with antibacterial agents at two concentration levels (20 and 100 μg l^{-1}), corresponding to extracts obtained

Table 3
Outline of the soil extraction method (PLE), solid-phase extraction (SPE) and the two HPLC methods (methods I and II), used for analysis of the antibacterial agents in soil samples

	Method I: tetracyclines and sulfonamides (CTC, OTC and SDZ)	Method II: macrolides (TYL and ERY)
Soil extraction	PLE: 1500 psi, room temperature; extraction buffer: 50% methanol and 50% 0.2 M citric acid (pH 4.7)	
SPE	PLE extract diluted to a methanol content <10%; SAX and HLB SPE cartridges in tandem	
HPLC	Column: Waters Xterra MS-C18, 100 mm × 2.1 mm, 3.5 μm, temperature 13 °C	
Mobile phases	A: 5% methanol + 80 mM formic acid B: 95% methanol + 80 mM formic acid	A: 20% methanol + 80 mM formic acid B: 95% methanol + 80 mM formic acid
Flow	200 μl min^{-1}	250 μl min^{-1}
Gradient	0–2.5 min: 98% A; 2.5–8 min: linear decrease to 50% A; 8–23 min: 50% A; 23–26 min: linear increase to 98% A; 26–30 min: 98% A	0–2 min: 96% A; 2–5 min: linear decrease to 30% A; 5–15 min: 30% A; 15–17 min: linear increase to 96% A; 17–20 min: 96% A
Injection volume	5 μl	
MS–MS	MRM detection, see Table 4	

Table 4
Precursor masses, product ions and optimised parameters for mass spectrometry MRM analysis of the antibacterial agents used in method I and II

MRM	Precursor mass (*m*/*z*)	Product ions (*m*/*z*)
Chlortetracycline (CTC)	479.0	444.2
Oxytetracycline (OTC)	461.0	426.0
Sulfadiazine (SDZ)	251.3	156.0
Tylosin A (TYL A)	916.0	772.0
Tylosin B (TYL B)	772.0	174.0
Tylosin C (TYL C)	902.5	758.5
Tylosin D (TYL D)	918.0	774.0
Erythromycin (ERY)	734.0	158.0
MS parameters	Method I: tetracyclines and sulfonamides (CTC, OTC and SDZ)	Method II: macrolides (TYL and ERY)
Nebulizer gas (NEB) ($l\,min^{-1}$)	5	10
Curtain gas (CUR) ($l\,min^{-1}$)	9	6
Collision gas (CAD) ($l\,min^{-1}$)	7	11
Ionspray voltage (IS) (V)	5500	3000
Temperature (TEM) (°C)	350	550
Declustering potential (DP)	41	100
Focusing potential (FP)	230	170
Entrance potential (EP)	−4.5	−15
Collision energy (CE)	30	40
Collision cell exit potential (CXP)	15	25

from extraction of soil samples with antibacterial agent contents of approximately 40 and 200 $\mu g\,kg^{-1}$ soil, respectively. The fortified samples were diluted to 300 ml with Milli-Q water and passed through the SAX–HLB SPE cartridges as described above.

2.5. LC–ESI-MS–MS analysis

Analysis of the resulting SPE extracts required two slightly different LC–ESI-MS–MS methods (method I for CTC, OTC and SDZ and method II for TYL and ERY), which mainly differ in the MS settings (Tables 3 and 4). For both quantification methods (methods I and II), the analytical system consisted of an Agilent 1100 series HPLC system (Agilent Technologies, Palo Alto, CA, USA), equipped with a degasser, a cooled autosampler (4 °C) and a cooled column oven (13 °C). Mass spectrometry detection was achieved using a Sciex API 3000 triple quadropole detector (Applied Biosystems, Foster City, CA, USA) equipped with an ESI source (Turbo Ionspray). Collection and treatment of data were performed using Analyst software (Applied Biosystems) in Windows NT platform-based data processing.

HPLC separations in both methods I and II were achieved using an Xterra MS-C_{18} analytical column (100 mm × 2.1 mm, particle size 3.5 μm) from Waters and gradient elution. The composition of mobile phases, gradient elution, flow rates and injection volume for methods I and II, respectively, are listed in Table 3. Mobile phases were prepared by mixing methanol and 308 μl formic acid with Milli-Q water in a 1 l volumetric flask, followed by degassing by ultrasonication for 10 min.

For MS detection, the instrument was operated in the positive ion mode and detection of the antibacterial agents was obtained using MRM detection. Precursor mass and product ion mass for the MRM detection are listed in Table 4, together with the MS–MS settings for methods I and II, respectively.

