1.0 INTRODUCTION

Veterinary drugs are used in chicken farms to control disease of laying hens. However, these compounds can be transferred to and accumulate in the eggs. The presence of veterinary drug residues in eggs is a potential health risk for the consumer because the residual drugs can provoke allergic reactions or induce pathogen resistance to antibiotics used in human medicine.

Eggs are among the highest food sources of lecithin (phospholipids) and also have significant amounts of fats. These co-extracted substances can lead to interference and ion suppression in the LC-MS analysis. Eight classes of veterinary drugs are included in this procedure.

2.0 PRINCIPLE

Samples are treated with an acidified acetonitrile/water solvent to precipitate proteins, release bound residues and to extract the veterinary drugs from the egg. It is followed by simple pass-through Oasis Prime HLB cartridge clean-up to remove fats and phospholipids. The extracted residues are examined using UPLC-MS-MS, a triple quadrupole mass spectrometer under electrospray ionization (ESI) conditions.

3.0 GENERAL PRECAUTION

1. All operations using solvents must be conducted in a fume cupboard. Rinse all glassware used before putting it in the washing up tray.

2. Avoid inhalation of and skin contact with the standard powders and the stock solutions.

3. Other hazards, refer to 10.0.

4.0 EQUIPMENT

Note: Equivalent equipment may be substituted.

4.1 Apparatus

1. Rotary stirrer - Heidolph Reax 2
2. Centrifuge - Jouan CR3i and microcentrifuge type Jouan A14
3. Balance - Mettler Top Loading Model PB1502 capable of weighing 2 g ± 0.01 g
4. Balance Analytical - Mettler Model AX2
5. Centrifuge tubes - Polypropylene (PP), 50 mL
6. Centrifuge tubes - Polypropylene (PP), 15 mL
7. Magnetic stirrer and stirbars, volumetric flasks, graduated cylinders, Pasteur pipettes, repeating pipettes and tips, beakers, bottles, weigh boats, spatulas, funnels, and bottle top volumetric dispensors.
8. LC vials with screw cap lids - 2 mL
9. Plastic screw cap vials - Polypropylene, 4 mL
10. Solid Phase Extraction – Oasis PRIME HLB, 3cc, 60 mg

4.2 Instrumentation

1. Waters Xevo TQ-XS or TQS MS/MS

2. Waters Acquity I-Class with MassLynx operating software

3. UPLC column – Acquity UPLC® BEH C_{18}, 2.1 x 100 mm, 1.7 µm

5.0 CHEMICALS, REAGENTS AND SOLUTIONS

Note: Equivalent reagents / solutions may be substituted.

5.1 Chemicals/Reagents

1. Acetonitrile OPTIMA™ LC/MS Grade
2. Methanol OPTIMA™ LC/MS Grade
3. Formic acid
4. Ammonium acetate
5. Water OPTIMA™ LC/MS Grade or house deionized water passed through Milli-Q Integral 5 A10 Pure (Millipore) Ultra Filtration System.

5.2 Solutions

1. 80% acetonitrile/water

Add 80 ml acetonitrile and make up to 100 ml with water in 100 ml volumetric flask and mix.

2. 0.2% formic acid in 80% acetonitrile/water

Add 0.2 ml formic acid to 100 ml volumetric flask. Dilute to volume with 80% acetonitrile/water and mix.

3. **UPLC mobile phase A: 2 mM ammonium acetate in water**

Place 1 ml of formic acid in a 1 liter volumetric flask and make up to the mark with water. Mix and filter through a 0.4 µm filter under vacuum. Store in reagent bottle at room temperature. Stable for 3 months.

(i) 5M ammonium acetate

Weigh 96.35 g of ammonium acetate into a beaker. Dissolve the salt with about 200 mL water (HPLC grade). Pour into 250 volumetric flask and make up to volume to 250 with water.

(ii) 2 mM ammonium acetate

Add 40 µl of the above 5M stock solution into 100 ml volumetric flask. Bring to volume with water.

4. UPLC mobile phase B: 2 mM ammonium acetate in methanol

Add 40 µl of the above 5M stock solution into 100 ml volumetric flask. Dilute to volume with methanol.

