DETERMINATION OF TETRACYCLINE, OXYTETRACYCLINE, DOXYCYCLINE AND CHLORTETRACYCLINE IN EGGS, MILK AND ANIMAL TISSUES USING UPLC

CFIA Saskatoon Laboratory
Centre for Veterinary Drug Residues

REFERENCES:
Oka, H.; Matsumoto, H.; Uno, K. J. Chromatogr., 325 (1985), 265-274
AOAC Official Methods of Analysis 995.09
UPLC transfer, Summary report on file, Sept 6, 2019 (V.Muir); Addendum, Oct 25, 2019 (C. Neiser).

1. Scope:

This method permits the detection and identification of tetracycline (TCS), oxytetracycline (OXY), doxycycline (DOXY) and chlortetracycline (CLI) in eggs, milk, muscle, liver and kidney of food animals; as well as detection and identification of the target analytes in equine kidney.

2. Principle:

The tetracyclines are extracted from the samples with a buffer (pH = 4.0) and the filtered extract is then passed through a conditioned C-18 solid phase extraction (SPE) column. After a water rinse, the tetracyclines are eluted from the SPE column with methanolic oxalic acid. The organic layer is removed through nitrogen-evaporation in a water bath set at 50°C to a final 200µL volume. The eluate is made to 2mL final volume with 90/10 water/acetonitrile. An aliquot of the eluate is PTFE filtered and injected into an ultra high performance liquid chromatograph equipped with a PDA detector run in 2D mode.
3. **Apparatus**

3.1 Notes:
   3.1.1 Suppliers listed for reference only. Other brands of equivalent performance may be substituted.
   3.1.2 All volumetric glassware used throughout this method is Class A.

3.2 Balance, electronic, 0.01 g and 0.0001 g sensitivity.

3.3 Centrifuge, with 50 and 15 mL tube carriers, capable of 6100 x g.

3.4 Centrifuge tubes, polypropylene, 15 mL and 50 mL disposable (#CA21008-951, Falcon Brand, VWR, Mississauga, ON).

3.5 Erlenmeyer flask, sidearm, 125 mL

3.6 Filter, Glass Fibre, Fisher brand, 5.5 cm (#09-804-55B, Fisher Scientific, Nepean, ON) or equivalent.

3.7 Funnel, Buchner, 5.5 cm, ceramic glass.

3.8 Homogenizer - Polytron Model PT 3100 (Brinkman Instruments Ltd., Rexdale ON).

3.9 Liquid chromatography column, analytical: Waters Acquity UPLC BEH C18 1.7µm, 2.1 x 50mm, Part Number 186002350 (Waters Ltd.).

3.10 Liquid Chromatography system, Waters Acquity UPLC H-Class, Waters Acquity photodiode array detector (instrument model 066M) with Empower Chromatography Data Software or equivalent.

3.11 Liquid dispenser, adjustable, 2-10 mL and 2-20 mL

3.12 Mechanical shaker, two-speed, flat bed (Eberbach 6010, VWR).

3.13 Nitrogen evaporator with heating bath, N-Evap Model 112 (Organization Assoc. Inc., Berlin, MA) or equivalent.

3.14 pH meter, ThermoScientific Orion 2 Star with an Accumet single junction, gel filled electrode (catalogue 13-620-108A), or equivalent.

3.15 Pipettors, adjustable, positive displacement 3-25 µL, 10-100 µL, 50-250 µL, 100-1000 µL, plus associated tips (Rainin, Gilson).

3.16 Repeater Pipettor, Handy Step or equivalent and associated tips.

3.17 Solid phase extraction columns, Bond Elut C18, 6 mL, 500 mg (#1210-2052, Varian, Harbor City, CA).
3.18 Solid phase extraction reservoir, 75 mL with adaptors (#9434, 9430 VWR).

3.19 Syringe and Syringe Filters: 3mL Syringe, disposable (Becton Dickson brand, Fisher Scientific); Acrodisc syringe filters, PTFE membrane, 0.2 μm (Acrodisc-13, Gelman Sciences, Inc., Montreal, PQ).

3.20 Transfer pipettes, 3 mL, disposable polyethylene (Falcon brand, VWR) or equivalent.

3.21 Vacuum manifold, for solid phase extraction (#5-7030-u (12 port), 57250-u (24 port), Supelco, Oakville, ON) or equivalent.

