ELSEVIER

Journal of Chromatography B, 783 (2003) 11–23

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Determination of oxytetracycline and its degradation products by high-performance liquid chromatography–tandem mass spectrometry in manure-containing anaerobic test systems

Marie-Louise Loke[a,*], Sonja Jespersen[b], Rob Vreeken[c], Bent Halling-Sørensen[a], Jette Tjørnelund[a]

[a]*Department of Analytical and Pharmaceutical Chemistry, Royal Danish School of Pharmacy, Universitetsparken 2, 2100 Copenhagen, Denmark*

[b]*TNO Nutrition, Utrechseweg 48, Zeist, The Netherlands*

[c]*Micromass B.V., Transistorstraat 18, 1322 CE Alemere, The Netherlands*

Received 22 August 2001; received in revised form 24 June 2002; accepted 3 July 2002

Abstract

This paper describes the development of a HPLC–MS–MS (ESI) method with baseline separation of oxytetracycline, 4-epi-oxytetracycline, α-apo-oxytetracycline and β-apo-oxytetracycline using an XTerra column and an MeOH–MilliQ-water (containing 8 m*M* formic acid) mobile phase. Limits of quantification for aqueous standards were in the range of 0.004 to 0.008 μ*M*. The linear range tested was 0.003 to 0.5 μ*M* and in one case up to 17 μ*M*. An experiment simulating the degradation of oxytetracycline in manure was set up and free concentrations of the four antibiotics were determined during 6 months. Oxytetracycline (>0.02 μ*M*) was observed up till 6 months after spiking. No important increase in free concentrations of the degradation products was observed.

Keywords: Antibiotics; Oxytetracycline; 4-Epioxytetracycline; Apooxytetracycline

1. Introduction

Oxytetracycline (OTC) is a broad-spectrum antibiotic widely used for treatment or prevention of infections in pig productions. Pig manure is led to manure tanks and eventually spread on agricultural land. Thus oxytetracycline as well as its degradation products—if formed in manure—could be exposed to the environment resulting in a threat to human health [1,2]. Oxytetracycline is known to have a number of antimicrobial active degradation products, which have earlier been found in soil interstitial water, dosage forms and bulk drug substances, porcine muscle and hen's eggs and plasma [3–6]. The three compounds are 4-epi-oxytetracycline (EOTC), α-apo-oxytetracycline (α-Apo-OTC) and β-apo-oxytetracycline (β-Apo-OTC) (Fig. 1).

The bioavailable part of a compound in a specific matrix is considered to be the free fraction. Thus bioavailability, and with that determination of the

*Corresponding author. Tel.: +45-35-30-6453; fax: +45-35-30-6013.

E-mail address: mlc@dfh.dk (M.-L. Loke).

1570-0232/02/$ – see front matter
PII: S1570-0232(02)00468-3

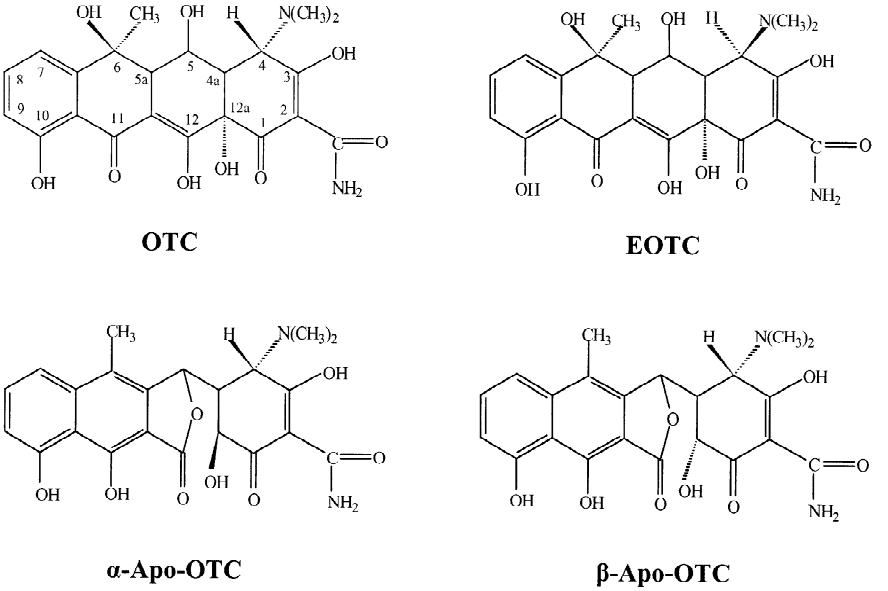

Fig. 1. Chemical structures of oxytetracycline (OTC), 4-epi-oxytetracycline (EOTC), α-apo-oxytetracycline (α-Apo-OTC) and β-apo-oxytetracycline (β-Apo-OTC).

free fraction, of a compound in a biological matrix is important in order to predict the possible uptake of the compound in the environment [7,8]. Thus, knowledge about the degradation of OTC and the degree of sorption of OTC and its degradation products to particles in the manure, are important factors when evaluating the fate of OTC [9].

A robust and sensitive method for the separation and quantification of low amounts of OTC, EOTC, α-Apo-OTC and β-Apo-OTC in matrices containing manure was therefore needed.

A few antibiotics have been determined in manure-containing matrices by HPLC–UV [1,2,10,11]. It is evident from these studies that the determination of oxytetracycline and its degradation products in low concentrations requires a more sensitive and selective detector, such as a mass spectrometer, if extensive sample preparation is to be avoided.

The two compounds EOTC and OTC have the same molecular masses and so does α-Apo-OTC and β-Apo-OTC. Thus the use of mass spectrometry as a sensitive detection method for the four compounds requires not only a mobile phase compatible with the MS-instrument, but also a chromatographic separation of EOTC and OTC as well as α-Apo-OTC and β-Apo-OTC, respectively.

A few publications describe the separation of OTC, EOTC, α-Apo-OTC and β-Apo-OTC by HPLC [4–6,12,13], but none of these methods offer baseline separation of EOTC and OTC.

Mass spectrometric analysis of oxytetracycline in different biological matrices using an electrospray interface on a variety of instruments like triple quadrupoles, ion traps or single quadrupole instruments has been reported by several authors [2,6,14–18]. Hamscher et al. detected low concentrations of OTC in liquid manure and soil using an ion trap instrument (ES, positive mode). However, neither separation nor detection of EOTC, α-Apo-OTC or β-Apo-OTC—which are known to have some potency compared to OTC [3]—was presented in their work [2]. Oka and Weimann both separated different tetracyclines via HPLC–MS (ESI, positive ionisation) but their work did not include detection of degradation products from these antibiotics [14,15]. Weimann and Bojesen also detected OTC in urine via column switching HPLC and tandem mass spectrometry [16]. For the tandem mass spectrometry analysis of OTC using electrospray and operation in the positive mode, Zurhelle et al. used a mobile phase consisting of acetonitrile–aqueous 0.5% formic acid [6]. However, no indications of separation

of compounds were stated using this HPLC-system. Blanchflower et al. separated tetracycline, oxytetracycline and chlortetracycline in muscle and kidney using MS-detection (APCI) and operation in a positive ion mode, but OTC could not be separated from EOTC in this HPLC–MS-system [18].

