CLG-MAL1.02		Page 1 of 20
Title: Confirmation of Macrolide/Lincosamide Antibiotics by Ion Trap HPLC/MS/MS		
Revision: 02	Replaces: CLG-MAL1.01	Effective: 07/30/2007

Contents

A. INTRODUCTION

1. Theory

This method utilizes the weakly basic character of macrolide and lincosamide (mac/linc) antibiotics. Sample tissue is made basic and extracted with ethyl acetate. Analytes are then partitioned into an acidic buffer and further cleaned up by extraction of the buffer solution with an organic solvent. The buffer is then made basic and analytes are re-extracted into ethyl acetate, evaporated to dryness, redissolved in mobile phase, and analyzed by ion trap HPLC/MS/MS. Confirmation is based on comparison of sample and MS/MS spectral data with that of a fortified tissue standard or external standard.

2. Applicability

The method is applicable to confirmation of macrolides in liver, kidney and muscle tissues of avian, porcine, and bovine origin at levels ≥ 0.1 ppm for Lincomycin, Clindamycin, Erythromycin, Tylosin, Tilmicosin, and Pirlimycin. The method is also applicable to confirmation of Tulathromycin at levels ≥ 1 ppm in liver and kidney of porcine and bovine origin.

B. EQUIPMENT

Equivalent apparatus may be substituted for those listed below.

1. Apparatus

a. Centrifuge - IEC-HN-S11.

b. Waring Blender - Cat. No. 33BL79 equipped with a 40 mL blending jar.

c. Vortex mixer - Genie 2, Fisher Scientific.

d. pH Electrode - Cat. No. 215 with a Accumet micro combination electrode, Cat. No.13-620-95, Denver Instrument Co.

e. pH meter - Cat. No 370 with ATC probe, Orion.

f. Balance - accurate to 0.01 g, Cat. No. PB 302, Mettler Toledo.

g. Balance - accurate to 0.001 mg, Cat. No. MT 5, Mettler Toledo.

h. Nitrogen evaporator - TurboVap LV, Zymark.

i. Volumetric flasks - 100 mL and 10 mL class A volumetric flasks.

j. 15 mL glass centrifuge tubes for Zymark TurboVap - Cat. No. 73790, Kimble.

k. 15 mL glass centrifuge tubes - Cat. No. 73785, Kimble.

l. Autosampler vials - 1.8 mL wide mouth glass, Cat. No. 5182-0543, Agilent.

m. Micropipetters capable of delivering 100 µL, 400 µL, 500 µL, 1000 µL.

n. Pasteur pipettes 5 3/4 in - Cat. No. 53283-910; Lab Depot.

2. Instrumentation

a. Ion trap mass spectrometer - Finnigan LCQ-deca equipped with an APCI LC interface and LCQ Xcalibur data system, or equivalent.

b. LC system - Quaternary pump equipped with degassing capability and autosampler. Thermo-Finnigan Surveyor HPLC and autosampler.

c. LC column - Zorbax SB-C18 2.1 x 150 mm containing 5 µm particles, preceded by a 0.2 µm frit filter.

C. REAGENTS AND SOLUTIONS

Equivalent reagents and solutions may be substituted for the following unless otherwise indicated:

1. Reagents

a. Methanol (MeOH), LC grade - Mallinckrodt UltimAR grade.

b. Water, LC grade - House distilled water passed through Waters MilliQ deionization system.

c. Acetonitrile - UV grade, Cat. No. 015-4, Burdick & Jackson.

d. Phosphoric acid - ACS grade, Cat. No. P6560, Sigma.

e. Ethyl acetate - UltimAr grade, Cat. No. U-553-10, Mallinckrodt.

f. Hexane - Cat. No. HX0296-1, EM OmniSolv.

g. Potassium phosphate monobasic (KH_2PO_4) - HPLC grade, Cat. No. P286-1, Fisher.

h. Formic acid - ACS grade, Cat. No. F-4636, Sigma.

i. Potassium carbonate - ACS grade, Cat. No. P6037, Sigma.