6.0 STANDARDS

Note: Equivalent standards / solutions may be substituted. Purity and counterions are to be taken into account when calculating standard concentrations

6.1 Source of Standards

Table 1: List of Standards & Manufacturer

Standard Analyte	Manufacturer	Standard Analyte	Manufacturer
Chloramphenicol	Dr. Ehrenstorfer	Dimetridazole	Fluka
Thiamphenicol	Dr. Ehrenstorfer	Ipronidazole	Witega
Florfenicol	Dr. Ehrenstorfer	Ronidazole	Fluka
Ciprofloxacin	Fluka	CAP-D_5	Cambridge Isotope
Enrofloxacin	Witega	DMZ-D_3	Witega
Norfloxacin	Fluka	IPZ-D_3	Witega
Tylosin	Dr. Ehrenstorfer	RNZ- D_3	WItega
Erythromycin	Sigma-Aldrich	Enrofloxacin-D_5	Witega
Tilmicosin	Dr. Ehrenstorfer	Norfloxacin-D_5	Dr. Ehrenstorfer
Oxytetracycline	Sigma-Aldrich	SMZ-C_{13}	Dr. Ehrenstorfer
Chlortetracycline	Sigma-Aldrich	Penicillin V	Dr. Ehrenstorfer
Doxycycline	Dr. Ehrenstorfer	Demeclocycline	Dr. Ehrenstorfer
Ampicillin	Dr. Ehrenstorfer		
Penicillin G	Dr. Ehrenstorfer		
Sulfadiazine	Dr. Ehrenstorfer		
Sulfathiazole	Dr. Ehrenstorfer		
Sulfamethazine	Dr. Ehrenstorfer		
Sulfaquinoxaline	Sigma-Aldrich		

Standard Analyte	Manufacturer	Standard Analyte	Manufacturer
Sulfadimethoxine	Fluka		

6.2 Preparation of Standard Solution(s)

6.2.1 Stock Standard Solutions and Internal Standard Stock Solutions

(i) Prepare each stock solutions and internal standard stock solutions at approximately 1.0 mg/mL when adequate material is available in appropriate solvent.

(ii) For each stock solution, calculate the amount of base material needed (eg. accounting for purity and/or water and sulfate content) to prepare at the required concentration.

6.2.2 Intermediate Standard Solutions

(i) Prepare each group of intermediate standard and internal standards solutions for the analytes below in 10 mL volumetric flasks.

(ii) Make up volume to 10 ml for each standard group in acetonitrile except for β-lactam group, dissolve in water.

Table 2: Mixed Intermediate Standard for 10 Groups in Acetonitrile

No	Standard Group	Analyte	Stock Standard Conc. (µg/ml)	Volume Stock Std. (ml)	Mixed Intermediate Std Conc. (µg/ml)
1	Amphenicol	Chloramphenicol	1000	0.36	36
		Thiamphenicol	1000	5	500
		Florfenicol	1000	2.5	250
2	Quinolone	Ciprofloxacin	1000	0.25	25
		Enrofloxacin	1000	0.25	25
		Norfloxacin	1000	0.125	12.5
3	Macrolide	Erythromycin	1000	0.5	50
		Tylosin	1000	0.25	25
		Tilmicosin	1000	0.125	12.5
4	Sulfonamide	Sulfamethazine	1000	0.25	25
		Sulfadiazine	1000	0.25	25
		Sulfathiazole	1000	0.25	25
		Sulfaquinoxaline	1000	0.25	25
		Sulfadimethoxine	1000	0.25	25
5	Dapsone	Dapsone	1000	1.25	125
6	Tetracycline	Oxytetracycline	1000	1	100
		Chlortetracycline	1000	1	100
		Doxycycline	1000	0.125	12.5

No	Standard Group	Analyte	Stock Standard Conc. (µg/ml)	Volume Stock Std. (ml)	Mixed Intermediate Std Conc. (µg/ml)
7	Nitroimidazole	Dimetridazole	1000	0.5	50
		Ipronidazole	1000	0.5	50
		Ronidazole	1000	0.5	50