The response for each of the antibacterial agents detected in the LC–ESI-MS–MS methods was evaluated for linearity, and the limits of detection and quantification for the instrument ($LOD_{instrument}$ and $LOQ_{instrument}$) were determined, using calibration curves for the concentration range 1–500 $\mu g\,l^{-1}$. Stock solutions of the antibacterial agents were prepared in methanol, wrapped in tinfoil and stored at 5 °C for a maximum of 1 month. From two separate stock solutions duplicate standards were produced in methanol at eight concentration levels (1, 5, 15, 35, 70, 135, 270 and 500 $\mu g\,l^{-1}$) and duplicate calibration curves for CTC, OTC, SDZ, TYL A and ERY were produced, by alternating the number of injections between two and six. To evaluate the method applicability to ECTC and the baseline separation of CTC and ECTC, the procedure was repeated for ECTC.

$LOD_{instrument}$ and $LOQ_{instrument}$ were determined using the standard deviation of the response (σ) and the slope of the calibration curves (S) [21]:

$$LOD_{instrument} = \frac{3.3\sigma}{S}$$

$$LOQ_{instrument} = \frac{10\sigma}{S}$$

2.6. Validation of soil extraction procedure (PLE–SPE–LC–ESI-MS–MS)

Recoveries for the entire PLE–SPE–LC–ESI-MS–MS procedures were determined for the loamy sand soil (Askov) and the sandy soil (Lundgaard). Soil samples from both soils were fortified with CTC, OTC, SDZ, TYL (A, B, C and

D) and ERY on four concentration levels (approximately 5, 25, 75 and 100 $\mu g\,kg^{-1}$ soil) and six replicates at each level. The fortified samples were extracted and analysed using the entire procedure. Recoveries were calculated as the percentage of extracted antibacterial agents compared to spiked level.

To compare recoveries obtained when combining three PLE extracts prior to SPE pre-concentration with recoveries obtained when only one PLE-extract were analysed, the recovery experiment for the 25 $\mu g\,kg^{-1}$ soil concentration level were performed using both procedures (six replicates, loamy sand soil only).

Day-to-day variations for the extraction procedure were determined for the loamy sand soil (Askov), by repeating the recovery experiment for concentration levels 5, 25 and 100 $\mu g\,kg^{-1}$ soil after 3 days.

Furthermore, the linearity range for the entire soil extraction procedure (PLE–SPE–LC–MS–MS) and limits of detection (LOD_{soil}) and quantification (LOQ_{soil}) were determined for the antibacterial agents for both soil types. Soil samples were fortified on six concentration levels (1, 5, 25, 50, 75 and 100 $\mu g\,kg^{-1}$ soil) with CTC, OTC, SDZ, TYL A and ERY to produce calibration curves. For the concentration levels 1, 25 and 75 $\mu g\,kg^{-1}$ soil, six replicates were produced, while duplicate samples were made for concentration levels 5, 50 and 100 $\mu g\,kg^{-1}$ soil. LOD_{soil} and LOQ_{soil} were determined using the standard deviation on the response (σ) and the slope of the calibration curves (S) as described in Section 2.5.

For TYL B, C and D, $LOD_{soil,estimated}$ and $LOQ_{soil,estimated}$ were estimated using the signal-to-noise ratio as $S/N = 3$ and $S/N = 10$, respectively, for soil samples containing 5 $\mu g\,kg^{-1}$ TYL B, C or D.

2.7. Extraction of antibacterial agents from barley grains

The PLE-method for soil extraction was applied to extraction of antibacterial agents from grains. Ripe grains of spring barley were freeze-dried and ground in an electronic coffee mill to coarse flour. Equivalent to soil samples, 10 g flour was mixed with 10 g Ottawa sand in the PLE-extraction cell and PLE was performed following the procedure described in Section 2.3. The PLE extracts were filtered through a 0.45 μm filter before combining two extracts and diluting with Milli-Q water to 500 ml. Tandem-SPE (SAX–HLB) were performed equivalent to soil samples (Section 2.4.), but it was necessary to replace the SAX-cartridge for every 200 ml sample due to clogging of the sorbent. Quantification of the SPE-extracts was achieved using the LC–ESI-MS–MS methods described in Section 2.5.

Recoveries for the extraction of antibacterial agents from grain were determined for eight replicate flour samples fortified with CTC, OTC, SDZ, TYL A and ERY to a concentration of 10 $\mu g\,kg^{-1}$ flour. Limits of detection ($LOD_{grain,estimated}$) and quantification ($LOQ_{grain,estimated}$) for the grain extraction were estimated using the signal-to-noise ratio (S/N), as $S/N = 3$ and 10, respectively.