2. Solutions

Note: Solutions may be stored at room temperature unless otherwise noted.

a. 50:50 (v/v) methanol/water:

Mix 50 mL methanol with 50 mL of water. Stable for 6 months.

b. 5:95 (v/v) acetonitrile/water + 0.1% formic acid:

Mix 50 mL acetonitrile, 950 mL water and 1.0 mL formic acid in a 1 liter graduated cylinder. Filter. Stable for 6 months.

c. 95:5 (v/v) acetonitrile/water + 0.1% formic acid:

Mix 950 mL acetonitrile, 50 mL water and 1.0 mL formic acid in a 1 liter graduated cylinder. Filter. Stable for 6 months.

d. 0.2 M potassium phosphate monobasic, pH 4.00:

Weigh 13.6 g of KH_2PO_4 into a 500 mL volumetric flask and dilute to volume with water. Adjust the pH to 4.00 with 1:20 phosphoric acid. Store refrigerated. Stable for 3 months. Warm to room temperature before use.

e. 1:20 (v/v) phosphoric acid/water:

Dilute 5 mL of conc. phosphoric acid to 100 mL with water in a volumetric flask.

f. 1:1 (v/v) ethyl acetate:hexane:

Mix equal volumes of ethyl acetate and hexane.

g. 2 M potassium carbonate:

Weigh 27.64 g of K_2CO_3 into a 100 mL volumetric flask and dilute to mark with water.

D. STANDARDS

1. Names & Sources

Name	**Cat. No.**	**Source**
Lincomycin hydrochloride	L6004	Sigma
Clindamycin hydrochloride	C5269	Sigma
Erythromycin	E0774	Sigma-USP
Tylosin tartrate	T6134	Sigma
Tilmicosin		Lilly Research Labs
Pirlimycin hydrochloride		Pfizer Corp.
Tulathromycin		Pfizer Corp.

2. Preparation of Standard Solutions

Note: Equivalent standards and solutions may be substituted for any of the following.

a. Individual drug stock standard solutions (100 µg/mL):

Using vendor's stated purity, or water and salt content, calculate the amount of material which contains 10 mg drug base. Weigh out approximately this amount for each drug, accurately recording weight to nearest 0.1 mg. Transfer to 100 mL glass volumetric flask and dilute to volume with methanol. Calculate exact concentration based on purity and actual weight. This solution is stable for 6 months at < -10ºC.

b. Mixed standard fortification solution (5 µg/mL Lincomycin, Clindamycin, Erythromycin, Tylosin, Tilmicosin, and Pirlimycin; 50 µg/mL Tulathromycin):

Add 500 µL of each of the above 100 µg/mL Lincomycin, Clindamycin, Erythromycin, Tylosin, Tilmicosin, and Pirlimycin drug stock solutions to a 10 mL volumetric flask. Add 5 mL of the Tulathromycin stock solution (100 µg/mL) to this flask and dilute to volume with methanol. This solution is stable for 6 months at < -10 ºC.

c. Preparation of mixed external standard solution (1 µg/mL Lincomycin, Clindamycin, Erythromycin, Tylosin, Tilmicosin, and Pirlimycin; 10 µg/mL Tulathromycin):

Using a micropipettor, transfer 2 mL of mixed fortification solution (D.2.b) to a 10 mL volumetric flask. Dilute to volume with 50% methanol in water (C.2.a) and mix by vortexing. This solution is stable for 3 months at < -10 ºC.

E. SAMPLE PREPARATION AND CLEANUP

1. Blend sufficient whole liver, muscle or kidney tissue for each sample in a 40 mL blending jar. A separate sample holding/receiving section may homogenize sample using their equipment.

   Note - After each blending and weighing, rinse the blending jar with hot tap water, D.I. water, methanol, hexane and finally with methanol.

2. Weigh 5.00 ± 0.10 g tissue into a 50 mL disposable polypropylene centrifuge tube. If muscle tissue is to be analyzed, add approximately 3.0 mL of water and mix well with a microspatula.

   Note: Prepare Controls (to be included as part of each sample batch) at this time:

   a. Negative controls are tissues from animals known to be free of drugs. If these are not available, tissue from an unknown source may be used provided it is first tested and shown to be free of contaminants. Store tissue frozen, preferably at < -10 ºC prior to analysis.

   b. Positive controls are negative tissues that have been fortified with mac/linc's before extraction. To prepare a fortified sample, add 100 µL mixed fortification solution (D.2.b.), then vortex 10 to 20 seconds (If muscle tissue, stir with a microspatula instead).