HPLC–MS–MS has not previously been used to separate and determine both oxytetracycline and its degradation products at the same time in manure or manure-containing samples. The HPLC–MS–MS method described in this paper makes it possible to baseline separate and quantify very low free concentrations of OTC and its three degradation products EOTC, α-Apo-OTC and β-Apo-OTC in manure-containing matrices.

2. Experimental

2.1. Antibiotics and chemicals

Oxytetracycline hydrochloride (M_w=496.9 g/mol) (Ph. Eur. 2nd. Ed) (CAS no. 2058-46-0) was purchased from Nomeco, UNIKEM (Copenhagen, Denmark). Oxytetracycline (M_w=460.4 g/mol) (CAS no. 79-57-2), 4-epi-oxytetracycline (M_w=460.4 g/mol) (CAS no. 35259-39-3), alpha-apo-oxytetracycline (M_w=442.4 g/mol) (CAS no. 18695-01-7) and beta-apo-oxytetracycline (M_w=442.4 g/mol) (CAS no. 18751-99-0) were purchased from Acros Organics (Geel, Belgium) and were all of 96–98% purity. Methanol of analytical grade was purchased from Merck (Darmstadt, Germany). Acetonitrile, HPLC-S, gradient grade was purchased from Biosolve (Valkenswaard, The Netherlands). Formic acid, pro analysis 98–100% was from Merck. The water used in the experiments was purified with a Millipore system (Bedford, MA, USA).

All salts used for the test medium in the anaerobic biodegradation experiment were purchased from Merck.

2.2. HPLC system

A Waters 2690 Alliance system (Milford, MA, USA) equipped with a cooled autosampler (10 °C) controlled the binary gradient system. A Waters XTerra RP18 column (150×3.0 mm I.D., 3.5 μm particles) was coupled with an XTerra RP18 guard column (20×3.0 mm I.D., 3.5 μm particles). Column temperature was 22(±2) °C.

The separation was performed by gradient elution. Mobile phase A was made by mixing 200 ml MeOH and 308 μl formic acid with MilliQ-water in a 1-l volumetric flask. Mobile phase B was made by mixing 950 ml MeOH and 308 μl formic acid with MilliQ-water in a 1-l volumetric flask. Both mobile phases were degassed by ultrasonication for 3 min. The gradient was as follows: 0–3.5 min 96% A and 4% B, 3.5–5.5 min a linear gradient to 10% A and 90% B, 5.5–13.0 min 10% A and 90% B, 13.0–13.5 linear gradient to 96% A, and 4% B, this mixture was maintained until 20.0 min. The injection volume was 40.0 μl and the flow-rate 0.40 ml/min.

2.3. MS system

All mass spectrometric measurements were performed on a Finnigan ion trap mass spectrometer (Thermo Finnigan, San Jose, CA, USA) equipped with an electrospray source. The instrument was operated in the positive ion mode and coupled to the outlet of the HPLC column via PEEK tubing. The temperature of the heated capillary was 220 °C. The nitrogen sheath and auxiliary gas flow was set at 80 and 20 (arbitrary units), respectively. Source voltage was set at 4.2 kV. Full scan data as well as centroid product ion scan data (dwell time 0.6 s) were collected when analysing samples.

Formation of product ions in the tandem MS experiments was done in two scan events—one for the precursor ions with m/z 461 (EOTC and OTC) and one for the precursor ions with m/z 443 (α-Apo-OTC and β-Apo-OTC). For mass 461 m/z the collision energy was set at 30.0% and the isolation width at 3.0. For mass 443 m/z the collision energy was set at 18.0% and the isolation width at 3.0.

Identification of the four oxytetracyclines was done using the retention time of each compound and the individual product ion fingerprint formed during fragmentation of the precursor ion, at that specific time. This relationship is illustrated in Fig. 2.

The product ions, and their proposed structures, used for identification and ratio measurements of