2.8. Application to field samples

To demonstrate the applicability of the soil extraction procedure to real field samples, soil samples were taken from two agricultural fields (loamy sand soil (Askov) and sandy soil (Lundgaard)) on several occasions after manure application in the period May to October 2000. The fields were located within two kilometres of each other, but represent to different soil types (Table 2). Liquid manure was obtained from a piglet-breeding farm where the animals were treated with CTC and TYL A and the content of antibacterial agents in the manure were estimated to be 300–500 $mg\,kg^{-1}$ dry mass manure, according to information from veterinary reports on treatments within the study period. The manure was applied according to Danish legislation to a level of 100 kg N ha^{-1} per year and ploughed to a depth of 20 cm, and spring barley was grown on the fields.

Duplicate soil samples were taken from three plots on each field, using a soil auger to a depth of 20 cm. The soil samples were air dried and sieved through a 2 mm sieve and the antibacterial agents were extracted and analysed as described in Sections 2.3–2.5.

The grain extraction method (Section 2.7) was applied to grains of spring barley harvested from plants grown on the agricultural fields (Askov and Lundgaard). The grains were harvested in August 2000 and stored at −20 °C until analysis. Pressurised liquid extraction of the antibacterial agents was performed after grinding of the grain samples, as described in Section 2.7.

3. Results and discussion

3.1. Soil extraction method optimisation

In development and optimisation of the PLE method several parameters need to be considered such as soil sample size, soil moisture content, extraction solvent polarity and pH, and PLE-settings such as pressure, temperature and number of solvent cycles.

In order to obtain the highest possible concentrations of the antibacterial agents in soil extracts, the performance of the PLE-system was investigated for soil samples weighing up to 25 g. However, samples greater than 10 g soil caused clogging of the PLE extraction cell. To increase contact-surface between soil particles and extraction buffer and prevent clogging of the extraction cell, optimum moisture content of approximately 5% was found and 10 g Ottawa sand standard was mixed into 10 g soil sample before extraction.

Extraction buffer for extracting the antibacterial agents from soil was selected in accordance with the physico-chemical properties of the compounds (Table 1). Many

different soil-adsorption mechanisms, such as hydrophobic interactions, hydrogen bonding, complexation and cation exchange, may affect the extraction of the compounds from soil. This was found particularly important for the tetracyclines as they form strong complexes with di- and trivalent cations in the clay mineral inter-layers or to hydroxy-groups at the surface of the soil particles [22–25] and therefore a complexation agent was added to the extraction buffer. As a starting point a combination of McIlvaine buffer (citric acid and potassium phosphate), EDTA and methanol was applied as extraction buffer, as previously employed for food analysis [26] and soil extractions [27]. However, this buffer precipitated within few hours, on occasions causing blockage in the tubes and valves of the PLE system. An extraction buffer containing methanol and only citric acid buffer (pH adjusted to 4.7) as complexation agent was therefore assessed for extraction efficiency. Recoveries achieved using this method were above 40%, which were comparable to recoveries for the McIlvaine + EDTA combination (<25–70%) and method development continued with the citric acid + methanol combination. At pH 4.7 CTC, OTC and SDZ are overall neutral and therefore have reasonably high affinity for the moderately hydrophobic extraction buffer, while TYL A and ERY are positively charged but due to their hydrophobicity they are extractable using the same buffer (see Table 1 for pK_a and log K_{ow} values).

The influence of the citric acid concentration in the extraction buffer was investigated by performing the PLE-extraction for the concentration range 0.2–0.5 M citric acid adjusted to pH 4.7. No significant difference in recovery was found (ranging from 24–33 to 30–35%). Correspondingly, the relative content of methanol in the buffer in the range 50–75% was tested and a slightly higher recovery was found when using 50% methanol (40–43% as compared to 36–39% for 75% methanol). Furthermore, increasing the methanol content resulted in increased colouring of the PLE-extract, which required additional sample clean-up. Hence, the buffer composition used in subsequent analysis was 50% methanol and 50% 0.2 M citric acid with pH adjusted to 4.7.

The PLE extraction conditions were set to 10 min extraction at 1500 psi at room temperature, followed by another flush of solvent for 3 min. No additional extraction of the antibacterial agents was achieved using a second and third flush. Optimum conditions are listed in Section 2.3 and summarised in Table 3.

3.2. LC–ESI-MS–MS methods

Two different LC–ESI-MS–MS methods (methods I and II) were required for the analysis of the antibacterial agents due to differences in physico-chemical properties (Table 1). In Fig. 1, chromatograms for MRM analysis of soil samples containing 25 $\mu g\,kg^{-1}$ soil of the antibacterial agents show negligible baseline noise when using MRM detection and that only analyte peaks occur in the chromatograms. Epi-chlortetracycline (ECTC) and CTC have the same precursor and product ions masses and therefore occur in the same chromatogram, but the peaks are separated.