3. Prepare an aqueous tissue suspension having a pH of 9.8 – 10.2 by adding aliquots of 2M K_2CO_3 and mixing until a stable pH in that range is obtained (usually requires 300-500 µL of 2M K_2CO_3). Vortex or stir with a microspatula (muscle) after each addition. Measure pH using a micro pH electrode just touching the surface of the solution.

4. Add 30 mL of ethyl acetate, cap and shake 3 min. Centrifuge for 10 min at approximately 2000 rpm. (~800 g - rcf). Pour the supernatant into a second 50 mL polypropylene centrifuge tube.

5. Repeat the above step with 15 mL of ethyl acetate and add to the polypropylene centrifuge tube. Remove any oily residue at the bottom of the tube of the combined ethyl acetate extracts with a Pasteur pipette (occasionally seen with muscle samples).

6. To the approximately 45 mL of combined organic phase, add 2.0 mL of 0.2M KH_2PO_4. Shake 3 min. and centrifuge at approximately 2000 rpm for 6 min. Transfer the bottom aqueous layer with a Pasteur pipette and bulb to a 15 mL glass centrifuge tube.
7. Repeat step 6 twice. Retain each sample's Pasteur pipette and use for all three transfers and then discard.
8. To the approximately 6 mL of combined aqueous solution, add 5 mL of a 1:1 ethyl acetate: hexane solution and invert the tube to mix 10 times. Centrifuge at 2000 rpm for 4 min. Discard the top organic layer. Note: Be sure to remove the entire top organic layer.
9. Adjust the pH of the aqueous solution to 9.8-10.2 by adding approximately 900 to 1200 µL of 2M K_2CO_3 (check the pH using a micro pH electrode just touching the surface of the solution)
10. Add 4.0 mL of ethyl acetate to the aqueous solution and shake 3 min. Centrifuge at 2000 rpm for 4 min. Transfer the upper organic layer to a 15 mL disposable glass centrifuge tube (Zymark tube) with a Pasteur pipette.
11. Repeat step 10 twice. Retain each sample's Pasteur pipette and use for all three transfers and then discard.
12. Evaporate the combined organic solution to near dryness (approximately 200 µL) in a TurboVap maintained at approximately 40 ºC. Take the remainder of the solution to dryness at room temperature.
13. Dissolve the residue in 500 µL of 50:50 (v/v) methanol/water. Mix for a total of 30 seconds prior to filtering through a 0.2 µm PTFE syringe filter into a 1.8 mL autosampler vial.

F. ANALYTICAL PROCEDURE

Note: Instrumental parameters yielding equivalent analytical results may be used.

1. Instrument Operating Parameters - LC System
   a. Install and degas mobile phases and install column and guard cartridge per manufacturers' instructions. Set initial composition to flow 5/95 acetonitrile/water+0.1% formic acid at 300 µL/min.
   b. Set-up the HPLC to run the following linear gradients:

Time in min.	Flow in mL/min.	A (5/95 A/W+0.1%fa)	B (95/5 A/W+0.1%fa)
0.00	0.30	100%	0%
15.00	0.30	0%	100%
15.10	0.30	100%	0%
25.00	0.30	100%	0%

c. Set injection volume to 20 µL.

d. Use a needle wash step with MeOH.

2. Instrument Operating Parameters – Mass Spectrometer

a. Calibrate the Finnigan LCQ ion trap mass spectrometer with electrospray interface according to the manufacturer's specifications.

b. Set Capillary Temp to 150 °C.

c. Operate in Pos mode.

d. Flow inject the external standard through a 5 µL loop and obtain the precursor ion centroids The following settings resulted in optimal ion intensities:

Scan range for data dependant aq.	400-950 d
Capillary temperature	150 ºC
APCI vaporizer temperature	450 ºC
Sheath gas flow	60
Aux gas flow	5
Capillary voltage	10 V
Tube lens offset	2 V
Micro scans	1
Ion time	100 msec
Source current	5.00 µA

