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ORIGINAL PAPER

Development of a UHPLC-MS/MS method for the measurement of chlortetracycline degradation in swine manure

Weilin L. Shelver · Vincent H. Varel

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Abstract An ultra-high-performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) method was developed capable of simultaneously measuring chlortetracycline (CTC), epi-chlortetracycline, and isochlortetracycline (ICTC), as well as other structurally related tetracyclines in swine manure. A simple sample preparation was used consisting of extraction, dilution, centrifugation, and ultrafiltration. The concentrations of analyte were calculated using d_6-tetracycline as an internal standard in the matrix-matched standard curve. A solvent gradient resolved the compounds in 3.5 min with an additional 1.5 min of re-equilibration allowing the analyses of a large number of samples in a short period of time. MS/MS was used as the detection method giving analyte confirmation in addition to a large dynamic range and low detection limit. The UHPLC-MS/MS method successfully resolved multiple degradation products of CTC from the complex manure matrix. The method detection limits ranged from 1.9 pg/μL for CTC to 7.3 pg/μL for ICTC, and the calibration curve was linear from 1 to 10,000 pg/μL. The method was tested by measuring CTC and its degradation products as a function of time in incurred swine manure that had been incubated at three different temperatures (22 °C, 38 °C, and 55 °C). CTC concentration at 22 °C decreased 44% after 25 days; greater percentage decreases were observed when the manure was stored at elevated temperatures (96% and 98% for 38 °C and 55 °C, respectively). The concentration of the microbiologically inactivate isomer, ICTC, increased over the incubation period. At 22 °C, ICTC continued to increase through 25 days of incubation; at 38 °C, ICTC concentration plateaued on day 14 while at 55 °C ICTC concentration plateaued on day 7, with concentration increases of 198%, 374%, and 282% for 22 °C, 38 °C, and 55 °C, respectively.

Keywords Analysis · Measurement · Manure treatment · Tandem MS

Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the product to the exclusion of others that may also be suitable. USDA is an equal-opportunity provider and employer.

W. L. Shelver (✉)
USDA-Agricultural Research Service,
Biosciences Research Laboratory,
1605 Albrecht Boulevard,
Fargo, ND 58102, USA
e-mail: Weilin.Shelver@ars.usda.gov

V. H. Varel
USDA-Agricultural Research Service,
U.S. Meat Animal Research Center,
P. O. Box 166, Clay Center, NE 68933, USA

Introduction

Tetracyclines are a group of antibiotics that are commonly used in animal husbandry for prophylaxis or treatment of infections and as a growth promoter. Among the tetracyclines, chlortetracycline (CTC) is most commonly used in swine production in the USA [1], although oxytetracycline and tetracycline are also frequently used [2]. In addition to the uses stated above, chlortetracycline also decreases fecal shedding of *Escherichia coli* O157:H7 by swine [3]. Animal wastes containing excreted antibiotics such as CTC are often stored in lagoons for a period of time before the lagoon contents are applied to nearby fields. The persistence of antibiotics can lead to environmental problems such as antimicrobial resistance [4] or even genetic toxicity in plants [5]. Sensitive analytical methods have been used to demonstrate the presence of CTC in manure, agriculture wastewaters, and in ground water near hog-raising facilities

[2, 6, 7]. The rate of CTC decomposition in manure is not well characterized, and more work needs to be done in order to minimize the probability of environmental CTC contamination.

The structure of CTC (Fig. 1) allows facile epimerization to form epi-chlortetracycline (epi-CTC) as well as an intramolecular rearrangement resulting in the formation of ICTC. Consequently, aqueous samples of CTC will often contain varying amounts of all three compounds. In addition, each form can undergo keto-enol tautomerization, making a total of six species potentially present in the analysis of samples containing CTC. The disappearance of CTC in aqueous solution is a function of ionic strength, pH, and temperature [8]. Rapid transformation occurs at alkaline pH, and decomposition is markedly accelerated as the temperature increases from 7 °C to 35 °C. Under acidic conditions (pH 2–6), epimerization can occur at the C-4 position producing 4-epi-chlortetracycline. Under basic conditions, CTC rearranges with nucleophilic attack of the C-6 hydroxyl group on the C-11 carbonyl group to form ICTC as well as the corresponding epimer, epi-ICTC, as was noted in the analysis of chicken eggs [9]. ICTC is inactive in microbiological growth inhibition tests, but its toxicological properties have not been explored [9]. Most of the early analyses for CTC did not specifically determine all of the CTC forms [10] and sometimes focused on epi-CTC and CTC only [11]. Others [12] have simultaneously quantified enol-CTC, keto-CTC, enol-4-epi-CTC, and keto-4-epi-CTC in milk. A limitation of the method was that ICTC and keto-CTC were not resolved. Since ICTC was not thought likely to occur in milk, the poor resolution was considered unimportant [12]. However, the substantial quantities of ICTC that accumulated in the hen's egg [9] demonstrated the importance of CTC and ICTC separation/quantification in the analytical method.

