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Detection of Tetracyclines in an Anaerobic Waste Digester Using Solid Phase Extraction and High-Performance Liquid Chromatography Mass Spectrometry

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DETECTION OF TETRACYCLINES IN AN ANAEROBIC WASTE DIGESTER USING SOLID PHASE EXTRACTION AND HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY MASS SPECTROMETRY

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with Honors College Graduate Distinction at

Western Kentucky University

By

Courtney Cruse

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*****

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ABSTRACT

Antibiotics are introduced to livestock to encourage growth and for the treatment of diseases. These antibiotics are not completely metabolized by swine, and thus these antibiotics are excreted with their waste. This poses a potential risk to human health as these antibiotics, a potential link to antibiotic resistant bacteria, then enter the surface water, ground water, and soil. In collaboration with the US Department of Agriculture (USDA) in Bowling Green, Kentucky, this research is concerned with analyzing the degradation of tetracyclines in swine waste from an anaerobic digester. Waste samples obtained from a digester and swine waste at the USDA lab are analyzed using a solid phase extraction method with a weak cation cartridge followed by analysis with High-Performance Liquid Chromatography Mass Spectrometry (HPLC-MS). Particular interest is in the degradation of three tetracyclines (tetracycline, oxytetracycline, and chlorotetracycline). Analyses reveal the presence of low concentrations (ppb) of tetracycline and chlortetracycline in the digester samples; oxytetracycline was below the level of detection. The aim is to compare tetracycline concentrations over a period of time. Thus, providing the ability to investigate the correlation of tetracycline concentrations to the concentrations of antibiotic resistant genes.

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1.1 Background

In recent years, there has been growing concern regarding the impact of confined animal feeding operations (CAFO) on antibiotic resistance and subsequent consequences on the environment and human health.[1-3] These antibiotics are introduced to the livestock to encourage growth and to prevent or treat diseases.[1, 4] In the United States, approximately 91% of CAFOs use antibiotics during their production process and when they are administered, only a portion of the antibiotic is absorbed by the animal's gastrointestinal tract.[4] As a result, anywhere from 30% to 90% is not absorbed by the animal; thus leading to the excretion of the antibiotic, or its metabolites, in their feces or urine.[4-5] The use of antibiotics in this manner allows for the selection of resistant bacteria in the gastrointestinal tracts through horizontal gene transfer or spontaneous mutation.[2] These antibiotics and antibiotic resistant genes are subsequently disseminated into the environment. Antibiotics have been found in surface water, ground water, and soils posing important questions regarding its impact on human health and the emergence of antibiotic resistant bacteria.[5]

1.2 Anaerobic Digestion

Anaerobic digestion is a process that involves bacteria breaking down organic matter in a closed system, known as a digester, without oxygen.[6] Organic matter can include manure, food scraps, fats and oils, and sewage sludge (biosolids). Anaerobic digestion systems can minimize odors, decrease the number of pathogens, generate

biogas, produce liquid and solid digestate, and decrease the amount waste volumes.[7] The bacteria anaerobically digest the organic matter and generate biogas, which consists of methane, carbon dioxide, water vapor and other gases. The production of methane is of interest as it is the main component of natural gas and can be used as a source of energy for electricity, heating and transportation fuel, while the remaining digestate material can be used as fertilizer (Figure 1).[7]

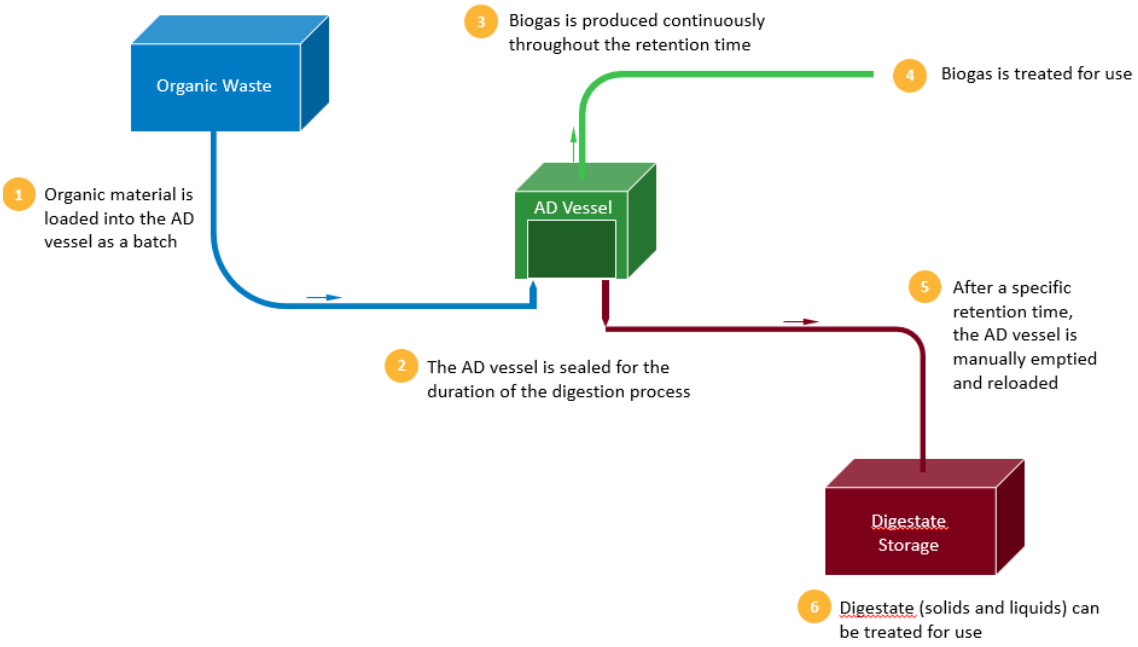


Figure 1. Batch Digester with single vessel.[6]

Digesters can be described by their operating temperature, wet or dry, and batch or continuous flow. There are two temperature ranges that digesters are operated; 86-100°F (mesophilic) and 122-140°F (thermophilic).[7] The different temperature ranges allow different populations of anaerobic microbes to thrive. A wet or dry digester refers to the amount of moisture. Wet digesters, also known as low solid digesters, process

organic matter that has less than 15% solids content in slurry formed by pumping, while dry digester, high solids digester, process organic matter that has greater than 15% solids.[7] In batch digesters, digesters are administered all organic matter all at once, and then periodically emptied and reloaded at a set time for digestion to occur. Continuous flow digesters are continuously fed organic matter and continuously emptied of digested material.[7]

There are four types of chemical processes that the introduced organic matter undergoes during the digestion process (Figure 2). These processes are hydrolysis, fermentation, acetogenesis, and methanogenesis.[6] During acetogenesis, soluble organic compounds and short-chain organic acids are generated. Then in methanogenesis, the acetic acid, carbon dioxide, and hydrogen are converted into biogas by the bacteria. [6]

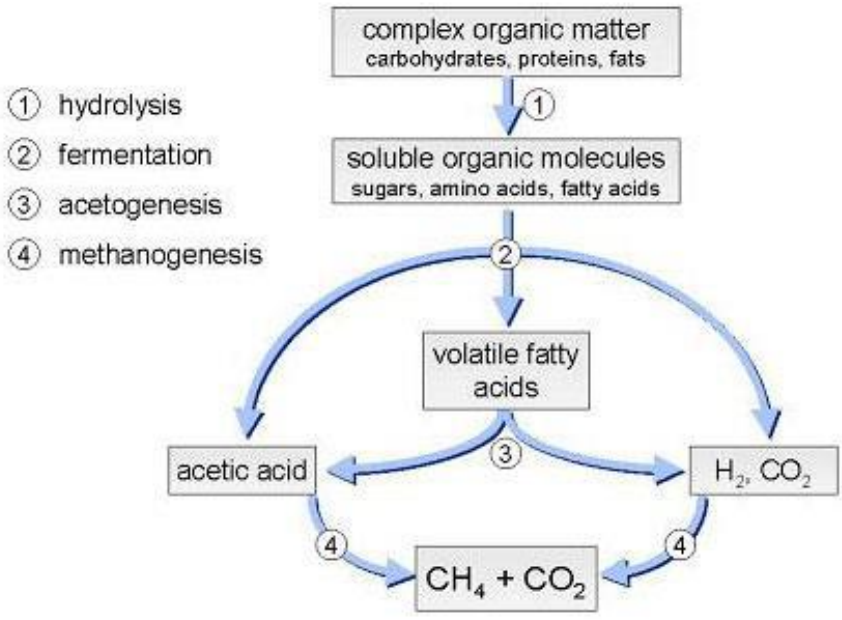


Figure 2. Anaerobic digestion chemical processes.[6]

1.3 Solid Phase Extraction

Solid Phase Extraction (SPE) is a sample preparation technique, typically for liquid samples, that ideally yields quantitative extractions.[8] Compounds of interest are retained by a sorbent housed in the cartridge. These sorbents can be reverse phase, normal phase, ion exchange, and adsorption. Specifically, ion exchange SPE is utilized when the compounds of interest are charged. The electrostatic interactions between the compound's charged functional group and the silica surface's bonded charged group is one method used to retain compound(s) on the cartridge. Thus, the pH of the sample solution must be one that allows for the compound of interest and the bonded silica phase to remain charged.[8] When either of the charged compounds are neutralized, the compound of interest is eluted from the cartridge, due to the disruption in the electrostatic interaction.[8]

There are two types of ion exchange sorbents, anion and cation exchange, either with a strong or weak exchanger bonded.[8] Strong ion exchange surfaces remain charged from about pH 1-12. Thus, the process of an SPE is dependent on the type and strength of the ion exchange cartridge. The generic SPE process is shown in Figure 4.