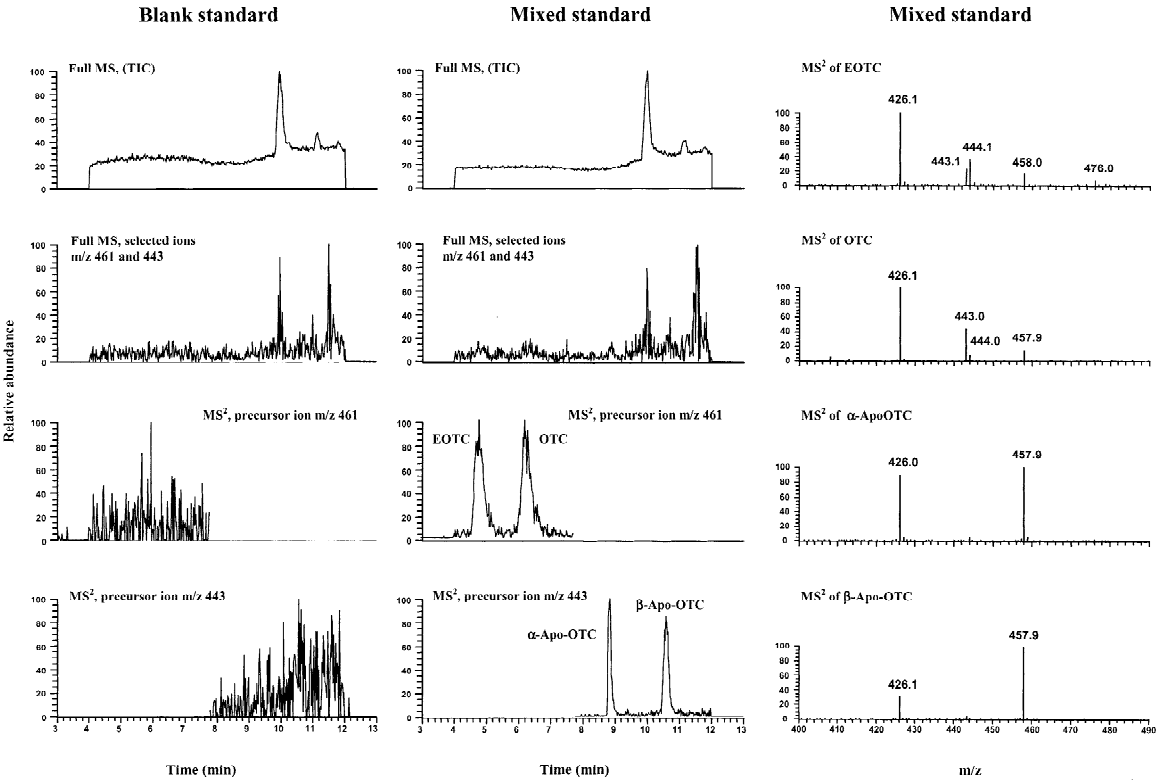

Fig. 2. HPLC–MS and HPLC–MS–MS chromatograms (data not smoothed) of a blank standard of 25% (v/v) MeOH–MilliQ-water and a mixed standard of EOTC and OTC (0.054 μ*M*) and α-Apo-OTC and β-Apo-OTC (0.057 μ*M*) along with the individual MS–MS-spectra of EOTC, OTC, α-Apo-OTC and β-Apo-OTC in the mixed standard.

EOTC, OTC, α-Apo-OTC and β-Apo-OTC are shown in Table 1.

Collection and treatment of data were done by use of Finnigan Xcalibur™ instrument software, version 1.0 SR1 (Thermo Finnigan, San Jose, CA, USA). All chromatograms were smoothed by a factor of 5.

Table 1
Molecular masses and proposed structures of the precursor and product ions of EOTC, OTC, α-Apo-OTC and β-Apo-OTC produced using the described positive ESI, tandem MS conditions

	Compound				Proposed structures of ions
	EOTC	OTC	α-Apo-OTC	β-Apo-OTC	
Precursor ion (*m*/*z*)	461	461	443	443	$[M+H]^+$
Most abundant product ions (*m*/*z*)	426	426			$[M+H-NH_3-H_2O]^+$
	443	443			$[M+H-H_2O]^+$
	444	444	426	426	$[M+H-NH_3]^+$
	458	458			$[M+H-NH_3-H_2O+CH_3OH]^+$
	476		458	458	$[M+H-NH_3+CH_3OH]^+$

2.4. Standards and recovery samples

Stock solutions of EOTC, OTC (434.3 μ*M*), α-Apo-OTC and β-Apo-OTC (452.1 μ*M*) were made in methanol and kept at −18 °C for a maximum of 1 month. Mixed or single standard solutions of the four compounds were made in 25% (v/v) MeOH in MilliQ-water or a mixture of (MeOH–MIM–MilliQ-water) (1:1:2, v/v) prior to the specific analysis of samples.

Non-spiked manure-containing matrix (e.g. from the control test bottles) made in the anaerobic degradation experiment (see Section 2.6) was used for the recovery experiments. This matrix contains 7.4% manure and is called manure incubation medium and henceforth this matrix will be referred to as MIM. To study the recovery of the four compounds in the manure-containing matrix (MIM), stock solutions of EOTC (434.4 μ*M*), α-Apo-OTC and β-Apo-OTC (452.1 μ*M*) were made in MeOH and a stock solution of OTC–HCl (1610.0 μ*M*) was made in MilliQ-water. Stock solutions were diluted in MilliQ-water resulting in OTC–HCl standards of 402.5, 40.3 and 4.0 μ*M* and EOTC standards of 43.4 and 4.3 μ*M* and α- and β-Apo-OTC standards of 45.2 and 4.5 μ*M*. For EOTC, α-Apo-OTC and β-Apo-OTC recovery was tested on two concentration levels, each with six replicates.

The recovery of OTC–HCl (i.e. OTC) was tested on four concentration levels each with six replicates. In all cases 2.00 ml of MIM were mixed with a specific volume of standard (40.0, 100.0 or 160.0 μl). This resulted in spiked concentrations of 3.62 and 0.36 μ*M* for EOTC, 80.50, 8.05, 0.81 and 0.32 μ*M* for OTC and 0.90 and 0.09 μ*M* for both α-Apo-OTC and β-Apo-OTC.

The tubes were closed and the samples left protected from light at room temperature (18 °C) for 30 min. Then MeOH was added to each sample making the total volume 4.00 ml. The samples were mixed on a whirl-mixer and filtered through a syringe filter 0.45 μm pore size (Minisart, Sartorius AG, Göttingen, Germany) into new plastic tubes. Then, 600.0 μl of the filtered sample were mixed firmly with 600.0 μl of MilliQ-water in 1.5-ml dark glass vials and stored at −18 °C until analysis.

Blank control samples were made by adding 40.0, 100.0 or 160.0 μl of MilliQ-water to 2.00 ml MIM instead of standard solution. For each added volume of water, six replicates were made and treated like the rest of the recovery samples.

2.5. Manure

Fresh pig-manure was obtained from a farm in the northern part of Zealand, Denmark. The manure was collected from a tank, where it had been kept for no more than 7 days. Antibacterial agents were not used in the production, or for treatment, of the pigs prior to the collection of manure. Thus the manure collected did not contain any of the investigated antibacterial agents. The raw manure was filtered through a 1 mm sieve into 1-l serum bottles where nitrogen was bubbled through manure for 15 min before closure of the bottles. The manure was stored for no more than 2 weeks at 4 °C before use. Every third day of storage gas was let out of the bottles by injecting a thin needle through the rubber in the cap of the bottles.

The pH in raw manure was 8.4 (21 °C) and the pH in fresh autoclaved MIM was 7.5 (21 °C) both measured with a PHM 95 pH/ion meter (Radiometer Denmark). The pH-meter was calibrated at pH 4.00 and pH 7.00 using buffers from Radiometer Analytical (Villeubanne, France).

2.6. Anaerobic degradation experiment

The anaerobic degradation experiment was based on an ISO-standard for evaluation of anaerobic biodegradation of organic compounds in sludge [19], and a method for determination of the anaerobic biodegradation potentials in sludge developed and validated by Madsen et al. [8].

The experiment was performed in 1-l serum bottles with a total liquid volume of 680 ml and a headspace of 100% N_2. The mineral medium used was prepared as described in ISO standard no. 11734 [19], however the reducing agent sodium sulfide nonahydrate was substituted by titanium(III) citrate and trace metals were added as described by Madsen et al. [8]. Resazurin (1.0 mg/l) was added as a redox indicator. As no reddish colouring of the test bottles from the Resazurin occurred and methane gas was

produced continually during the experiment, the test conditions were confirmed to be strictly anaerobic.

