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A. INTRODUCTION

1. Summary of Procedure

Partially thawed samples are extracted with McIlvaine Buffer/EDTA solution. After centrifuging, the extracts containing the tetracyclines are cleaned-up by passing through C18 SPE cartridges. Tetracyclines are eluted from the cartridge with methanolic oxalic acid, evaporated and reconstituted with aqueous methanol and analyzed by reverse phase High Performance Liquid Chromatography (HPLC).

2. Applicability

This method is suitable for the identification of tetracyclines in bovine, porcine, ovine muscle, kidney and liver as well as poultry muscle and kidney at levels ≥ 0.5 ppm.

Note: Refer to 21CFR for tolerance values set by U.S. FDA and 40CFR for tolerance values set by U.S. EPA.

B. EQUIPMENT

Note: Equivalent equipment may be substituted.

1. Apparatus

a. Analytical balance - 0.001 g sensitivity, Cat. No. PG5-0025, Mettler Toledo.

b. Buchner Funnel - 5.5 cm diameter, Cat. No. 60240, Coors.

c. Centrifuge - refrigerated, accommodating 50 mL, tubes at a minimum of 2500 rpm, Cat. No. Gentra GP8R, IEC.

d. Centrifuge tubes - polypropylene, 50 mL, disposable, Falcon Blue Max, Cat. No. 2098, Becton Dickinson.

e. Centrifuge tubes - glass, 15 mL, graduated at 0.1 mL intervals, with stoppers, Cat. No. 4515315 and 4515315, Kimble.

f. Filter paper - glass microfiber, grade GFB, 5.5 cm, Cat. No. 1821-055, Whatman.

g. Sidearm Erlenmeyer flask - 250 mL, Cat. No. 27060-250, and 27060-250, Kimble.

h. Mechanical shaker - flatbed, 2 speed, Eberbach.

i. N-Evap - with heated water bath, Model No. 112, Organomation Associates.

j. pH meter - readable and accurate to within 0.05 units, Model No. 611, Orion.

k. Sample filter cartridge - 13 mm diameter x 0.2 - 0.45 micron, acrodisc, Cat. No. LC13 PVDF, Gelman.

l. SPE Cartridge - 6 mL, 500 mg C18 packing, Bond-Elut, Cat. No. 1210-2052, Varian.

m. SPE vacuum manifold, (Supelco Visiprep 5-7030).

n. Two-way Stopcocks - Cat. No. 7241-00, J.T. Baker.

o. SPE reservoirs - 75 mL, Cat. No. 7120-03, J.T. Baker.

p. SPE adapters - Cat. No. 7122-00, J.T. Baker.

q. Vortex mixer - Vortex Genie-2, Scientific Industries.

r. Syringes, disposable - 3 mL, Cat. No 309586, Becton Dickinson.

s. Volumetric flask - 15 mL and 2 mL.

2. Instrumentation

a. Liquid chromatograph consisting of:

Pump DIONEX HPG-3400RS HPG

DIONEX TCC-3000 column oven

DIONEX VWD-3400 detector .

Autosampler

DIONEX WPS-3000 T SL 6.80 SR5 Build 2413 Chromeleon Datasystem

Syncronis C8, 1.7um,100 x2.1,part # 97202-102130

C. REAGENTS AND SOLUTIONS

Note: Equivalent reagents / solutions may be substituted. The stability time frame of the solution is dependant on the expiration date of the components used or the listed expiration date, whichever is soonest.

1. Reagents

a. Acetonitrile - HPLC grade, Cat. No. 015-4, Burdick & Jackson.

b. Methanol - HPLC grade, Cat. No. A452-4, Fisher.

c. Oxalic acid - dihydrate, Cat. No. 2752, Mallinckrodt.

d. Citric acid - monohydrate, Cat. No. C-712, Sigma.

e. Sodium phosphate - dibasic, anhydrous, Cat. No. 3828-01, J.T. Baker.

f. EDTA - disodium dihydrate, Cat. No. 4931, Mallinckrodt.

g. Ammonium acetate - Cat. No. 0596-01, J.T. Baker.

h. Trifluoroacetic acid - Cat. No. 9470-01, J.T. Baker.

i. Ammonium hydroxide - Cat. No. 9721-01, J.T. Baker.