Table 3: Mixed Intermediate β-lactam Standard and Internal Standard in Water

No	Standard Group	Analyte	Stock Standard Conc. (µg/ml)	Volume Stock Std. (ml)	Mixed Intermediate Std Conc. (µg/ml)
1	β-lactam Std	Ampicillin	1000	0.5	50
		Penicilin G	1000	0.125	12.5
		Penicillin V	1000	500	50

Table 4: Mixed Intermediate Internal Standards in Acetonitrile

No	Standard Group	Analyte	Stock Standard Conc. (µg/ml)	Volume Stock Std. (µl)	Mixed Intermediate Std Conc. (µg/ml)
1	Amphenicol	CAP-D_5	1000	50	5
2	Quinolone	Enrofloxacin-D_5	1000	500	50
		Norfloxacin-D_5	100	1000	10
3	Sulfonamide	SMZ-$13C_6$	1000	500	50
4	Tetracycline	DMC	1000	500	50
5	Dapsone	Dapsone-D_8	1000	50	5
6	β -lactam	Penicillin V	1000	500	50
7	Macrolide	Roxythromycin	1000	500	50
8	Nitroimidazole	IPZ-D_3	1000	50	5
		IPZ-OH-D_3	1000	50	5
		DMZ-D_3	1000	50	5
		RNZ-D_3	1000	50	5

6.2.3 Working Standard and and Internal Standard for Spiking and Matrix Calibration

6.2.3.1 Prepare the composite "Acetonitrile Mix" working solution(s) for the veterinary drugs contained in the acetonitrile spiking solutions using the intermediate standard solutions above and the volumes listed in the Table 5 below.

(a) Pipet the calculated volume and as shown in Table 5 into a 10 mL volumetric flask.

(b) Dilute to 10 mL volume with acetonitrile.

(c) Cap flask and mix.

(d) Transfer solution into glass vials with screw cap lids.

Table 5: Mixed Working Standards for 10 Groups in Acetonitrile

No	Standard Group	Analyte	Mixed Intermediate Std Conc. (µg/ml)	Solution Volume, ml	Mixed Working Std Conc. (µg/ml)
1	Amphenicol	Chloramphenicol	36	0.1	0.36
		Thiamphenicol	500		5
		Florfenicol	250		2.5
2	Quinolone	Ciprofloxacin	25	1	2.5
		Enrofloxacin	25		2.5
		Norfloxacin	12.5		1.25
3	Macrolide	Erythromycin	50	1	5
		Tylosin	25		2.5
		Tilmicosin	12.5		1.25

No	Standard Group	Analyte	Mixed Intermediate Std Conc. (µg/ml)	Solution Volume, ml	Mixed Working Std Conc. (µg/ml)
1	Amphenicol	Chloramphenicol	36	0.1	0.36
		Thiamphenicol	500		5
		Florfenicol	250		2.5
2	Quinolone	Ciprofloxacin	25	1	2.5
		Tiamulin	25		2.5
		Lincomycin	25		2.5
4	Sulfonamide	Sulfamethazine	25	1	2.5
		Sulfadiazine			
		Sulfathiazole			
		Sulfaquinoxaline			
		Sulfadimethoxine			
5	Dapsone	Dapsone	125	0.1	1.25
6	Tetracycline	Oxytetracycline	100	1	10
		Chlortetracycline	100		10
		Doxycycline	12.5		1.25
7	Nitroimidazole	Dimetridazole	50	0.1	0.5
		Ipronidazole			
		Ronidazole			
		Metronidazole			

3. Prepare the composite working solution(s) for the veterinary drugs contained in the “Beta Lactam mix” using the intermediate standard solutions above and the volumes listed in the Table 6 below.

(a) Pipet the calculated volume of intermediate standards and internal standard into a 10 mL volumetric flask.

(b) Dilute to 10 mL volume with water.

(c) Cap flask and mix.

(d) Transfer solution into plastic screw cap vials.