Two stock solutions of OTC–HCl (40.9 μ*M* and 27.8 μ*M*, respectively) were made in mineral medium under N_2. Sixteen test bottles containing 525.0 ml of mineral medium and 50.0 ml manure were prepared under N_2 and closed. Four of these bottles were spiked with 100.0 ml of the strongest of the two OTC–HCl stock solutions, and four bottles were spiked with the weaker OTC–HCl stock solution. This resulted in two times four test bottles with a total start concentration of 60.4 μ*M* OTC–HCl (30.0 mg/l) and 4.0 μ*M* OTC–HCl (2.0 mg/l), respectively. The remaining eight non-spiked bottles were autoclaved three times in 30 min at 120 °C. After cooling they were spiked with OTC–HCl similar to the non-autoclaved flasks. Prior to spiking, 5.0 ml of 0.2 m*M* titanium(III) citrate [20] were added to all bottles in order to reduce the medium. Two sets (autoclaved and non-autoclaved) of non-spiked control bottles were prepared as described above. However, these bottles were spiked with 100.0 ml of mineral medium instead of OTC–HCl stock solution.

To ensure that anaerobic conditions were maintained in the test bottles during the hold experiment, a syringe flushed three times with N_2 was used for sampling. Samples of 5.0 ml were taken from each test flask and mixed with 5.0 ml MeOH. Then the samples were centrifuged 5 min at ca. 10,000 *g* and filtered through a syringe filter 0.45 μm pore size (Minisart) into 1.5-ml glass vials. The vials were stored at −18 °C immediately, and kept there until analysed on the HPLC–MS-system.

To prevent changes in peak shape and retention times when analysing in the HPLC–MS–MS system all samples containing 50% MeOH were diluted 1:1 in MilliQ-water before analysing.

3. Results and discussion

3.1. Chromatography

In earlier publications the detection of OTC, EOTC, α-Apo-OTC and β-Apo-OTC caused problems with very long overall run time or insufficient separation of OTC and EOTC [6,7,13]. A few methods for separation of OTC and EOTC have been published but none of these shows the baseline separation needed when two compounds with the same masses and fragmentation pattern are to be detected via mass spectrometry [6,12,13].

Oxytetracycline has three pK_a values; 3.5, 7.6 and 9.2, where pK_{a1} = 3.3 is connected with the OH-group on C-3, pK_{a2} = 7.6 with the C-11–C-12 keto-enol system and pK_{a3} = 9.5 is associated with C-4 dimethyl amine [21]. Tetracyclines are known as strong metal chelating agents, which very easily form complexes with di- and trivalent metal ions [22–25], thus EDTA and oxalic acid are often added to the mobile phase to improve the chromatographic resolution [26].

In order to inhibit peak tailing and enhance separation of the four antibiotics, the column material should have a high purity and no or very low access to free silanol groups. Furthermore, it should have a high pH stability in order to make the use of acidic mobile phases possible over long periods of time. The silica-based column material Spherisorb 5-Phenyl from Chrompack and shielded hybrid materials XTerra MS and XTerra RP18 from Waters were tested along with the polymeric PLRP-S material. A variety of aqueous mobile phases containing either oxalic acid, formic acid or trifluoracetic acid were tested in combination with the organic modifiers methanol and acetonitrile. The XTerra materials showed excellent peak shapes and good baseline separation of all of the oxytetracyclines when using both oxalic and formic acid in concentrations of both 40 and 8 m*M*. The XTerra RP18 column with an inner diameter of 3.0 mm was chosen for the HPLC–MS–MS experiments as this gave very good conditions for operating with a flow of 0.4 ml/min—ideal for the selected MS-conditions.

Oxalic acid is a non-volatile compound thus capillary temperature below 300 °C could cause problems with clogging of the interface of the MS-instrument [14,16,26]. In order to prevent this, the mobile phase chosen was a mixture of MilliQ-water and MeOH with added formic acid to a concentration of 8 m*M*. Methanol was chosen as the organic modifier, as its capacity for ionisation of the oxytetracyclines was superior to MeCN. By using the low concentration of formic acid the mobile phases were kept at low pH (3.8), which prevented peak

tailing and enhanced the positive ionisation of the oxytetracyclines, without introducing problems such as solvent-induced suppression of the ionisation. Detection and separation of the four oxytetracyclines on the XTerra RP18 column is illustrated in Fig. 2 by a full scan, a HPLC–MS and a HPLC–MS–MS chromatogram of a standard mixture of EOTC, OTC, α-Apo-OTC and β-Apo-OTC. Individual MS–MS spectra of the antibiotics are also shown in Fig. 2.

The developed chromatographic system is very robust and small changes of the combination of the mobile phases or the gradient can be done without losing baseline separation or peak shape of the four compounds. However, the time of equilibration with low concentrations of organic modifier is crucial for obtaining reproducible chromatograms with baseline separated non-tailing peaks.

3.2. HPLC–MS–MS

The first step in the development of the HPLC–MS–MS procedure was to determine the type of ionisation. Pure standards of OTC, EOTC, α-Apo-OTC and β-Apo-OTC in MeOH–MilliQ-water (1:3, v/v) and in MeOH–MIM–MilliQ-water (1:1:2, v/v) were injected into a flow of 0.4 ml/min of mobile phase and tested on atmospheric pressure chemical ionisation (APCI) and electrospray ionisation (ESI). ESI in positive ion mode was found to be the best method for ionisation of the oxytetracyclines under the given conditions. This confirms the various reports in the literature, where mostly the positive ion mode is reported for the analysis of tetracyclines [14,15,27–31].

Proposed structures of the product ions observed in the HPLC–MS–MS spectra (Fig. 2) are given in Table 1. α-Apo-OTC and β-Apo-OTC are aromatic degradation products of OTC (see Fig. 1) thus loss of water from the C-6 and C-5a position, as observed for OTC and EOTC, is not possible. This corresponds nicely with the observed MS^2 spectra, showing only the $[M+H-NH_3]^+$ and $[M+H-NH_3+CH_3OH]^+$ ions for both apo-compounds.

The identity of ions with m/z 458 and m/z 476 has not been fully confirmed, but could be accounted to the formation of methanol adducts such as $[M+H-NH_3-H_2O+CH_3OH]^+$ and $[M+H-NH_3+CH_3OH]^+$ in the ion trap. The theory of this uncommon reaction is supported by the increased intensity of m/z 458 found in relation to the retention time, i.e. the increased percentage of methanol in the gradient, as well as the fact that these masses are not found when samples are analysed on a triple quadrupole instrument (API 3000) using the presented analytical method.

In order not to overload the MS-instrument with water-soluble ions from the matrix, viz. manure, the HPLC-flow was diverted to waste during the first 4 min of analysis.

