

**United States Department of Agriculture
Food Safety and Inspection Service, Office of Public Health Science**

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Title: Qualitative Identification of Tetracyclines in Tissues		
Revision: 01	Replaces: NA	Effective: 9/25/03

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A. INTRODUCTION

This method is used for qualitative identification of Tetracyclines (tetracycline, chlortetracycline and oxytetracycline) in tissues by HPLC analysis.

1. Theory

Partially thawed samples are blended with McIlvaine Solution (McIlvaine Buffer/EDTA). After centrifuging, the extracts 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 HPLC.

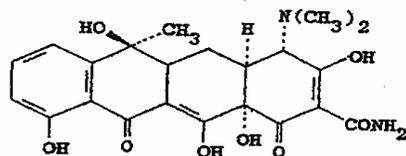
2. Applicability

Applicable to beef, pork, lamb, and poultry muscle, as well as beef, lamb, and pork kidney and liver.

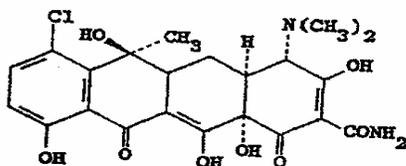
3. Structure

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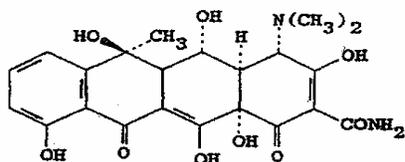
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Tetracycline



Chlortetracycline



Oxytetracycline

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B. EQUIPMENT

1. Apparatus

Note: An equivalent apparatus may be substituted.

- 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 (IEC Genra GP8R).
- 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, Kimble 4515315.
- 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, Kimble 27060-250.
- h. Homogenizer – Model No. PT 10/35, Brinkmann model PT 10/35.
- i. Mechanical shaker - flatbed, 2 speed, Eberbach.
- j. N-Evap with heated water bath, Model No. 112, Organomation Associates.
- k. pH meter readable and accurate to within 0.05 units – Model No. 611, Orion.
- l. Sample filter cartridge - 13 mm diameter x 0.2-0.45 micron, acrodisc, Cat. No. LC13 PVDF, Gelman.
- m. SPE Cartridge - 6 mL, 500 mg C18 packing, Bond-Elut, Cat. No. 1210-2052, Varian.
- n. SPE vacuum manifold, (Supelco Visiprep 5-7030).
- o. Two-way Stopcocks – Cat. No. 7241-00, J T Baker.
- p. SPE reservoirs - 75 mL. Cat. No. 7120-03, J T Baker.
- q. SPE adapters - (Baker 7122-00).
- r. Vortex mixer - Scientific Industries Vortex Genie – 2.
- s. Syringes, disposable - 3 mL (Becton Dickinson 309586).
- t. Volumetric flask - 15 mL and 2 mL.

2. Instrumentation

Note: An equivalent instrument may be substituted.

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- a. Liquid chromatograph consisting of
 - i. Solvent delivery system capable of providing pulse-free flow rates of 0.5-2 mL/minute.
 - ii. Column heater, with ability to maintain system at approximately 35°C.
 - iii. UV detector for HPLC capable of monitoring wavelengths of 350-390 nm.
 - iv. Autosampler or manual injector capable of injection volumes up to 100 microliters.
 - v. Integrator, or chromatography workstation to record detector information.
 - vi. Column: Prodigy C8, 5 micron, 250 x 3.2 mid-bore (Phenomenex OOG-3301- RO).

C. REAGENTS AND SOLUTIONS

1. Reagents

Note: Equivalent reagents or solutions may be substituted.

- 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, 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.

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- 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 21.01 g 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 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.
- f. Dilution Solution:
Mix 100 mL MOX and 100 mL deionized water.

D. STANDARDS

Equivalent standards or solutions may be substituted.

- 1. Source:
Chlortetracycline hydrochloride (CTC), oxytetracycline hydrochloride (OTC), and tetracycline hydrochloride (TTC) are available from Sigma Chemical Company and U.S. Pharmacopeia, 12601 Twinbrook Parkway, Rockville, MD 20852.
- 2. Preparation of Stock and Intermediate Standards:
 - 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.
 - b. Mixed Intermediate Standard (125µg/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.
- 3. Preparation of Working Standards:

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- a. Mixed Working Standards (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.
Note: Use 100 µL of this solution to fortify 5 g sample for 0.5 ppm recovery.
 - b. HPLC standard – Prepare daily HPLC standard in 15 mL 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, and transfer to syringe and filter into HPLC vial.
4. Storage conditions: Store all standards at < -10°C. The stability of the standards is:
- a. Stock standards - 6 months.
 - b. Intermediate - 6 months.
 - c. Working - One week.

E. SAMPLE PREPARATION

Samples are not homogenized before analysis.