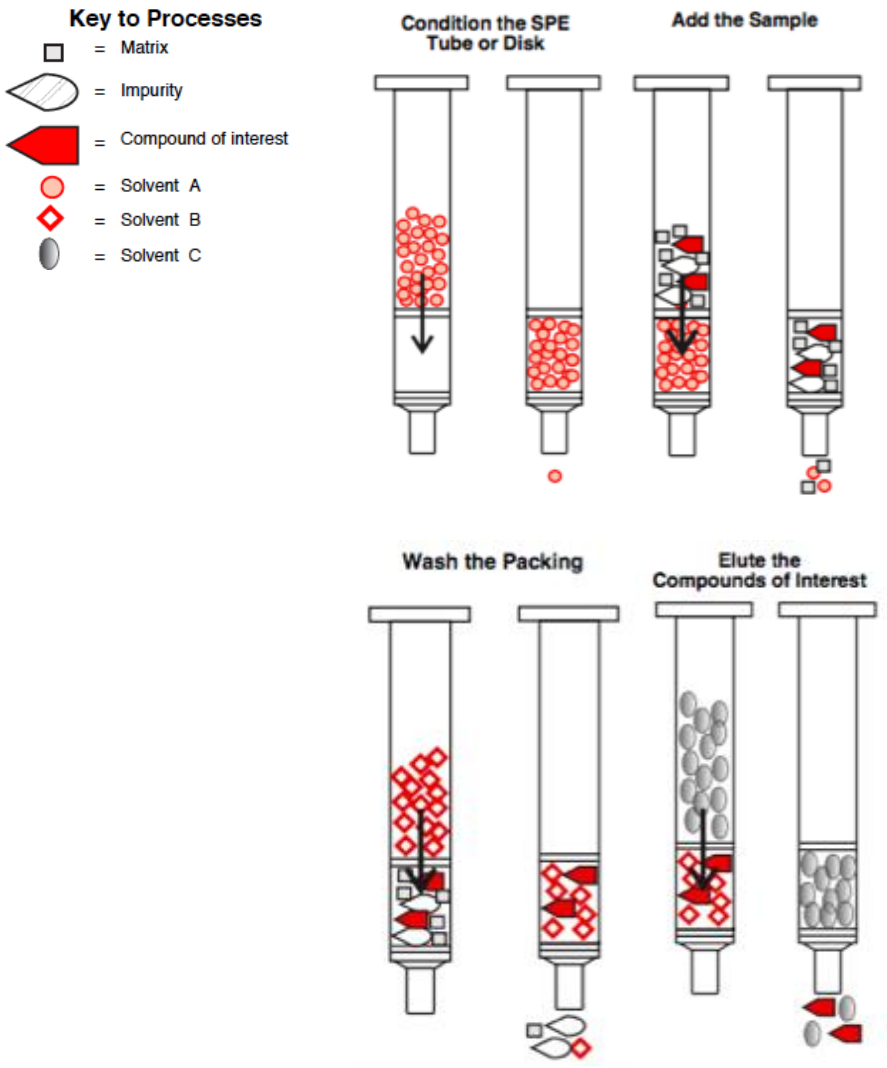


Figure 3. Generic SPE Procedure.[8]

1.4 High-Performance Liquid Chromatography

High-Performance Liquid Chromatography (HPLC) is a technique used for the separation and analysis of non-volatile or thermally-unstable compounds, using high pressure to move a mobile phase through a packed column containing a stationary phase (typically 3 to 5 μm in diameter).[9] Due to physical and/or chemical interactions between the component molecules being analyzed and packed particles of the stationary phase, the components are separated and detected as they exit the column (Figure 1). The resulting

chromatogram can be used for qualitative and/or quantitative analysis. The retention time (the time required for the molecule to exit the column after the injection) of a molecule is used in qualitative analysis to identify a particular compound present in the sample.[9] In quantitative analysis, the peak area is used to determine the concentration of the molecule of interest.[9]

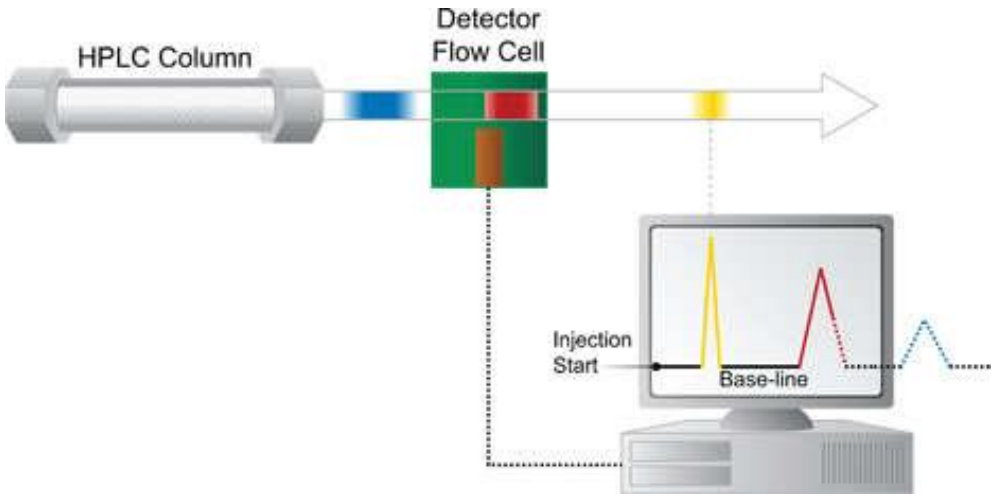


Figure 4. Component separation and detection.[10]

The most common method used in HPLC is reverse phase chromatography. In this method, the column packing is non-polar and the mobile phase consists of two solvents: aqueous and organic.[9] Typically, the method begins with a higher percentage of a water-based (aqueous) mobile phase with an optional buffer, which, over time, increases the percentage of water miscible organic solvent (known as a gradient method).[9]

For reversed-phase chromatography, a commonly employed column is the C18 column. A C18 column (octyldecylsilane, ODS) contains octadecyl groups chemically bonded to silica packed columns.[11] Silica is a popular material for packing in bonded

phase HPLC columns as they are rigid and resist compaction resulting from high flow rate and pressure. The silanol (Si-OH) groups on the packed particle surface serve as bonding sites for octadecyl groups of a C18 (Figure 2).[11]

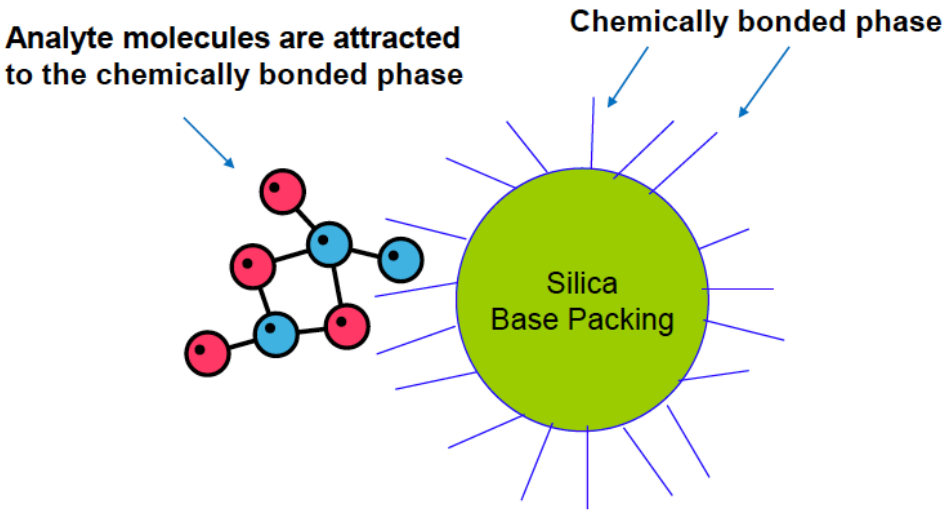


Figure 5. C18 column.[9]

