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

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

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. An aliquot of the eluate is injected into a high performance liquid chromatograph equipped with a Penta Fluorophenyl analytical column and a UV detector.
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 sensitivity.

3.3 Centrifuge, with 50 mL tube carriers, capable of minimum 3800 up to 6100 X G.

3.4 Centrifuge tubes, polypropylene, 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 Filters: Acrodisc syringe filters, PTFE membrane, 0.2 μm (Acrodisc-13, Gelman Sciences, Inc., Montreal, PQ); Nylaflo ® 0.2 μm nylon filters, 47 mm (P/N 66602, LifeScience)

3.8 Funnel, Buchner, 5.5 cm, ceramic glass.

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

3.10 Liquid chromatography column, analytical: Luna PFP, Penta Fluorophenyl, 5 μm, 4.6 mm x 150 mm (Phenomenex, Torrance, CA).

3.11 Liquid Chromatography system: Waters Alliance 2695, Waters 2487 UV detector and Waters 2996 photodiode array detector with Empower Chromatography Data Station or equivalent.

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

3.13 Mechanical shaker, two-speed, flat bed (Eberbach, #51105, VWR).

3.14 Pasteur pipette, disposable.

3.15 Solid phase extraction columns, Bond Elut C18, 6 mL, 500 mg (#1210-2052, Varian, Harbor City, CA).

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

3.17 Vial, LC, 2 mL, disposable.

3.18 Volumetric flasks, 5, 10, 500, 1000, 2000 mL

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

4.3 Disodium ethylenediamine tetraacetate (Na$_2$ EDTA), reagent grade (#S311, Fisher Scientific).

4.4 McIlvaine Buffer: Dissolve 56.82 g dibasic sodium phosphate (Na$_2$HPO$_4$) 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.

4.5 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.

4.6 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.

4.7 Mobile phase: Acetonitrile and 0.1% trifluoroacetic acid are mixed by the LC to create a gradient mobile phase (see 7.3).

4.8 Oxalic acid dihydrate, reagent grade (#A219, Fisher Scientific).

4.9 Sodium phosphate, dibasic, reagent grade (#S374, Fisher Scientific).

4.10 Sodium tungstate dihydrate, ACS reagent grade, >99% (Fisher Scientific, Acros Organics, AC42447-1000). Used only for the analysis of eggs.

4.11 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.

4.12 Solvents: Acetonitrile and methanol, distilled in glass.

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

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

4.16 Trifluoroacetic acid, 0.1% solution: Add 1 mL trifluoroacetic acid to a 1 L volumetric flask containing approximately 900 mL water. Mix, bring to volume and mix again. Filter through 0.20 µm filter.

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 Job Hazard Analysis (JHA) for preparation of standards and relevant Material Safety Data Sheets (MSDS’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:

\[
\text{Example 1} \quad \frac{\text{Corrected mass}}{\text{mass}} = \frac{\text{Target Mass x 100}}{\text{Purity, % age}} \times \frac{\text{Molecular Weight (chemical form, salt)}}{\text{Molecular Weight (free base)}}
\]

\[
\text{Example 2} \quad \frac{\text{Corrected mass}}{\text{mass}} = \frac{\text{Target Mass}}{\text{Purity, µg base per mg}} \quad (Watch \ the \ units)
\]

5.1.5 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.

5.2 Stock Solutions (1000 µg/mL):
Dissolve 25 mg (refer to notes section, 5.1) of each tetracycline in methanol in separate 25mL volumetric flasks. Dilute to volume with methanol and mix. Prepare every six months OXY, TCS, CLI); every 2 years (DOXY). Store in a freezer set at approximately -20°C.
5.2.1 Stocks of differing concentration can be prepared. Stock have been assessed for suitability at concentrations up to 5000 μg/mL. (March 2015, standard validation results on file).

5.3 Mixed Working Solution (10μg/mL): Add 0.5 mL of each 1000 μg/mL Stock Solution to one 50mL volumetric flask. If a stock of a different concentration is used, modify the dilution accordingly. Dilute to volume with methanol and mix. Prepare every six months. Store in a freezer set at approximately -20°C.

5.4 Calibration Curve Solutions (0.1 μg/mL and 0.5 μg/mL): Add 100 and 500μL of the Mixed Working Solution to separate 10mL volumetric flasks, add 6.0 mL methanolic oxalic acid to each flask, bring to volume with water and mix. Inject 10, 20 and 40 μL of each solution to give 0.05, 0.10, 0.20, 0.25, 0.5 and 1.0μg/g tetracyclines tissue equivalency. Prepare daily as needed.