2. Solutions

a. Mobile phase:

Prepare 0.05 M ammonium acetate solution by weighing 3.85 g ammonium acetate into a 1 L beaker and dissolving it in 900 mL distilled water. Adjust pH of solution to 3.0 ± 0.5 with trifluoroacetic acid. Transfer to a 1 L volumetric flask and dilute to volume. To prepare mobile phase, combine 770 mL ammonium acetate solution with 230 mL acetonitrile, filter and degas.

Note: If pH of mobile phase drops below 2.95, it can be raised by drop-wise addition of dilute ammonium hydroxide.

b. McIlvaine Buffer:

Dissolve 28.41 g anhydrous dibasic sodium phosphate in distilled water in a 1 L volumetric flask, dilute to volume, and mix. Dissolve 21.01g citric acid, monohydrate in distilled water in a 1 L volumetric flask, dilute to volume, and mix. Combine 1 L citric acid solution with 625 mL phosphate solution in a 2 L flask. Check pH which should be 4.00 ± 0.05.

c. McIlvaine/EDTA Solution (McIlvaine Buffer/0.1 M EDTA):

Add 60.49 g disodium EDTA dihydrate to 1.625 L McIlvaine buffer.

d. Elution solution (Methanolic oxalic acid, 0.01M):

Add 1.26 g reagent grade oxalic acid dihydrate to a 1 L volumetric flask. Dissolve in HPLC grade methanol, dilute to volume, and mix.

e. MOX (Methanolic oxalic acid solution, 0.12M):

Add 1.50 g reagent grade oxalic acid dihydrate to a 100 mL volumetric flask. Dissolve in HPLC grade methanol, dilute to volume, and mix. Prepare fresh daily.

f. Dilution Solution:

Mix 100 mL MOX and 100 mL deionized water.

D. STANDARD(S)

Note: Equivalent standards / solutions may be substituted. Purity and counter ions are to be taken into account when calculating standard concentrations. The stability time frame of the solution is dependant on the expiration date of the components used or the listed expiration date, whichever occurs first.

1. Source:

   a. Chlortetracycline hydrochloride (CTC), Sigma Chemical Company and U.S. Pharmacopeia, Rockville, MD.

   b. Oxytetracycline hydrochloride (OTC), Sigma Chemical Company and U.S. Pharmacopeia, Rockville, MD.

   c. Tetracycline hydrochloride (TTC), Sigma Chemical Company and U.S. Pharmacopeia, Rockville, MD.

2. Preparation of Standard Solution(s)

   a. Stock standards (2.5 mg/mL) prepare every six months:

   Accurately weigh the equivalent of 250 mg (weights must be corrected for assayed content) of each tetracycline (OTC, TTC, CTC) hydrochloride into separate weighing dishes. Transfer to separate 100 mL volumetric flasks with methanol, mix until dissolved, and dilute to volume. Stock standards are stable for 6 months at $<$ -10 °C unless the note under Section D heading applies.

   b. Mixed Intermediate Standard (125 µg/mL), prepare every six months:

   Add 5.0 mL of each tetracycline stock solution to a 100 mL volumetric flask. Mix, and dilute to volume with methanol. Intermediate standards are stable for 6 months at $<$ -10 °C unless the note under Section D heading applies.

c. Mixed Working Standards (to fortify 5 g sample for 0.5 ppm recovery; 25 µg/mL), prepare weekly:

Pipet 2 mL of 125 µg/mL mixed intermediate solution to a 10 mL volumetric flask and dilute to volume with methanol. Mixed working standards are stable for one week at < -10 °C unless the note under Section D heading applies.

Note: Use 100 µL of this solution.

d. HPLC standard – Prepare daily HPLC standard in 15mL tube as follows:

Add 100 µL mixed working standard (25 µg/mL), 400 µL MeOH, 500 µL MOX solution and 1000 µL deionized water. Vortex, transfer to syringe, and filter into HPLC vial.