1.5 Mass Spectrometry

Mass spectrometry (MS) can be coupled to an HPLC to provide additional qualitative and quantitative data by measuring the mass of a molecule. For MS, the molecule must first be converted into a gas-phase ion. There are several ways to ionize a molecule in MS, including electrospray ionization (ESI).[12] In ESI, ions are formed from an aerosol when the eluent from the HPLC is introduced to a high voltage (Figure 3).[12] This is a type of atmospheric pressure ionization (API) that ionizes at atmospheric pressure instead of in a vacuum. It is a continuous technique through an electrochemical process where electrons are transferred to a conductive surface. In positive mode, the

droplets leaving the stainless-steel capillary are positively charged and electrons are accepted by the conductive surface.[12]

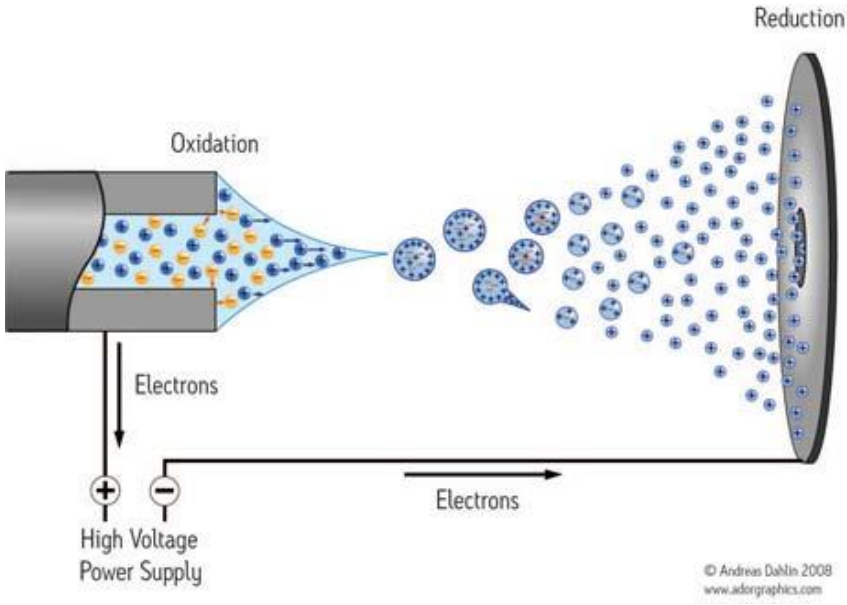


Figure 6. ESI formation of ions and movement through an electric field to the counter plate.[12]

As the eluent from the HPLC exits the capillary, it is aerosolized and the charged ions enter the mass spectrometer. Then a counter-current gas (a cone) is applied to help the desolvation of the droplets as they enter the gas vacuum region of the mass spectrum (Figure 4). These ions are then separated and detected based on their mass-to-charge ratio (m/z) due to the electrostatic interactions and the vacuum effects of the mass spectrum.[12] The mass spectrum graphs the relative ion signal vs. the m/z. If operated in positive mode, the molecular ion will be detected as the mass plus a hydrogen ion (M^+).

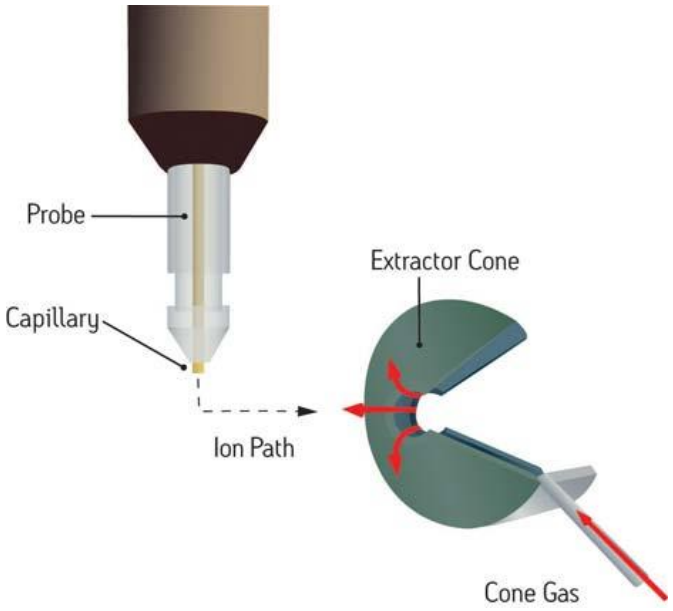


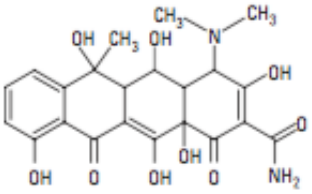
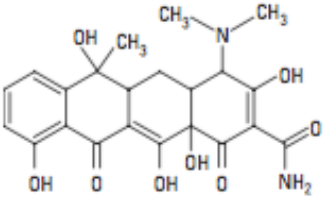
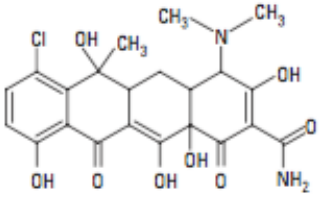
Figure 7. ESI to MS diagram.[12]

1.6 Proposed Research

This research is concerned with developing an HPLC method that can effectively separate and detect three types of tetracyclines: tetracycline (TC), oxytetracycline (OTC), and chlortetracycline (CTC). Tetracyclines are a group of antibiotics that are amphoteric and are characterized by a partially conjugated, four ring structure.[13] They are able to form stable complexes with multivalent cations and are soluble in polar organic solvents.[13] These antibiotics are commonly used in response to human and animal infections (Table 1).[3, 14] In collaboration with the US Department of Agriculture (USDA) in Bowling Green, Kentucky, this research is concerned with analyzing the degradation of tetracyclines in swine waste from an anaerobic digester. Waste samples obtained from a digester and swine waste at the USDA lab are analyzed using a solid phase extraction method with a weak cation cartridge followed by analysis with High-Performance Liquid Chromatography (HPLC). The aim is to compare tetracycline concentrations over a

period of time. Thus, providing the ability to investigate the correlation of tetracycline concentrations to the concentrations of antibiotic resistant genes.

Table 1. Tetracyclines of interest.[14]

Name	CAS #	pKa	Structure
Oxytetracycline	6153-64-6	3.3/7.3/9.1	
Tetracycline	60-54-8	3.3/7.7/9.7	
Chlortetracycline	57-62-5	3.3/7.4/9.3	

2 Experimental

2.1 Sample Preparation

Digester samples corresponding to different days of the digestion process were obtained from the USDA anaerobic digester. The initial swine waste introduced to the digester was obtained from a local farm in Bowling Green, KY. A single sample analysis required 10 mL of digester waste delivered into a 45 mL centrifuge vial. Then 10 mL EDTA buffer and 10 mL methanol were added. The solution was vortexed for 1 minute, then sonicated for 20 minutes, and then vortexed for 1 minute. This was to ensure the

tetracyclines were released from the solid material. The solid material was separated out by centrifugation for 10 minutes at 4,500 rpm. The supernatant was decanted and concentrated sulfuric acid was used to adjust the pH to 4.00.

The 0.1 M EDTA buffer was prepared by mixing 9.306 g of EDTA with 96 mL of 0.2 M Na_2HPO_4 and 154 mL of 0.1 M citric acid. The 0.4 M citric acid used for elution was 95% methanol and 2.10 g citric acid.

Digester waste was spiked at 2.4 ppm of tetracycline, oxytetracycline, and chlortetracycline and were added to the centrifuge vial prior to the addition of 10 mL of 0.1 M EDTA buffer and 10 mL of methanol. The tetracycline standards were purchased from Sigma-Aldrich.

2.2 Solid Phase Extraction

A Phenomenex X-CW, Weak Cation Mixed Mode Phase SPE cartridge was utilized for the SPE procedure.

SPE Procedure:

Condition: The cartridge was conditioned with 5 ml of methanol followed by 5 mL nano-water, each time, running the solution through the cartridge until the meniscus sits above the top of the solid phase.

Load: The sample was then loaded onto the cartridge. Making sure to not allow the meniscus to fall below the solid phase.

Wash: The cartridge was washed with 10 mL of 10% methanol/water followed by 5 mL methanol. The cartridge was then allowed to dry for 20-30 minutes under high pressure.

Elution: The tetracyclines were eluted with 4 mL of 95% methanol in 0.04 M citric acid by gravity. This was performed twice and collected for analysis by HPLC-MS.

2.3 HPLC-MS Analysis

The eluent from the SPE procedure was evaporated with nitrogen and reconstituted in 1 mL of methanol. The samples were then injected into the HPLC-MS and run individually in Full Scan MS and MS/MS modes for each analyte. The m/z for tetracycline, oxytetracycline, and chlortetracycline are 445, 461, and 479, respectively. The HPLC-MS utilized was an Agilent-500 ESI with separation using a Kinetex C18 LC Column from Phenomenex.

Table 2. HPLC-MS settings.