3. Procedure for Instrumental Analysis of Samples, Controls and Standards

a. Turn on pump and set up mass spectrometer. Equilibrate column in mobile phase at 0.30 mL/min for at least 30 min.

b. Flow inject the external 100 ppb standard through a 5 µL loop and obtain the precursor centroids.

c. Inject the external standard through the HPLC system and acquire spectra using data dependant scanning under the following procedure:

Scan	**Event**	**Details**
1	Pos	Full scan 400-950 D
2	Pos	Dep MS^2 most intense ion from parent mass list (1)
3	Pos	Dep MS^2 2^{nd} most intense ion from parent mass list (1)
4	Pos	Dep MS^2 3^{rd} most intense ion from parent mass list (1)

Dependent Data Settings			
Segment time:	6.3 to 7.5 minutes	7.5 to 9.1 minutes	9.1 to 12.5 minutes
1. Parent mass list	407.1, 411.1, 425.1, 734.2, 806.0, 869.5, 916.3	407.1, 411.1, 425.1, 734.2, 806.0, 869.5, 916.3	407.1, 411.1, 425.1, 734.2, 806.0, 869.5, 916.3
2. Charge State	1	1	1
3. Isolation Width	3.0	4.0	3.0
4. Activation Amplitude	34	34	34
5. Activation Q	0.25	0.25	0.25
6. Activation Time	30	30	30
7. Min. Signal Required	5000	1000	5000
8. Min. MSn Signal Required	500	100	100

d. Inject the recovered standard and verify retention time, and divert valve switching time.

e. Inject the negative control and the sample extracts. If necessary to control carryover, precede each sample analysis with a sample diluent injection.

f. Column, Pump, and APCI Interface Care. At the end of set of analyses, flush the column for 30 min with acetonitrile + water (60 + 40) at 0.30 mL/min.

G. CONFIRMATION

1. Data Processing.

View total ion current, base ion chromatogram, and/or a reconstructed ion chromatogram for each drug for each data file. Note retention time of any visible peaks in a drug window. Generate averaged spectra across the retention time window for each drug. This is usually from near the start to near the end of the peak visible in the chromatograms, though a smaller range may be used to avoid a spurious ion spike. Where no peak is visible, use the same settings as in a contemporaneous spiked or positive control extract.

2. Confirmation Criteria.

   a. Retention times of extract peaks must match the peak retention time in a contemporaneous (within same analysis set on same day) fortified control extract chromatogram within 0.2 min.

   b. The mac/linc peak in the total ion chromatogram (TIC) is present at a S/N ratio of at least 3/1. This is estimated by visual inspection of the TIC.

   c. The spectra from the extract must visually match spectra from external standards in the same data set. The base ion must be the same. The base ion, two qualifying ions and at least an additional product ion shall be present and readily distinguished from background and matrix ions. There should be a general absence of nonspecific ions. Major specific ions for each mac/linc are listed below:

Analyte	Approx. retention time (min)	Precursor ion	Spectra Range	Base Production	Product Ions
Lincomycin	7.19	407.1	100-420	359	126*, 172, 389*
Tulathromycin	8.50	805.95	200-820	577	420*, 703*, 576, 559
Pirlimycin	9.15	411.1	100-425	363	327*, 375, 393*
Clindamycin	9.44	425.1	105-440	377	172, 126*, 389*, 407
Tilmicosin	10.11	869.5	225-880	696	522*, 678*, 738
Erythromycin	10.69	734.2	190-745	576	522*, 558*, 698, 716
Tylosin	11.00	916.3	240-930	772	407*, 598*, 754

*Recommended qualifying ions.

   d. The quality assurance positive and negative control samples confirm and fail to confirm, respectively, for the presence of the appropriate drug.

3. Criteria for Repeating an Analysis.

   Sample analyses may be repeated under the following conditions:

   a. The conditions described in G.2.d are not met.

   b. The instrument is suspected to be malfunctioning, as demonstrated by: clearly aberrant

standard spectra; failure of a calibration check performed shortly after analysis of the sample set; instrumental parameters, especially vacuum readings, outside of normal operating range; or other conditions noted and documented by the analyst.

c. There is suspected carryover from a previous high concentration sample or standard. In this case, the sample should be reanalyzed after the cause of the carryover has been identified and measures taken to prevent its recurrence.

d. There is strong evidence of mac/linc presence, but multiple extraneous ions with relative abundance exceeding that of mac/linc's base ion prevent unambiguous confirmation. In this case, it may be appropriate to reanalyze the suspected positive sample together with a chromatographic standard, and negative and positive QA controls.