3.22 Vials, LC, 2mL clear glass with blue cap, PTFE slit (Canadian Life Sciences, VT009/C397B (vials/caps)).

3.23 Volumetric flasks, various volumes, glass with stoppers.

3.24 Vortex mixer, variable speed.

4. Reagents

4.1 Notes:
4.1.1 Manufacturers listed for reference only. Other brands of equivalent (or better) grade may be substituted.
4.1.2 All water used throughout the method was purified by reverse osmosis followed by deionization, adsorption and filtration.
4.1.3 Preparation instructions are provided for guidance purposes only and, unless noted otherwise, the volume required can be adjusted to allow for more or less solution as required. Preparation details are to be recorded in the reagent preparation log.

4.2 Acetonitrile-190, HPLC grade, Caledon - preferred supply. An alternate supply (Fisher Optima HPLC grade) has been observed to contribute to increased baseline noise with the PDA.

4.3 Citric acid monohydrate, reagent grade (#A104, Fisher Scientific, Edmonton, AB).

4.4 Disodium ethylenediamine tetraacetate (Na₂ EDTA), reagent grade (#S311, Fisher Scientific).

4.5 McIlvaine Buffer: Dissolve 56.82 g dibasic sodium phosphate (Na₂HPO₄) in distilled water in a 2 L flask. Dilute to volume and mix. Dissolve 42.02 g citric acid monohydrate in distilled water in a 2 L flask. Dilute to volume and mix. Combine and mix 2 L of the citric acid solution with 1250 mL of the sodium phosphate solution in a 4 L beaker. Check the pH of this mixture; it should be 4.0 ± 0.05.

4.6 McIlvaine Buffer/EDTA solution: Make the McIlvaine Buffer to contain 0.1 M disodium EDTA by adding the appropriate weight and dissolving. For example, 37.224 g EDTA/L x 3.250 L = 120.98 g. Store at room temperature for up to 3 months.

4.7 Methanol, distilled in glass, Caledon.
4.8 Methanolic oxalic acid, 0.01 M: Dissolve 0.63 g oxalic acid dihydrate in approximately 400 mL methanol in a 500 mL volumetric flask. Dilute to volume with methanol and mix.

4.9 Mobile phase: 0.1% trifluoroacetic acid in acetonitrile and 0.1% trifluoroacetic acid in water are mixed by the LC to create a gradient mobile phase (see 7.3).


4.12 Sodium tungstate dihydrate, ACS reagent grade, >99% (FisherScientific, Acros Organics, AC424471000). Used only for the analysis of eggs.

4.13 Sodium tungstate dihydrate, 10% (w/v) solution: In a 100mL volumetric flask, dissolve 10g sodium tungstate dihydrate into approximately 80mL polished water. Make to 100mL final volume with water and mix. Used only for the analysis of eggs.

4.14 Sulfuric Acid, 50% (v/v), approximately 9M (FisherScientific, Ricca Chemicals, 8180-32). **CAUTION: Severe corrosive hazard. See SDS for more information.** Used only for the analysis of eggs.

4.15 Sulfuric acid, 0.34M solution: In a 100mL volumetric flask, transfer 3.78mL or 5.6g of the 50% sulphuric acid to approximately 80mL polished water. Dilute to volume with water and mix. Used only for the analysis of eggs.

4.16 Trifluoroacetic acid, reagent grade (#T6508, Sigma Aldrich). **CAUTION: severe corrosive hazard. See SDS for more information.**

4.17 Trifluoroacetic acid, 0.1% solution (in water): Add 1 mL trifluoroacetic acid to a 1 L volumetric flask containing approximately 900 mL water. Mix, bring to volume and mix again.

4.18 Trifluoroacetic acid, 0.1% solution (in acetonitrile): Add 1 mL trifluoroacetic acid to a 1 L volumetric flask containing approximately 900 mL acetonitrile. Mix, bring to volume and mix again.

4.19 Water/Acetonitrile, 90/10 (diluent): Add 5 mL acetonitrile to 45mL water in a 50mL centrifuge tube. Vortex mix. Prepare fresh as needed.