Setting	
Flow Rate	0.2 mL/min
RF Loading	55%
Ionization Type	ESI
Polarity Mode	Positive
Capillary Voltage	80.0 Volts
Needle Voltage +, -	±5000 Volts
Scan Range	200-1500
Nebulizer Gas	Nitrogen
Nebulizer Gas Pressure	40.0 psi
Drying Gas Pressure	15.1 psi
Drying Gas Temperature	400 °C
Spray Shield Voltage +, -	±600.0 Volts

Table 3. HPLC-MS method.

Time (min)	0.1% Formic acid/acetonitrile	0.1% Formic acid/water
0.00	10%	90%
5.00	30%	70%
8.10	50%	50%
11.0	10%	90%

2.4 Digester Parameters

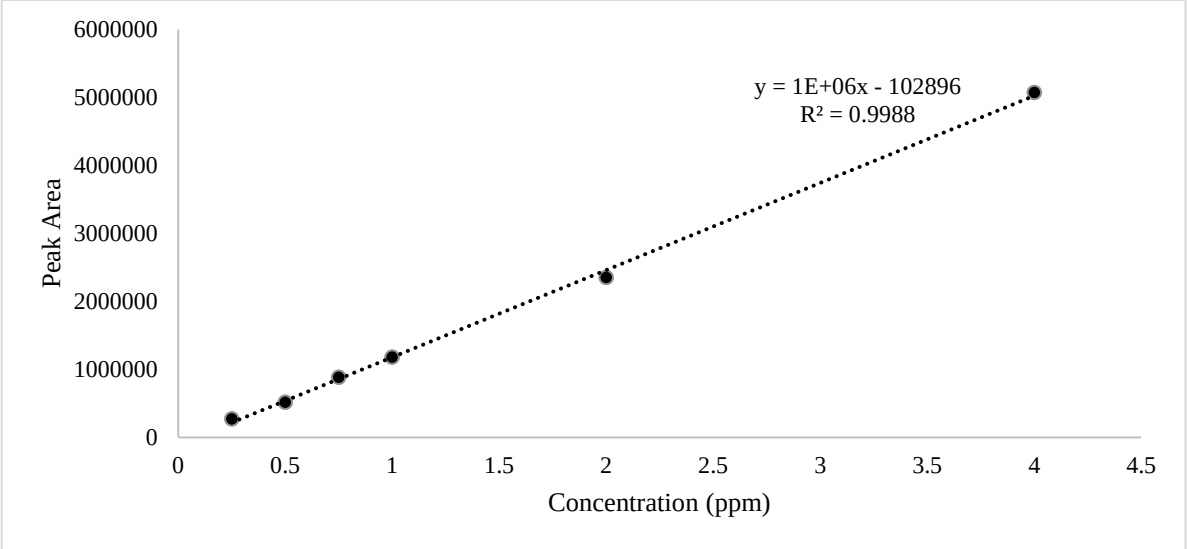
The batch digesters were fed tetracycline free corn manually throughout the experimental period. Samples were collected on various days during a 100-day study.

3 Results and Discussion

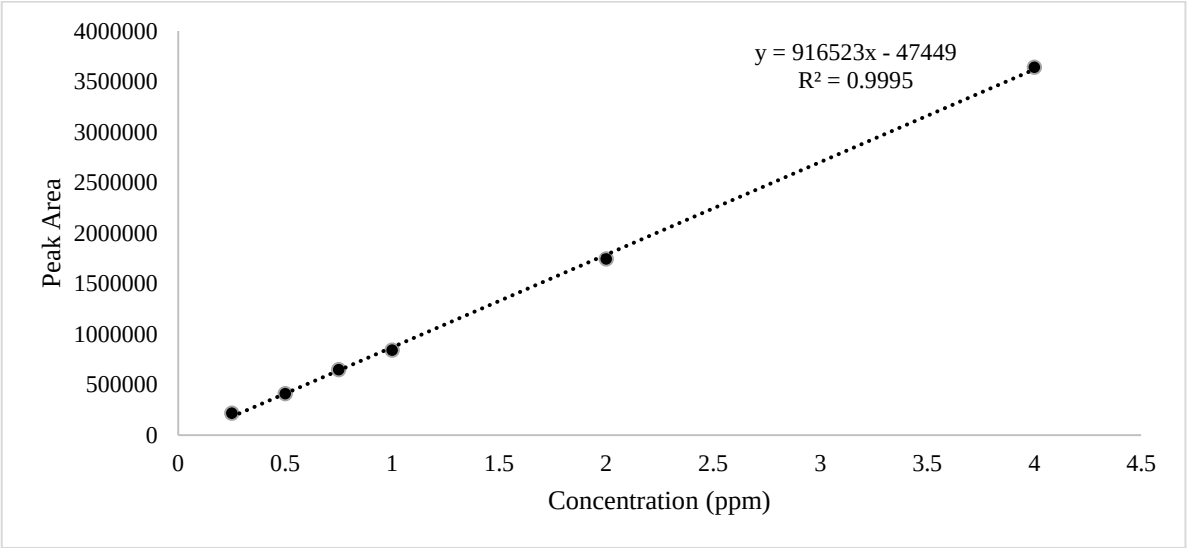
3.1 Calibration Curves

Calibration curves were developed for tetracycline, oxytetracycline, chlortetracycline. Concentrations of 0.250 ppm, 0.500 ppm, 0.750 ppm, 1 ppm, 2 ppm, and 4 ppm were developed by serial dilution and analyzed by HPLC-MS for each tetracycline. There is a linear correlation between the peak area of a chromatogram and the concentration of the species identified. For a given tetracycline, the peak area of the six working standards were graphed against the known concentration analyzed to create a calibration curve (Figure 8). Because of this linear relationship, the calibration curves can be used to calculate the concentration of an unknown waste solution by using the equation of the line.

A



B



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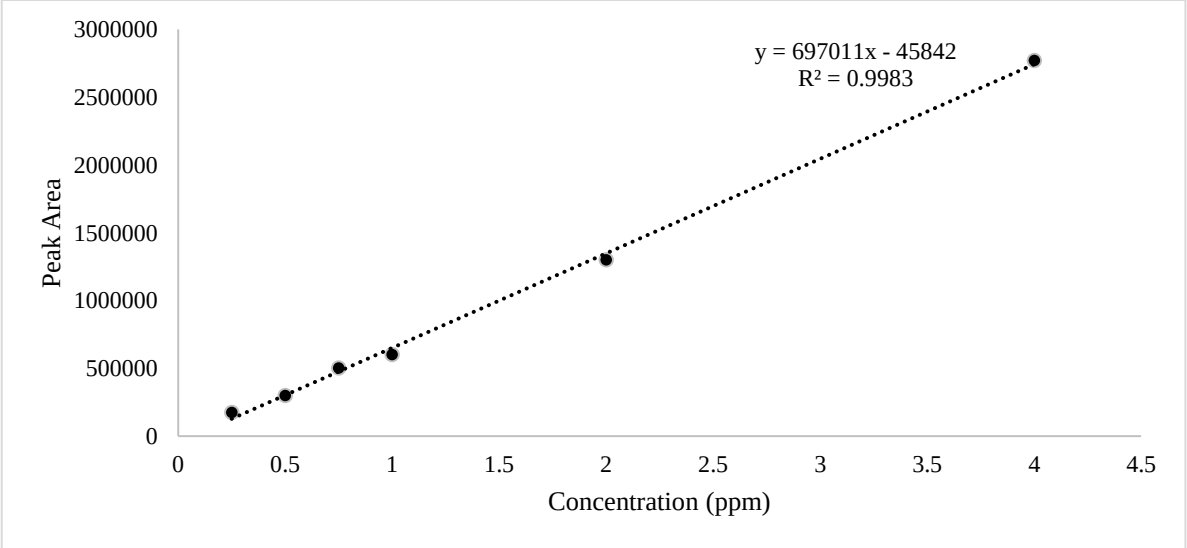


Figure 8. Calibration Curves. A. 445, TC. B. 461, OTC. C. 479, CTC.

3.2 Limit of Detection

The limit of detection (LOD) of the HPLC-MS was determined by preparing seven 1.0 ppm standards consisting of each tetracycline; tetracycline, oxytetracycline, chlortetracycline. Each peak area was integrated and the concentration was calculated from the calibration curves. The standard deviation of the seven analyses was multiplied by three to obtain the LOD.

Table 4. Limit of Detection determination.