The developed method was evaluated on analytical criteria such as linear range and limit of quantification. Validation data for the analytical system are presented in Table 2. All data are based on standards made in 25% (v/v) MeOH–MilliQ-water solutions, and used for the determination of free concentrations of antibiotics—if not otherwise mentioned. The data clearly show a linear behaviour of the calibration curve over the range tested, viz. three orders of

Table 2
Analytical data of the HPLC–MS–MS-method used to analyse the antibiotics EOTC, OTC, α-Apo-OTC and β-Apo-OTC

Analytical parameters	EOTC	OTC	α-Apo-OTC	β-Apo-OTC
Retention time[a] (min)	6.00	7.30	8.90	10.60
Linear concentration range[b] (μ*M*) (RSD%, $N=3$)	0.003–0.543 (49.1–3.2)	0.003–17.375 (37.0–2.9)	0.003–0.565 (48.8–1.7)	0.003–0.565 (15.0–0.4)
Correlation coefficient R^2, ($N=21$)	0.9539	0.9915	0.9972	0.9964
LOQ[c] (±SD, $N=3$)	0.004 (0.001)	0.004 (0.000)	0.006 (0.001)	0.008 (0.000)

[a] The retention time of the hold-up volume is 2 min.
[b] Standard solutions made in 25% MeOH–MilliQ-water (v/v).
[c] Lowest measured values where RSD<20%.

magnitude (0.003 μ*M* up to 0.565 μ*M*) with the exception of OTC, where 4–5 orders of linearity were measured. In the last case a weighting factor of $1/x$ was applied to reduce the influence of the highest concentration tested on the slope of the calibration curve.

LOQs for the test compounds were defined as the lowest concentrations measured where the relative standard deviation (RSD) was less than 20%.

3.3. Application to real-life samples

An anaerobic degradation experiment was performed over 6 months, simulating the degradation of OTC in a manure tank. When studying samples of environmental origin it is very often the free fraction of compound, i.e. the concentration of compound found in the aqueous phase that is of interest. The free fraction is considered as the bioavailable part of the chemical compound [7] and is important when estimating sorption coefficients, run-off and leaching properties as well as effects of the compounds on bacteria and the microflora in general [1,7].

The applicability of the analytical method was demonstrated by analysing the free concentration of OTC, EOTC, α-Apo-OTC and β-Apo-OTC in manure-containing samples.

3.3.1. Sample preparation

The biological activity in manure is extremely high. In order to stop all biological activity after collection of samples, the manure samples were mixed 1:1 with MeOH. Furthermore, these relatively high amounts of MeOH were necessary in order to induce flocculation of manure particles and increase the stability of the oxytetracyclines during storage. Flocculation of the manure particles made precipitation by centrifuging possible, after which filtration of the supernatant could be performed. Filtration was done to prevent changes in the distribution of antibiotics between the solid and the aqueous phase of the samples during storage.

3.3.2. Recovery in MIM

The recovery experiment was carried out using single standards of EOTC, OTC–HCl, α-Apo-OTC and β-Apo-OTC. In Fig. 3 HPLC–MS–MS chromatograms and MS^2-spectra of four spiked MIM samples are shown.

The recovery data of EOTC, OTC, α-Apo-OTC and β-Apo-OTC in MIM is shown in Table 3. In general very low recoveries were observed for all the tested antibiotics. Only samples spiked with 80.50 μ*M* OTC and 0.90 μ*M* β-Apo-OTC had recoveries above 70%. Especially, the recovery of EOTC was very low, even for high concentrations of compound, thus the spiked concentrations of EOTC had to be higher than for α-Apo-OTC and β-Apo-OTC in order to achieve reliable data.

The recovery percentages depended on the total amounts of antibiotic added, and all of the oxytetracyclines show decreasing recoveries with decreasing amounts of compound added. Therefore, it was not possible to specify one single overall recovery percentage for any of the test compounds.

Pig manure is a very heterogeneous and complex matrix containing metal ions such as K^+, Ca^{2+}, Mg^{2+}, Cu^{2+}, Zn^{2+}, Fe^{3+} and Na^+ as well as, ammonium ions, acetate ions and bicarbonate [32–34], and raw manure as well as MIM had a pH value between 7.4 and 8.5.

OTC is well-known for the ability of forming strong complexes with divalent cations like Mg^{2+} and Ca^{2+} [35] and its capacity of binding to humic acids, proteins and organic matter as well as anionic groups (e.g. silanol groups) in sand and soil [24,27,36]. Looking at the structure of EOTC, α-Apo-OTC and β-Apo-OTC it is clear that these compounds must also possess some of the strong abilities of complex binding. Reduced recoveries of OTC on C_{18} cartridges have been reported [12], and can probably be caused by secondary interactions of ionic or hydrogenic bonding [5]. Furthermore, specific drug–matrix or cartridge–matrix interaction may also reduce drug recovery [37].

In Table 3 the recoveries found in MIM when using 25% (v/v) MeOH–MilliQ-water standard solutions for calibration curves are shown along with recoveries calculated from standard solutions prepared in filtered matrix standard solutions (MeOH–MIM–MilliQ-water (1:1:2)). The calculation using matrix standards gave higher recoveries than when using water standards for the calibration curve. However, the overall picture of recoveries being below 50% and only samples spiked with 80.50 μ*M*