As shown in Table 5, the methods were linear in the complete concentration range tested (1 and 500 $\mu g\,l^{-1}$, equivalent to 0.07–33 $\mu g\,kg^{-1}$ soil) and covers the requirements for most environmental soil samples containing antibacterial agents. The limits of detection ($LOD_{instrument}$) and quantification ($LOQ_{instrument}$) are listed in Table 5. These values correspond to soil concentrations of 0.5–1.5 and 1.5–5 $\mu g\,kg^{-1}$ soil, respectively, assuming that 3 × 10 g soil is extracted and concentrated to 2 ml SPE extract.

3.3. Clean-up and pre-concentration using SPE

The combination of the SAX and the HLB (polymeric) SPE sorbents act as both clean-up and pre-concentration. The SAX column reduces matrix interferences by adsorbing anionic humic particles from the soil extracts, avoiding contamination, blocking and overloading of the HLB sorbent. At a buffer pH of 4.7, the antibacterial agents are overall neutral or cations (Table 1) and are therefore not retained on the SAX cartridge, while the polymer based HLB cartridge simultaneously retains neutral polar and non-polar compounds, including the studied antibacterial agents.

Dilution of the PLE extracts with Milli-Q water to a methanol content below 10%, were primarily performed to avoid continuous elution of the antibacterial agents from the HLB sorption material, but additionally the buffer capacity (20 mM citric acid) remained sufficient to keep a sample pH of 4.7 after dilution.

Recoveries and corresponding 95% confidence intervals for the SPE method at two concentration levels (approximately 20 and 100 $\mu g\,l^{-1}$ in the final extract) are listed in Table 6. The SPE method is optimised for tetracyclines, i.e. sorbent material and sample pH, and recoveries for these compounds (CTC and OTC) are above 80%. The method is also applicable for the macrolides (TYL A and ERY) with recoveries above 80% and for the degradation products, with recoveries of approximately 100% for TYL B and TYL D and approximately 70% for TYL C. The sulfonamides are a more hydrophilic group of antibacterial agents and recoveries of approximately 85% for SDZ were found. SDZ is one of the more hydrophilic sulfonamides (log $K_{ow} = -0.092$), but some sulfonamides are even more hydrophilic, i.e. sulfanilamide (SUL, log $K_{ow} = -0.719$) [28].

3.4. Validation of the soil extraction procedure

Mean recoveries and corresponding confidence intervals for the PLE-extraction of six replicate soil samples are listed in Table 7. Recoveries for all antibacterial agents are satisfactorily high and the standard deviations on six replicate samples are low, as demonstrated by narrow confidence intervals.