E. SAMPLE PREPARATION

Freshly collected samples must be kept cold before and during shipping to laboratory. Once received at laboratory, samples must be frozen (< -10 °C) prior to mincing/grinding if they cannot be prepared on the day of receipt. If sample is frozen, allow to thaw, but keep as cold as possible. Dissect away fat and connective tissue from kidney or liver. Mince finely or grind tissue in blender or vertical cutter-mixer. Store frozen (< -10 °C) prior to analysis.

F. ANALYTICAL PROCEDURE

1. Preparation of Controls

a. Depending on the type of tissue to be analyzed for sample(s) weigh 5.0 ± 0.1 g of partially thawed appropriate blank tissue (previously analyzed and found to contain no tetracyclines) separately into two 50-mL polypropylene tubes. Use one tube as the negative control and fortify the other tube with 100 µL of mixed standard (D.2.c.) for 0.5 ppm recovery.

2. Extraction Procedure

a. Weigh 5.0 ± 0.1 g of each homogenized sample into 50 mL polypropylene centrifuge tubes.

b. Add 20 mL McIlvaine/EDTA solution to each sample and controls.

c. Cap tube and shake 10 minutes on a flat bed shaker at high speed.

Note: Liver tissues may not clean-up satisfactorily unless deproteinized. If needed, the following extra step is recommended: Uncap, add 5 mL 0.34 M sulfuric acid and 5 mL 7% sodium tungstate. Cap and shake vigorously for 30 seconds.

d. Centrifuge contents of tube at a minimum of 2500 rpm for 10 minutes at approximately 15 °C. Pour supernatant into a second centrifuge tube, being careful not to transfer any tissue. Refrigerate at 2 to 8 °C.

e. Add 20 mL McIlvaine/EDTA solution to the residue in the first centrifuge tube and cap securely. Re-suspend solids by shaking vigorously. Shake 10 minutes on a flat bed shaker set at high speed, then centrifuge and decant as described in step F.2.d.

f. Centrifuge combined extracts at a minimum of 4000 rpm for 20 minutes at approximately 15 °C. This is an appropriate stopping point in the analysis.

g. Place a single GFB filter paper into a 5.5 cm Buchner filtering funnel, attach to a 250 mL sidearm flask, and apply vacuum. Moisten the paper with McIlvaine/EDTA solution to assure that the filter paper is well-seated, and then filter the combined sample extracts. Rinse centrifuge tube with 4 mL McIlvaine/EDTA solution and filter into flask.

h. Attach an SPE cartridge to an SPE vacuum manifold. (Warning: The SPE cartridge must not be allowed to go dry between pre-wash, sample addition, and sample wash steps. When multiple samples are run, it may be necessary to stop flow through the column until the next solution can be conveniently added, then reapply vacuum.) Condition the cartridge with 10 mL methanol followed by 15 - 20 mL distilled water, at approximately 1.5 - 2.5 mL/minute. Apply vacuum as necessary. Discard eluate.

   Note: Test new lots of SPE cartridges for suitability at 0.5 ppm [(~ tissue equivalent in a 5 g sample)/actual concentration 1.25 µg/mL]: Condition a new cartridge as in F.2.h. Deliver a 100 µL aliquot of the mixed standard [(D.2.c.) 25 µg/mL] to the cartridge. Elute tetracyclines from the cartridge as in steps F.2.j-l. Inject eluate onto the HPLC and calculate percent recoveries using peak heights/area of the same concentration of mixed standards (see Note D.2.c.). Recoveries should be greater than 95%.

i. Connect a 75 mL reservoir to the cartridge. Add the filtered sample extract to the SPE reservoir. Rinse the flask with approximately 4 mL McIlvaine/EDTA solution and add the rinses to the reservoir. Drain extract through the column by gravity. If gravity is not sufficient for some slow samples, gently apply vacuum and adjust stopcocks to achieve a flow rate of 1.5 - 2.5 mL/minute. After sample has been applied to column, rinse the sidearm flask with 20 mL distilled water, and add to reservoir. Drain under 5 -10 mm Hg vacuum. Allow cartridge to go dry after the water rinse is completed, and continue to draw air through the cartridge for at least 2 minutes. Discard eluate.

