1.0 INTRODUCTION

This method can be used to monitor for residues of the metabolites of macrolide drugs in animal and fish tissues, and also in processed foods.

2.0 PRINCIPLE

Tissue samples are blended with EDTA-McIlvaine buffer solution. After centrifuging, the extracts containing the macrolide are cleaned-up by passing through MCX SPE cartridges. Macrolides are eluted from the cartridge with 5% ammonia in methanol. The eluate is evaporated to dryness and reconstituted with 0.1% formic acid. The extracted residues are examined using LC-MS/MS a triple quadrupole mass spectrometer under electrospray ionization (ESI).

3.0 GENERAL PRECAUTION

3.1 Wear disposable gloves when handling all types of tissue sample. Wash hands thoroughly with soap and water after handling tissue samples.

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

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

3.4 Other hazard, refer to Appendix II.

4.0 EXTRACTION APPARATUS/EQUIPMENT

4.1 Glassware/plastic ware

1. Beakers, various sizes.
2. Duran bottles, 1000 mL with solvent bottle dispenser (10 mL).
3. Volumetric flasks, various sizes.
4. Falcon (centrifuge) tubes (50 mL) with lids.
5. Test tubes (borosilicate glass) – 10 mL capacity.
6. HPLC insert, vials and caps.

4.2 Apparatus

Note: Equivalent apparatus may be substituted

1. Homogeniser, type Silverson L5M-A.
2. Mincer, type Kenwood 8B07.
3. Centrifuge, type Jouan CR3i and microcentrifuge type Jouan A14.
4. Top pan balance, type Mettler PB1502.
5. Analytical balance, type Mettler AX26.
6. Nitrogen evaporator, Turbo-Vap LV, (Zymark) Caliper Life Science.
7. Water purification system, Milli-Q Integral 5 A10 Pure (Millipore).
8. Millipore filter apparatus.
9. Water bath, Memmert
10. Vortex mixer, type Thermolyne.
11. pH meter, type Crison 2001.
12. Pipettor, microadjustable, 50 µl, 100 µl, 500 µl and 1000 µl and tips.

4.3 Solid Phase Extraction

1. OASIS MCX SPE column, 60 mg/3 ml, Waters or equivalent.
2. SPE vacuum manifold and tank.
3. Reservoir adaptor caps.
4. Syringes, plastic disposable (10 mL).
5. Vacuum flask.
6. Vacuum pump.

4.4 Equipment

1. Micromass Quattro Ultima Pt LC/MS-MS, fitted with an ESP probe.
2. HPLC system, Waters 2695.
3. HPLC Column: Columbus 150 x 2 mm, 5 µm, (Phenomenex) or equivalent.

5.0 REAGENTS

Chemicals and solvents are analytical and HPLC grade respectively except where stated. Distilled / de-ionised water is used throughout.

5.1 Reference Standards

1. Tiamulin fumarate (Sigma–Aldrich).
2. Lincomycin (Sigma–Aldrich).
3. Spiramycin (Dr. Ehrenstorfer, Augsburg, Germany).
4. Tylosin (Dr. Ehrenstorfer, Augsburg, Germany).
5. Tilmicosin (Dr. Ehrenstorfer, Augsburg, Germany).

6. Roxithromycin (Dr. Ehrenstorfer, Augsburg, Germany).

7. Josamycin (Dr. Ehrenstorfer, Augsburg, Germany).

5.2 Chemicals

1. Citric acid monohydrate (Ajax, AR grade) or equivalent.
2. Disodium hydrogen phosphate dihydrate (BDH, AnalaR).
3. Disodium ethylenediaminetetraacetate dihydrate (disodium salt) (EDTA) (Ajax, AR grade) or equivalent.
4. Methanol.
5. Acetonitrile.
6. Ammonia solution, 32%.

5.3 Solutions

1. Extraction solution: McIlvaine/buffer

1. Phosphate buffer 0.2 M: Dissolve 35.6 g of disodium hydrogen phosphate (5.2.2) in water and make up to 1 litre.
2. Citric acid 0.1M: Dissolve 21.01 g of citric acid monohydrate (5.2.1) in about 300 ml water and make up to 1 litre.
3. Mix 385.5 ml citric acid 0.1M with 614.5 ml phosphate buffer 0.2M.

4. Check the pH is at 4.0 ± 0.05. Adjust if necessary. To the combined solution add 37.22 g of EDTA and dissolved. Store at 2 to 8°C in a refrigerator for a month.