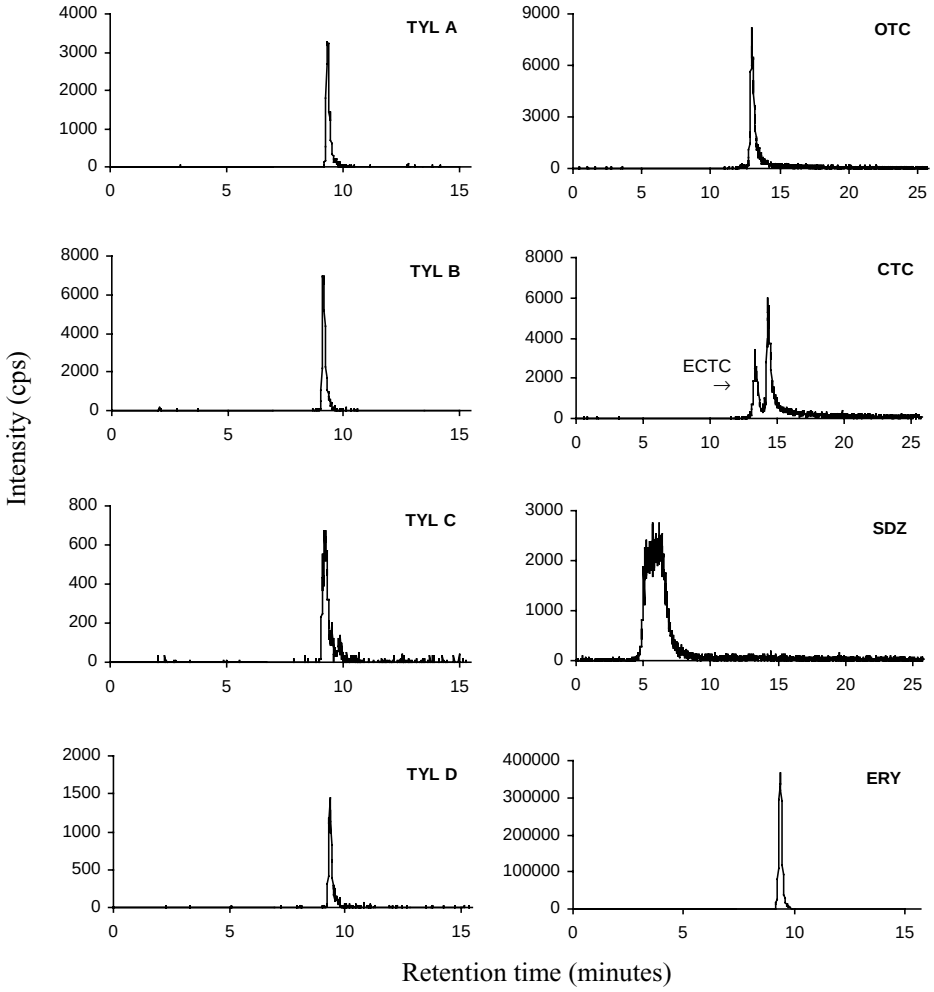


Fig. 1. Chromatograms corresponding to LC–ESI-MS–MS analysis (MRM positive mode) of soil sample containing 25 μg kg^{-1} soil of the antibacterial agents. Method I for CTC, OTC and SDZ, and method II for TYL A, B, C, D and ERY.

For the tetracyclines (OTC and CTC) recoveries of approximately 50–70% are achieved, which is lower than recoveries obtained for the SPE method, indicating that the compounds are not fully extracted from the soil. This is probably due to the many sorption mechanisms involved in the binding of tetracyclines to soil, resulting in very strong sorption (Table 1). Recoveries for the two soil types are comparable, but with a tendency towards higher recoveries in the sandy soil. This corresponds well with previous studies indicating that tetracyclines primarily sorb to the clay fraction of the soil by complexation and hydrogen bonding [24]. However, almost identical K_d values for OTC for the loamy

Table 5
Method validation parameters for the LC-MS–MS methods (methods I and II) for analysis of antibacterial agents

Compound	t_R (min)	Linearity range (μg l^{-1})	Linear regression coefficient, R^2	$LOQ_{instrument}$ (μg l^{-1})	$LOD_{instrument}$ (μg l^{-1})
Method I					
Chlortetracycline (CTC)	14.4	1.02–542	0.9991	63.3	19.0
Epi-chlortetracycline (ECTC)	13.4	5.01–133.6	0.9926	49.2	14.8
Oxytetracycline (OTC)	12.9	1.09–583	0.9995	49.7	14.9
Sulfadiazine (SDZ)	5.6	0.84–450	0.9998	25.7	7.7
Method II					
Tylosin A (TYL A)	9.3	1.10–586	0.9990	73.9	22.2
Erythromycin (ERY)	9.4	1.01–540	0.9998	27.7	8.3

Calibration curve produced using standard solutions of the antibacterial agents in methanol in the range 1–500 μg l^{-1}. Limits of detection and quantification ($LOD_{instrument}$ and $LOQ_{instrument}$) were estimated from the standard deviation and slope of the calibration curves.

Table 6
Recovery of antibacterial agents for the HLB–SAX SPE method

Spiked concentration ($\mu g\,l^{-1}$)	Concentration in water sample ($\mu g\,l^{-1}$)	Recovery (%)	95% Confidence level (%)
Chlortetracycline			
21.6	0.144	84.7	75.3–94.1
108.1	0.720	109.4	101.5–117.2
Oxytetracycline			
21.5	0.143	113.3	104.3–122.3
107.7	0.718	125.1	117.1–133.1
Sulfadiazine			
21.7	0.145	84.1	75.7–92.4
108.6	0.724	85.4	79.1–91.8
Erythromycin			
25.7	0.171	92.8	79.2–106.3
128.5	0.857	79.9	73.7–86.2
Tylosin A			
20.1	0.134	112.6	103.0–122.1
100.3	0.669	106.5	99.1–113.9
Tylosin B			
20	0.133	105.4	95.2–115.6
100	0.667	104.1	97.7–110.6
Tylosin C			
20	0.133	61.5	51.9–71.0
100	0.667	77.3	74.2–80.4
Tylosin D			
20	0.133	96.4	88.6–104.2
100	0.667	92.5	87.0–97.9

Soil extracts (six replicates) from non-spiked soil fortified on two concentration levels with the antibacterial agents and concentrated using SPE.

sand and sandy soil have been determined [14], which may correspond to binding to acid sites in organic fraction in the soil, i.e. humic acid [25].