4.20 Water/Acetonitrile, 90/10, Wash solution: Add 100 mL acetonitrile to 900mL water in a beaker. Prepare as needed.
5. Standard Solutions:

5.1 Notes:

5.1.1 Avoid direct contact with analytical standards. Wear disposable nitrile gloves, lab coat and protective eyewear. **In addition to following the safety procedures outlined in the Agency’s Laboratory Safety Manual and the Saskatoon Laboratory’s Safe Work Practices and Handling, Storage and Disposal of Chemicals and Hazardous Waste, the analyst must review the CVDR safe work practices procedure for preparation of standards and relevant Safety Data Sheets (SDS’s).**

5.1.2 Supplier Information is provided for guidance purposes only. For current supplier details, contact the program supervisor.

5.1.2.1 Doxycycline Hyclate was obtained from MP Biomedicals.

5.1.2.2 Chlortetracycline hydrochloride, oxytetracycline hydrochloride, and tetracycline hydrochloride were obtained from Sigma-Aldrich Canada Ltd.

5.1.3 Standard preparation instructions are provided for guidance purposes only and, unless noted otherwise, the volume required can be adjusted to allow for more or less solution as required. Preparation details are to be recorded in the standards preparation log.

5.1.4 To determine the weight of the standard required, the analyst must know the chemical form (hydrochloride, sodium salt, etc.) and assayed purity of the analytical standard material, taking both into account when determining the actual amount to weigh for a given concentration.

- Calculate target mass (non-corrected) using the calculation noted below.

\[
\text{Target mass, mg} = \text{Concentration (µg/mL)} \times \text{Final Volume, mL} \times 0.001 \text{mg/µg}
\]

Example (assumes 1000 µg/mL target concentration, 10 mL final volume):

\[
\text{Target mass, mg} = 1000 \text{ µg/mL} \times 10 \text{ mL} \times 0.001 \text{ mg/µg} = 10 \text{ mg}
\]

- Correct the calculated target mass for material purity and formulation.

Example (assumes 90% purity, hydrochloride form of residue = 515.34 g/mol; Base form of residue = 478.88 g/mol):

\[
\text{Corrected mass, g} = 10 \text{ mg} \times \frac{100}{90} \times \frac{515.34}{478.88} \times 0.001 \text{g/mg} = 0.01196 \text{ g}
\]

5.2 Stock Solutions (1000 µg/mL, target concentration):

5.2.1 In separate 10 mL volumetric flasks, dissolve target mass (10mg target, adjusted for purity and salt content, as applicable) of each tetracycline in methanol. Bring to final volume with methanol and mix. Prepare every six months (OXY, TCS, CLI); every year (DOXY). Store in a temperature monitored freezer set at approximately -20°C.

5.2.2 The actual stock standard concentration may vary slightly from the target concentration. In that event, the amount required to prepare a given concentration of a working standard solution will need to be adjusted accordingly to ensure that the working solution
concentration is maintained at the target value.

5.3 Mixed Working Solution (10μg/mL):
Add 500 μL (adjust volume as required for actual stock concentration) of each Stock Solution to a 50mL volumetric flask. Dilute to volume with methanol and mix. Prepare every six months. Store in a temperature monitored freezer set at approximately -20°C.

5.4 Calibration Curve Set, prepare fresh for each run, levels set up to bracket the level(s) of interest, for example: **Low range, <500 ppb**: Pipette 5, 10, 20, 50 or **High range, ≥ 200 ppb**: Pipette, 20, 50, 75 and 100 μL of the 10 μg/mL working mix directly into 2mL LC vial. Make to 1 mL volume with 90/10 (water/acetonitrile). Vortex mix. Inject 20 μL of each to yield calibration curves: Low range [50, 100, 200, 500]; High range [200, 500, 750, 1000] ppb tissue equivalents (TE). **For sets requiring the full range, prepare the calibration set to include 50, 200, 500, 750, 1000 ppb, TE.**

6. Extraction procedure:

6.1 Notes:
6.1.1 In addition to following the safety procedures outlined in the Agency's Laboratory Safety Manual and the Saskatoon Laboratory's Safe Work Practices and Handling, Storage and Disposal of Chemicals and Hazardous Waste, the analyst must review the relevant CVDR Safety Safe Work Practices procedures and Safety Data Sheets (SDS’s).