2. Ammonia solution pH 12.0

Pipet 5 ml ammonium solution 32% in a flask. Add 90 ml of water and adjust to pH 12.0 with ammonium solution 32%.

3. Methanol/Ammonium solution pH 12.0 (10/90 v/v)

Add 10 ml of methanol and and fill up till 100 ml with ammonia solution pH 12.0.

4. Ammonia solution pH 12.0/methanol (5/95 v/v)

Pipet 5 ml ammonia solution pH 12.0 in a volumetric cylinder. Make up to 100 ml with methanol.

5. HPLC mobile phase A: 0.1% formic acid

Place 1 ml of formic acid in a 1 litre cylinder 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.

6. **HPLC mobile phase B: 0.1% formic acid in acetonitrile**

Place 1 ml of formic acid in a 1 litre cylinder and make up to the mark with acetonitrile. Filter through a 0.4 µm filter under vacuum and store in a reagent bottle at room temperature. Stable for 3 months.

6.0 STANDARDS

N.B. Supplies of both standard material and deuterated internal standards are extremely limited. Fresh stock solutions should only be prepared when absolutely necessary.

6.1 Stock standards

6.1.1 Macrolide Standard Solutions: 1 mg/ml in methanol

Weighed out the appropriate amount of macrolide standard and internal standard, taking into account of the purity of the standard material in a 10 ml flask. Dissolve and make up to the mark with methanol. Transfer to a brown glass vial and store in a refrigerator at -20°C.

2. **Internal Standard Solutions: 100 µg/ml in methanol**

Weigh 2.5 ± 0.025 mg of roxithromycin into a 25 ml glass volumetric flask. Dissolve and make up to the mark with methanol. Transfer to a brown glass vial and store in a refrigerator at -20°C.

6.2 Intermediate Standard

1. Macrolide Intermediate Standard Solutions: 100 µg/ml in methanol

Pipette 1 ml of each stock solution (6.1.1) into individual 10 ml volumetric flasks. Add methanol, mix and make up to the mark. Transfer to a brown glass vial and store in a refrigerator at -20°C.

3. **Working Standard and Internal Standard Solution for Matrix Calibration**

1. Mixed Marolide Working Standard for Poultry Muscle

Pipette appropriate volume of 100 µg/ml of individual intermediate standard (6.2.1) into a 10 ml volumetric flask. Add methanol, mix and make up to the mark. Transfer to a brown glass vial and store in refrigerator at 4°C.

Compound	Initial conc. of individual intermediate std. (µg/ml)	Vol. of individual intermediate std. (µl)	Volume of methanol (mL)	Final conc. of mixed working std. (µg/ml)
Tilmicosin	100	200	10	2.0
Tiamulin		200		2.0
Tylosin		200		2.0
Erythromycin		200		2.0
Lincomycin		400		4.0
Spiramycin		400		4.0

2. Mixed Marolide Working Standard for Porcine Kidney

Pipette appropriate volume of 100 µg/ml (6.2.1) and 1 mg/ml (6.1.1) into a 10 ml volumetric flask. Add methanol, mix and make up to the mark. Transfer to a brown glass vial and store in refrigerator at 4°C.

Compound	Initial conc. of std. solution (µg/ml)	Vol. of individual intermediate std. (µl)	Volume of methanol (mL)	Final conc. of mixed working std. (µg/ml)
Tiamulin	100	200	10	2.0
Tylosin	100	200		2.0
Erythromycin	100	400		4.0
Spiramycin	100	600		6.0
Tilmicosin	1000	200		20
Lincomycin	1000	300		30

3. Internal Standard Solution: 5 µg/ml in methanol

Pipette 500 µl of roxithromycin stock solution (6.1.2) into 10 ml volumetric flasks. Add methanol, mix and make up to the mark. Transfer to a brown glass vial and store in a refrigerator at -20°C.

1. SAMPLING

For analysis sub samples are taken from thin slices through the frozen sample to gain as representative a sample as possible. Homogenization of whole sample is best avoided as loss of analyte through increased enzymatic activity may result.

1. SPIKING PROCEDURE

1. Quality Control

1. Weigh out 5 portions (2.00 ± 0.05 g) of negative minced tissue into a 50 ml centrifuge tube.

(i) Blank Samples (2 portions)

Add 100 µl and 200 µl of 5 µg/ml internal standard into a blank tissue pellet poultry muscle and porcine/bovine kidney respectively. Vortex mix for homogenisation. Allow to stand for ~10 minutes. Continue the step from 9.1.5 till the end.