For the macrolides (TYL A and ERY), the recoveries range from 50 to above 100%, depending on spike concentration and soil type. Recoveries for ERY are highest in the sandy soil, in which the content of clay and organic carbon is lower than the loamy sand soil. This indicates that ERY mainly sorbs to the organic fraction or clay by hydrophobic interactions. Contrary, recoveries for TYL A are higher in the loamy sand soil, which is inconsistent with the partition coefficients determined by [14], showing much stronger sorption to the loamy sand soil as compared to the sandy soil (Table 1). Possibly the PLE solvent (citric acid + methanol) is not optimal for extracting TYL from soil and higher extraction efficiency may be achieved by using a more hydrophobic solvent. However, for the degradation products TYL B, C, and D, satisfactory recoveries are obtained, indicating that the soil extraction method is also applicable for these compounds.

For SDZ recoveries of approximately 80% for the complete soil extraction method are comparable to the recoveries achieved for the SPE method only, indicating that SDZ are fully extracted from the soil using the PLE procedure, but is partly lost during the SPE concentration.

It was examined whether comparable results were obtained when combining three PLE extracts prior to the SPE concentration rather than working with only one extract, by performing the recovery experiment for the 25 $\mu g\,kg^{-1}$ soil level using both procedures. No significant deviation between procedures was found, as the 95% confidence intervals for recoveries overlap (Table 7). Furthermore, the calibration curve for the entire soil extraction method (see Section 3.5.) consisted of three points where three extracts were combined (1, 5 and 25 $\mu g\,kg^{-1}$) and three points where only one extract were concentrated (50, 75 and 100 $\mu g\,kg^{-1}$), and the calibration curves were linear in the complete range (Table 8). This also demonstrates that no matrix effect or breakthrough of the SPE cartridges happened when a 1 l sample was extracted.

Day-to-day variation was investigated for the loamy sand soil and the results are shown in Table 7. Significant day-to-day variation is determined as not-overlapping 95% confidence intervals for recoveries obtained on the same concentration level on different days. For some experiments day-to-day variation is demonstrated even though the recoveries are comparable, which is due to the low standard deviations obtained for six replicate samples and corresponding narrow confidence intervals.

Calibration curves were produced for the entire soil extraction procedure, by fortifying soil samples from the loamy sand soil and the sandy soil with the antibacterial agents at six concentration levels, Validation results are shown in Table 8. Linear calibration curves were obtained for all

Table 7
Recovery for soil samples (loamy sand and sandy soil) spiked with the antibacterial agents in concentration levels ranging from 5 to 100 μg kg^{-1} soil

Spiked concentration (μg kg^{-1} soil)		Sandy soil (Lundgaard)		Loamy sand soil (Askov)			
		Recovery (%)	95% CI (%)	Day 1		Day 2	
				Recovery (%)	95% CI (%)	Recovery (%)	95% CI (%)
Chlortetracycline							
5.4	3 ex.	33.0	31–35	42.5	35–50	65.8	62–70
27.0	3 ex.	–	–	–	–	31.3	29–34
27.0		66.8	61–73	48.5	46–51	33.2	31–35
81.2		72.9	68–78	–	–	44.8	43–47
108.1		75.9	75–77	51.3	48–54	45.7	44–48
Oxytetracycline							
5.4	3 ex.	277.0	145–409	63.3	57–69	88.1	81–95
26.9	3 ex.	–	–	–	–	45.1	43–47
26.9		102.0	88–116	69.9	64–76	54.9	53–57
80.9		134.4	108–161	–	–	81.6	76–87
107.7		119.0	106–133	80.7	74–87	82.1	79–86
Sulfadiazine							
5.4	3 ex.	66.9	48–86	48.5	44–53	70.4	62–78
27.2	3 ex.	–	–	–	–	64.4	62–67
27.2		84.6	79–90	52.3	50–54	67.2	66–69
81.2		74.8	74–76	–	–	85.8	83–89
108.6		71.3	70–73	64.4	62–67	83.6	83–85
Tylosin A							
5.0	3 ex.	71.8	58–86	123.7	119–129	98.0	87–109
25.1	3 ex.	–	–	–	–	112.3	80–144
25.1		50.0	41–59	92.6	91–94	126.6	108–145
75.3		47.0	42–52	–	–	121.2	115–127
100.3		45.4	22–69	94.3	91–98	109.4	105–114
Tylosin B							
5		67.6	48–88	97.4	92–102	128.8	109–149
25		85.3	60–110	98.7	97–101	–	–
75		95.3	84–107	–	–	–	–
100		94.3	57–151	108.3	104–112	117.3	115–120
Tylosin C							
5		56.3	53–60	143.1	132–154	185.7	168–203
25		63.8	54–74	69.6	42–97	–	–
75		64.3	52–76	–	–	–	–
100		59.0	29–89	81.2	78–85	153.3	149–158
Tylosin D							
5		84.9	66–104	103.2	98–109	105.3	91–120
25		77.4	63–92	49.1	30–68	–	–
75		77.3	68–87	–	–	–	–
100		70.1	35–106	60.3	58–63	83.5	80–87
Erythromycin							
6.4	3 ex.	114.2	87–141	78.0	72–84	82.1	72–93
32.1	3 ex.	-	–	–	–	47.3	39–55
32.1		104.4	38–171	61.1	59–63	57.3	56–57
96.5		99.2	72–126	–	–	65.6	53–79
128.5		52.8	36–70	68.6	66–71	83.5	82–85