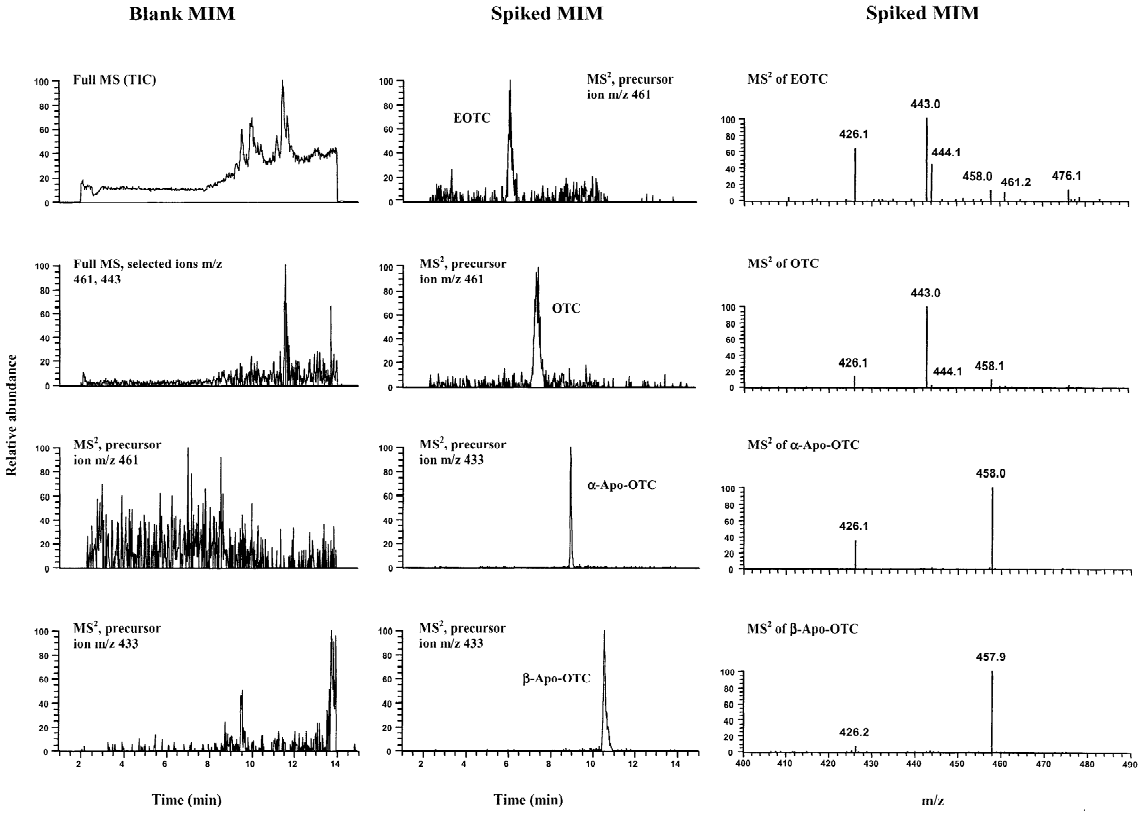

Fig. 3. HPLC–MS and HPLC–MS–MS chromatograms (data not smoothed) of single compound samples of EOTC, OTC, α-Apo-OTC and β-Apo-OTC along with individual MS–MS-spectra of the antibiotics observed in the MIM-containing recovery samples. Blank MIM was spiked with 40 μl MilliQ-water instead of antibiotics. All samples contain 25% (v/v) MeOH. Spiked concentrations of antibiotics in the samples presented are for EOTC = 0.36 μ*M*; OTC = 0.81 μ*M*; α-Apo-OTC and β-Apo-OTC = 0.90 μ*M*. Free concentrations of antibiotics found in the recovery experiment are presented in Table 3.

Table 3
Recovery of EOTC, OTC, α-Apo-OTC and β-Apo-OTC in the manure-containing matrix (MIM)

Compound	Added in MIM (μ*M*)	Found in MIM (μ*M*) based on standards in:		Recovery in MIM (%) based on standards in:		SD		RSD (%) MilliQ-w/matrix
		MilliQ-w	Matrix	MilliQ-w	Matrix	MilliQ-w	Matrix	
EOTC	3.62	0.54	1.19	14.9	33.0	2.1	4.6	13.9
	0.36	0.03	0.07	8.9	19.7	1.9	4.2	21.3
OTC	80.50	57.18	72.28	71.0	90.4	6.4	8.2	9.1
	8.05	2.66	3.38	33.0	42.0	1.8	2.3	5.5
	0.81	0.15	0.19	18.6	23.7	5.2	6.7	28.1
	0.32	0.06	0.16	18.0	51.8	4.5	12.9	24.9
α-Apo-OTC	0.90	0.29	0.59	31.9	65.7	7.3	15.0	22.8
	0.09	0.008	0.017	8.9	18.3	2.6	5.4	29.8
β-Apo-OTC	0.90	0.72	0.78	79.0	86.4	6.9	7.5	8.7
	0.09	0.05	0.051	51.7	56.5	18.3	20.0	35.3

Data in columns marked ''MillliQ-w'' are calculated via calibration curves made from standard solutions in MeOH–MilliQ-water. Data in columns marked ''matrix'' are based on standard solutions made in filtered manure-containing matrix (MeOH–MIM–MilliQ-w) (1:1:2). All data derive from six replicates.

OTC and 0.90 μ*M* β-Apo-OTC having recoveries above 70% remained the same. Comparison of the recoveries calculated from pure standards and standards made in filtered matrix indicates that the low recovery rates are caused mainly by strong sorption and complex binding of the test compounds to particles in the non-filtered MIM.

As the manure matrix will always contain colloids and metal ions capable of binding oxytetracyclines, it is not possible to define whether the enlarged concentrations of compound, found when using matrix standards, is due to compensation of suppression of ionisation in the MS-instrument or binding of test compound to organic colloids and metal ions in the filtered matrix.

However, as very high distribution coefficients of OTC to solids were also previously found for soils [38] and manure [1] it is not surprising that the recovery of the four oxytetracyclines in MIM is low, and most likely depends on the degree of binding of the antibiotics to mineral-cations and organic matter in the matrix or the medium used for preparation of the stock solution of OTC–HCl.

Interday recovery data are presented in Table 4 and show that the test compounds are not degraded in the manure-containing solutions during storage.

Thus we believed that the low recovery of EOTC, OTC, α-Apo-OTC and β-Apo-OTC is due to high amounts of oxytetracyclines being bound to particles in the manure matrix (MIM) rather than fast degradation to unknown compounds. A final confirmation of this hypothesis can only come from an extensive extraction procedure development for OTC in manure, which is outside the scope of this paper.

3.3.3. Data from the degradation experiment

The information of interest in this specific experiment was the free (i.e. bioavailable) concentration of OTC as well as the identity and free concentrations of degradation products formed in the manure matrices. By using the sensitive and very selective HPLC–MS–MS method developed, these data were collected successfully.

Fig. 4A illustrates the average free concentration of OTC found in the four different test settings during the experiment. On the first day of sampling (i.e. shortly after spiking) the free concentration of OTC found in the autoclaved and non-autoclaved bottles spiked with 60.4 μ*M* OTC was 13.0 and 6.0 μ*M*, respectively. These concentrations account for 21% and 10% of the added amount of OTC, and are lower than expected from the results of the recovery experiment. In these bottles, OTC was found in concentrations between 1.0 and 13.0 μ*M* during the first week. Hereafter OTC was measured in concentrations between 0.74 and 0.02 μ*M* during all 6 months of sampling. In bottles spiked with 4.0 μ*M* of antibiotic, free concentrations of OTC between 0.52 and 0.01 μ*M* were found during the first 3 weeks. After this the average concentrations of OTC were no longer significantly different from zero. Also in this case the recovery found shortly after spiking was extremely low (4–13%).

