METHOD: RTETE 025

DETERMINATION OF TETRACYCLINES IN EGGS

1. INTRODUCTION

Tetracyclines are a common antibiotic used in poultry production. Tetracyclines are extracted from eggs with EDTA-McIlvaine buffer pH 4.0 and cleaned up by tandem solid phase extraction with C18 and PRS SPE columns. The eluate is analyzed by HPLC with UV detection at 375 nm. Quantification is accomplished via standard addition calibration curves and detection levels of 25 ppb can be reached with this method.

2. EQUIPMENT

Centrifuge: Beckman Coulter Allegra 21R.

Analytical column: Luna C8(2) Column: 3.0 x 150 mm, 5 µm particle size, or similar equivalent column.

Guard Column: Security Guard cartridge system with C18 cartridge, or similar.

Solid-Phase extraction (SPE) cartridges: Sep-Pak Vac (500mg)/3cc C18 cartridges (Microsep), or similar.
Solid-Phase extraction (SPE) cartridges: Bond-Elut (500mg)/3cc PRS cartridges (Set point), or similar.

SPE vacuum manifold: Supelco 24 port model, or similar.

Whirl-mixer: Vortex Mixer VM-300, or similar.

Ultrasonication Bath: MRC D150H or similar.

Mechanical shaker: Labotech, or similar.

Analytical Balances: MettlerScale AJ100, Mettler DeltaRange Scale, or similar.

pH meter: Bench pH/MV0C meter (HI 9321), or similar.

Glassware: 50 mL polypropylene centrifuge tubes; 50 mL solvent reservoirs; 250 mL side-arm flasks; 15 mL conical glass tubes; 10, 25, 500, 1000 mL measuring cylinders; reagent bottles; plastic Pasteur pipettes.

Water purifier: Elgastat UHQ, or similar

3. INSTRUMENTATION

Liquid chromatograph: Chemstation consisting of a Series 1200 binary pump, vacuum degasser, auto sampler, column heater and diode array detector, or similar. Data processing done by Series 1200 Chemstation software, or equivalent.

4. REAGENT AND SOLUTION LIST

REAGENTS

Acetonitrile, methanol and ethyl acetate: HPLC grade supplied by Riedel-de Haën, or similar.

HPLC Water: Obtain from an Elgastat UHQ water purifier system.

Glass Fibre Pre-filters: supplied by Millipore-Microsep.

Di-Sodium hydrogen phosphate anhydrous (Na₂HPO₄): AR grade supplied by Merck, or similar

Citric acid monohydrate: AR grade supplied by Merck, or similar

Ethylene diamine tetra acetate diodium (EDTA): AR grade supplied by Merck, or similar

Oxalic acid dehydrate: AR grade supplied by Merck, or similar

Ammonium acetate: Ultra grade supplied by Fluka, or similar.
Trifluoroacetic acid (TFA): Spectrophotometric grade supplied by Sigma-Aldrich, or similar

Reference standards:
- Oxytetracycline – Fluka
- Tetracycline hydrochloride – Fluka
- Chlortetracycline hydrochloride – Fluka
- Doxycycline hydrochloride – Sigma

SOLUTIONS

McIlvaine buffer: Weigh 28.4 g anhydrous dibasic sodium phosphate into a plastic weighing boat. Transfer the powder into a 1000ml volumetric flask.

Ensure that all the powder is transferred into the flask before rinsing the weighing boat and funnel with distilled water as the powder solidifies when it comes in contact with water. Dissolve the powder in distilled water by sonification.

Place 21.0 g citric acid monohydrate into another 1000 mL volumetric flask and dilute it to volume with distilled water. Combine 1 L citric acid solution with 625 mL sodium phosphate in 2 L flask. The pH of the mixture must to be 4.0±0.05, if not refer to troubleshooting of the method.

McIlvaine buffer-EDTA solution: Adjust McIlvaine buffer to contain 0.1 M disodium ethylene diamine tetra acetate (EDTA) as follows: To 1.625 L McIlvaine buffer, add 60.5 g disodium EDTA dihydrate and mix until solid dissolves. (Prepare fresh solution weekly)

(90:10, v/v) Ethyl acetate: methanol: Mix 900 ml of ethyl acetate with 100 ml of methanol.

1 M Oxalic acid dihydrate: Weigh 12.607g of oxalic acid dihydrate and dissolve in 100 ml water.

Methanol:Acetonitrile-Mix equal volumes of methanol and acetonitrile in a volumetric flask.

Elution Solvent: (8:1:1, v/v/v) 1M oxalic acid: Methanol: Acetonitrile – mix 100 mL of 1 M oxalic acid with 25 ml of 1:1 methanol – acetonitrile solution.