UHPLC-MS has gained popularity in residue analyses because of efficient analysis times, minimal sample size requirements, and reduced solvent use compared with traditional HPLC [11]. Tandem mass-spectrometric detection is very specific and sensitive, providing positive confirmation of the analyte. Because of these properties, UHPLC-MS/MS methods are increasingly used for environmental and food safety studies. Few UHPLC analyses for CTC have been reported. Although UHPLC-MS/MS analysis was applied to CTC in plasma, the epimer or tautomers of CTC were not quantified by the analysis [12]. Likewise, the development of a UHPLC-UV method for tetracyclines, including chlortetracycline and its epimer, has been recently reported [13], but the iso-forms of CTC were not measured.

The objective of this study was to develop a rapid UHPLC-MS/MS method capable of analyzing CTC, epi-CTC, and ICTC with a low detection limit. A secondary objective was to detect other tetracyclines commonly used in the swine industry such as tetracycline and oxytetracyline. The UHPLC-MS/MS method was then applied to measure CTC and its degradation products in incurred swine manure

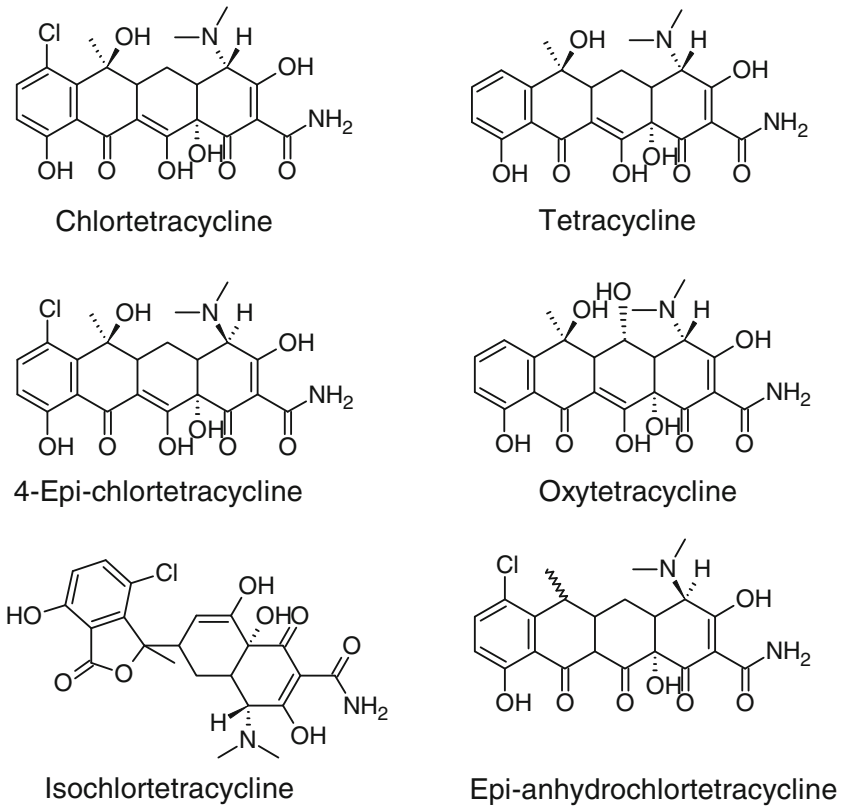

Fig. 1 Structures of tetracyclines used in this study

samples from digesters incubated at three different temperatures as a function of time to provide information to minimize potential environmental impact of CTC in swine manure.

Materials and methods

Materials

Nanosep centrifugal filters with a 10 K omega cutoff were obtained from Pall Life Sciences (P/N OD010C35, Port Washington, NY, USA). Oasis HLB cartridges were purchased from Waters Corporation, Milford, MA, USA. Chlortetracycline hydrochloride was obtained from Fluka Chemie AG (Buchs, Switzerland). Oxytetracycline hydrate and tetracycline were purchased from Sigma-Aldrich (St. Louis, MO, USA). Epi-CTC, iso-CTC, epi-anhydro-CTC, and d_6-tetracycline were obtained from Toronto Research Chemicals (North York, Ontario, Canada). All other chemicals and solvents were obtained from common laboratory suppliers.