6.2 Weigh 2.0± 0.02 g of test material into a 50 mL polypropylene centrifuge tube.
6.2.1 Test Material weighing notes:
   - (Kidney, muscle and liver) tissue – chop coarsely at weighing. If chopped too fine, this may contribute to an “over-blended” sample at the polytron step. Even with centrifugation, fines/sediment may carryover when the solution is decanted onto the GF/B filter and/or at the SPE step causing those to plug.
   - Carcass held, muscle samples – are received whole and processed as per CVDR-S-0036, Handling and Processing of Samples for Analysis. The carcass held muscle, processed through a blender, is then sub-sampled in duplicate for analysis. Do not polytron the blended tissue.
   - For Proficiency Testing/Quality control material received as lyophilized tissue, follow the hydration instructions provided from provider prior to analysis.
   - Pre-homogenized or lyophilized tissue. Do not polytron these samples as the fines generated from over-blending may plug the GF/B filters and/or SPE columns.
   - Milk and egg samples are typically received frozen. Thaw (either several hours at room temperature or overnight in a refrigerator) and gently mix (for example by inversion) prior to subsampling. Neither of these sample types require polytronning as the shake step is adequate for blending.
   - NOTE: Egg samples are received and blended at sample reception prior to splitting for analysis by the various programs. If not received as a blended composite (non-routine submission for example), contact CVDR Sample Receiving for preparation instructions.
6.3 Prepare the matched (to test material type) Recovery and Quality control samples.

6.3.1 Low Range set:
- Positive Control Sample (0.100 μg/g TE) Using a control material different from that used to prepare the recovery sample (same matrix type/different source), weigh a 2.0± 0.02 g sample and spike with 20 μL of the mixed working solution. Include one positive control sample for each matrix type included in the run.
- Recovery sample (0.250 μg/g TE) Weigh 2.0± 0.02 g of blank control tissue and fortify with 50 μL of the Mixed Working Solution. Include one recovery sample for each matrix type included in the run.

6.3.2 High Range/Full range sets:
- Positive Control Sample (0.250 μg/g TE) Using a control material different from that used to prepare the recovery sample (same matrix type/different source), weigh a 2.0± 0.02 g sample and spike with 50 μL of the mixed working solution. Include one positive control sample for each matrix type included in the run.
- Recovery sample (0.500 μg/g TE) Weigh 2.0± 0.02 g of blank control tissue and fortify with 100 μL of the Mixed Working Solution. Include one recovery sample for each matrix type included in the run.

6.3.3 Negative Control Sample: Weigh 2.0± 0.02 g of blank control. Do not fortify. Include at least one negative control per batch of samples.

6.4 To each, add 15mL McIlvaine Buffer/EDTA solution. Briefly vortex mix any included lyophilized tissues to fully resuspend the material prior to the shaking step (6.6).

6.5 Polytron blend coarsely chopped tissue samples/controls.

6.5.1 Caution: Do not over-blend as this may cause a plugging issue at the filtration and/or SPE steps. Rinse the Polytron probe with 2 x 2 mL buffer solution.

6.5.2 DO NOT POLYTRON Milk/Eggs/Lyophilized sample (rehydrated)/Pre-homogenized material. Proceed to 6.6.

6.6 Cap and shake the tubes for 5 minutes on low speed.

6.6.1 EGGS ONLY: After the shake step, add 2 mL of the 0.34M sulphuric acid and 2 mL of the 10% sodium tungstate solutions to each egg sample. Re-cap and mix by inversion for approximately 30 seconds (approximately 20 times).

6.7 Centrifuge at 6100 x g (operating temperature set at 20 °C) for 5 min.

6.8 Pour the supernatant into a fresh 50mL centrifuge tube, being careful not to include any of the pelleted sample.

6.9 Repeat extraction of the remaining pelleted sample with another 10mL of buffer/EDTA solution. Cap tube and ensure the remaining pelleted solids are fully resuspended by vortexing prior to shaking. Shake on low speed for 5 minutes.

6.10 Centrifuge at 6100 x g (operating temperature set at 20 °C) for 5 minutes.

6.11 Pool the supernatant with the first aliquot.
6.12 Prepare the Buchner Funnel/Erlenmeyer flasks. Place a GF/B filter into a Buchner funnel and set onto a 125 mL Erlenmeyer flask. Moisten the filter with buffer/EDTA solution and start the vacuum (It is essential that the vacuum be established before the sample is poured on the filter paper).