0.05 M Ammonium acetate: Weigh 3.854 g of ammonium acetate and dissolve in 1 L deionized water.

Mobile phase: Solvent A: Adjust pH of 0.05 M ammonium acetate solution to 3.0±0.05 with trifluoroacetic acid. Filter through 0.2 µm nylon filter and degas. Solvent B: Acetonitrile.
**Primary standards:**

1. Accurately weigh the correct masses of each tetracycline standard into four 100 mL volumetric flasks and make to volume with methanol.

2. Store standards in amber bottles. Prepare fresh primary standards every three months and store at -20°C.

Refer to form 27A attached to the certificate of analysis of each standard for the calculation of the corrected masses. **Working standards:**

1. Prepare a 10 ppm mixed working standard by diluting the primary standards 10 times. That is, accurately measure 1 ml of each primary standard, using a micro syringe, into a 10 ml A-grade volumetric flask and dilute to the mark with methanol. 2. Prepare a 1 ppm working standard by diluting the 10 ppm working standard 10 times. That is, accurately measure 1 ml of the 10 ppm working standard, using a micro syringe, into a 10 ml A-grade volumetric flask and dilute to the mark with elution solvent.

3. You may also prepare the 1 ppm working standard by diluting the primary standards 100 times. That is, accurately measure 0.1 mL of the primary standard, using a micro syringe, into a 10 ml A-grade volumetric flask and dilute to the mark with elution solvent.

4. Prepare fresh working standards weekly and store them at approximately 4°C in the walk-in fridge.

---

**5. SAMPLE FORTIFICATION**

5.1 Accurately weigh 5 g homogenized control egg sample (drug free) into each of 50 mL polypropylene centrifuge tubes. Include three blank samples into the range. 5.2 Fortify according to table below:

<table>
<thead>
<tr>
<th>Fortification Level (mg/kg)</th>
<th>Fortification Standard (mg/l)</th>
<th>Fortification Volume (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.025</td>
<td>10</td>
<td>12.5</td>
</tr>
<tr>
<td>0.050</td>
<td>10</td>
<td>25</td>
</tr>
<tr>
<td>0.100</td>
<td>10</td>
<td>50</td>
</tr>
<tr>
<td>0.200</td>
<td>10</td>
<td>100</td>
</tr>
<tr>
<td>0.300</td>
<td>10</td>
<td>150</td>
</tr>
<tr>
<td>0.400</td>
<td>10</td>
<td>200</td>
</tr>
<tr>
<td>0.500</td>
<td>10</td>
<td>250</td>
</tr>
</tbody>
</table>
5.3 Fortify the control sample to obtain at least 5 points for the calibration curve around the MRL in the linear range, including $\frac{1}{2}$MRL, MRL and 2MRL (where applicable).

5.4 MIX THEN VORTEX SAMPLES FOR 30 SECONDS. ALLOW SAMPLES TO STAND IN THE DARK FOR 20 MIN.

6. EXTRACTION OF SAMPLES:

6.1 Accurately weigh 5 g test sample into a labeled 50 ml polypropylene centrifuge tube.

6.2 Add 30 mL EDTA-McIlvaine buffer pH 4.0 and vortex-mix until dissolved (if not dissolved insert in the ultrasonic bath).

6.3 Shake the samples on mechanical shaker for 10 min.

6.4 Filter the extract using glass fibre pre-filters, into 250 ml side-arm flask with vacuum.

7. CLEANUP ON C18 AND PRS CARTRIDGES:

7.1 Condition a C18 cartridge equipped with 50 mL reservoir, with 10 mL of methanol followed by 10 mL of water (DO NOT ALLOW THE CARTRIDGE TO GO DRY – Leave headspace of cartridge filled with water).

7.2 Load the filtered extract onto the conditioned cartridge at 1-2 drops/s. 7.3 Rinse flask with 5 ml distilled water and add to the cartridge. Then before the cartridge dries up rinse the walls of the reservoir with another 5ml distilled water and allow it also to go through the cartridge at 1-2 drops/sec.

7.3 After everything has passed through the cartridge, apply full vacuum and dry the cartridge for at least 5 min.

7.4 Condition a PRS cartridge with 10 mL of (90:10, v/v) ethyl acetate – methanol (DO NOT ALLOW THE CARTRIDGE TO GO DRY – Leave headspace of cartridge filled with solution).

7.5 Place the C18 cartridge on top of the conditioned PRS column and elute tetracyclines from C18 onto PRS with 50 mL of ethyl acetate : methanol (90:10).

7.6 Discard C18 cartridge and wash PRS cartridge with 10 ml methanol.

7.7 Apply full vacuum and dry the cartridge for at least 5 min.

7.8 Accurately measure 5 ml of elution solvent and elute into 15 ml conical glass tubes.
7.9 Mix using plastic Pasteur pipette, filter through 0.2 µm nylon filter and inject.