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 Job Hazard Analyses (JHAs) and Material Safety Data Sheets (MSDSs).

6.1.2 Test Material weighing notes:
  o (Kidney, muscle and liver) tissue at weighing is to either be prepared whole (finely diced) or freshly homogenized. Pre-homogenized tissue tends to cause the SPE columns to become plugged.
  o Milk samples are typically received frozen. Thaw and gently mix (for example by inversion) prior to subsampling.
  o Egg samples are received whole at sample reception and are blended prior to splitting for analysis by the various programs. If not blended at receipt for analysis, contact CVDR Sample Receiving for preparation instructions.
  o If frozen as a bulk sample, the eggs will need to be thawed overnight at 4°C to allow for adequate mixing prior to subsampling for analysis.

6.2 Weigh 5.0 ± 0.05 g of test material into a 50 mL polypropylene centrifuge tube. See 6.1.2.

6.2.1 Recovery sample (0.5 μg/g, tissue equivalent, TE) Weigh 5.0 g ± 0.05 g of blank control tissue and fortify with 250 μL of the Mixed Working Solution. Include one recovery sample for each matrix type included in the run.

6.2.1 Positive Control Sample (0.1 μg/g, TE) Using a control material different from that used to prepare the recovery sample (same matrix type/different source), weigh a 5.0 g ± 0.05 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.

6.2.1 Negative Control Sample: Weigh 5.0 ± 0.05 g of blank control. Do not fortify. Include at least one negative control per batch of samples.

6.3 To each, add 20mL McIlvaine Buffer/EDTA solution and homogenize for 10 sec with a Polytron blender. Rinse the Polytron probe with 2 x 2 mL buffer solution. Cap and shake the tubes for 5 minutes on high speed.
6.3.1 EGGS ONLY: After the high-speed shake, add 5mL of the 0.34M sulphuric acid and 5mL of the 10% sodium tungstate solutions to each egg sample. Re-cap and shake by hand for approximately 30 seconds.

6.3 Centrifuge at 6100 x g for 5 min.

6.4 Pour the supernatant into a second 50mL centrifuge tube, being careful not to include any of the tissue. Add 20mL of buffer/EDTA solution to the first tube, cap the tube and resuspend the tissue plug by mixing on a vortex mixer. Shake for 5 minutes.

6.5 Centrifuge at 6100 x g for 5 minutes.

6.6 Add the supernatant to the second 50 mL centrifuge tube. Resuspend the tissue plug with a further 10mL buffer/EDTA solution and shake for 5 minutes.

6.7 Centrifuge both the first and second centrifuge tubes at 6100 x g for 5 minutes.

6.8 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).

6.8.1 Filter both of the supernatants from each sample into the same flask. Changing the GF/B filter in between each of the two centrifuge tubes might also help if the filter paper appears to be plugging up. A poorly filtered sample will plug the SPE columns. Plugged filters can also be avoided by slowly pouring the supernatant onto the centre of the moistened filter paper instead of pouring the entire contents of the centrifuge tube on all at once.

6.8.2 Rinse the centrifuge tubes with 2 x 2 mL buffer/EDTA solution and pass through the filter.

6.9 Use adaptors to mount 75 mL reservoirs onto the Bond Elut solid phase extraction columns, and place onto the vacuum block.

6.10 Wash the columns with 10mL methanol followed by 10mL water. Add the extract to the reservoir. Rinse the sidearm flask with 2 x 2mL buffer and add to the reservoir (see Note below). As required, use vacuum assist to maintain a minimum flow rate of 2-3 mL/min.

**Critical Control Point:**

The SPE columns should not be allowed to go dry from the initial methanol wash until the sample and sample wash have passed through. This requires that the elutions be closely monitored. If several columns are running low simultaneously, the flow can be interrupted until they are filled with the next wash. Consult the manufacturer's SPE column guide before attempting this method. 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.

6.11 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.

6.12 Drain and clean the extraction system and place 10mL volumetric flasks into position. Elute the tetracyclines from the column with 6.0mL methanolic oxalic acid solution into the 10 mL flasks.
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.

6.13 Bring the samples to volume with distilled water, cap and mix thoroughly.

6.14 Filter the samples and standards through the Acro filters 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.

7.2 System preparation:
   7.2.1 Filter mobile phase and needle and seal wash components. Turn on the degassing unit and prime the LC system lines and needle and seal wash lines. Rinse the analytical column with approximately 15 mL water/acetonitrile (80/20) followed by approximately 20 mL mobile phase. During each rinse purge the injector at least once.