(ii) Spike Samples (3 portions)

a) Add 100 µl and 200 µl of 5 µg/ml internal standard into a blank tissue pellet poultry muscle and porcine/bovine kidney respectively.
b) Add 100 µL of mixed working standard with appropriate concentrations into a blank tissue pellet.
c) Vortex mix for homogenisation.
d) Allow to stand for ~10 minutes.
e) Continue the step from 9.1.5 till the end.

2. Matrix Calibration Curve

1. Weigh out 6 portions (2.00 + 0.05 g) of negative minced tissue into a 50 ml centrifuge tube.

2. Add appropriate volume of mixed working standard and internal standard with appropriate concentration as for poultry muscle and kidney porcine/bovine as below;

a) Chicken Muscle

Label	Spike concentration (µg/kg)		Spike concentration (µg/ml)		Mix WS, volume (µl)	Mix working I.S, 5 µg/ml volume (µl)
	Set 1	Set 2	Set 1	Set 2		
Std 0	0	0	0	0	0	100
Std 1	25	50	250	500	25	100
Std 2	50	100	500	1000	50	100
Std 3	100	200	1000	2000	100	100
Std 4	150	300	1500	3000	150	100
Std 5	300	600	2250	6000	300	100

Set 1 (Tilmicosin, tiamulin, tylosin, erythromycin), Set 2 (Lincomycin, Spiramycin),

b) Porcine Kidney

Label	Spike concentration (µg/kg)					Mix WS, volume (µl)	Mix working I.S, 5 µg/ml volume (µl)
	Tiamulin, Tylosin	Erythro	Spira	Linco	Spira		

Std 0	0	0	0	0	0	0	200
Std 1	25	50	75	250	375	25	200
Std 2	50	100	150	500	750	50	200
Std 3	100	200	300	1000	1500	100	200
Std 4	150	300	450	1500	2250	150	200
Std 5	300	600	900	3000	4500	300	200

3. Vortex mix for homogenization.

4. Allow to stand for ~ 10 minutes. Continue the step from 9.1.5 till the end.

1. SAMPLE PREPARATION PROCEDURE

2. Sample Extraction

1. Weigh 2.0 ± 0.05 g of minced sample into a plastic Falcon tube.

2. Add 100 µl and 200 µl of 5 µg/ml internal standard into a blank tissue pellet poultry muscle and porcine/bovine kidney respectively.

3. Vortex mix for homogenisation.

4. Allow to stand for at least 10 minutes.

5. Add 20 ml EDTA-Mcllvain buffer (5.3.1).

6. Homogenize at full speed for at least 20 second.

7. Mix with a rotative stirrer at 230 rpm for 10 minutes.

8. Centrifuge the sample at 4000 rpm for 15 minutes.

9. Apply supernatant to SPE for further clean up.

2. SPE Cleanup

1. Condition the OASIS MCX SPE cartridges (60 mg/3 ml) with 3 mL methanol followed by 3 mL water, then 3 ml EDTA-Mcllvain buffer (if the tubes are to be left for more than 5 minutes after conditioning, close the tap and leave at least 200 µL of liquid covering the sorbent bed).

2. Fit a plastic syringe barrel and syringe adaptor to the top of the SPE tube. Load the sample supernatant into the SPE tube. Allow the solution to drip through the sorbent bed under gravity.

3. Wash the SPE tube with 3 ml MeOH / ammonia solution pH 12 (10/90, v/v) allow to flow through under gravity.

4. Apply full vacuum for ~ 10 minutes or 1-2 min by nitrogen in order to dry the SPE tubes.

5. Place labeled 10 mL test tubes into the SPE tank rack under the SPE tubes.

6. Elute the samples with 2 x 6 ml ammonia solution pH 12/methanol (5/95 v/v). (A flow rate of not more than 1 mL/ minute should be used).

7. Place the test tube containing sample extract into a pre-heated evaporation bath at ~ 45°C. Adjust the nitrogen flow such that the surface of the liquid ripples gently. (Avoid an excessive nitrogen flow rate as this could cause analyte loss).

8. Allow the sample solutions to evaporate to dryness.

9. Add 200 µL of 0.1% formic acid to the residue and mix the contents on a vortex mixer for 10 - 15 seconds and sonicate for ~ 5 min.

10. If the sample is turbid or contains particulates, centrifuge at 13000 rpm. Sample extracts should be stored at +4 °C if not to be run immediately. Transfer the sample extract to a labeled LC vial.