8. **CHROMATOGRAPHIC CONDITIONS:**

Flow rate: 0.8 mL/min

Injection volume: 100 µl

Detector: UV set at 375 nm

Column temperature: 40 °C

Mobile phase: Solvent A – 0.05 M Ammonium acetate, pH 3.0 with TFA
Solvent B – Acetonitrile

Gradient Elution:

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>B (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>20</td>
</tr>
<tr>
<td>4</td>
<td>20</td>
</tr>
<tr>
<td>10</td>
<td>60</td>
</tr>
<tr>
<td>11</td>
<td>20</td>
</tr>
<tr>
<td>15</td>
<td>20</td>
</tr>
</tbody>
</table>

Run time: 15 min

The elution order of analytes is indicated below. With this mobile phase the following retention times can be expected:

Oxytetracycline - 2.9 min
Tetracycline – 3.8 min
Chlortetracycline – 7.7 min
Doxycycline- 8.2 min
9. TROUBLESHOOTING:

9.1 If the required separation of the analytes and/or the matrix peaks is not achieved, the following parameters may be optimized:

- Flow rate
- Column temperature
- Mobile phase composition
- Gradient
- Analytical Column

9.2 When the above chromatographic conditions are changed for troubleshooting, the retention times will also change and they must be verified with a standard. Re-run the sequence and re-quantify the analyte peaks.

9.3 McIlvaine: If the pH of the solution is not 4.0±0.05 the following must be done:

i) Ensure that the pH electrode is clean and recalibrate the pH meter.

Measure the pH of the solution again.

If the required pH of the solution is still not achieved discard the solution and prepare fresh solution.

9.4 C8 and C18 columns may be used interchangeably but the type of column must be the same. Evaluate column efficiency by calculating the number of theoretical plates using the following equation:

\[ N_{\frac{W}{2}} = 5.54 \left( \frac{t_R}{W^{\frac{1}{2}}} \right)^2 \]

If a significant decrease in the number of theoretical plates is observed, replace the column.

10. QUANTITATION:

10.1 Create a fortified calibration curve with at least six points, including \( \frac{1}{2} \) MRL, MRL and 2MRL (where applicable) using the Excel programme, and calculate the concentration of the unknown sample according to the fortified calibration curve. The calibration curve can also be created on the instrument software (if there are no blank peaks at the analytes’ retention time) and the concentration transferred on Excel program to be reported. The square of the correlation coefficient for the fortified calibration curve must be \( \geq 0.98 \).
10.2 Prepare the external calibration curve using the same points as in the fortified calibration curve in the elution solvent.

Use the table below to prepare the standards in 1ml vials. Conc. (ppm) | Standard Conc. (ppm) | Volume of std (µl) | Volume of elution solvent (µl)
--- | --- | --- | ---
0.025 | 1 | 25 | 975
0.050 | 1 | 50 | 950
0.100 | 1 | 100 | 900
0.200 | 1 | 200 | 800
0.300 | 1 | 300 | 700
0.400 | 1 | 400 | 600
0.500 | 1 | 500 | 500

11. QUALITY ASSURANCE PLAN

A. Performance standards

Refer to the Quality Manual of the Residue Laboratory

B. Critical Points and Control Specifications

Record Sample Weight Acceptable Control
5.000 g ± 0.100g

C. Readiness to Perform

a) Prepare an internal standard calibration curve for each batch of 50 samples. The square of the correlation coefficient must be ≥ 0.98. If this is not achieved, redo the calibration if the fault is known, or consult the Technical Manager.

b) Verify the separation between different analytes and ensure that separation between the matrix (interference peaks) and the analyte(s) is achieved. If not, consult the Technical Manager. Refer to the troubleshooting section of the method.

c) All deviations from the method must be noted in the laboratory notebook and signed by the Technical Manager.

d) Calculate the recovery of the method by plotting the slope of the fortified calibration curve over the slope of the external calibration curve and divide by the concentration factor.
Note to use approximately the same range in both calibration curves and include the concentration factor in the calculation ($V_f/V_i$).

D. Sensitivity:

a) Lowest limit of detection (LDL) : 0.025 ppm  
b) Lowest limit of quantification (LLQ) : 0.050 ppm  
c) Minimum proficiency level : 0.100 ppm  

(The combined total residues of all substances within the tetracycline group)

E. Sample Acceptability

Matrices: Eggs  
Condition upon receipt: Normal  
Sample receipt size, minimum: at least 50 g per sample  
Sample storage: frozen/room temperature

F. Reporting of results

Results are reported to three decimal places and indicated in the units of mg/kg.

12. SAFETY

12.1 Wear safety glasses or goggles when using the manual homogenizer during sample extraction.

12.2 Work in the fume hood when handling trifluoroacetic acid.

12.3 Observe all safety rules at all times (GSOP 004) and discard all waste, solvents and reagents as specified in GSOP 003.

13. REFERENCES


14. HISTORY


Revision 2: March 2010, Re-wrote method in point format; added troubleshooting section and safety section; Changed mobile phase and the gradient program; Changed pH of McIlvaine buffer from 4.5 to 4.0; Changed sample mass from 10 g to 5 g; Added centrifugation step and filtration step; Added rinsing of flask with McIlvaine buffer.