Sample preparation

Solid-phase extraction

Samples (1 g) were added to McIlvaine buffer with 0.1 M EDTA (each liter contains 28.4 g of disodium phosphate, 19.2 g of citric acid, and 29.2 g of disodium EDTA), pH 4, (10 mL), centrifuged at 1,300×*g* for 15 min, and the supernatant was loaded onto an Oasis HLB column (60 mg). The column was pre-conditioned with 1.2 mL each of MeOH, 0.5 M HCl, and H_2O. The cartridges were then washed with 1.2 mL of H_2O and 10% MeOH and eluted with 4 mL of CH_2Cl_2/acetone (3:2). After evaporating the solvent, samples were reconstituted with 200 μL of 10% MeOH with 0.2% formic acid.

Filtration

One gram of digester contents was combined with 4 mL of nanopure water, the sample vortexed, and then centrifuged at 1,300×*g* for 5 min. The supernatant was removed, centrifuged at 18,000×*g* for 15 min, and the resulting supernatant filtered through a Nanosep 10 K omega centrifuge filter. The filtrate was used to measure the CTC concentration.

Standard preparation

Method of standard addition

Standard addition samples were prepared by adding chlortetracycline standards (final concentrations 200, 500, 1,000, 2,000 pg/μL) together with 500 pg/μL of d_6-tetracycline in 10% MeOH with 0.2% formic acid to each sample. The unknown concentration was computed from the linear regression line *x* intercept in the usual method for standard addition computations.

Matrix-matched standards

Matrix-matched standards were prepared from swine waste filtrate from animals not treated with chlortetracycline. For the standard curve, CTC, epi-CTC, ICTC, epi-anhydro-CTC, oxytetracycline, and tetracycline were added giving final concentrations of 0, 1, 5, 10, 50, 100, 500, 1,000, 5,000, and 10,000 pg/μL, and each sample contained 500 pg/μL of d_6-tetracycline as the internal standard. The unknown concentrations were computed from the standard curve.

UHPLC-MS/MS instrumental analysis

The UHPLC-MS/MS system consisted of a Waters ACQUITY™ LC system in combination with a Waters ACQUITY™ Triple Quadrupole Mass Spectrometer using MassLynx™ 4.1 with TargetLynx™ for acquisition, processing, and quantitation (Waters Corporation, Milford, MA, USA). A Waters ACQUITY™ UPLC BEH C_{18} column (1.7 μM, 2.1×50 mm) with a Waters Vanguard precolumn (1.7 μM, 2.1×5 mm) was used to achieve analyte separation. The gradient system consisted of: solvent A= H_2O/MeOH 95:5 containing 0.2% formic acid; solvent B= CH_3CN/MeOH 40:60 containing 0.2% formic acid. The gradient was as follows—0–0.5 min, 10% B; 0.5–1.5 min, 10% B to 100% B; 1.5–3.5 min, hold at 100% B; 3.5–3.52 min, 100% B to 10% B, and 3.52–5 min, hold at 10% B. The column temperature was maintained at 40 °C, and the auto-sampler was maintained at 4 °C. The flow rate was 0.6 μL/min, and the injection volume was 10 μL with a total run time of 5 min per sample. The mass spectrometer was operated using electro-spray ionization in the positive ion mode with multiple reaction monitoring. The source temperature was set at 150 °C, and the desolvation temperature was set at 500 °C. Capillary voltage was set at 3.2 kV. Cone gas flow of nitrogen was set at 50 L/h, and desolvation gas flow was at 800 L/h while collision gas flow of argon was set at 0.17 mL/min. In preliminary experiments, each analyte was directly infused using electro-spray ionization in the positive mode to identify product ions; optimum cone voltage and collision energies were obtained using the Autotune function. For each analyte, the summed peak areas of three transitions were used for quantitation (Table 1). Compound concentration was computed using d_6-tetracycline as the internal standard with the equation: concentration=analyte area×(IS concentration/IS area) using TargetLynx™ software.