Furthermore, the table lists the day-to-day variation determined for the loamy sand soil and compares the recovery for the 25 μg kg^{-1} soil level, achieved by using one or three PLE extracts for each sample, respectively (3 ex. refers to three combined extracts).

compounds and for both soils, with regression coefficients (R^2) in the range of 0.94–0.99 and linearity range of approximately 1–100 μg kg^{-1}, which is the complete range tested. For the entire soil extraction method, LOD_{soil} and LOQ_{soil} were estimated from the standard deviation and slope of the calibration curves (Section 2.5). Values obtained for LOD_{soil} and LOQ_{soil} for the antibacterial agents were in the range <1–5 and 1–10 μg kg^{-1}, respectively, which covers the range expected for environmental soil samples [3,5,6]. For the degradation products TYL B, C and D, $LOD_{soil,estimated}$ and $LOQ_{soil,estimated}$ were estimated using the signal-to-noise ratio (S/N) and were in the same range

Table 8
Method validation for the entire soil extraction procedure (PLE–SPE–LC–MS–MS)

Compound	Linearity range ($\mu g\,kg^{-1}$ soil)	Correlation coefficient, R^2	LOD_{soil} ($\mu g\,kg^{-1}$ soil)	LOQ_{soil} ($\mu g\,kg^{-1}$ soil)
Loamy sand soil (Askov)				
Chlortetracycline (CTC)	1.2–108.2	0.9560	0.6	1.1
Oxytetracycline (OTC)	1.1–107.7	0.9568	1.9	4.0
Sufadiazine (SDZ)	1.1–108.6	0.9859	0.9	1.2
Tylosin A (TYL A)	1.0–75.3	0.9867	4.0	9.6
Tylosin B (TYL B)[a]	–	–	0.4	1.2
Tylosin C (TYL C)[a]	–	–	1.3	4.2
Tylosin D (TYL D)[a]	–	–	1.8	5.9
Erythromycin (ERY)	1.3–128.5	0.9836	2.4	4.3
Sandy soil (Lundgaard)				
Chlortetracycline (CTC)	1.2–108.2	0.9917	0.6	1.3
Oxytetracycline (OTC)	1.1–107.8	0.9566	5.6	12.8
Sufadiazine (SDZ)	1.1–108.6	0.9960	2.9	6.4
Tylosin A (TYL A)	1.0–75.3	0.9543	2.5	4.7
Tylosin B (TYL B)[a]	–	–	1.0	3.3
Tylosin C (TYL C)[a]	–	–	1.7	5.6
Tylosin D (TYL D)[a]	–	–	2.3	7.7
Erythromycin (ERY)[a]	1.3–96.5	0.9506	5.5	11.0

Soil samples spiked at six levels in the range 1–100 $\mu g\,kg^{-1}$ soil to produce calibration curves. LOD_{soil} and LOQ_{soil} were estimated from the standard deviation and slope of the calibration curves.
[a] LOD and LOQ determined as S/N = 3 and 10, respectively, for soil samples containing 5 $\mu g\,kg^{-1}$ of the antibacterial agents.

as the parent compound, TYL A (Table 8). These levels of quantitation are relatively low and make the analytical method applicable for analysing most environmental soil samples for the studied antibacterial agents.

3.5. Application to field samples

The liquid manure applied to the agricultural fields was obtained from pigs treated with CTC and TYL A and no other antibacterial agents (OTC, ERY or SDZ) were detected in the soil samples. The measured concentrations of CTC and TYL A in the soil samples taken from the two manure-amended fields are shown in Table 9. The first sample (9 May 2000) was obtained 9 days after manure application and show concentrations of approximately 10–15 $\mu g\,kg^{-1}$ soil for CTC in both soils and 25–55 and 10–20 $\mu g\,kg^{-1}$ soil for TYL A in the loamy sand and sandy soil, respectively. These concentrations decline continuously during the following 146 days period. Concentrations in samples taken on the last date of sampling (2 October 2000), was below or near the LOD_{soil} for CTC and approximately 15 and 3 $\mu g\,kg^{-1}$ for TYL A for the loamy sand and sandy soil, respectively. Hence, the method is applicable for monitoring of the antibacterial agents levels in field samples over time.