7.3 LC parameters:
   - Column temperature: 30°C
   - 2487 Detector λ: 350 nm
   - Run time: 24 min
   - Flow rate: 1.4 mL/min

   **LC Gradient Program:**

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7.4 System suitability:
   7.4.1 In some circumstances, while not ideal, there may not be adequate time to review the system suitability injections prior to the onset of the sample run. In those cases, a decision with respect to fitness of the system can be preliminarily based on the operators assessment of the instrument’s basic operating conditions (refer to the instrument specific
standard operating procedures for details).

7.4.2 When the system has equilibrated, inject either a chemical standard or a spiked sample in triplicate, prepared from a previous run. A prepared (positive) vial from the current run may be substituted if the sample from a previous run is not available.

7.4.3 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.

7.4.4 If the chromatography is not suitable, troubleshoot and resolve problem. If not readily resolved, notify program supervisor. If okay, inject 10, 20, and 40 µL of each Calibration Curve Solution and 40 µL of each sample.

7.5 Column conditioning, post run: at the end of each run, flush column with a minimum 30 mL water/acetonitrile (80/20) followed by 20 mL 20/80 water/acetonitrile and 30 mL 50/50 water/acetonitrile. Note: volumes are guideline amounts may be increased to address state of system at a given time. Store in 50/50 water/acetonitrile. The PFP column should never be stored in 100% organic.

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 sample weight and for recovery as follows:

\[
\text{Analyte level, } \mu\text{g/g, TE} = \frac{(\text{sample peak area}) - (\text{y-intercept})}{\text{Slope of calibration curve}} \times \frac{5.00 \text{ g}}{\text{Sample Wt (g)}} \times \frac{100}{\% \text{ Recovery}}
\]

9. Confirmation

9.1 Suspect positive samples are to be (have been) confirmed by LC-MS/MS. See CVDR-M-3031 for confirmation conditions applicable to the tetracyclines class of antibiotics.

9.2 The suspect test sample is confirmed to contain the specific analyte(s) if the following criteria are satisfied:

9.2.1 The retention time of the analyte(s) in the suspect sample match the retention time of the matrix-fortified standard run under the same experimental conditions to within ± 2.5%.

9.2.2 All the expected product ion transitions including the precursor ion must be present with measurable ion intensities.

9.2.3 The product ion intensity ratios (notably the primary ratio data) in the suspect test sample are to agree to those of included matrix-fortified control sample(s) to within approximately 20%.

10. Test Reporting

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

Analytical Range
- All species, tissues
  - Oxytetracycline, Tetracycline: 0.050 - 1.0 μg/g
  - Chlortetracycline: 0.10 - 1.0 μg/g
  - Doxycycline: 0.075 - 1.0 μg/g

Detection Limit
- All species, tissues
  - Oxytetracycline, Tetracycline: 0.025 μg/g
  - Chlortetracycline, Doxycycline: 0.050 μg/g

Reporting Limit
- All species, tissues
  - Oxytetracycline, Tetracycline: 0.050 μg/g
  - Chlortetracycline: 0.100 μg/g
  - Doxycycline: 0.075 μg/g

(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.

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

11. Quality Assurance Plan

11.1 Performance Standards

11.1.1 Quantitative Determinative Performance

Acceptable Repeatability, all analytes, all matrices: CV ≤ 20%

Acceptable Reproducibility, all analytes, all matrices: CV ≤ 30%

Acceptable Recovery (%): Tissues*/egg, Milk

*see 11.1.2 (preliminary data, Jan 2017)

- OXY: 95 ± 25%; 95 ± 10%
- TCS: 85 ± 20%; 90 ± 10%
- CLI: 75 ± 20%; 85 ± 10%
- DOXY: 75 ± 20%; 50±10%(egg) 90 ± 15%

Acceptable correlation coefficient, r ≥ 0.995, all analytes

No false positives.

No false negatives if OXY, TCS present ≥ 0.025 μg/g; if CLI, DOXY ≥ 0.050 μg/g

No systematic bias and no individual bias ≥30%.

11.1.2 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.

11.2 Critical Control Points:

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<th>Item</th>
<th>Acceptable Control</th>
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<td>Bond-Elut</td>
<td>Don’t let the C18's run dry, as this will impact recovery</td>
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11.3 Readiness to Perform (Training Plan)

Note: 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.

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.

11.3.1 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 standard curve, (0, 0.05, 0.10, 0.20, 0.25, 0.50, and 1.0 μg/g).

