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High-throughput analysis of tetracycline antibiotics and their epimers in liquid hog manure using Ultra Performance Liquid Chromatography with UV detection

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ABSTRACT

Antibiotics contained in animal manure can contaminate soil, groundwater and eventually surface and drinking water. To reduce the usage of antibiotics in livestock industry the EU banned their application as growth promoters in 2006. Even though the antibiotics are still used for this purpose and therefore it is necessary to control their applications.

An Ultra Performance Liquid Chromatography method (UPLC) with UV detection for determination of tetracycline (TC), oxytetracycline (OTC), chlortetracycline (CTC), and doxycycline (DOX) including their epimers in the liquid hog manure was developed. The antibiotics were extracted with ethyl acetate and separated on UPLC BEH Shield RP18 column. The validated method was selective for all analytes and system suitability was assessed. Calibration curves ranged from 7.8 to 250.0 μg mL^{-1} with determination coefficient of 0.9999. The method limits of quantification ranged from 0.9 to 1.6 mg kg^{-1}. Recoveries were 52.4 ± 3.8%, 72.4 ± 5.0%, 83.8 ± 5.7% and 95.9 ± 4.7% for TC, OTC, CT, and DOX, respectively. The method was used for the determination of TC, OTC, CT, and DOX in liquid hog manure samples.

1. Introduction

Antibiotics represent a large group of pharmaceuticals with still growing consumption in both human and veterinary medicine (McEvoy et al., 2004). There are three significant field of application of antibiotics for animal husbandry: treatment of infection in livestock, prevention of infection and growth promoters (Schlüsener et al., 2003). About 50–90% of the administered pharmaceutical dose is excreted rapidly after the treatment (Kroker, 1983). Little is known about the behavior, concentration and the fate of antibiotics in manure and soil (Nowara et al., 1997; Haller et al., 2002; Schlüsener et al., 2003). Excreted antibiotics may be mobile in soil and could be transported to the ground and surface water and consequently to the drinking water (Schlüsener et al., 2003; Thorsten et al., 2003). Regarding former studies, the presence of antibiotics in the environment can cause the development of antibiotics-resistant bacteria and can have an adverse effect on the water environment and animals (Lu et al., 2004). Their potential presence in source drinking water could have unknown health effect on humans and animals due to the chronic low-level exposure to these substances over the lifetime (Batt and Aga, 2005).

The most widely used groups of antibiotics in the European Union's animal husbandry are tetracyclines, macrolides, penicillines, aminoglycosides and sulfonamides (European Commision, 1999).

Tetracyclines both natural and semisynthetic form a large group of products produced mainly by *Streptomyces* spp. They have a broad-spectrum of activities including inhibition of many common Gram-positive and Gram-negative bacteria, chlamydia, rickettsie, etc.; they are distinguished mainly for bacteriostatic action caused by inhibition of protheosynthesis (Cooper et al., 1998; Debut, 1988).

Extraction procedures and chromatographic methods for determination of tetracyclines (oxytetracycline, tetracycline, chlortetracycline, and doxycycline) in liquid manure have been previously published. For extraction of tetracyclines liquid–liquid extraction (LLE) using various extraction solution and pressurized-liquid extraction (PLE) as well as solid-phase extraction (SPE) were described. Extraction solutions for LLE were, e.g. ethyl acetate (Hamscher et al., 2002), citric buffer (Kühne et al., 2000), mixture of acidified ACN and EDTA-McIlvaine buffer (Hu et al., 2008; Martínez-Carballo et al., 2007) mixture of citric acid, oxalic acid, methanol and water (Wang and Yates, 2008) or buffered methanol–water mixture (Aust et al., 2008). For PLE citric acid followed by mixture of methanol, water and citric acid (Jacobsen and Halling-Sørensen, 2006) was used as the extraction solution. SPE was developed either as the only extraction step (Thorsten et al., 2003; Kemper et al., 2008) or as the clean-up and pre-concentration step after LLE (Jacobsen and Halling-Sørensen, 2006; Martínez-Carballo et al., 2007; Aust et al., 2008; Hu et al., 2008).

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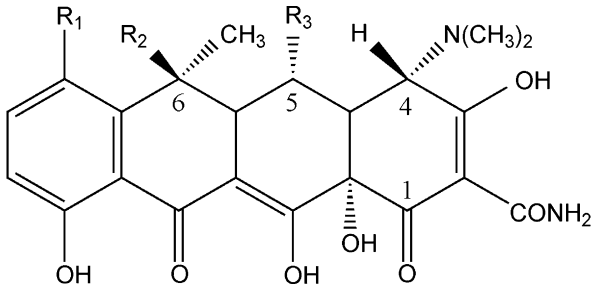

Fig. 1. Structures of tetracyclines.

	R_1	R_2	R_3
TC	H	OH	H
OTC	H	OH	OH
CTC	Cl	OH	H
DOX	H	H	OH

Determination of tetracyclines was performed on HPLC-UV (Hu et al., 2008; Kühne et al., 2000; Thorsten et al., 2003; Wang and Yates, 2008) or on HPLC–MS/MS (Aust et al., 2008; Hamscher et al., 2002; Jacobsen and Halling-Sørensen, 2006; Kemper et al., 2008; Martínez-Carballo et al., 2007) system.