1. LC-MS/MS ANALYSIS

2. HPLC Parameter (Inlet Method)

1. Inlet

1. Solvent and Flow

Pump A %	95 (Formic acid in H_2O, 100%)
Pump B%	5 (Formic acid in acetonitrile, 100%)
Pump C%	0
Pump D%	0
Run Time (mins)	25
Flow (ml/min)	0.25
Degasser	Normal
Flow Ramps (mins)	2

2. Column Setup

Temperature (°C)	30
Temperature Limit (°C)	20
Low Pressure (bar)	0
High Pressure (bar)	300
Pre-column volume (µl)	0

3. Pump Gradient

Time	A (%)	B (%)	C (%)	D (%)	Flow	Curve
0.00	95	5	0	0	0.25	1
10.00	60	40	0	0	0.25	6
15.00	20	80	0	0	0.25	6
18.00	20	80	0	0	0.25	6
20.00	95	5	0	0	0.25	6
25.00	95	5	0	0	0.25	6

4. Pump Events

Time	Event
0.00	Switch 1

Activate at no change

2. Autosampler

1. Autosampler Initial Conditions

Sample Heater	Injection Parameters
Temperature (°C) : 20	Injection Volume (µl) : 25
Temperature Limit (°C): 20	Needle Depth (mm) : 1
	Draw Speed (µl/sec) : Slow

2. Autosampler Purge

Loop Volumes : 0

3. Click at the tower (change mode) and load method

2. Mass Spectrometer Parameter

1. Electrospray Source (Tuning Parameter)

Capillary :	3.35 kVolts
Cone :	35 Volts
RF Lens 1 :	35
Aperture :	0.0
RF Lens 2 :	0.0
Source Temp (^{o}C) :	120
Desolvation Temp (^{o}C) :	350
Cone gas flow :	60 L/hr
Desolvation gas flow :	600 L/hr
Collision cell pressure :	3.00^{e-3} mbar

2. Analyser Parameters: (Tuning Parameter)

MS1

LM Resolution 1 :	13.0
HM Resolution 1 :	13.0
Ion Energy 1 :	1.0 Volts
Entrance :	1 Volts
Collision :	43 Volts
Exit :	1 Volts

MS2

LM Resolution 2 :	13.0
HM Resolution 2 :	13.0
Ion Energy 2 :	1.8 Volts
Multiplier :	650 Volts

10.3 MS Method Parameters (Experiment Setup)

10.3.1 Function 1

Type	MRM
Ionization Mode :	ES^+
Inter-Channel Delay (sec) :	0.1
Inter-Scan Time (sec):	0.02
Span (Da) :	0.0
Start Time (Mins):	4
End Time (Mins):	12
Cone Voltage (V)	35
Dwell Time (s)	0.2

Name	Precursor ion (m/z)	Product ion (m/z)*	Collision energy (eV)
Lincomycin	407.47	359.09	18
		126.18	26
Tilmicosin	435.43	174.32	21
		694.93	15
	869.19	132.00	40
		173.40	40
		696.10	39
Spiramycin	843.56	540.42	27
		174.15	41
		101.17	42
		318.02	42

*Most abundant product ion is in bold

10.3.2 Function 2

Type	MRM
Ionization Mode :	ES^+
Inter-Channel Delay (sec) :	0.1
Inter-Scan Time (sec):	0.02
Span (Da) :	0.0
Start Time (Mins):	10

End Time (Mins):	25
Cone Voltage (V)	35
Dwell Time (s)	0.2

Name	Precursor ion (m/z)	Product ion (m/z)*	Collision energy (eV)
Tiamulin	494.34	192.03	21
		119.03	36
Tylosin	916.77	772.46	28
		174.14	40
Erythromycin	734.50	558.10	17
		576.18	20
Roxithromycin	837.64	679.43	22