j. Place a 15 mL graduated centrifuge tube in the vacuum apparatus to serve as a collection vessel. Elute tetracyclines from the cartridge with 6 mL elution solution. Apply vacuum to initiate flow Continue elution. Once flow stops, apply vacuum to remove residual solvent from the cartridge. Remove tubes from

vacuum manifold and vortex.

k. Place the tube containing the methanolic eluate in an N-Evap with a water bath temperature of 40 - 50 °C. Reduce volume of the eluate to 0.5 - 1 mL under a stream of dry nitrogen. Do not allow going to dryness. Adjust volume to 1 mL with methanol and vortex briefly. Dilute to 2.0 mL with distilled water, stopper tube, and vortex.

l. Pour approximately 0.5 - 1.0 mL of extract into a 3 mL syringe and filter through an Acrodisc filter into an HPLC autosampler vial or other appropriate container. Store remaining extract at $<$ -10 °C.

Note: The extraction should be completed in one day. Injections should be started on the autosampler on the same day as the extraction

3. Instrumental Settings

Note: The instrument parameters may be optimized to ensure system suitability.

a. UHPLC Parameters

The following instrumental parameters using a DIONEX Ultimate 3000 UHPLC system and a UHPLC column. The analyst should optimize these parameters for the instrument being used.

Injection volume:	35 µL.
Flow rate:	0.5 mL/min.
Wavelength:	375 nm.
Column heater:	35 °C.
Run time:	5 min.

4. Injection sequence / Sample Set

a. Mixed standard

b. Negative Control (Blank)

c. Positive Control (Fortified Recovery)

d. Samples, up to maximum of 22

e. Mixed standard or Positive Control (note: Reinject 0.5 ppm mixed recovery after every 10 samples)

G. CALCULATIONS / IDENTIFICATION

1. Calculate the recovery of all three tetracyclines fortified at 0.5 ppm levels using peak height or area of chromatograms of same concentrations of standards (see Note D.2.c.).

2. The following criteria are used to identify a sample as positive (+):
   a. Recovery of each analyte at 0.5 ppm level should be ≥ 20%.
   b. Signal to noise ratio(s) at 0.5 ppm level should be greater than 3.
   c. Retention time of sample peak(s) should match with the retention time of tetracyclines recoveries within ± 0.2 min.

H. SAFETY INFORMATION AND PRECAUTIONS

1. Required Protective Equipment — Safety glasses, plastic gloves, and laboratory coat.

2. Hazards

Procedure Step	*Hazard*	*Recommended Safe Procedures*
Oxalic acid	Poisonous, caustic, and corrosive to skin and mucous membranes.	Exercise caution when weighing, preparing, and handling solutions.
Trifluoroacetic acid	This reagent can produce toxic effects through exposure to skin, eyes, and respiratory system.	Operations involving these solvents must be carried out in a well-ventilated fume hood, using protective clothing when applicable.
Acetonitrile Methanol	Harmful vapor.	Avoid breathing fumes.

3. Disposal Procedures

   Follow local, state and federal guidelines for disposal.

I. QUALITY ASSURANCE PLAN

1. Performance Standard
   a. Refer to Section G for identification criteria for the recovery.

b. No false positives for the negative control (blank).

2. Critical Control Points and Specifications

Record	*Acceptable Control*
a. Sample weight	5.0 ± 0.1 g
b. Temperature	Keep sample as cold as possible during analysis.
c. MOX (Methanolic Oxalic Acid Solution)	Prepare fresh with each set.

3. Intralaboratory Check Samples

a. System, minimum contents.

i. Frequency: One per week per analyst when samples analyzed.

ii. Records are to be maintained.

b. Acceptability criteria.