The average free concentration of OTC, EOTC, α-Apo-OTC and β-Apo-OTC found in test bottles containing non-autoclaved MIM spiked with 60.4 μ*M* OTC is shown in Fig. 4B. The total free concentration of antibiotics measured is also given in Fig. 4B. In all types of test bottles the picture was the same as shown in Fig. 4A, i.e. a drastically decreasing free concentration of OTC within the first hours and days but no increase of importance in the free concentrations of the degradation products. In general EOTC was not found in the test bottles, whereas low and slightly fluctuating average free concentrations of α-Apo-OTC (0.2–0.5 μ*M* in bot-

Table 4
Inter-day recovery data with 95% confidence intervals found for OTC in the manure-containing matrix using the described HPLC–MS–MS method. All data derive from six replicates

Compound	Spiked conc. in MIM (μ*M*)	Day 1		Day 3	
		Recovery (%)	95% Conf. (%)	Recovery (%)	95% Conf. (%)
OTC	80.5	54.3	47.9–60.7	71.0	58.2–83.8
	8.05	22.6	19.8–25.4	33.0	29.4–36.6
	0.81	17.3	13.1–21.5	18.6	8.2–29.0
	0.32	/	/	18.0	9.0–27.0

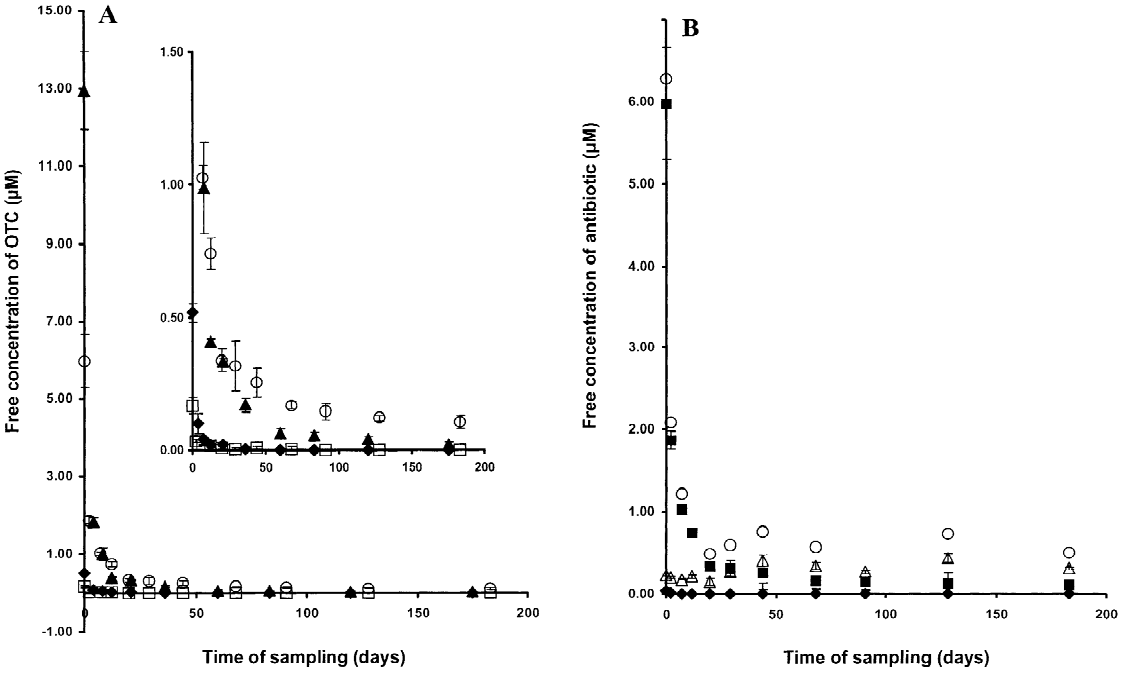

Fig. 4. (A) Free concentrations of OTC (μ*M*) found in the four different set-ups of the degradation experiment. Test bottles were: ♦ autoclaved, spiked with 4.0 μ*M* OTC; □ non-autoclaved, spiked with 4.0 μ*M* OTC; ▲ autoclaved, spiked with 60.4 μ*M* OTC and ○ non-autoclaved, spiked with 60.4 μ*M* OTC. First measuring point was 2 h after spiking of bottles. (B) Free concentrations (μ*M*) of: ♦ EOTC, ■ OTC, △ α-Apo-OTC, □ β-Apo-OTC and ○ total amount of antibiotics, found in the anaerobic degradation experiment where non-autoclaved test bottles were spiked with 60.4 μ*M* OTC. First time of measuring was 2 h after spiking. All values in (A) and (B) are average values of four replicates. Standard deviations are given as error bars.

tles spiked with 60.4 μ*M* OTC and 0.01–0.06 μ*M* in bottles spiked with 4.0 μ*M* OTC) were found during all 6 months of sampling. In the bottles spiked with 60.4 μ*M* of OTC very low average free concentrations of β-Apo-OTC (<0.10 μ*M*) were detected during all 6 months. No β-Apo-OTC was found in bottles spiked with low amounts of OTC.

As the binding abilities of each compound to manure particles are not finally clarified, it cannot be determined whether one of the degradation products is formed in superior amounts.

Due to the variations in the recoveries found for each of the oxytetracyclines it was not possible to calculate the total amount (bound and free) of antibiotics in the samples. However, as the recovery experiment confirmed that the recovery of all four oxytetracyclines in MIM—shortly after spiking—is very low, we believe that sorption (including chelation of OTCs with metals and organics present in the MIM) is the dominant reason for finding very low (bioavailable) free concentrations of antibiotics in the experimental samples. Thus consistent with Hamscher et al. [2] we expect OTC residues to be transported to the environment with the solid part of manure, after which OTC and its degradation products are expected to accumulate in the soil. Consequently there is no immediate risk of run-off to streams or leaching to the groundwater of free bioavailable OTCs.

4. Conclusion

A very robust chromatographic method with optimal baseline separation of OTC and its degradation products EOTC, α-Apo-OTC and β-Apo-OTC was developed using only MS-compatible volatile mobile phases containing MeOH, MilliQ-water and formic acid. A sensitive and very selective detection of the four antibiotics was performed on an ion trap instrument. The limits of quantification (LOQ) in

25% (v/v) MeOH–MilliQ-water standards were found to be: 0.004 (±0.000) μ*M* for OTC, 0.004 (±0.001) μ*M* for EOTC, 0.006 (±0.001) μ*M* for α-Apo-OTC and 0.008 (±0.000) μ*M* for β-Apo-OTC.

An anaerobic degradation experiment was performed over 6 months, simulating the degradation of OTC in a manure tank. The developed HPLC–MS–MS method was successfully used for quantification of the free concentration of OTC, EOTC, α-Apo-OTC and β-Apo-OTC in this very complex manure-containing matrix.

The recovery of the four antibiotics in the liquid part of the manure matrix (MIM) was studied and found to be very low (often <52%) and dependent on the spiked amount of compound.