Table 6: Mixed Working β-lactam Standards and Internal Standard in Water

No	Standard Group	Analyte	Stock Standard Conc. (µg/ml)	Volume Stock Std. (ml)	Mixed Intermediate Std Conc. (µg/ml)
1	β-lactam Std	Ampicillin	50	1	5
		Penicilin G	12.5		1.25

3. Prepare the composite working solution for the isotopically-labeled veterinary drugs used for internal standards concentrations shown in Table 7.

(a) Pipet the calculated volume of intermediate IS into a 10 mL volumetric flask.

(b) Make up volume to 10 mL with acetonitrile.

(c) Cap flask and mix.

(d) Transfer into 4 glass vials.

Table 7: Mixed Working Internal Standards in Acetonitrile

No	Standard Group	Analyte	Mixed Intermediate Std Conc. (µg/ml)	Solution Volume, ml	Mixed Working Std Conc. (µg/ml)
1	Amphenicol	CAP-D5	5	1	0.5
2	Quinolone	Enrofloxacin-D_5	50		5
		Norfloxacin-D_5	10		1
3	Sulfonamide	SMZ-13C_6	50		5
4	Tetracycline	DMC	50		5
5	Dapsone	Dapsone-D_8	5		0.5
6	β-lactam	Penicillin V	50		5
7	Macrolide	Roxythromycin	50		5
8	Nitroimidazole	IPZ-D_3	5		0.5
		IPZ-OH-D_3	5		0.5
		DMZ-D_3	5		0.5
		RNZ-D_3	5		0.5

7.0 SAMPLE EXTRACTION

7.1 Preparation of Controls and Samples

1. Weigh 2 g ± 0.1 g of homogenized whole chicken egg samples into labeled 50 mL polypropylene centrifuge tubes.

2. Sample for screening set consist of

   (i) Blank (negative control) – 1 portion
   (ii) Spike/recovery (positive controls) – 2 portions for high and low concentrations

   (iii) Unknown samples

7.1.3 Prepare blank, spikes and sample as in Table 8 below;

Table 8: Preparation of Controls and Samples

Label	Std Mix Series 1 in ACN (µl)	Std Mix Series 2 in ACN (µl)	Mixed Working Internal Std, (µl)
Blank	-	-	40
Spike 1	20	20	40
Spike 2	80	80	40
UnknownSample	-	-	40

4. Vortex all uncapped tubes 10 seconds to mix.

5. Allow to stand at least 10 minutes.

6. Continue with to extraction procedure 7.2.

7.2 Extraction Procedure

1. Add 8 ml of 0.2% formic in ACN/H_2O (80:20) to all tubes using a solvent dispenser. Cap tubes well.
2. Vortex tube for 30s and shake all tubes using overhead shaker for 30 minutes.
3. Centrifuge the tubes at 4,000 rpm for 10 minutes.
4. Load 0.5 ml supernatant through Oasis Prime HLB, 3cc, 60 mg without vacuum.
5. Discard extract.
6. Load another 0.5 ml through the same SPE.
7. Collect 0.2 ml filtrate.
8. Dilute filtrate with 0.6 ml water.
9. Vortex 30s, centrifuge high speed ~13,000 rpm for 5 min.
10. Inject into the LC/MS system.

8.0 INSTRUMENTAL SETTINGS

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

8.1 Instrument Operating Parameters - UPLC system

8.1.1 Mobile phase for Residue analysis:

Mobile Phase A - 2 mM amonium acetate in water

Mobile Phase B - 2 mM amonium acetate in methanol

Flush column with 1:1 A/B at a flow rate of 0.5 mL/min for three minutes. Change the mobile phase initial conditions to 100% A. Allow column to equilibrate until the “delta” value on the pressure reading is < 20.

8.1.2 UPLC gradient program:

Flow rate: 0.35 mL/min

Pressure Limits: 200 psi minimum; 18,000 psi maximum

Run time: 11 minutes

Table 10: UPLC gradient program

Time (Min)	% Mobile Phase A	% Mobile Phase B	Gradient
0	90	10	none
0.25	90	10	linear
6	10	90	linear
7.5	10	90	linear
7.6	90	10	linear
11	90	10	linear

8.1.3 Autosampler program:

i. Run time: 11

ii. Injection volume: 5 µL

iii. Weak wash solvent: 20/80 acetonitrile/water

iv. Strong wash solvent: 50/50 acetonitrile/water

v. Sample temperature: 15 °C

4. Column Manager

i. Column temperature: 45 °C

8.2 Instrument Operating Parameters - Mass Spectrometer

Mass Spectrometer calibration and resolution are to be done according to the manufacturer's specification using the manufacturer's supplied calibration solution.