Table 1 Mass spectrometer operating conditions, precursor ions, and product ions for tetracyclines

Compound	Precursor ion	Product ions	Collision energy (eV)	Cone voltage
4-Epi-anhydro-chlortetracycline	461.0	97.9	20	40
		154.0	40	
		444.0	30	
4-Epi-chlortetracycline	479.3	97.9	40	40
		444.0	20	
		462.0	20	
Chlortetracycline	478.9	154.0	30	30
		443.9	20	
		461.9	15	
Isochlortetracycline	479.1	154.0	25	35
		194.9	35	
		462.0	45	
d_6-Tetracyline	451.0	160.0	25	30
		416.0	20	
		433.2	15	
Oxytetracycline	461.0	201.1	45	30
		425.9	20	
		443.3	15	
Tetracycline	444.9	154.0	25	30
		410.0	20	
		427.3	15	

Assay parameter determination

Seven individual samples of diluted control swine manure spiked at 5 pg/μL for ICTC, 100 pg/μL for 4-epi-anhydro-CTC, and 10 pg/μL for CTC, oxytetracycline, and tetracycline were used to establish the method detection limit. Chlortetracycline (500 ng/mL) was stored in either a borosilicate or a high-density polyethylene container for 1, 2, and 5 days at 4 °C and the concentration determined. In a second set of experiments, a 500-ng/mL working solution was stored at −20 °C, 4 °C, and ambient temperature for 21 days and the concentration determined to measure stability. The percent CVs were determined utilizing samples spiked to a 500 ng/mL level of each analyte in a blank matrix. Each sample was injected three times in 1 day and the results used to determine within-run variation. The between-day variation was obtained from the average of three sets of injections analyzed on three separate days. Matrix interferences were determined by spiking all the analytes into mobile phase and into control manure extracts at concentrations of 500 ng/mL, analyzing the peak areas, and comparing the results. Calibration curves were obtained by fitting calculated concentrations with a linear function (1/× weighting).

Swine waste and their treatment

A composite cross of the Duroc, Landrace, and Large White ~180 lbs swine were used for this study. Swine were grouped into eight animals per pen, and ten pens were used. The animals were fed with a standard growing/finishing diet with or without 55 mg/kg of CTC as described by Yen [14]. Manure was collected from ten pens between 21 and 28 days after initiation of treatments and pooled between pens. Sets of digesters were adapted to 22 °C, 38 °C, or 55 °C in incubators, with three digester flasks (2 L) per temperature. Each digester was charged with approximately 6% of manure solids. After thorough mixing, a 10-g sample was removed from each digester, at 0, 1, 3, 7, 14, 21, and 25 or 28 days and stored at −80 °C until utilized for determination of CTC and CTC-degradation products. The pH of the manure slurry in the digesters ranged between 6.2 and 7.3 for the 22 °C treatment, 6.1–6.3 for the 38 °C treatment, and 6.2–6.6 for the 55 °C treatment.

Statistical analyses

A repeated-measure two-way ANOVA to test for significant differences was performed (SigmaPlot® ver. 11.2, Systat Software, Inc., San Jose, CA). Each pen was used as the repeated measure, with temperature and day as the treatments and the data being the concentration of CTC or ICTC. The data was transformed by taking the natural logarithm and the tests (Shapiro–Wilk) for normality and constant variance set at the 0.01 levels (all were >0.05 except CTC normality at $p=0.049$) demonstrating the critical assumptions (normal distribution and constant variance) needed for parametric statistics were met. All pairwise multiple comparison procedures (Holm–Sidak method) were tested for at

the 0.05 level. Both CTC and ICTC showed a normal distribution and constant variance, and each of the treatments showed significant differences, and their interactions were significant.

Results and discussion

LC-MS/MS development

We tested two different types of Waters UHPLC columns, ACQUITY™ UPLC BEH C_{18} column and ACQUITY™ UPLC HSS C_{18} column and have found that the BEH column was more suitable for the tested compounds.

Different solvents were tested for the best elution of the target compounds. Although an increase in methanol (constant formic acid at 0.2%) eluted the target compounds, peak broadening including front tailing for chlortetracycline and back tailing for oxytetracycline was observed. A 60/40 mixture of methanol and acetonitrile with 0.2% formic acid produced sharp and symmetric peaks during the elution process. A gradient using methanol with longer analysis time (6.5 vs 5 min) also produced satisfactory results, but we elected to use the former conditions because of the shorter analysis time.

As described by Tylová et al. [15], the UHPLC has greatly reduced the analysis time from previously reported HPLC studies (run time, 25–45 min) [16, 17]. Our analysis time was 5 min/sample compared with Tylová et al.'s 6.5 min. Unlike Tylová et al. who used UV detection and subsequently off-line MS detection after isolation of the UHPLC fraction, we utilized UHPLC-MS/MS to simultaneously separate and identify our analytes.