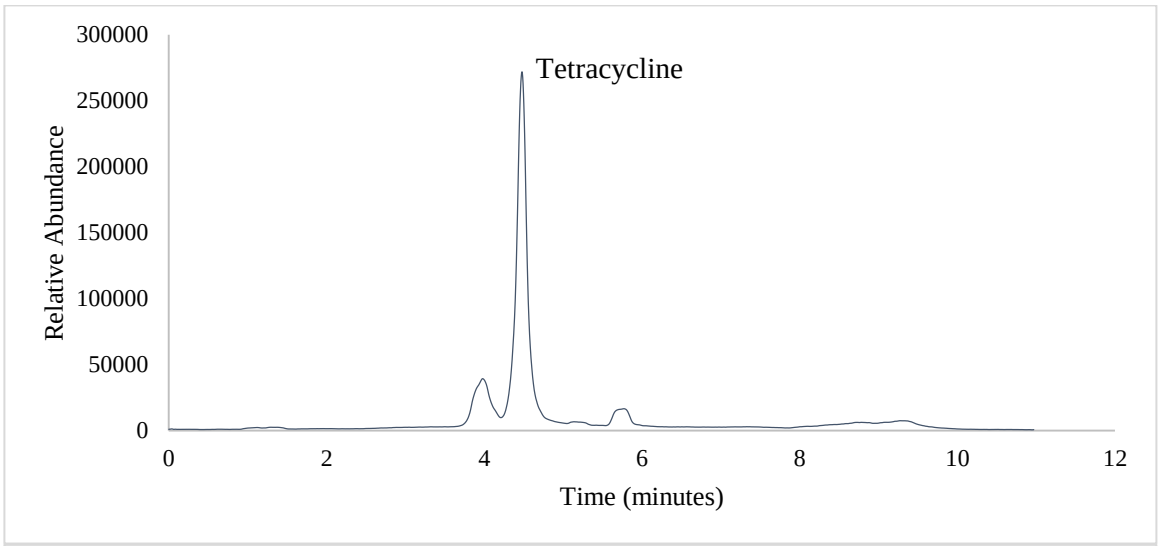
	Tetracycline (ppm)	Oxytetracycline (ppm)	Chlortetracycline (ppm)
Trial 1	0.920	0.957	0.923
Trial 2	0.871	0.894	0.874
Trial 3	0.859	0.907	0.863
Trial 4	0.895	0.841	0.914
Trial 5	0.903	0.915	0.911
Trial 6	0.854	0.877	0.874
Trial 7	0.869	0.874	0.873
Std. Dev.	0.024683714	0.036674242	0.024615133
LOD	74.051 ppb	110.023 ppb	73.845 ppb

3.3 Digester Sample Analysis

3.3.1 Control

A control consisting of 2.4 ppm of each tetracycline was prepared for the analyses of Digester A and 1.25 ppm of each tetracycline was prepared for Digester B. A MS/MS scan was performed by HPLC-MS for each of the precursor ions, 445, 461, and 479, for reference (Figure 9).

A



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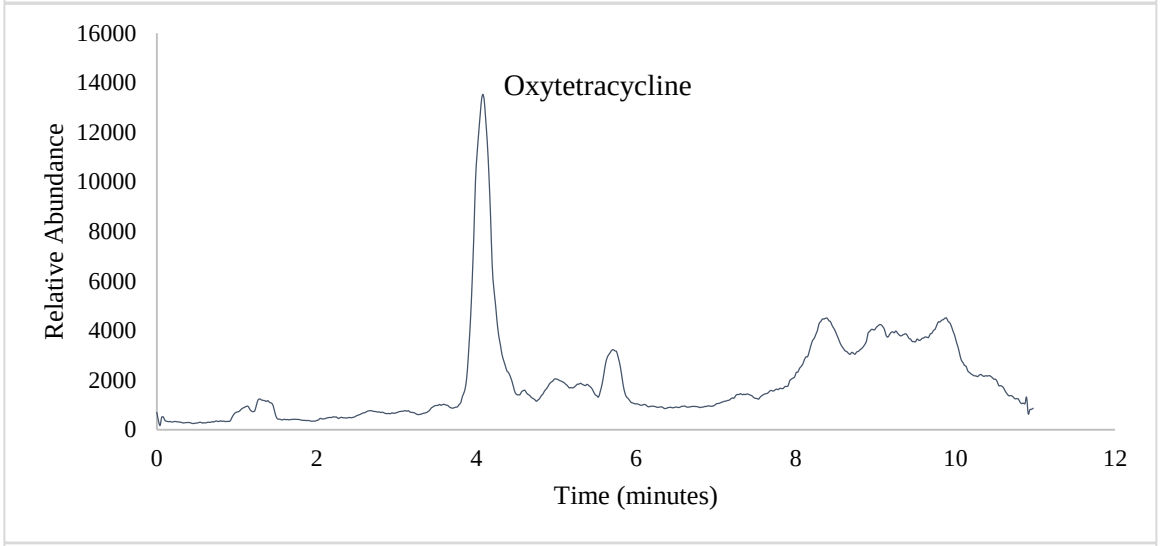

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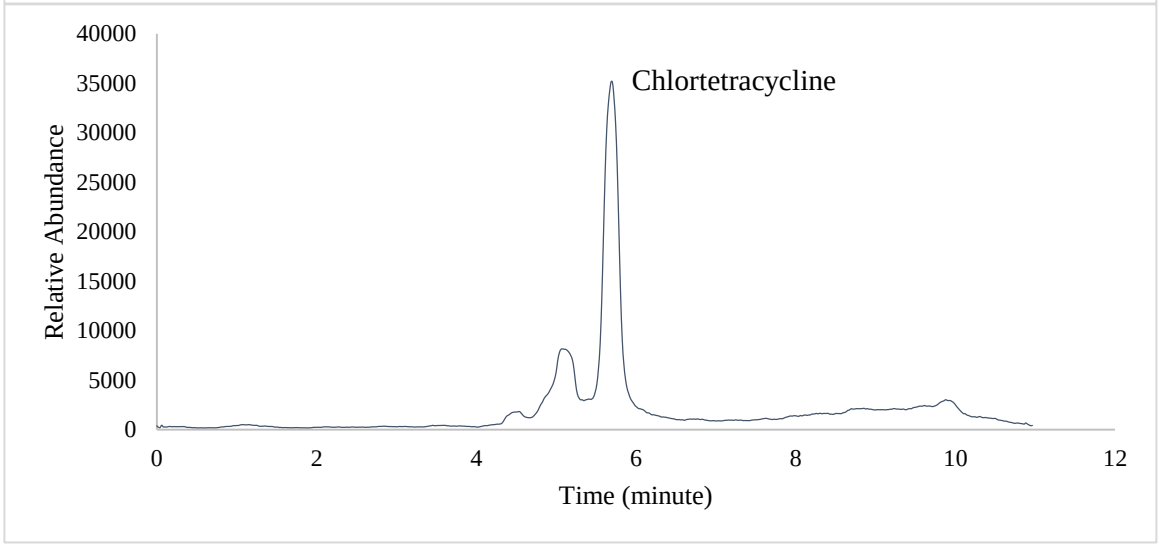


Figure 9. MS/MS Scan 2.4 ppm control. A. 445, TC. B. 461, OTC. C. 479, CTC.

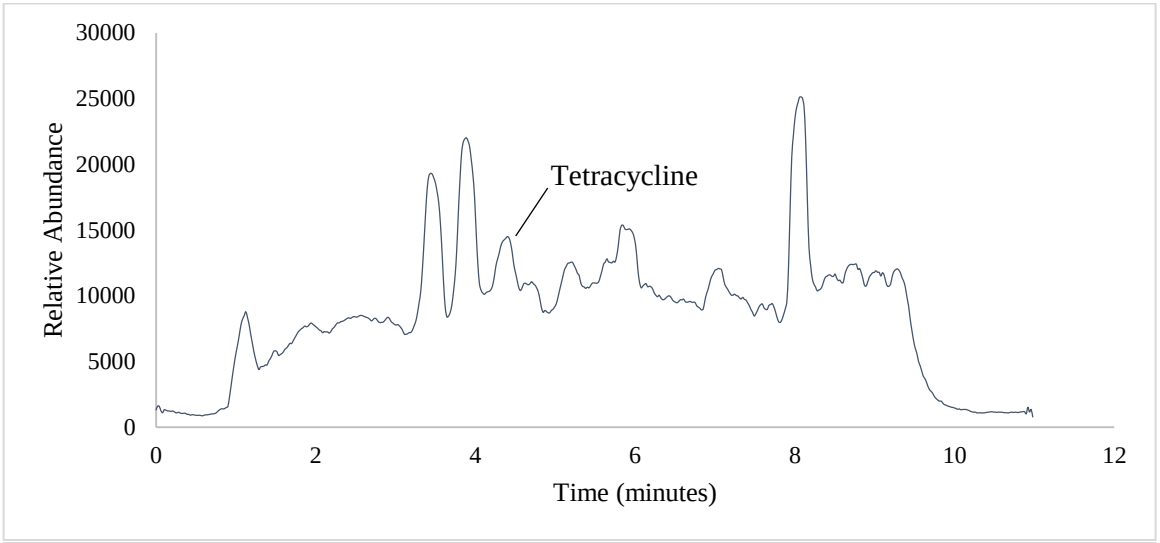
Peaks resulting from the HPLC-MS/MS scan can be integrated for all three tetracyclines of interest and each with different retention times. This allows for the qualitative and quantitative analysis of tetracycline, oxytetracycline, and chlortetracycline.