1. CALCULATION OF RESULTS

2. Calculation Using Response Factor

1. Calibrate the instrument by analysing a minimum of five calibration standards.

2. A response factor for macrolides metabolite is calculated as follows.

$$RF = \frac{A_{Std} \text{ x } C_{IS}}{A_{IS} \, x \, C_{Std}}$$

RF = Response factor

A_{Std} = Integrated abundance of the quantitation ion for macrolide standard

A_{IS} = integrated abundance of the quantitation ion for the internal standard

C_{std} = concentration of macrolide standard in µg/kg

C_{IS} = concentration of the internal standard in µg/kg

3. Calculate the mean response factor (RF_{mean}) of the five concentration levels.

4. Calculate the macrolide sample concentration, using following formula

$$C_{macrolide} = \frac{A_{macrolide}\, x\, C_{IS}}{A_{IS}\, x\ (RF_{mean})}$$

$C_{macrolide}$ = concentration of macrolide in µg/kg in the tissue sample

$A_{macrolide}$ = integrated abundance of the ion for macrolide sample

A_{IS} = integrated abundance of the quantitation ion for the internal standard

RF_{mean} = mean response factor of analyte

2. Calculation Using Linear Regression Equation

1. As an alternative to calculate the mean response factor, a linear regression curve can be generated from the initial calibration data by plotting the ratio of macrolide/IS-macrolide (response) versus macrolide concentration.

2. Using the response and associated ppb values, calculate slope, intercept, and correlation coefficient (r) of the regression line.

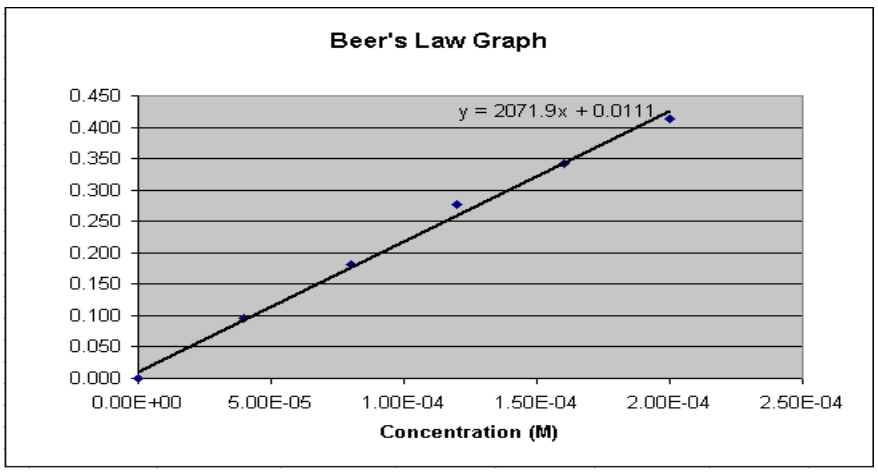
y = mx + c, where

m = slope of the calibration curve
c = intercept from the calibration curve

$$y = \frac{Macrolide_{peak\ area}}{IS - macrolide_{peak\ area}} = \text{response}$$

x = macrolide concentration in ug/kg (ppb)

3. The linear equation shown on the chart represents the relationship between Concentration (x-axis) and Response (y-axis) for the compound.



y = 2071.9 x + 0.111
y - 0.0111 = 2071.9x
x = (y - 0.0111) / 2071.9

m = 2071.9, c = 0.111

X = concentration of macrolide in µg/kg in the tissue sample

y = integrated abundance of the quantitation ion for macrolide sample/ integrated abundance of the quantitation ion for macrolide internal standard sample

4. Results are calculated using linear regression equation by Quanlynx software.

1. CRITERIA FOR ACCEPTIBILITY OF RESULTS

1. Residues of macrolide metabolites in samples are considered confirmed once **all** of the following method performance criteria are met:

(i) The relative retention time of macrolide metabolites in a sample obtained are corresponded to the ratio obtained in standard matrix, with a maximum variation of 2.5%.

(ii) The presence of the two products ion, from each macrolides metabolites molecular peak and presence of ion originating from their internal standards.

(iii) The signal-to-noise (S/N) for all diagnostic ions should be ≥ 3

(iv) The stability of the ion ratio between the two transitions for each macrolide metabolites in accordance with the tolerances recommended as showed in Table 1.

Table 1 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

2. The HU will ensure that blanks do not give rise to any signals that meet the appropriate identification point.

3. These analytes should not be present in food of animal origin. If the identified analyte concentration in a sample is equal to (or greater than) MRL, the sample shall be judged non-compliant. Any samples detected below this concentration shall be reported as negative.

4. If duplicate determinations are carried out and one replicate reveals the presence of macrolide and the other does not, the HS will require that the analysis be repeated.

5. The HS will ensure that percentage recovery values should fall within the ranges.

6. It is normal practice to run a number of recoveries (minimum 2) in each analytical batch. Ideally all of the individual recovery values should fall inside the acceptable range, defined above. If the recovery of analyte from fortified known negative tissue fall outside the acceptable range, the HS must exercise professional judgement in deciding whether or not to accept some/all of the results in the batch as follows:

- If percentage recoveries falls outside the acceptable range and sample is negative the HS may accept the results in the batch.