6.12.1 Slowly pour the supernatant from the centrifuged sample into the centre of the moistened filter paper, watching to see if the filter is plugging as you pour the liquid out of the tube. If the filter appears to be plugging, the GF/B filter can be changed before the full volume is transferred. Do a final rinse of the filter with approximately 2mL of McIlvaine buffer. Note: A poorly filtered sample will plug up the SPE columns.

6.13 Prepare the solid phase extraction (SPE) columns. Use adaptors to mount the 75 mL reservoirs onto the Bond Elut solid phase extraction columns, and place the assembled columns onto the vacuum block.

6.14 Condition the columns with 10mL methanol followed by 10mL water. Add the filtered extract to the reservoir. **Critical Control Point:** Do not let the SPE column run dry at any point in the column conditioning, sample loading and/or column rinse steps.

6.14.1 If several columns are running low simultaneously, stop the flow and fill the reservoir as applicable to the SPE step – ie condition, load, wash.

6.14.2 The flow rate through the column should not exceed a steady drip. The flow rate may decrease as more sample extract passes through the column, and vacuum assist may be required to maintain a rate of 2-3 mL/min (1 drop/sec = approximately 2 mL/min).

6.15 Rinse the sidearm flask with 20mL water and add to the reservoir when the extract is loaded on the column. Allow the column to run dry when the water rinse is completed. Draw air through the column for 5 minutes with vacuum at maximum.

6.16 Drain and clean the extraction system and place 15mL Falcon tubes into position. Elute the tetracyclines from the column with 6.0 mL methanolic oxalic acid solution into the 15 mL falcon tubes. As required, use vacuum assist to maintain a minimum flow rate of 2-3 mL/min. **Note:** The elution flow rate with the methanolic oxalic acid should be closely monitored, since the methanol will pass through the column more quickly than the aqueous extracts.

6.17 Evaporate samples to approximately 200 µL under gentle nitrogen flow in a water bath set at 50°C. Make to 2mL final volume using graduations on the Falcon tube with 90/10 water/acetonitrile.

**NOTE:** N-evaporator needles are to be cleaned between runs to ensure that cross contamination between runs is controlled.

6.18 Cap and vortex samples to mix. Filter the samples and standards through the Acro filters (PTFE) into LC vials.
7. **LC Determination:**

7.1 Instrument conditions are provided for reference purposes. For current run conditions contact the program supervisor. The run conditions are provided with the printed data package. Instructions are provided for the Waters Acquity H-class system, Empower version 3, build 3471, ACQ-PDA detector.

7.2 System preparation:

7.2.1 Prepare mobile phase and wash components (see section 4 for reagent preparation instructions).

7.2.2 Prepare the UPLC for analysis.

Note: The PDA lamp must NOT be turned on before establishing flow through the system. Similarly, at shut down, the lamp must be turned off first, then the flow stopped. The PDA flowcell will be compromised if the lamp is on without mobile phase flowing through the cell.

Typical system start procedure is:
1) In Sample Manager FTN, go to System>Control>Start Up System and check off A, B, C, D and seal wash to prime the LC system lines and seal wash lines, 3 minutes per line at a flow rate of 4 mL/min. NOTE: This turns off the column/sample temperature control, which can be manually turned back on after start up initiates.
2) Load the TCS instrument method (see 7.5 for details) and hit SETUP. When the pressure has stabilized (delta psi readback will be approximately 50), proceed with system suitability analyses.

7.3 System Suitability

7.3.1 The system suitability injections are usually set up in their own sample queue to allow review prior to the onset of sample injections.

7.3.2 System suitability sample set includes the listed functions, to be injected in the order listed, using the TCS instrument method set:
   1. equilibrate
   2. clear calibration
   3. condition column, typical run time, one gradient cycle.
   4. inject samples (use one of the prepared calibration vials, typically the lowest calibration level), inject in triplicate

7.3.3 Suitability criteria for acceptability of chromatography:

Analytes are to be detected with retention parameters comparable to the presented typical chromatograms (see Appendices). Minor tailing is expected with tetracyclines, but excessive tailing is an indication of decreased efficiency of the analytical column, which should then be replaced.

7.3.4 If the chromatography is not suitable, troubleshoot and resolve problem. If not readily resolved, notify program supervisor. If okay, proceed with routine analyses.