Refer to I.1

If unacceptable values are obtained, then:

i. Investigate following established procedures.

ii. Take corrective action as warranted.

4. Sample Acceptability and Stability

a. Matrix: Bovine, porcine, ovine and poultry muscle and kidney, bovine, ovine and porcine liver.

b. Sample Receipt size: more than 10 g.

c. Condition upon receipt: cold or frozen.

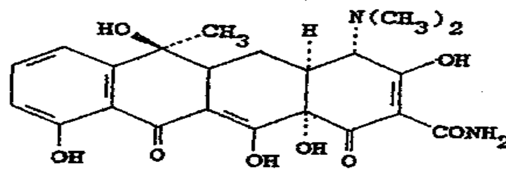
d. Sample storage:

i. Time: Indefinite

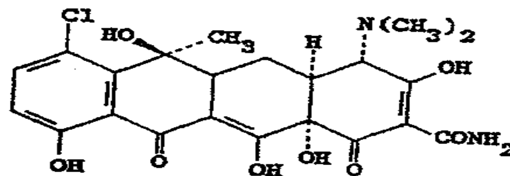
ii. Condition: Frozen at $<$ -10 °C.

J. APPENDIX

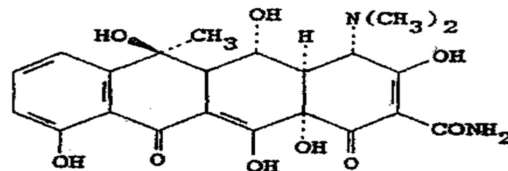
1. Structures



Tetracycline



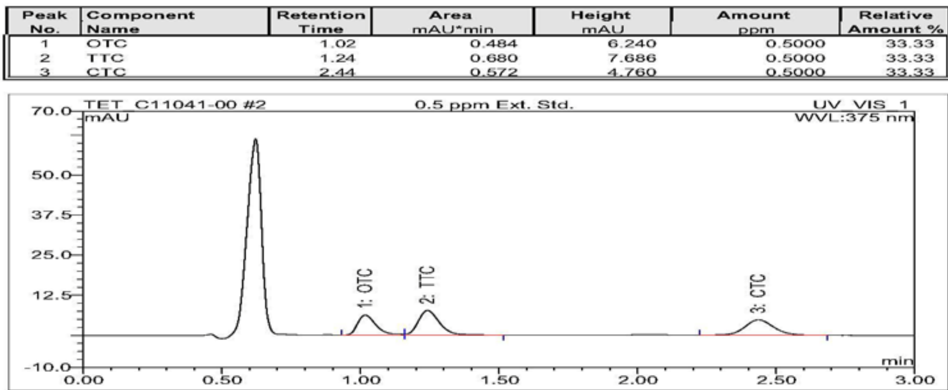
Chlortetracycline



Oxytetracycline

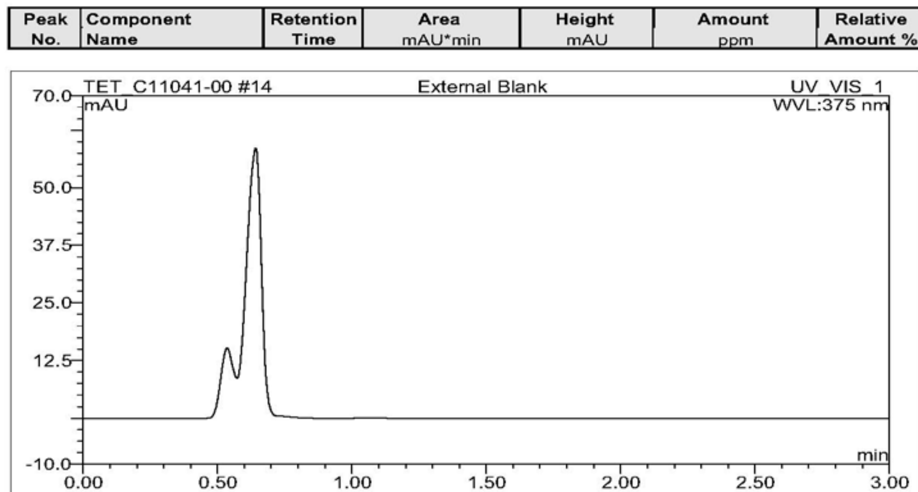
2. Examples of Chromatograms/spectra:

a. External Standard at 0.5ppm



Peak No.	Component Name	Retention Time	Area mAU*min	Height mAU	Amount ppm	Relative Amount %
1	OTC	1.02	0.484	6.240	0.5000	33.33
2	TTC	1.24	0.680	7.686	0.5000	33.33
3	CTC	2.44	0.572	4.760	0.5000	33.33

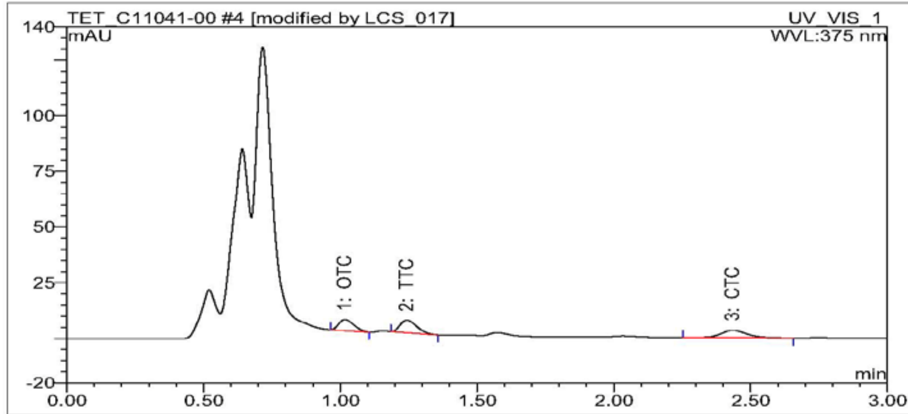
b. External Blank



Peak No.	Component Name	Retention Time	Area mAU*min	Height mAU	Amount ppm	Relative Amount %

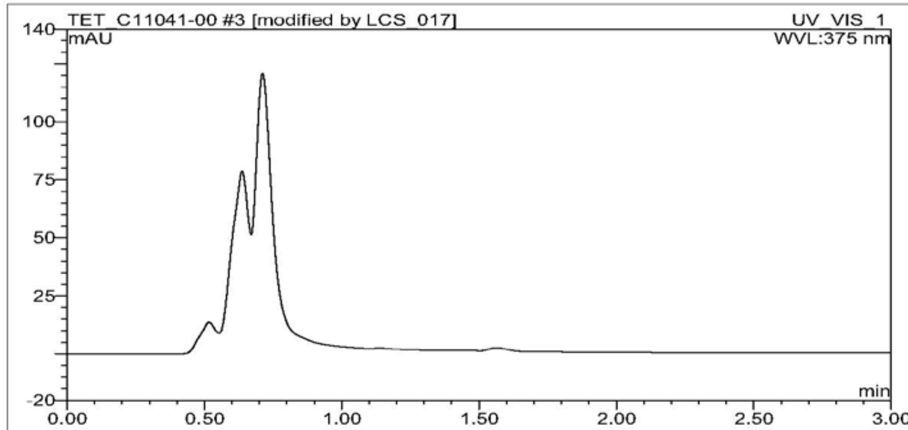
c. Recovery at 0.5 ppm

Peak No.	Component Name	Retention Time	Area mAU*min	Height mAU	Amount ppm	Relative Amount %
1	OTC	1.02	0.311	4.868	0.3933	35.53
2	TTC	1.24	0.393	5.570	0.3652	32.99
3	CTC	2.44	0.362	3.291	0.3485	31.48



d. Tissue Blank

Peak No.	Component Name	Retention Time	Area mAU*min	Height mAU	Amount ppm	Relative Amount %



K. APPROVALS AND AUTHORITIES

1. Approvals on file.

2. Issuing Authority: Director, Laboratory Quality Assurance Division.