Sample preparation and analysis

In this study, three different methods were tested for chlortetracycline analysis: solid-phase extraction, ultrafiltration with standard addition, and ultrafiltration with matrix-matched calibration curves. We have utilized HLB solid-phase cartridges with samples adjusted to either pH 4 or pH 7 and have found in most cases the recoveries are comparable, but pH 4 produced more consistent recoveries. Using different elution conditions (pure solvent or mixture of methanol, ethyl acetate, acetone, and methylene chloride with or without acid or base modifier) produced variable results, and we found that using CH_2Cl_2/acetone (3:2) as eluant produced the highest recoveries for spiking levels of 10, 50, and 200 ng/mL at percent recoveries of 108, 78, and 103, respectively, with an external calibration curve. Using internal standard adjustment, the recoveries ranged from 96.7% to 118%. The mean percent recoveries of d_6-tetracycline, the internal standard, were 45.6±6.1 (n=10).

We used an ultrafiltration device to remove the particulates and proteins in the digester fluids to minimize potential assay interferences. A similar approach of using ultrafiltration for sample treatment had been reported previously for measurement of CTC in milk [18]. This approach provided a simple cleanup with no prolonged sample handling to minimize epimerization and isomer formation during sample handling as CTC is prone to these transformations. We then used this filtrate to test the ability of the standard addition method to give reproducible results while avoiding background or matrix problems. Large variations of the slope among the samples were produced resulting in excessive variation of the x intercept, the value of the concentration of the unknown in the sample. Because of the inconsistent results, we abandoned the use of standard additions as a method of computing the concentration of the unknowns. Thus, we combined ultrafiltration with a matrix-matched calibration curve, and we were able to analyze chlortetracycline and its metabolites, oxytetracycline and tetracycline, simultaneously with more reproducible results. Since the filtration method is more efficient in comparison to the SPE method, we elected to use this approach to determine the chlortetracycline and its degradation products from the digesters.

Assay performance

Selectivity

The UHPLC-MS/MS method resolved CTC, 4-epi-CTC, ICTC and 4-epi-anhydro-CTC, oxytetracycline, tetracycline, and the internal standard, d_6-tetracycline. Control digester samples contained no quantifiable CTC or its derivatives (data not shown) demonstrating the assay would be highly specific for the outlined analytes and would be unlikely to show false-positives.

Method detection limits and spiked recoveries

Tetracycline had a MDL of 2.0 pg/μL; oxytetracycline had a MDL of 2.6 pg/μL; 4-epi-CTC had a MDL of 1.9 pg/μL; ICTC had a MDL of 7.3 pg/μL; CTC had a MDL of 3.4 pg/μL, and 4-epi-anhydro-CTC had a MDL of 6.5 pg/μL. Recoveries were 74%, 64%, 102%, 112%, 106%, and 71% for tetracycline, oxytetracycline, 4-epi-CTC, ICTC, CTC, and 4-epi-anhydro-CTC, respectively. The method showed a low detection limit for all the tetracyclines, including CTC and its decomposition products, as well as oxytetracyline and tetracycline.

Linearity

The calibration curve for all analytes was linear over a wide dynamic range from 1 to 10,000 pg/μL and in general, with r^2>0.99 for all the tested compounds.