- If 2 recoveries fall within the acceptable range, 1 recovery falls outside the acceptable range, and the sample is positive average mean recovery shall be taken. If the mean recovery falls within the acceptable range, results for that batch shall be accepted.
- If only one recovery falls within the acceptable range, 2 recoveries fall outside the acceptable range and the sample is positive, the HS will reject the entire batch and the analysis be repeated.

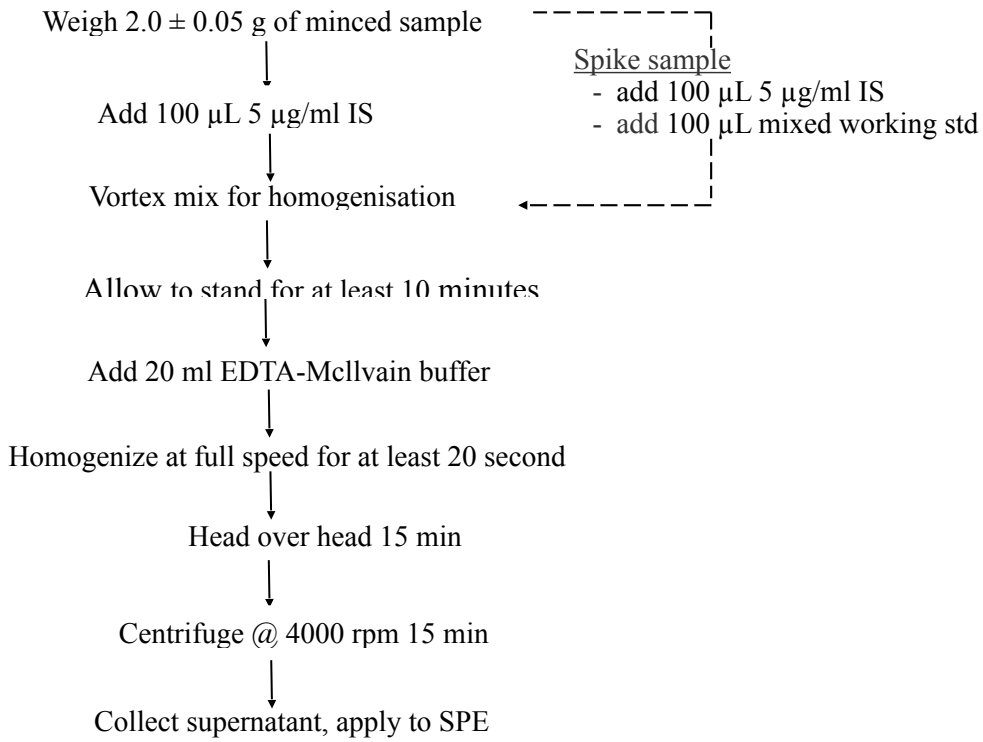
7. Refer to SOP MKAV/C033, "Guidelines for analyte identification and approval of test results" for further performance criteria and requirements for mass spectrometric detection.

1. REFERENCE

13.1 EU DG Trade. (2012). Training Course on Macrolides and Triphenylamines In Food. Food Safety and Quality Laboratory of Selangor.

Appendix 1

DETERMINATION OF MACROLIDE RESIDUES IN ANIMAL TISSUES USING



#SPE Clean up: (OASIS MCX SPE column, 60 mg/3 ml)

- Condition : 3 ml methanol
  3 ml water
  3 ml EDTA McIllvaine buffer
- Load sample : All
- Washing : 3 ml methanol/ammonia solution pH 12(10/90 v/v)

↓

Eluate

↓

- Evaporate using N_2-evap at ~ 45°C
- Add 200 µl 0.1% formic acid
- Vortex ~ 15 seconds & sonicate ~ 5 min.

↓

Transfer solution into microcentrifuge tube

↓

Centrifuge at 13000 rpm for 5 minutes

↓

Collect clear solution & ready for LCMS

Appendix II

HAZARD ANALYSIS

Procedure Step	Hazard	Recommended Safe Procedures
Macrolide standards	Can cause kidney damage	Where PPE when handling standards.
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.
Formic acid, concentrated	Harmful if inhaled. Causes skin and eye burns.	Use only in fume hood. Wear personal protective equipment, avoid skin contact.