F. ANALYTICAL PROCEDURE

1. Extraction

- a. Weigh 5.0 ± 0.1 g of partially thawed intact samples separately into 50 mL polypropylene centrifuge tubes.
- b. 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 control and fortify the other tube with 100 µL of mixed standard (D.3.a) for 0.5 ppm recovery.
- c. Add 20 mL McIlvaine solution to each sample. Homogenize using Polytron until sample is uniformly blended (15-30 seconds). Rinse probe with 4 mL of McIlvaine, adding rinses to centrifuge tube.
- d. 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.

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- e. 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.
- f. Add 20 mL McIlvaine 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.1.e.
- g. 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.
- h. 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 solution to assure that the filter paper is well-seated, and then filter the combined sample extracts. Rinse centrifuge tube with 4 mL McIlvaine solution and filter into flask.
- i. 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.
- j. Connect a 75 mL reservoir to the cartridge. Add the filtered sample extract to the SPE reservoir. Rinse the flask with approximately 4 mL buffer 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.
- k. 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.
- l. 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

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tube, and vortex.

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

2. Instrument Conditions

a. HPLC Parameters

The following instrumental parameters using a Hewlett Packard Series 1050 HPLC system and a Phenomenex Prodigy HPLC column. The analyst should optimize these parameters for the instrument being used.

- i. Injection volume: 35 µL
- ii. Flow rate: 0.6 mL/min
- iii. Attenuation: 2⁷ or 2⁴ for low level standards and samples
- iv. Sensitivity: 2.0 AUFS
- v. Wavelength: 375 nm
- vi. Column heater: 35°C
- vii. Run time: 10 - 14 min

- b. Inject blank, mixed recovery, 0.5 ppm level mixed standard (see Note D.3.a) and samples.

Note: Reinject 0.5 ppm mixed recovery after every 10 samples.

Method Notes:

- i. Test new lots of SPE cartridges for suitability at 0.5 ppm: Condition a new cartridge as in F 1.i. Deliver a 100 µL aliquot of the mixed standard [(D.3.a) 100 µg/mL] to the cartridge. Elute tetracyclines from the cartridge as in steps F.1.k-m. Inject eluate onto the HPLC and calculate percent recoveries using peak heights/area of the same concentration of mixed standards (see Note D.3.a). Recoveries should be greater than 95%.
- ii. The extraction should be completed in one day. Injections should be started on the autosampler on the same day as the extraction.
- iii. If pH of mobile phase drops below 2.95, it can be raised by drop-wise addition of dilute ammonium hydroxide.

3. Sample and standard chromatograms

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(See appendix).

G. CALCULATION

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.3.a).

H. HAZARD ANALYSIS

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.

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3. Disposal Procedures

Procedure Step	Disposition	Notes
Mcllvaine solution	Pour down drain	
Mobile Phase Methanol extracts	Collect in lab solvent waste container	Dispose as per local, state and federal guidelines.

I. QUALITY ASSURANCE PLAN

1. Performance Standard:

- a. Signal to noise ratio(s) at 0.5 ppm level should be greater than 3.
- b. Recovery of each analyte at 0.5ppm level should be $\geq 20\%$.
- c. Retention time of sample peak(s) should match with the retention time of tetracyclines recoveries within ± 0.2 min.
- d. If all three criteria above are satisfied, the sample is considered positive (+).

2. Critical Control Points and Specifications

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

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3. Readiness To Perform

a. Familiarization

i. Phase I: Generate chromatograms of mixed standards in duplicate on three working days at the following levels:

- (a) 0 ppm.
- (b) 0.5 ppm.

ii. Phase II: Generate chromatograms of mixed tetracyclines fortified samples in duplicate over a period of 3 working days at the following levels:

- (a) 0 ppm muscle blank.
- (b) 0.5 ppm muscle recovery.
- (c) 0 ppm liver blank.
- (d) 0.5 ppm liver recovery.
- (e) 0 ppm kidney blank.
- (f) 0.5 ppm kidney recovery.

iii. Phase III Check samples for analyst accreditation:

- (a) 14 samples of muscle, liver and kidney (5 muscles, 5 liver and 4 kidney samples) fortified with various tetracyclines. At least one sample should be blank. Samples given by supervisor or Quality Assurance Manager (QAM).
- (b) Report analytical findings to supervisor and QAM.
- (c) Letter from QAM is required to commence official analysis.

b. Acceptability criteria.

Refer to section I.1 above.

4. Intralaboratory Check Samples

a. System, minimum contents.

- i. Frequency: One check sample per week if samples are analyzed.
- ii. Records are to be maintained for:

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- (a) All findings.

- b. Acceptability criteria.
If unacceptable values are obtained, then:
 - i. Stop all official analyses by that analyst.
 - ii. Take corrective action.

- 5. Sample Acceptability and Stability
 - a. Matrix: Applicable to beef, pork, lamb and poultry muscle, beef, lamb and pork kidney and liver.
 - b. Sample size for analysis: more than 10 g.
 - c. Condition upon receipt: cold or frozen.
 - d. Sample storage: <-10°C.

- 6. Sample Set
 - a. One blank.
 - b. One recovery at 0.5 ppm level.
 - c. Samples.

- 7. Sensitivity
 - a. Minimum proficiency level - 0.5 ppm.