8.2.1 Electrospray Source Parameters:

(a) Capillary (kV): 0.5
(b) Cone (V): Variable - analyte dependent
(c) Source Temperature (°C): 500
(d) Desolvation Temperature (°C): 800
(e) Cone Gas Flow (L/hr): 150
(f) Desolvation Gas Flow (L/hr): 1000
(g) Collision Gas Flow (mL/min): 0.15

8.2.2 Analyzer Parameters:

(a) LM 1 Resolution: 2.73
(b) HM 1 Resolution: 14.74
(c) Ion Energy 1: 0.1
(d) LM 2 Resolution: 2.73
(e) HM 2 Resolution: 15
(f) Ion Energy 2: 0.5

8.2.3 MS Method Parameters:

(a) Type: MRM
(b) Ion Mode: ES+ and ES-
(c) Dwell (s):
(d) Start time (min):

(e) End time (min):

8.2.4 Instrumental Settings - MRM Parameters

Table 11 Instrument Settings

Analyte	Ionisation Mode	RT (min)	Window (min)	Dwell Times (ms)	Precursor Ion(m/z)	Product Ions (m/z	Cone (V)	Collision Energy (V))
Chloramphenicol	Negative	3.60	3.3-4.0	0.2	321	152, 257	70	18, 12
Thiamphenicol	Negative	2.38	2.0-3.0	0.2	354	185, 290	52	22, 12
Florfenicol	Negative	2.92	2.6-3.2	0.1	356	185, 336	56	22, 10
CAP-D_5	Negative	3.58	3.3-4.0	0.2	326	157	54	18
Ciprofloxacin	Positive	2.85	2.5-3.2	0.001	332	288, 314	32	18, 22
Enrofloxacin	Positive	4.97	4.6-5.5	0.01	360	245, 316	32	20, 22
Norfloxacin	Positive	2.85	2.5-4.5	0.001	320	233, 276	32	25, 20
Enrofloxacin-D_5	Positive	4.89	4.6-5.4	0.01	365	245	16	26
Norfloxacin-D_5	Positive	2.85	2.5-4.5	0.001	325	281	30	18
Erythromycin	Positive	5.28	4.6-5.8	0.001	734	158, 576	30	30, 20
Tylosin	Positive	5.71	5.1-6.2	0.001	916.5	101, 174	60	45, 40
Tilmicosin	Positive	5.77	5.4-6.4	0.001	869	174, 696	25	45, 40
Sulfamethazine	Positive	2.61	2.3-3.0	0.001	279	156, 186	46	18, 16
Sulfadiazine	Positive	1.59	1.2-2.2	0.001	251	108, 156	32	24, 14
Sulfathiazole	Positive	1.93	1.6-2.3	0.001	256	108, 156	12	22, 14