Additionally, a blank consisting of nano-water was run through the sample preparation, SPE and HPLC-MS procedures. This was done to confirm the absence of contamination in the procedure. Tetracycline, oxytetracycline, and chlortetracycline were not detected in the resulting MS/MS chromatograms. Thus, the procedure followed did not contain tetracyclines interferences that may have affected the concentration quantification.

3.3.2 Native and Spiked Sample Analysis

Each digester sample analyzed followed the same sample preparation, SPE procedure, and HPLC-MS method. The first trial consisted of the native digester sample (Figure 10) and the second trial a 2.4 ppm spiked digester sample (Figure 11). These samples allowed for the evaluation of the percent recovery and calculation of the concentration of the tetracyclines detected in the digester samples.

A



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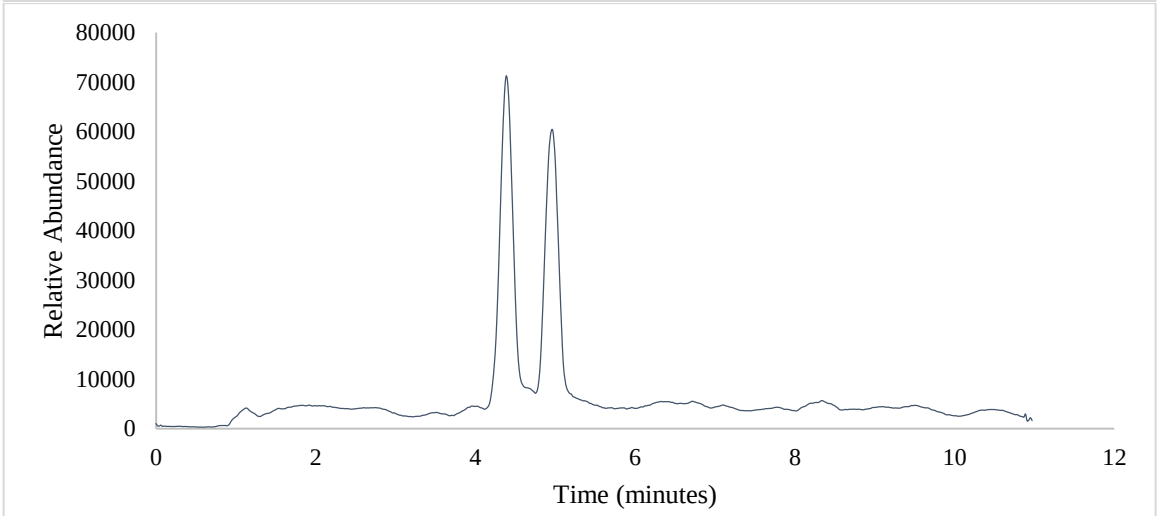

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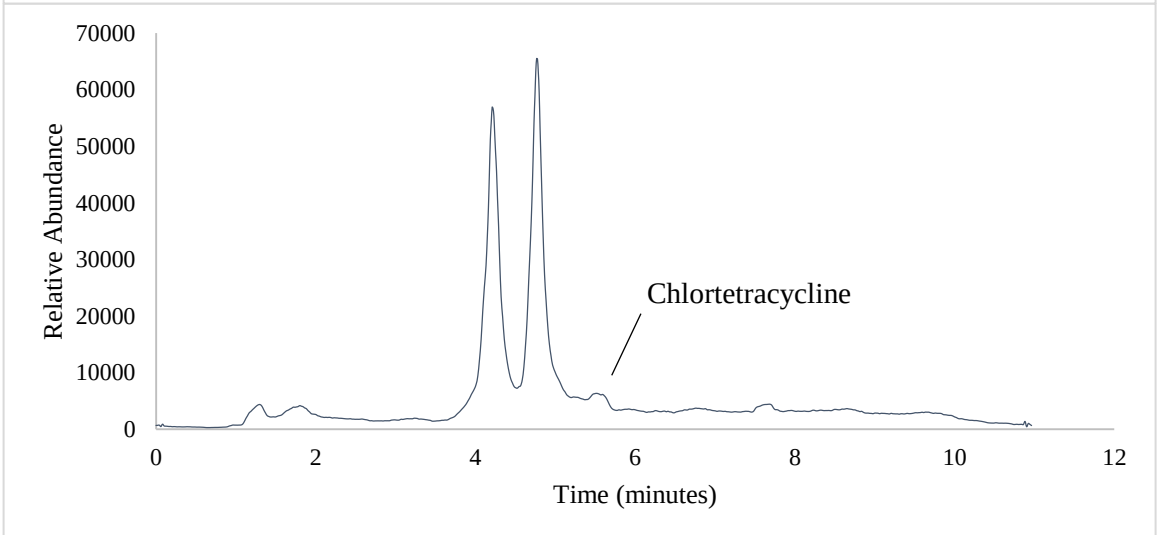
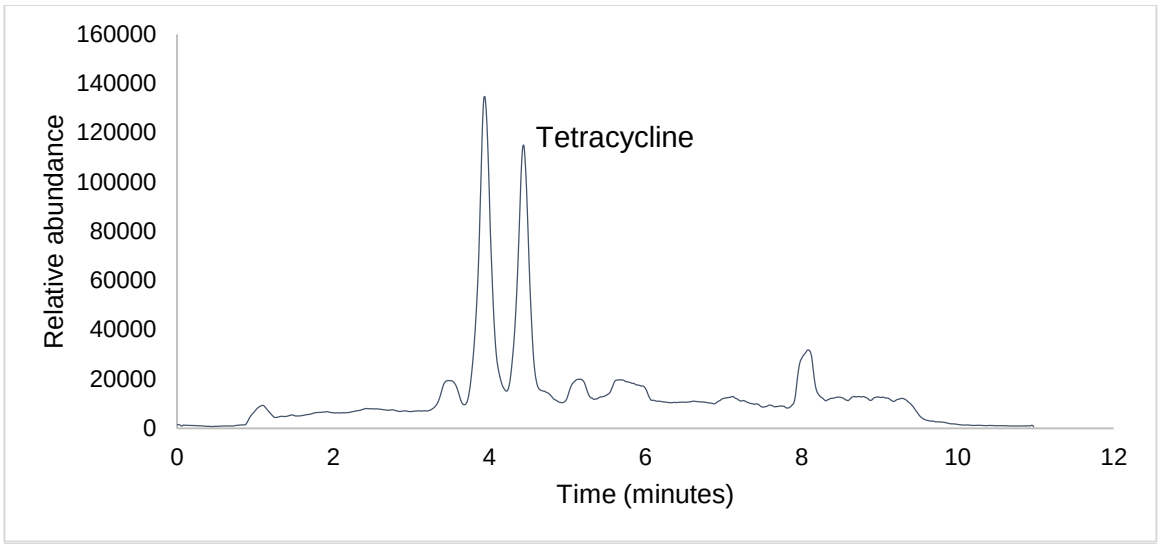


Figure 10. MS/MS Scan, Native Sample BBP-A 10/24/16. A. 445, TC. B. 461, OTC. C. 479, CTC.

A



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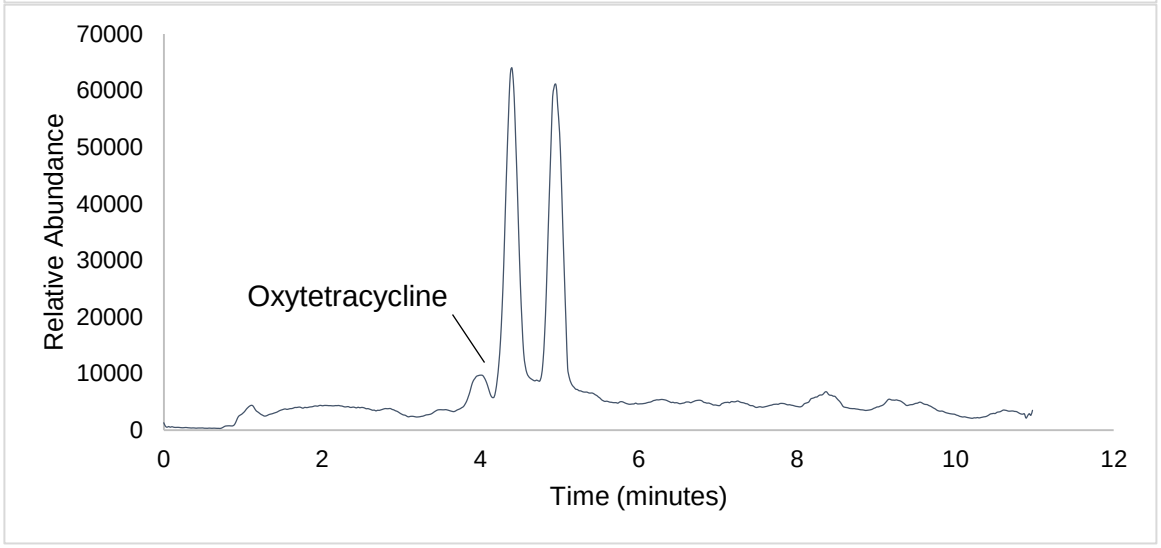