To date no studies about determination of tetracyclines in any matrix using Ultra Performed Liquid Chromatography (UPLC) has been published. UPLC is a modern separation technique providing considerable high-throughput analysis compared to HPLC. UPLC allows separations on column materials at high pressures up to 100 MPa using sub-2 μm particles, which yields significantly higher separation efficiencies and shorter run times compared to HPLC technique (Bendahl et al., 2005).

In this study, the validated UPLC method with LLE pre-concentration for analysis of tetracycline antibiotics, i.e. tetracycline (TC), oxytetracycline (OTC), chlortetracycline (CTC), doxycycline (DOX) and their epimers (TC_{ep}, CTC_{ep}, and DOX_{ep}) in liquid manure is described (for structures see Fig. 1). Because both the LLE procedure and UPLC analysis were performed under acidic conditions, the formation of the epimers was considered (Cooper et al., 1998; Halling-Sørensen et al., 2002; Hamscher et al., 2002; Skúlason et al., 2003). The developed method was applied for analysis of liquid hog manure samples from five different localities in the Czech Republic.

2. Experimental

2.1. Chemicals, reagents, and glassware

TC was purchased from Spofa (Prague, Czech Republic), OTC from VUAB (Roztoky u Prahy, Czech Republic), CTC from Sigma–Aldrich (Steinheim, Germany) and DOX from Calbiochem (San Diego, USA). The acetonitril (ACN) used as the chromatographic mobile phase was LC/MS grade and was obtained from Chromservis (Prague, Czech Republic). Methanol HPLC grade used for standards and manure samples preparation was purchased from Merck (Darmstadt, Germany). Ethyl acetate p.a. and citric acid were purchased from Lach-Ner (Neratovice, Czech Republic). Acetic acid and Na_2EDTA were purchased from Sigma–Aldrich (Steinheim, Germany).

To avoid formation of complexes of tetracyclines with metal ions, proteins, and silanol groups (Thorsten et al., 2003; Batt and Aga, 2005) all glassware used was rinsed with saturated solution of Na_2EDTA in methanol–water (50:50, v/v) and air-dried before use.

2.2. Standards and samples preparation

2.2.1. Stock solution

Individual antibiotic standards (1 mg) were diluted in 1 mL of methanol–acetic acid (99:1, v/v) and stored at −20 °C. Stock mixture solution was prepared by mixing of equal volumes of TC, OTC, CTC, and DOX standard solutions to final concentration of 250.0 μg mL^{-1} and were stored at −20 °C for maximum of 14 d (see Section 3.3.6).

2.2.2. Manure sampling and pretreatment

Liquid manure samples were collected (1 L amber glass bottles) in five different animal farming areas (one sample from each area) in the Czech Republic in autumn 2008 and stored at −20 °C before use.

2.2.3. Spiked matrix preparation

Liquid manure (5 g) free of targeted antibiotics (blank) was spiked with stock solution (0.5 mL) to required concentration for validation tests (see Section 2.6) and kept at 4 °C for period of 3 d prior to analysis (simulation of natural conditions).

2.3. Liquid–liquid extraction

LLE procedure was similar to previously published one (Cooper et al., 1998; Hamscher et al., 2002). Briefly, 6 mL of citrate buffer (pH 4.7) was vortexed intensively with 5 g of liquid manure sample. Then 1 mL of Na_2EDTA solution (5 mg mL^{-1}) was added and vortexed intensively again for 1 min. After that 35 mL of ethyl acetate was added, vortexed intensively for 1 min, slightly for 15 min and centrifuged. The organic phase was separated and the procedure was repeated with another 1 mL of Na_2EDTA solution and 35 mL of ethyl acetate. The organic phase was evaporated to dryness and reconstituted in 0.5 mL of methanol–acetic acid (99:1, v/v).

2.4. UPLC

UPLC analysis with UV detection was carried out on a Waters Acquity UPLC System ("W", Czech Republic, Prague) consisted of Acquity UPLC Solvent Manager, Acquity UPLC Sample Manager, Acquity UPLC Column Heater/cooler, Acquity UPLC Diode Array Detector (PDA, set at 200–400 nm, 350 nm used for chromatogram extraction). Empower 2 software was used for data processing. The analyses were performed on Acquity UPLC BEH Shield RP18 column (50 × 2.1 mm i.d.; particle size, 1.7 μm; Waters); flow rate, 0.4 mL min^{-1}; column temperature, 22 °C. The temperature of the sample manager was set to 10 °C for analysis of standards and 22 °C for manure samples to avoid matrix precipitation (for details see Section 3.2.2). The mobile phase A, formic acid–water (0.1:99.9, v/v); B, ACN; gradient elution (min/%A): 0/95, 2.3/92, 2.8/80, 4.0/75, followed by 1.5 min equilibration step (total run time 5.5 min). For analysis of manure samples the gradient procedure was supplemented with 1.0 min wash step with 100% ACN.

2.5. Off-line MS detection

UPLC fractions evaporated to dryness and reconstituted in methanol–water–formic acid (50:50:0.1, v/v/v) were analyzed by direct infusion on an APEX-Ultra FTMS instrument equipped with a 9.4 T superconducting magnet and an Dual II electrospray ionization (ESI) ion source (Bruker Daltonics, Billerica, MA). The cell was