Analyte	Ionisation Mode	RT (min)	Window (min)	Dwell Times (ms)	Precursor Ion(m/z)	Product Ions (m/z	Cone (V)	Collision Energy (V))
Sulfaquinoxaline	Positive	2.98	2.6-3.4	0.001	301	108, 156	54	26, 16
Sulfadimethoxine	Positive	3.01	2.6-3.4	0.001	311	108, 156	38	26, 20
SMZ-13C_6	Positive	2.61	2.4-2.9	0.001	285	186	60	16
Dapsone	Positive	2.04	1.9-2.28	0.003	249	108, 156	48	20, 14
Dapsone-D_8	Positive	2.04	1.9-2.28	0.003	257	160	40	14
Oxytetracycline	Positive	2.98	2.7-3.3	0.01	461.2	426.2, 443.1	30	19, 13
Chlortetracycline	Positive	3.85	3.6-4.3	0.001	479.3	444.2, 462.2	30	20, 18
Doxycycline	Positive	3.13	2.7-3.6	0.01	445.2	154, 428.2	30	28, 20
DMC	Positive	3.36	3.0-4.4	0.001	465.2	448.2	30	18
Ampicillin	Positive	2.77	1.0-5.0	0.001	350	160, 174	35	12, 15
Penicillin G	Positive	4.00	3.4-4.5	0.01	335	160, 176	64	14, 10
Penicillin V	Positive	4.08	3.9-4.6	0.01	351	160	23	10
Dimetridazole	Positive	2.24	1.8-2.6	0.05	142	81, 96	54	14, 14
Ipronidazole	Positive	.351	3.1-3.8	0.001	170	109, 124	2	24, 18
Ronidazole	Positive	1.81	1.6-2.3	0.05	201	55, 140	32	20, 10
IPZ-D_3	Positive	3.50	3.1-3.8	0.001	173	127	54	20
DMZ-D_3	Positive	2.23	1.8-2.6	0.001	145	99	54	14

Analyte	Ionisation Mode	RT (min)	Window (min)	Dwell Times (ms)	Precursor Ion(m/z)	Product Ions (m/z	Cone (V)	Collision Energy (V))
RNZ-D_3	Positive	1.79	1.6-2.3	0.001	204	143	35	10

8.3 Sample Set

i) External standard

ii) Blank sample (negative control)

iii) Spike sample at low concentration (positive control)

iv) Spike sample at high concentration (positive control)

v) Up to 27 samples

vi) External standard, spike sample

Note: Placing solvent blanks in the sample injection sequence is prudent in case a high finding leads to carry-over. Additionally, one may want to include an additional external standard or spike sample within the sample injection sequence to verify retention time and instrument response stability.

9.0 CALCULATIONS / IDENTIFICATION

9.1 Monitored ions for each analyte will be assessed as follows:

i. The ion for each analyte must be present. The required ion for each compound is listed in Table 11.

ii. All product ions specified for ratio matching are present with a signal-to noise ratio ≥ 3.

iii. Retention time for ions in the samples must match the retention time of the positive control within 2.5%.

iv. Ion ratios are calculated by dividing the area count of each diagnostic ion by the area count of the base ion. Ion ratios should be less than 1. If the ratio is not less than 1 for a sample set, the inverse of this ratio may be used.

v. The level of the ion in the blank (negative control) must be less than 10% of the spiked 1 (positive control).

9.2 A sample is screened positive for an analyte as follows;

i. Sample ion ratio match the ratio of the positive control sample.

ii. The stability of the ion ratio between the two transitions for sample in accordance with the tolerances recommended as showed in Table 12 below;

Table 12 Maximum permitted tolerances (%) for relative ion intensities using LC-MSMS (Decision 2002/675/EC)

Relative intensity	Tolerances in LC–MS/MS (%)
≥ 50%	±20
≥20% - <50%	±25
≥10% - <20%	±30
<10%	±50

9.3 If samples meet screen positive criteria of as in 9.2, further confirmation test should be conducted.

10. SAFETY INFORMATION AND PRECAUTIONS

Procedure Step	Hazard	Recommended Safe Procedures
Antibiotic standards	Some individuals may have allergic reactions to certain βlactams, sulfa, or other drugs.	Wear appropriate personal protective equipment to avoid dermal contact.
Formic acid, concentrated	Harmful if inhaled. Causes skin and eye burns.	Use only in fume hood. Wear personal protective equipment, avoid skin contact.
Acetonitrile	Flammable, toxic, may be fatal if inhaled or absorbed.	Use only in a fume hood. Avoid breathing fumes. Keep away from flame or heat.
Methanol	Flammable, harmful if swallowed.	Use only in a fume hood. Avoid breathing fumes. Keep away from flame or heat.

11.0 REFERENCE

11.1 Waters Application Note: Simple and Effective Clean-up for UPLC-MS/MS Determination of Veterinary Drug Residues in Egg

Appendix 1

Determination of Multi-class Veterinary Drugs in Egg Using LC-MS/MS