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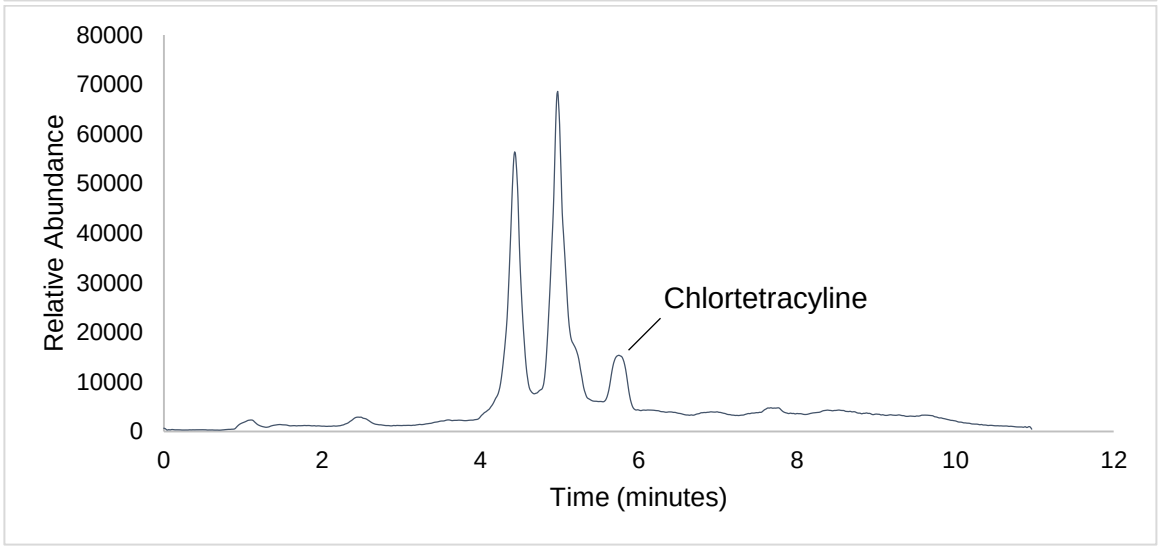


Figure 11. MS/MS Scan, Spiked Sample BBP-A 10/24/16. A. 445, TC. B. 461, OTC. C. 479, CTC.

Tetracycline and chlortetracycline are present in digester samples, while oxytretracycline is below the limit of detection. The presence of each tetracycline is confirmed by the same retention time and m/z ratio (MS/MS) scan.

Two sample from Digester A were analyzed by HPLC-MS. Each sample was analyzed twice, once spiked with 2.4 ppm standard and once as native. The tetracycline HPLC-MS peaks were integrated and the percent recovery and detected concentration of the native samples were calculated for Digester A (Table 5). Four Digester B samples were analyzed. Two native (unspiked) samples were processed and injected once and two additional samples were run in duplicate as a spike and native sample and the detected concentrations of the native samples were calculated (Table 6).

Table 5. Digester Sample Results BBP-A.

	Sample	Control Peak Area	Native Peak Area	Detected Native Conc.	Spiked Peak Area	Recovery
TC	BBP-A 10/24/16	2510000	55472	123.6 ppb	972387	63.5%
	BBP-A 10/10/16	2510000	30348	104.0 ppb	976700	62.3%
OTC	BBP-A 10/24/16	207318	0	Below LOD	88656	42.8%
	BBP-A 10/10/16	207318	0	Below LOD	101072	48.8%
CTC	BBP-A 10/24/16	419856	42076	113.1 ppb	150529	74.2%
	BBP-A 10/10/16	419856	0	Below LOD	140687	66.5%

Table 6. Digester Sample Results BBP-B.

	Sample	Trial	Control	Detected Native Conc.	Spiked Peak Conc.
TC	BBP-B 10/10/16	1	1.25 ppm	155 ppb	-
	BBP-B 10/31/16	1	1.25 ppm	232 ppb	-
	BBP-B 11/07/16	1	1.25 ppm	218 ppb	826 ppb
		2	1.25 ppm	206 ppb	662 ppb
	BBP-B 11/14/16	1	1.25 ppm	210 ppb	708 ppb
		2	1.25 ppm	175 ppb	563 ppb
OTC	BBP-B 10/10/16	1	1.25 ppm	Below LOD	-
	BBP-B 10/31/16	1	1.25 ppm	Below LOD	-
	BBP-B 11/07/16	1	1.25 ppm	Below LOD	986 ppb
		2	1.25 ppm	Below LOD	1.238 ppm
	BBP-B 11/14/16	1	1.25 ppm	Below LOD	1.150 ppm
		2	1.25 ppm	Below LOD	1.131 ppm
CTC	BBP-B 10/10/16	1	1.25 ppm	272 ppb	-
	BBP-B 10/31/16	1	1.25 ppm	695 ppb	-
	BBP-B 11/07/16	1	1.25 ppm	758 ppb	1.524 ppm
		2	1.25 ppm	533 ppb	1.382 ppm
	BBP-B 11/14/16	1	1.25 ppm	566 ppb	1.153 ppm
		2	1.25 ppm	501 ppb	1.006 ppm

Two additional Digester A samples were analyzed by HPLC-MS (Table 7). Each were run in triplicate and the average peak area and standard deviation were calculated. This was performed to analyze the repeatability of the procedure.

Table 7. Replication Analysis

Sample			Trial 1	Trial 2	Trial 3	Avg. ppm	Std. Dev.
TC	BBP-A 11/14/16	Area	68837	75932	72184	-	-
		ppm	134.0	139.6	136.6	136.7	2.8
	BBP-A 10/17/16	Area	12685	54793	55666	-	-
		ppm	90.2*	123.1	123.7	123.4	0.5
OTC	BBP-A 11/14/16	Area	0	0	0	-	-
		ppm	-	-	-	-	-
	BBP-A 10/17/16	Area	0	0	0	-	-
		ppm	-	-	-	-	-
CTC	BBP-A 11/14/16	Area	43180	49359	419856	-	-
		ppm	126.9	141.8	132.1	133.6	7.6
	BBP-A 10/17/16	Area	42632	53020	46226	-	-
		ppm	127.7	136.6	145.8	136.7	9.0

Deviations in the analysis of the two Digester A samples were lower for the tetracycline analysis than for the chlortetracycline analysis. *Trial 1 for sample BBP-A 10/17/16 was considered an outlier and was neglected from the average and standard

deviation calculations. The average standard deviation for tetracycline was 1.65 ppm and for chlortetracycline was 8.3 ppm.

The detected concentrations of the native digester samples are calculated per 1 mL of methanol. The tetracycline concentrations must be corrected to represent the 10 mL digester samples (Table 8). If duplicates or triplicates were analyzed, the average detected native concentrations were used to calculate the concentration in the digester waste sample.

Table 8. Corrected Digester Sample Concentrations.

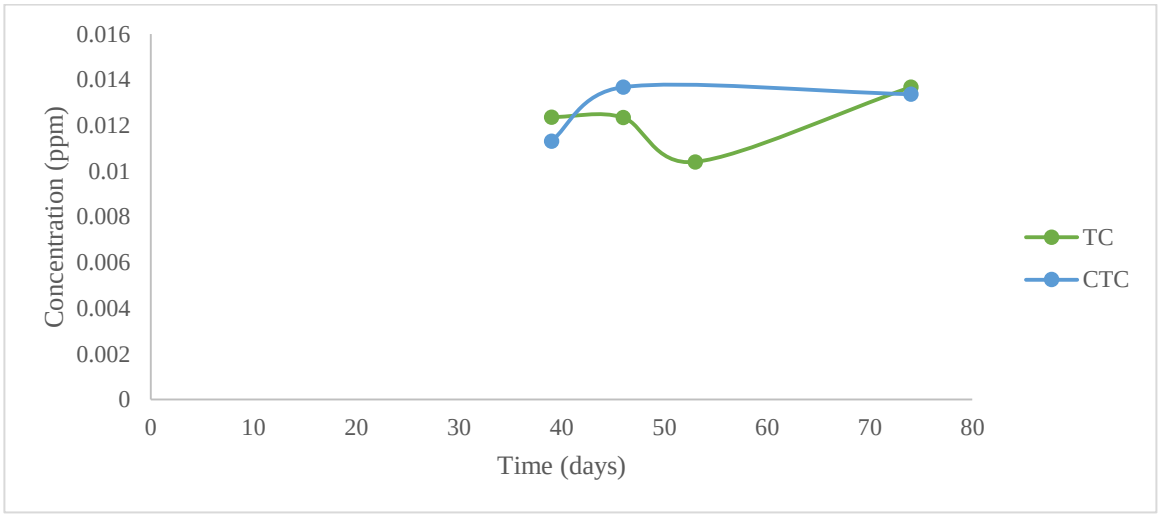
	Sample	Concentration
TC	BBP-A 10/10/16	12.36 ppb
	BBP-A 10/17/16	12.34 ppb
	BBP-A 10/24/16	10.40 ppb
	BBP-A 11/14/16	13.67 ppb
	BBP-B 10/10/16	15.50 ppb
	BBP-B 10/31/16	23.20 ppb
	BBP-B 11/07/16	21.20 ppb
	BBP-B 11/14/16	19.25 ppb
CTC	BBP-A 10/10/16	11.31 ppb
	BBP-A 10/17/16	13.67 ppb
	BBP-A 10/24/16	Below LOD
	BBP-A 11/14/16	13.36 ppb
	BBP-B 10/10/16	27.20 ppb
	BBP-B 10/31/16	69.50 ppb
	BBP-B 11/07/16	64.55 ppb
	BBP-B 11/14/16	53.35 ppb