7.4 Run samples:

7.4.1 Following review and acceptance of the system suitability results, proceed with the planned run. Include mid run and bracketing injections of known positives (typically the QC and one of the calibration standards) to monitor for retention time and response drift.
7.5 UPLC run conditions:
  7.5.1 LC parameters, routine analyses:

- **Column temperature:** 45°C
- **ACQ-PDA Detector λ:** Normal acquisition rate/5 pps/3.6 nm bandwidth
  2D mode,
  channel 1: 350 nm (OXY, TCS and DOXY);
  channel 2: 370nm (CLI)
- **Run time (per injection):** 10 min
- **Solvent A Name:** 0.1% TFA in Water
- **Solvent B Name:** 0.1% TFA in Acetonitrile
- **Solvent C Name:** Acetonitrile
- **Solvent D Name:** Water

<table>
<thead>
<tr>
<th>Table 1: LC Gradient Program, TCS routine instrument method</th>
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<tbody>
<tr>
<td><strong>Step</strong></td>
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7.5.2 LC parameters, Shut down:
  Column conditioning, post run: at the end of each run, add the instrument shutdown method
  (See Table 2) which will 1) shut down the lamp and 2) flush the column through to a non-
  TFA based storage mix, Acetonitrile/Water (90/10).

<table>
<thead>
<tr>
<th>Table 2: LC Gradient Program, TCS shut down method (PDA lamp off)</th>
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<tbody>
<tr>
<td><strong>Step</strong></td>
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</table>

8. Calculations:

8.1 Calibration curve generation and quantitation calculations are done using Empower software. A
  linear curve function is used to produce the calibration curve for each analyte.

8.2 The calculation for the concentration of analyte in the samples includes a correction for recovery
  (inherent to the Empower software, set up based on “spike” defined at sample set preparation – see 7.4.1). For results to be reported into Sample Manager (where >LOQ), correction for the
exact mass (as recorded at weighing) is done manually.

<table>
<thead>
<tr>
<th>EMPOWER RESULT</th>
<th>WEIGHT CORRECTED</th>
</tr>
</thead>
<tbody>
<tr>
<td>Analyte level, μg/g, TE = (sample peak area) - (y-intercept) x 100 % Recovery</td>
<td>x 2.00 g Sample Wt (g)</td>
</tr>
</tbody>
</table>

9. **Confirmation**

9.1 Suspect samples, with retention times within 2.5% of the fortified samples, at or above the detection limit (as noted in 10.2), are to be confirmed by LC-MS/MS. See CVDR-M-3031 for typical confirmation conditions applicable to the tetracyclines class of antibiotics.

9.2 CVDR-M-3011 typically receives samples for quantitative review which have already been screened and confirmed as positive from the CVDR-M-3031 method. No further confirmation analyses are required in those situations.

10. **Test Reporting**

10.1 Results are reviewed by program supervisor before reporting or re-analysis of samples.

10.2 Method Characteristics (1)

<table>
<thead>
<tr>
<th>Analytical Range</th>
<th>All species, tissues</th>
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<tbody>
<tr>
<td>Oxytetracycline, Tetracycline</td>
<td>0.050 - 1.0 μg/g</td>
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<tr>
<td>Chlortetracycline</td>
<td>0.10 - 1.0 μg/g</td>
</tr>
<tr>
<td>Doxycycline</td>
<td>0.075 - 1.0 μg/g</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Limit of Detection</th>
<th>All species, tissues</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxytetracycline, Tetracycline</td>
<td>0.025 μg/g</td>
</tr>
<tr>
<td>Chlortetracycline, Doxycycline</td>
<td>0.050 μg/g</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Limit of Quantitation</th>
<th>All species, tissues</th>
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</thead>
<tbody>
<tr>
<td>Oxytetracycline, Tetracycline</td>
<td>0.050 μg/g</td>
</tr>
<tr>
<td>Chlortetracycline</td>
<td>0.100 μg/g</td>
</tr>
<tr>
<td>Doxycycline</td>
<td>0.075 μg/g</td>
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</table>

Tolerances: See Health Canada’s website for the most current information regarding (Proposed) Maximum Residue limits ((P)/MRL’s), and/or banned substances.