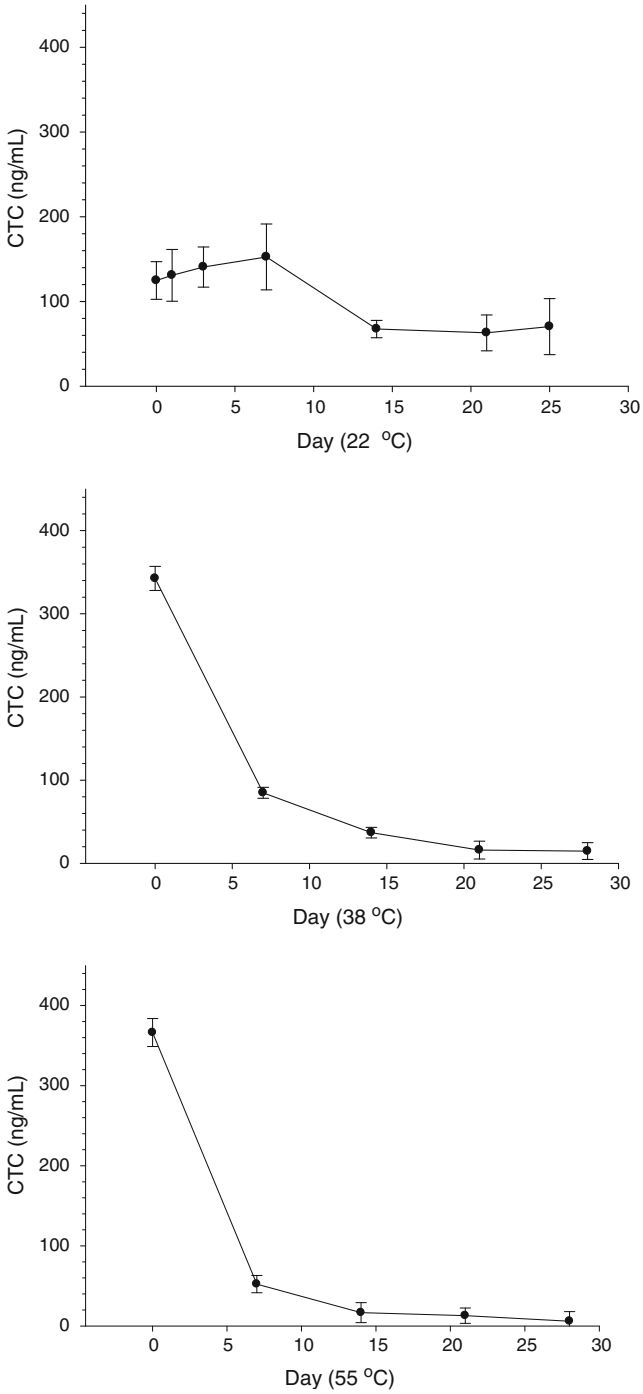

Fig. 2 Chlortetracycline concentrations in digesters at three temperatures as a function of time

Reproducibility

The method showed acceptable within-day and between-day precision with the exception of 4-epi-anhydro-CTC, where the percent CVs were <15%. The percent CV of within-day and between-days is as follows: tetracycline (1.4; 3.0), d_6-tetracycline (2.5; 6.5), oxytetracycline (4.3; 4.5), CTC (4.9; 6.0), 4-epi-CTC (1.2; 2.2), ICTC (6.1; 11.6), and 4-epi-anhydro-CTC (23, 19.7).

Stability

Tylová et al. [15] found that there was no significant degradation or epimerization in CTC stocks (1 mg/mL) or working

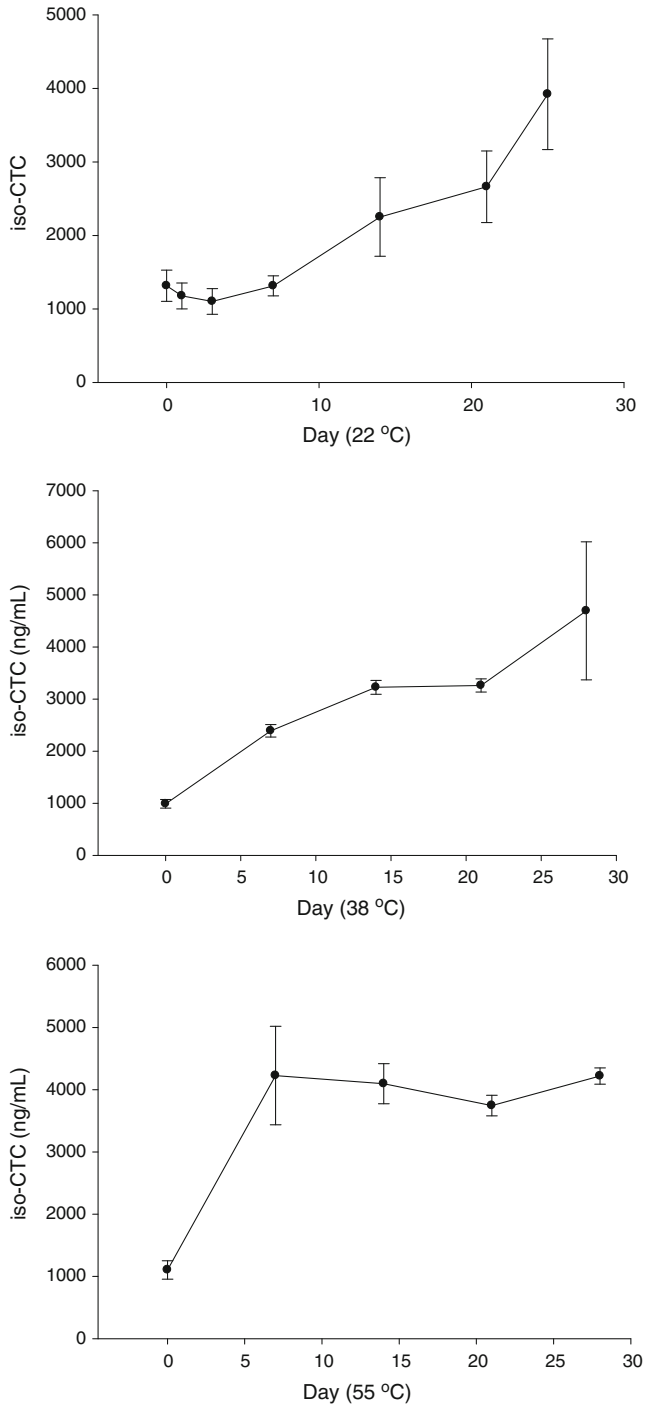

Fig. 3 Iso-chlortetracycline concentrations in digesters at three different temperature digesters as a function of time