3.3.3 Tetracycline Concentration Over Time

Samples of digester waste were extracted from the digesters and at different times during the digestion process. The samples analyzed were used to investigate the change in tetracycline concentrations during the experiment by graphing the time vs. the

concentrations (Figure 12). Time is defined as the number of days since the initial addition of swine waste to the digester. The concentrations of each tetracycline for Digester A and Digester B used were from the native samples (non-spiked samples). If multiple trials were run for a given sample, the average concentration was used for the graph.

A



B

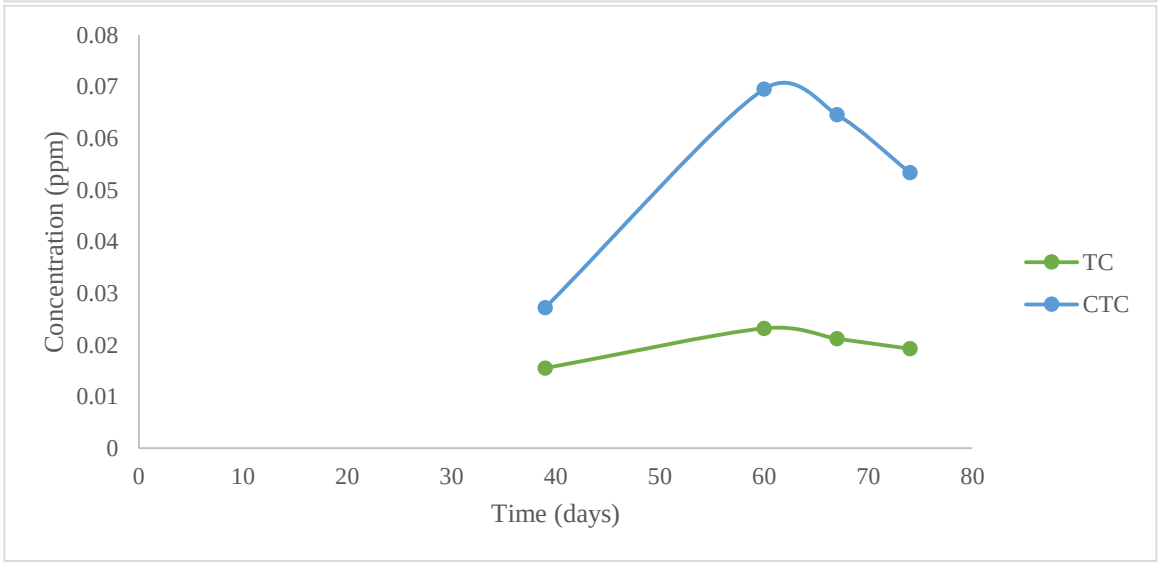


Figure 12. Tetracyclines (445 and 479 m/z ion trace) vs. Time. A. Digester A; B. Digester B.

The concentration was expected to decrease through the experimental period due to the degradation of tetracycline and chlortetracycline in the digester. Oxytetracycline was included in this analysis, as it was under the limit of detection (LOD) for the native samples analyzed. For tetracycline and chlortetracycline, there is a nonlinear relationship between the number of days and concentration. This could be attributed sampling heterogeneity because the digester cannot be stirred. However, even in low concentrations, tetracycline and chlortetracycline persist in the anaerobic digesters for at least 76 days after the initial introduction of swine water to the digesters.

3.3.4 Gene Expression

Samples collected from the digesters periodically throughout the experiment were also analyzed for the presence of antibiotic resistant genes by polymerase chain reaction (PCR). The genes analyzed were Tet(Q), Tet(O), and Tet(W). All samples analyzed by HPLC-MS were also tested for the presence of these three antibiotic resistant genes. Results for these analyses, all samples reveal that all samples were tested for the three antibiotic resistant genes.

4 Conclusions

In conclusion, analyses of samples originating from two digesters (Digester A and Digester B) at the USDA in Bowling Green, KY were completed to better understand the degradation of tetracyclines in an anaerobic waste digester. It was found that tetracyclines persisted over a 76 day period. Analysis confirmed the presence of

tetracycline and chlortetracycline in low concentrations (ppb), while oxytetracycline was below the limit of detection; the limit of detection for tetracycline, oxytetracycline, and chlortetracycline are 74.1 ppb, 110.0 ppb, and 73.8 ppb, respectively. These samples also tested positive for the antibiotic resistant genes (Tet(Q), Tet(O), and Tet(W)) using PCR.

Limited sample availability prevented a comprehensive understanding of the concentration changes of tetracycline and chlortetracycline in the digesters. The samples obtained were extracted from Digester A and Digester B between day 39 and day 74. After this period of time, if the tetracyclines were present, low and steady concentrations of the tetracyclines in the digester waste would be expected. However, analysis of concentrations over the experimental period was nonlinear; there were fluctuations in the concentrations detected. Additional samples extracted from the digesters at earlier days in the experimental period are required to better understand the initial degradation changes.

5 References

1. Joy, S. R.; Bartelt-Hunt, S. L.; Snow, D. D.; Gilley, J. E.; Woodbury, B. L.; Parker, D. B.; Marx, D. B.; Li, X., Fate and Transport of Antimicrobials and Antimicrobial Resistance Genes in Soil and Runoff Following Land Application of Swine Manure Slurry. *Environmental Science & Technology* **2013,** *47* (21), 12081-12088.
2. Peng, S.; Wang, Y.; Zhou, B.; Lin, X., Long-term application of fresh and composted manure increase tetracycline resistance in the arable soil of eastern China. *Science of The Total Environment* **2015,** *506–507*, 279-286.
3. Montes, N.; Otero, M.; Coimbra, R. N.; Méndez, R.; Martín-Villacorta, J., Removal of tetracyclines from swine manure at full-scale activated sludge treatment plants. *Environmental Technology* **2015,** *36* (15), 1966-1973.
4. Chen, Y.; Zhang, H.; Luo, Y.; Song, J., Occurrence and assessment of veterinary antibiotics in swine manures: A case study in East China. *Chinese Science Bulletin* **2012,** *57* (6), 606-614.
5. An, J.; Chen, H.; Wei, S.; Gu, J., Antibiotic contamination in animal manure, soil, and sewage sludge in Shenyang, northeast China. *Environmental Earth Sciences* **2015,** *74* (6), 5077-5086.
6. Costa, A.; Ely, C.; Pennington, M.; Rock, S.; Staniec, C.; Turgeon, J., Anaerobic Digestion and its Applications. United States Enviornmental Protection Agency Office of Research and Development, 2015.
7. USEPA Types of Anaerobic Digesters. https://www.epa.gov/anaerobic-digestion/types-anaerobic-digesters - DigesterDisc.
8. Co., S.-A., Suplico Guide to Solid Phase Extraction. **1998,** (Bulletin 910).
9. Technologies, A. HPLC Basics. http://polymer.ustc.edu.cn/xwxx_20/xw/201109/P020110906263097048536.pdf.
10. Waters How Does High Performance Liquid Chromatography Work. http://www.waters.com/waters/en_US/How-Does-High-Performance-Liquid-Chromatography-Work%3F/nav.htm?cid=10049055&locale=en_US (accessed March 19).
11. Technologies, A., The LC Handbook: Guide to LC Columns and Method Development.
12. Waters What is MS and How does it Work? http://www.waters.com/waters/en_US/What-is-MS-and-How-does-it-Work%3F/nav.htm?cid=10073253&locale=en_US.
13. Fedeniuk, R. W.; Shand, P. J., Theory and methodology of antibiotic extraction from biomatrices. *Journal of Chromatography A* **1998,** *812* (1–2), 3-15.
14. Zhai, C.-H.; Zou, Y., Determination of Tetracyclines in Chicken by Solid-Phase Extraction and High-Performance Liquid Chromatography: Application Note. **2008**.