(1) The Measurement Uncertainty is to be re-calculated whenever a change that affects method accuracy, precision or sensitivity occurs. This information is to be prepared by the responsible program chemist and a copy forwarded to the Section Head.
11. Quality Assurance Plan

11.1 Performance Standards

11.1.1 Determinative Performance

Acceptable Repeatability,
all analytes, all matrices \( CV \leq 20\% \)

Acceptable Reproducibility,
all analytes, all matrices \( CV \leq 30\% \)

Acceptable Recovery (%)

<table>
<thead>
<tr>
<th>Tissues (^{(2,4)})</th>
<th>egg (^{(4)})</th>
<th>Milk (^{(3)})</th>
</tr>
</thead>
<tbody>
<tr>
<td>OXY: 95 ± 25%; 85 ± 20%; 95 ± 10%</td>
<td></td>
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<tr>
<td>TCS: 90 ± 25%; 75 ± 20%; 90 ± 10%;</td>
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<tr>
<td>CLI: 80 ± 20%; 60 ± 15%; 85 ± 10%</td>
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<tr>
<td>DOXY: 80 ± 20%; 50 ± 15%; 90 ± 15%</td>
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\(^{(2)}\) A limited set of performance data in liver was reviewed (May 2008) and results suggest that in liver, all analytes have slightly lower recoveries and higher repeatability between replicates.

\(^{(3)}\) Milk, Recovery data - preliminary data, Jan 2017

\(^{(4)}\) Recovery data updated based on 2017-2019 charted results (summary on file)

Acceptable correlation coefficient, \( r \geq 0.995 \), all analytes
No false positives.
No false negatives if \( \geq LOQ \) (see Section 10.2).
% Trueness, 100 ± 30%.

11.2 Critical Control Points:

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Item</th>
<th>Acceptable Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.14</td>
<td>Bond-Elut</td>
<td>Don’t let the C18's run dry, as this will impact recovery</td>
</tr>
</tbody>
</table>

11.3 Readiness to Perform (Training Plan)

Note: Refer to CVDR-S-0001 and QM-S-0010 for additional training details. Initiate CVDR-A-0046, analyst qualification flowchart, at the onset of analyst qualification process.

As part of the analyst qualification process, an observation run is to be completed, whereby the analyst has the opportunity to work with an experienced analyst.

11.3.1 Full qualification requires an observation run to be completed, in which the analyst has the opportunity to observe and work with an experienced analyst who is experienced in the application of the procedure.

11.3.2 Phase I and II

At the discretion of the program supervisor, phase I and II may be combined, for each run, setting up the calibration and system suitability standards required for phase I along
with the required spikes for phase II.

Phase I provides analyst with the opportunity to demonstrate competency on instrument setup and evaluation of instrument system suitability data and is to include:
two runs, setup and run on separate days. Each run is to include the system suitability injections, as noted in Section 7 and a calibration set.

Phase II provides analyst with the opportunity to demonstrate competency on the analytical procedure, evaluation of results and reporting and is to include:
two runs, done on separate days. Each run to include the system suitability injections, the calibration set and for each of kidney and egg, runs one and two respectively, a negative control, a recovery sample plus six analyst spikes, 3 levels in duplicate.

Submissions for program supervisor review and approval to proceed to phase III include:

For each run, the worksheet/report which is to include the system suitability evaluation (analyst comments) as they relate to acceptance of instrument output, the run sequence, regression analysis(es), all chromatograms, as well as a summary of the analyst spike recoveries and precision.

All runs must be accounted for (including those which did not meet the test method acceptability criteria).

11.3.3 Upon approval, proceed to Phase III. Phase III provides for an evaluation of the analyst’s ability to obtain and produce an analytical result which is unknown to them and is to include:
Two quantitative determinative runs set up on separate days. Each run to include the system suitability injections, a calibration curve, the recovery sample, positive and negative quality control samples, and 6 check samples (whose levels are to be blind to the analyst). This program regularly participates in proficiency testing rounds. When available, one phase III set should include PT material.

11.3.4 Phase III submissions to program supervisor include
For each run, the worksheet and associated Empower generated report. The data package is to include the system suitability evaluation (analyst comments) as they relate to acceptance of instrument output, the run sequence, regression analysis(es), all chromatograms, as well as a quantitative presentation of the check sample results.

All runs must be accounted for (including those which did not meet the test method acceptability criteria).