11.3.2 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 phase I requirements (standard curve and system suitability injections) and for each of muscle and kidney, a negative control, the positive quality control, and six analyst spikes, 3 levels in duplicate.

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

For each run, the worksheet/report (reviewed and approved by the supervisor) 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 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).
For each run, the worksheet/report (reviewed and approved by the supervisor) 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 quantitative presentation of the check sample results.

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

11.3.4 Submissions for review of the analyst qualification include:

A completed analyst qualification flow chart (CVDR-A-0046)

For the phase I and II data, summaries of the analyst spike results (including an assessment of the recovery and precision data).

For the phase III data, a quantitative presentation of the check sample results.

All runs must be accounted for. The summaries are to be traceable to the run identifiers and traceable to the analytical instrument used at analysis.

11.3.5 Acceptability criteria. See 11.1, Performance Standards.

11.3.6 Upon successful completion of the requirements for qualification, the Section Head will authorize the analyst to perform the specific test method via a “Readiness to perform” letter, CVDR-A-0009. Records of completion are to be maintained by the respective program supervisor. See QM-S-0006.

11.4 Intralaboratory Check Samples:

11.4.1 System, minimum requirements

Recovery sample analyzed with each run, used to correct for recovery.

Repeat samples - one for every 20 samples analyzed, set up in the same 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 for trends. SPC review criteria - results are within calculated control limits.

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:

Check samples are obtained from the Proficiency Testing Unit, CVDR, and when available, from the United Kingdom's FAPAS program.

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 of the prepared stocks (prepared at the working standard concentration) relative to the current/old mixed working standard solution.

11.6.2 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. To each, add 6 mL of methanolic oxalic acid. Make to volume with water. Filter into LC vials and inject 20 μL.

11.6.2.1 Retention times for the old and new solutions must not vary by more than 2% 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 Working standards are validated against the previous working standard.

11.6.3.1 Add 500 μL of each old and new mixed working solution to separate 10 mL volumetric flasks. To each, add 6 mL of methanolic oxalic acid. Make to volume with water. Filter an aliquot into an LC vial and inject 40 μL.

11.6.3.2 See CVDR-S-0014 for repeatability criteria of responses. If the Relative Percent Difference (RPD) 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. Re-evaluate the solutions. Possible sources of variability include the instrumentation, possible errors at dilution and/or a preparation error at the stock.
11.6.4 Records of the preparation details, the supporting chromatograms from the 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 in the Standards log book.

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

12. Revision Status

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<th>Previous Version Revision Date</th>
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<td>CVDR-M-3011.18</td>
<td>2017/02</td>
<td>Title, scope, Sections 4, 6, 10, 11</td>
<td>Re-instated egg analyses (previously included in this method; see last use with version 11, archived 2006/05). Performance review data on file with program.</td>
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<td>Section 4</td>
<td>Updated to include sodium tungstate and sulfuric acid reagents and prepared solutions (required for analysis of eggs)</td>
<td>C. Neiser</td>
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<td></td>
<td>6.1.2</td>
<td>Deleted “samples must be frozen prior to analysis”. Storage conditions (11.7, speaks to this requirement). Moved weighing notes from 6.2 into “notes section”. Added handling instructions for egg samples.</td>
<td>C. Neiser</td>
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<td></td>
<td></td>
<td>6.2</td>
<td>Removed heading “all matrices” as this was a carryover from a prior version. Renumbered remaining items.</td>
<td>C. Neiser</td>
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<td></td>
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<td>6.3.1</td>
<td>Updated to include additional extraction step required for egg analyses (treatment with solutions of sulphuric acid and sodium tungstate).</td>
<td>C. Neiser</td>
</tr>
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<td></td>
<td></td>
<td>Section 9; Appendix II</td>
<td>Revised reference to more clearly link to confirmation method (CVDR-M-3031). Deleted Appendix II, confirmation instrument conditions. See instead CVDR-M-3031.</td>
<td>C. Neiser</td>
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<td></td>
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<td>Section 10</td>
<td>Updated to include egg matrix and respective performance specifications; Amended accuracy criteria from 20% to 30% tolerance to reflect actual performance, all analytes (based on review of control charts, 2016-2017; 2017-2018).</td>
<td>C. Neiser</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Section 11</td>
<td>Updated to include egg matrix</td>
<td>C. Neiser</td>
</tr>
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Appendix I: TYPICAL CHROMATOGRAM

Sample Name: CHEM STD 0.5