solutions (250 μg/mL) when stored at −20 °C for up to 14 days. They also observed that CTC can epimerize rapidly at room temperature (42% at 6 h). We tested the stability of working solutions at 500 ng/mL, much lower than Tylová et al.'s study, to determine the stability of concentrations closer to real manure samples during sample handling. We found that CTC concentration can change depending on the storage conditions. It was found that, when stored at 4 °C in a high-density polyethylene container, the concentration decreased 2.0%, 3.6%, and 6.6% for days 1, 2, and 5 respectively (n=2) while, in glass containers, the concentration decreased 14.6%, 25.2%, and 39.6% for days 1, 2, and 5, respectively (n=2). When stored in high-density polyethylene for 21 days, the samples stored at 4 °C decreased 5.6% compared with samples at −20 °C, while the samples at ambient temperature showed a decrease in concentration of 39.5% (n=2).

Therefore, we minimized the usage of glass containers for extractant storage and sample handling. In general, the processed samples were analyzed by LC-MS/MS within 2 days. A more extensive stability test has been described by Søeborg et al. [19] in Milli Q water and soil water. However, they did not differentiate between the storage containers.

Matrix effect

Wei et al. [2] noted that their method did not overcome the matrix effects of animal waste water despite the use of solid-phase extraction to purify samples. Our results clearly indicated that matrix interference occurred in the sample filtrate compared with analytes dissolved in mobile phase. Ion suppression (−) or ion enhancement (+) occurred as follows: tetracycline (+ 21.2%), d_6-tetracycline (+ 24.6%), oxytetracycline (+ 26.7%), 4-epi-CTC (− 10.6%), ICTC (+ 35.9%), CTC (+ 16.0%), and 4-epi-anhydro-CTC (− 87.5%). Therefore, matrix-matched calibration curves were employed for the UHPLC-MS/MS measurement of all unknowns to minimize the potential for misleading results.

Digester samples

As analytical techniques have improved, the detection of agrochemicals in the environment [20] has increased, documenting the need for consideration of how contaminated materials are disposed of and what constitutes safe practices. Recent reports [2, 6, 7] have clearly demonstrated CTC contamination of water near agricultural facilities. The presence of large amounts of CTC in swine manure warrants further studies in regards to the safe disposal or recycling of this material. Consequently, analytical procedures for determining CTC and its degradation products in swine manure are needed to document the chemical fate of CTC. The analytical method must be capable of at least modest throughput in addition to being sensitive and capable of resolving the common degradation products associated with CTC. The use of the simple suspension followed by filtration gives only water-soluble forms of CTC including its epimer and isomer forms, but these are the most available forms involved in transport into ground or surface waters or for uptake by plants.

CTC concentrations decreased over time at all temperatures in the manure digesters, with the elevated temperatures exhibiting more uniform and rapid decreases (Fig. 2). At 22 °C, the concentration of CTC did not decrease until day 14 and remained relatively constant until the end of the study period. In contrast, the data at the higher temperatures showed a sharp decrease followed by a more gradual decrease to the end of the study. These changes were substantiated by statistics with large changes in concentration leading to statistically significant concentration difference (<0.05) between those days. Loftin [8] demonstrated that, in aqueous solution, CTC spontaneously degrades quite rapidly at alkaline pHs, and this degradation markedly increases with increasing temperature at all pHs tested (although ionic strength changes had little effect). They measured half-lives of 16 h at pH 7.0 and 22 °C and less than 6 h at pH 7.0 and 35 °C. At pH 5.0, the corresponding half-lives were 293 and 125 h, respectively. In our experiments, the half-lives could not be accurately assessed due to non-first-order kinetics degradation but were greater than 21 days (504 h) at 22 °C and less than 5 days (120 h) at the elevated temperatures. Our 38 °C digester which had a pH of 6.1–6.3 produced comparable half-life results with that observed by Alvarez [21], who studied the decomposition of CTC in an anaerobic digestion of pig manure at 35 °C and pH 7.0 and observed half-lives varying from 3.2 to 4.1 days. Another batch study of anaerobic digestion [22] showed gradual disappearance of CTC with a half-life estimated around 25 days with an initial pH of about 8.0 (falling to about 7.4 during the