11.3.5 Acceptability criteria. See 11.1, Performance Standards.

11.3.6 Upon successful completion of the requirements for qualification, program supervisor is to forward the completed CVDR-A-0046 and the CVDR-A-0009 forms to the Section Head for review and signature. Records of completion are to be maintained by the respective
program supervisor as per QM-S-0006.

11.4 Intralaboratory Check Samples:

11.4.1 System, minimum requirements
- Recovery sample analysed with each run, used to correct for recovery.
- Repeat samples – given the infrequent run nature of this method, include a duplicate analysis within each run.
- Positive Control Samples - at least one positive control is included in every run. The level is as defined within the text of the method.
- Negative Control Samples - at least one negative control is included in every run.

11.4.2 Records are to be updated and reviewed for trends by the analyst with every run. Those records include:
- All repeat sample results.
- Positive Quality control results are recorded in a table and when sufficient data is available to generate control limits, the positive QC results are plotted into Individual, X and moving Range, R, Statistical Process Control (SPC) charts. Analyst qualification and validation data may be used to supplement charts.
- Recovery sample results are expressed as a percentage and are recorded for every run. This data may be used to prepare Individual, X and moving Range, R, SPC charts.

11.4.3 Acceptability criteria.
See Section 11.1, Performance standards.

Positive Control and Recovery Sample results, when plotted into an SPC chart, are to be reviewed. See CVDR-S-0004, Test Method Quality Control Samples, for details.

When criteria are not met and/or trends observed, consult with the responsible supervisor. Investigate and identify probable cause, documenting this information with the run, noting actions taken on the necessary control charts/table.

11.5 Interlaboratory Check Sample:
11.5.1 Check samples are obtained from the Proficiency Testing Unit, CVDR, and when available, from the United Kingdom's FAPAS program, Rikilt and/or Progetto Trieste.
11.5.2 In the absence of external PT material, in-house check samples will be evaluated once per fiscal year.
11.5.3 See CVDR-S-0004 for additional details.

11.6 Uniform Analytical Standards:
11.6.1 When each new stock standard solution is prepared (Section 5) it is validated for use as per CVDR-S-0014 and is to include a review of the individual preparation for possible cross contamination review as well as a review of the concentration.
11.6.2 For retention and cross contamination review, prepare individual working solutions at 1.0 μg/mL by adding 10 μL of each old and new stock solution to separate 10 mL volumetric
flasks. Make to volume with water/acetonitrile (90/10). Filter into LC vials and inject 20 μL.

11.6.2.1 Compare the retention of the analyte from the new stock to that observed in a either a previously validated mixed standard or a single analyte preparation of the previous stock solution. Retention times for the old and new solutions must not vary by more than 2.5% difference to be acceptable.

11.6.2.2 Since the CVDR-M-3011 and CVDR-M-3031 programs share stocks, individual dilutions of the new stocks are to be evaluated for suitability (ie no cross contamination concerns) on the LC-MS/MS system, CVDR-M-3031 injection profile.

11.6.3 The quantitative review of the stock can be done with either the single analyte dilution or in a freshly prepared mixed working standard, comparing to a previously validated solution, prepared at an equivalent in-vial concentration for comparison purposes.

11.6.3.1 Prepare an in-vial dilution suitable for injection, old and new. Add 50 μL of each old and new mixed working solution to separate LC vials. Make to 1.0 mL volume with water/ACN (90/10). Inject 20 μL.

11.6.3.2 See CVDR-S-0014 for criteria of responses.

11.6.3.3 If the repeatability criteria between the analyte responses obtained for the old and new standard solutions exceeds tolerance, determine the source of the variance, taking necessary steps for correction. Possible sources of variability include the instrumentation, dilution errors and/or a preparation weighing or transfer error when preparing the stock.

11.6.4 Records of the preparation details, reference to the supporting chromatograms from the standard validation run(s), the calculation results (% difference of the retention times and the % RPD data for response comparisons) and conclusions with regards to acceptance of the new standard preparations are kept with the standards log book/binder.

11.7 Sample Acceptability and Stability:
Matrix: liver, muscle, kidney, milk, egg
Condition upon receipt: cold, not spoiled
Sample Storage: 8 weeks
Condition: frozen at approximately -20°C

Appendix I: TYPICAL CHROMATOGRAMS, Egg Positive and Negative Control Samples