During the anaerobic degradation experiment a drastic decrease of the free (i.e. bioavailable) concentration of OTC was observed within a few days. In bottles spiked with 60.4 μ*M* and 4.0 μ*M* OTC, very low free concentrations of OTC were measured during 6 months and 2 months, respectively. No increase of importance in the free concentrations of the degradation products was observed.

Literature studies and earlier performed experiments lead us to believe that the very low free concentrations of OTC, EOTC, α-Apo-OTC and β-Apo-OTC found in both the recovery study and the anaerobic degradation experiment is due to high amounts of oxytetracyclines being bound to particles in the manure matrix rather than degradation to unknown compounds.

Acknowledgements

All analytical work was done at TNO Nutrition in Zeist, The Netherlands. The author gratefully acknowledges the use of the laboratory facilities at TNO. A special thanks to Dr. Elwin R. Verheij at TNO Nutrition for making the collaboration possible. This investigation was funded by a grant from the Danish Centre for Sustainable Land Use and Management of Contaminants, Carbon and Nitrogen, under the Danish Strategic Environmental Research Programme, Part 2, 1997–2000—this is gratefully acknowledged.

References

[1] M.-L. Loke, J. Tjørnelund, B. Halling-Sørensen, Chemosphere 48 (2002) 351.
[2] G. Hamscher, S. Sczesny, H. Höper, H. Nau, Anal. Chem. 74 (2002) 1509.
[3] B. Halling-Sørensen, G. Sengeløv, J. Tjørnelund, Arch. Environ. Contam. Toxicol 42 (2002) 263.
[4] P.D. Bryan, J.T. Stewart, J. Pharm. Biomed. Anal. 5 (12) (1994) 675.
[5] R.W. Fedeniuk, S. Ramamurthi, A.R. McCurdy, J. Chromatogr. B 677 (1996) 291.
[6] G. Zurhelle, E. Müller-Seitz, M. Petz, J. Chromatogr. B 739 (2000) 191.
[7] C.J. van Leeuwen, J.L.M. Hermens, Risk Assessment of Chemicals: An Introduction, Kluwer Academic, The Netherlands, 1995.
[8] T. Madsen, H.B. Rasmussen, L. Nilsson, Chemosphere 31 (10) (1995) 4243.
[9] S.E. Jørgensen, B. Halling-Sørensen, Chemosphere 40 (2000) 691.
[10] M. Kühne, D. Ihnen, G. Möller, O. Agthe, J. Vet. Med. A 47 (2000) 379.
[11] M.-L. Loke, F. Ingerslev, B. Halling-Sørensen, J. Tjørnelund, Chemosphere 40 (2000) 759.
[12] E.J. Mulders, D. van de Lagemaat, J. Pharm. Biomed. Anal. 7 (12) (1989) 1829.
[13] N.H. Khan, E. Roets, J. Hoogmartens, H. Vanderhaeghe, J. Chromatogr. 405 (1987) 229.
[14] H. Oka, Y. Ikai, Y. Iyo, J. Hayakawa, K. Harana, M. Suzuki, H. Odani, K. Maeda, J. Chromatogr. B 693 (1997) 337.
[15] A. Weimann, G. Bojesen, Anal. Lett. 31 (12) (1998) 2053.
[16] A. Weimann, G. Bojesen, J. Chromatogr. B 721 (1999) 47.
[17] V.H. Vartanian, B. Goolsby, J.S. Brodbelt, Am. Soc. Mass Spectrom. 9 (1998) 1089.
[18] J.W. Blanchflower, R.J. McCracken, A.S. Haggan, D.G. Kennedy, J. Chromatogr. B 692 (1997) 351.
[19] ISO, Water Quality—Evaluation of the ''Ultimate'' Anaerobic Biodegradability of Organic Compounds in Digested Sludge—Method by Measurement of the Biogas Production, International Organisation for Standardisation, 1998, DS/EN ISO, 11734.
[20] A.J.B. Zehnder, K. Wuhrmann, Science 194 (1976) 1165.
[21] F.A. Hochstein, C.R. Stephens, L.H. Conover, P.P. Regna, P. Pasternack, P.N. Gordon, F.J. Pilgrim, K.J. Brunings, R.B. Woodward, J. Am. Chem. Soc. 75 (22) (1953) 5455.
[22] R.K. Blackwood, in: J.J. Hlavka, J.H. Boothe (Eds.), The Tetracyclines, Springer-Verlag, Berlin, 1985, p. 59.
[23] S.T. Day, W.G. Crouthamel, L.C. Martinelli, J.K.H. Ma, J. Pharm. Sci. 67 (11) (1978) 1518.
[24] B.B. Sithole, R.D. Guy, Air Soil Pollut. 32 (1987) 315.
[25] K. Florey (Ed.), Analytical Profiles of Drug Substances, Vol. 13, Academic Press, New York, 1984.
[26] H. Oka, Y. Ito, Y. Ikai, T. Kagami, K. Harada, J. Chromatogr. A 812 (1998) 309.
[27] H. Oka, H. Nakazawa, K. Harada, J.D. MacNeil (Eds.), Chemical Analysis for Antibiotics Used in Agriculture, AOAC International, Arlington, USA, 1995, p. 333.

[28] K. De Wasch, L. Okerman, S. Croubles, H. De Brabander, J. Van Hoof, P. De Backer, Analyst 123 (1998) 2737.
[29] H. Nakazawa, S. Ino, K. Kato, T. Watanabe, Y. Ito, H. Oka, J. Chromatogr. B 732 (1999) 55.
[30] K. Harada, K. Masuda, M. Suzuki, H. Oka, Y. Ikai, J. Hayakawa, Org. Mass Spectrom. 28 (1993) 1512.
[31] N. Van Eeckhout, J.C. Perez, J. Claereboudt, R. Vandeputte, C. Van Peteghem, Rapid Commun. Mass Spectrom. 14 (2000) 280.
[32] M. Choudhary, L.D. Bailey, C.A. Grant, Waste Manage. Res. 14 (1996) 581.
[33] A.I. De la Torre, J.A. Jiménez, M. Carballo, J.R. Fernandez, M.J. Muñoz, Chemosphere 41 (2000) 1629.
[34] J. Japenga, K. Harmsen, Neth. J. Agric. Sci. 38 (1990) 353.
[35] B.T. Lunestad, J. Goksøyr, Dis. Aquat. Org. 9 (1990) 67.
[36] H. Pouliquen, H. Le Bris, Chemosphere 33 (5) (1996) 801.
[37] B. Law, S. Weir, J. Pharm. Biomed. Anal. 10 (1992) 181.
[38] M. Rabølle, N.H. Spliid, Chemosphere 40 (2000) 715.