H. HAZARD ANALYSIS

1. Required Protective Equipment

   a. Personal protective equipment (PPE) includes gloves, safety glasses, and lab coat, where applicable.

   b. Fume hood.

2. Hazards

Procedure Step	Hazard	Recommended Safe Procedures
Mac/Linc antibiotic Standards	Can cause kidney damage.	Wear PPE when handling standards.
Methanol Hexane Ethyl acetate	Highly flammable, and may produce toxic effects to skin, eyes and the respiratory system.	Use under a fume hood away from all electric devices and open flames. Avoid breathing vapors.
Acetonitrile	Highly flammable and toxic liquid. May cause skin irritation.	Use under a fume hood away from all electric devices and open flames. Treat as cyanide. Avoid breathing vapors
Formic acid, phosphoric acid	Corrosive. Danger of chemical burns.	Prepare solutions in a fume hood. Wear PPE, and avoid contact with skin.

3. Disposal Procedures

Procedure Step	Hazard	Recommended Safe Procedures
Methanol/acetonitrile Hexane, Ethyl acetate	See section 2 above	Collect waste in a sealed container and store in a cool, well ventilated, flammable liquid storage area/cabinet for disposal in accordance with local, state, and Federal regulations.
Acids and acidic reagents.	See section 2 above	Collect waste in a sealed container and store in a cool, well ventilated, acid liquid storage area/cabinet for disposal in accordance with local, state, and Federal regulations.

I. QUALITY ASSURANCE PLAN

1. Performance Standard

   Refer to Section G.2 for Confirmation Criteria.

2. Readiness to Perform

   a. Familiarization

      i. Phase I: Standards - Inject external standard solutions (D.2.c) in duplicate on at least three different days, and verify instrument response is adequate for confirmatory purposes.

      ii. Phase II: Fortified samples - Analyze one fortified bovine kidney, liver, and muscle and one blank bovine kidney, liver, and muscle. On a subsequent separate day analyze one fortified porcine kidney, liver, and muscle and one blank porcine kidney, liver, and muscle. On a subsequent separate day analyze one fortified poultry kidney, liver, and muscle and one blank poultry kidney, liver, and muscle. (total 18 samples)

         Note: Phase I and Phase II may be performed concurrently.

      iii. Phase III: Check samples for analyst accreditation.

         (a) 6 check samples fortified at levels between 1–2 times minimum proficiency level (MPL, see I.6 below) using analytes and concentrations unknown to the analyst. These six unknowns shall use two bovine kidney, two bovine liver, and two bovine muscle tissues. Each set must include a positive

control and a negative control.

(b) Report analytical findings to Supervisor and Quality Assurance Manager (QAM).

(c) Approval from the Supervisor and the QAM is required to commence official analysis.

b. Acceptability criteria.

Refer to section I.1 above

3. Intralaboratory Check Samples

a. System, minimum contents.

i. Frequency: One per week per analyst when samples analyzed.

ii. Records are to be maintained for review.

b. Acceptability criteria.

If unacceptable values are obtained, then:

i. Stop all official analyses by that analyst.

ii. Take corrective action.

4. Sample Acceptability and Stability

a. Matrices: Bovine, porcine and poultry liver, muscle, and kidney.

b. Sample receipt, minimum weight: approximately 50 grams.

c. Condition upon receipt: chilled or frozen.

d. Sample storage:

i. Time: 2 weeks for blended/ homogenized samples

ii. Condition: frozen (less than -10 °C)