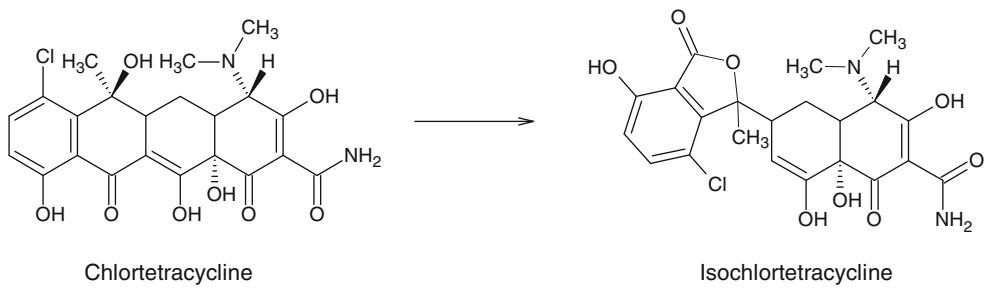

Fig. 4 Conversion of chlortetracycline to isochlortetracycline as observed in the digestors. Other compounds such as the 4-epimers and anhydrochlortetracycline are not shown

study) and with a temperature increase from 10 °C to ~20 °C over about 75 days. However, CTC concentrations remained constant at about 10 mg/L from day 60 to day 225 with little or no change. Stone et al.'s low-temperature half-life results were similar to our 22 °C digester results, although our manure pHs (6.3–7.2) were lower than their pHs. Decomposition under aerobic composting conditions [23] gave a half-life of 86.6 days under conditions where the pH ranged from 6.5 to 7.8 and the temperature from 10 °C to nearly 60 °C. Chlortetracycline in manure samples did not disappear as rapidly compared with an aqueous solution, possibly due to particulate matter providing some protection. It is difficult to compare these studies because of the differences in temperature and pH, both of which markedly affect the decomposition of CTC, and both factors were allowed to change in some of the studies in order to mimic real life conditions. In most of the studies, CTC was degraded rather slowly in hog manure, but high temperatures markedly increased this decomposition. The in vitro studies also demonstrated a marked dependence on temperature with much of CTC's decomposition due to its instability in aqueous solution. These degradation studies often counted CTC and its epi/isomers as a single entity and did not distinguish the varying contributions of CTC, epi-CTC, or ICTC to the total concentration.

Isochlortetracycline concentration was observed to increase over time. The rate of ICTC formation markedly increased with higher temperatures (Fig. 3). The increase in rate of formation with increasing temperature indicates an energy barrier to the formation typical of chemical reactions. The repeated-measure two-way ANOVA revealed significant interaction and showed the differences in formation at 22 °C were significant for all comparisons except between 0 and 7, and 14 and 21 days, caused by a slow start and equilibrium at later times. At 38 °C, all differences were significant except days 14 and 21 where the reaction had plateaued. At 55 °C, the reaction plateaued earlier making the differences between days 7, 14, and 21 not significant. At days 7 and 14, there were significant differences in ICTC formed between all temperatures, but not at day 0 or day 21. In all cases, ICTC had much higher concentrations compared with the CTC independent of temperature and treatment days. At treatment day 0, ICTC concentration was approximately nine times higher than CTC, which confirmed the results reported by Grote [24]. Apparently, ICTC accumulated as CTC decomposed, indicating ICTC is at least moderately stable and more stable than CTC. Although CTC can be epimerized under the mild acidic condition in the mobile phase, we have found only small concentrations of epi-CTC and were not able to reliably quantify it. Our study indicated ICTC is a major product of CTC decomposition (Fig. 4), with temperature being a major factor in its formation as expected of a chemical structure rearrangement. ICTC is not active as an antibacterial and would not result in antimicrobial resistance; however, the lack of data on its toxicity indicates further safety study is needed.

In conclusion, the UHPLC-MS/MS system was able to provide detailed epimer and isomer information for samples containing CTC. In addition, this method is capable of being adapted to the measurement of tetracycline and oxytetracycline in swine manure. Chlortetracycline degradation was temperature-dependent, and the higher the temperature, the faster the degradation. We found that, in swine manure, the majority of CTC degraded to ICTC which is a biologically inactive form. The environmental load of the biologically active form of CTC, therefore, may be overestimated if the method of analysis does not distinguish between CTC and ICTC.

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