Some deviation is seen between the concentrations measured on the three different plots on each field at each sampling day. This is probably due to heterogeneous mixing of soil and manure, and it is seen that the deviation declines during the period of sampling. Deviations between measured concentrations in field samples were also demonstrated by Hamscher et al. [6], who measured high concentrations of CTC in heterogeneously distributed manure aggregates.

Flour of grinded barley grains was extracted to evaluate whether any detectable uptake of antibacterials agents by barley plants occurred. The PLE extraction procedure was applied with only few modifications, i.e. a filtration step prior to SPE clean-up and replacement of the SAX-cartridge

Table 9
Antibacterial agent content ($\mu g\,kg^{-1}$soil) measured in soil samples from two agricultural fields, Askov (loamy sand soil) and Lundgaard (sandy soil)

Sampling day	Chlortetracycline						Tylosin A					
	Loamy sand, plot no.			Sandy soil, plot no.			Loamy sand, plot no.			Sandy soil, plot no.		
	1	2	3	1	2	3	1	2	3	1	2	3
9 May 2000	15.5	13.9	11.5	10.7	11.0	8.9	57.4	45.5	24.1	20.0	21.3	8.0
10 July 2000	2.9	3.2	–	5.1	11.7	5.1	34.1	26.6	6.4	19.2	20.4	7.5
5 September 2000	–	0.6	–	0.6	3.4	0.8	10.7	16.1	12.6	3.8	3.7	1.8
2 October 2000	–	–	–	–	0.9	0.7	15.0	11.9	18.0	3.0	–	2.9

(–) Below LOD_{soil} (see Table 8). Samples were taken from three plots on each field, shortly after amendment with liquid manure from a piglet-breeding farm and throughout the growth season until October.

for every 200 ml sample. Recoveries for the grain extraction were determined for spiked grain samples and were 42.3% (34–50) for CTC, 31.8% (28–36) for OTC, 53.5% (47–60) for TYL A, 56.5% (52–61) for ERY and 39.9% (38–42) for SDZ (95% CI in brackets). These recoveries are equivalent to recoveries obtained for soil extractions and demonstrate that the antibacterial agents are not lost due to the considerable amounts of suspended flour particles in the samples or during filtration.

$LOD_{grain,estimated}$ and $LOQ_{grain,estimated}$ for the grain extraction were estimated using the signal-to-noise ratio for flour samples fortified to 10 $\mu g\,kg^{-1}$ of each of the antibacterial agents. Estimated values for $LOD_{grain,estimated}$ were 0.3, 0.4, 1.4, 1.5 and 0.2 $\mu g\,kg^{-1}$ grain for CTC, OTC, SDZ, TYL A and ERY, respectively. Corresponding $LOQ_{grain,estimated}$ values were in the range 0.6–5.0 $\mu g\,kg^{-1}$ grain.

To test the procedure on real field samples, non-spiked grain samples were harvested from fields amended with liquid manure and extracted using the procedure described in Section 2.7. None of the antibacterial agents were measured in concentration levels above $LOD_{grain,estimated}$. The grains were harvested in August when the soil content of CTC and TYL A had declined to below 10 $\mu g\,kg^{-1}$ soil (Table 9), so apparently the antibacterial agents have not been taken up by plants and accumulated in the grains in detectable amounts.

4. Conclusions

Simultaneous extraction of CTC, OTC, SDZ, TYL A and ERY from soil was obtained using a simple PLE method followed by clean-up and concentration using SPE and analysis by two slightly different LC–ESI-MS–MS methods (methods I and II). Recoveries and LOD_{soil} and LOQ_{soil} for the extraction procedure were satisfactory, demonstrating that the procedure is applicable for environmental samples.

Within each group of antibacterial agents, i.e. tetracyclines, macrolides and sulfonamides, there was some variation between the recoveries achieved for the representatives and interpretation of the applicability of the methods for other compounds in each group is difficult. However, the compounds examined in this study represent a wide range of physico-chemical properties and therefore the method is expected to be applicable to many of the antibacterial agents present simultaneously in manure.

The soil extraction procedure was optimised for tetracyclines and higher recoveries could probably be achieved for the macrolides and sulfonamides, if modifying the method with regards to these compounds. However, this procedure represents a useful compromise for simultaneous extraction of all three groups of antibacterial agents from soil.

Acknowledgements

The technical help and assistance provided by Susanne Hermansen, Danish University of Pharmaceutical Sciences, is gratefully acknowledged. We also thank Erik Damgaard and co-workers at Askov Experimental Station, Danish Institute of Agricultural Sciences, Denmark, for assistance with the field experiments and providing soil samples. The Danish Research Council (SJVF) is gratefully acknowledged for financial support to the project SOUND (molecular tools for sustainable land use—critical processes for soil functioning in presence of organic pollutants), project no. 23-02-0152.

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