J. WORKSHEET

Example of a worksheet is shown on the following page.

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TETRACYCLINES WORKSHEET

Analyst: _____
 Date Started: _____
 Date Completed: _____
 Species: _____
 Tissue: _____

Set # _____
 Reviewed By Initials and Date _____

Std. And Reagents	MWL ID #
OTC Standard	
TTC Standard	
CTC Standard	
Methicarb Buffer	
SPE Cartridges	
Methanol	
Elution Solution	
MOX solution	
Mechan Phase	

Equipment:	MWL ID #
Balance	
Microprocessor(s)	
N-Evap	
N-Evap Temp.	(40-50 C)

Sample Storage:	
Holding	From FRZ to FRZ Storage
From FRZ	to FRZ
Notes:	

Sample No.	Lab. Number	Form No.	Species	Tissue	Sample Analysis Data		BC Zone Size	Sample Wt. (5.00 ± 0.05 g)	OTC +/-	TTC +/-	CTC +/-
					AT Number						
1	Recovery										
2											
3											
4											
5											
6											
7											
8											
9											
10											
11											
12											

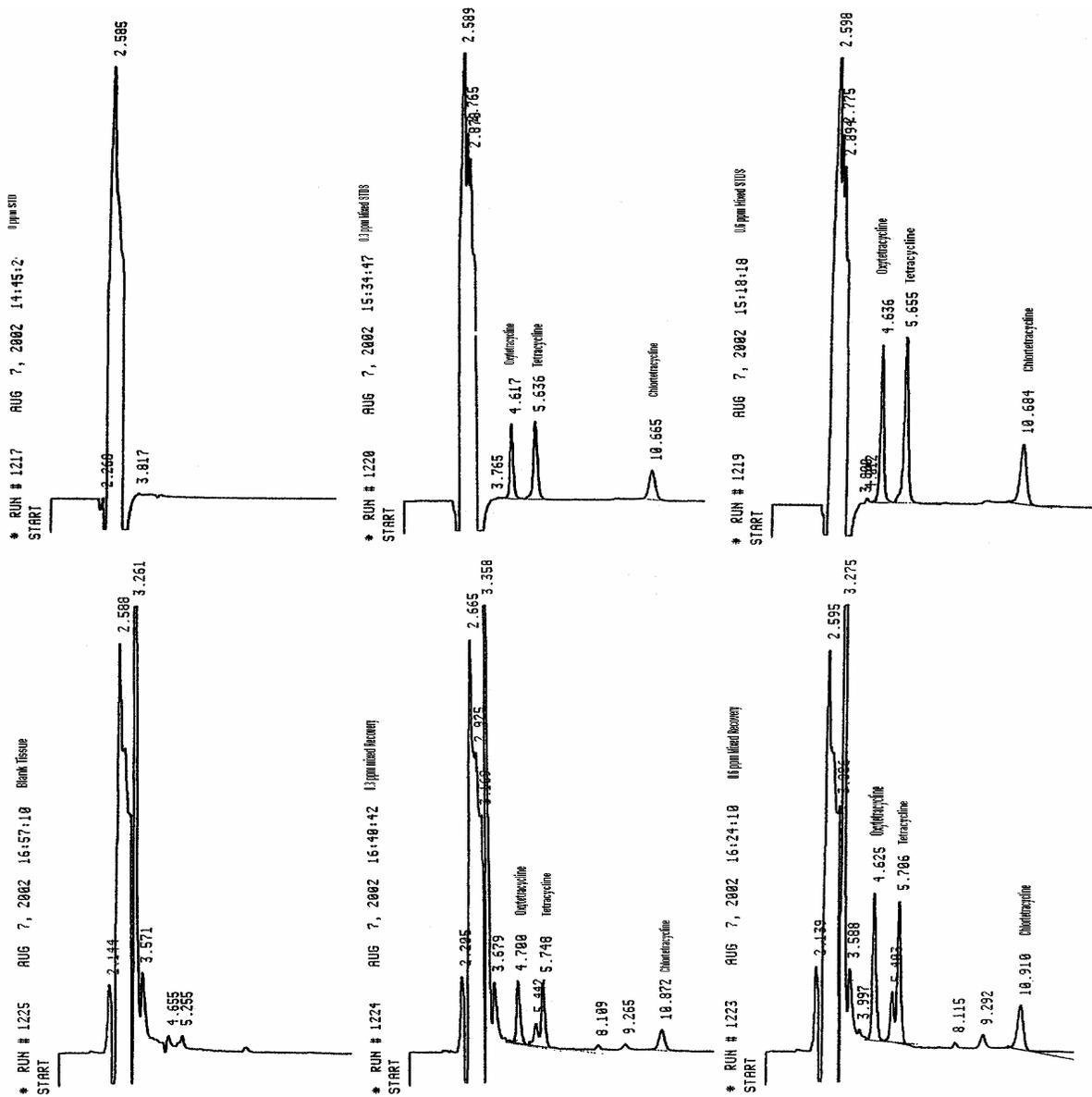
REMARKS:

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K. APPENDIX

Chromatograms of tetracyclines



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