5. Sample Set - Each sample set must include the following:

a. Negative control sample

b. Positive control sample

c. Samples

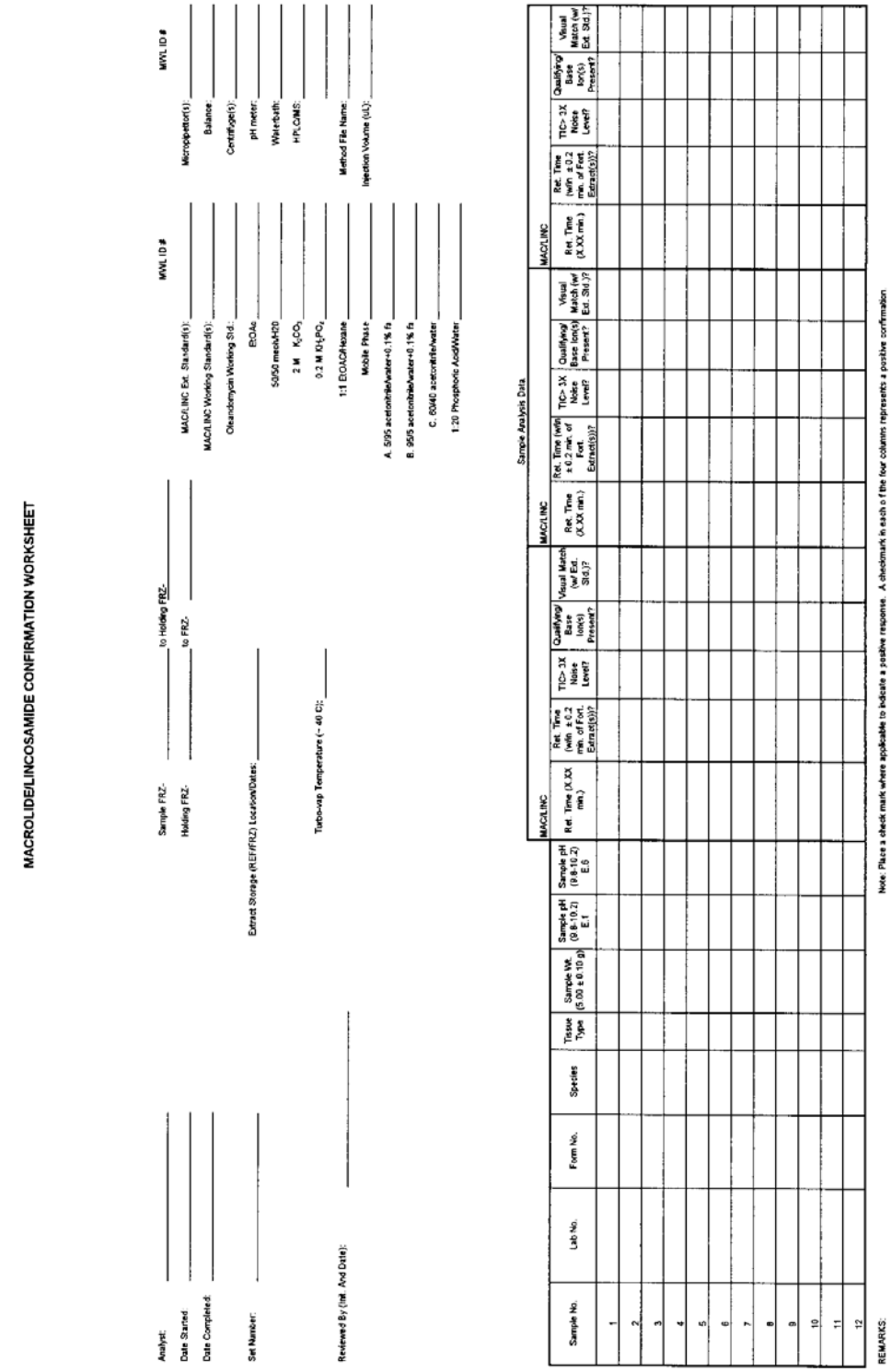
6. Sensitivity

Minimum proficiency levels (MPL), in ppm, by tissue.

	Liver	Kidney	Muscle
Lincomycin	0.1	0.1	0.1
Pirlimycin	0.1	0.1	0.1
Clindamycin	0.1	0.1	0.1
Tilmicosin	0.1	0.1	0.1
Erythromycin	0.1	0.1	0.1
Tylosin	0.1	0.1	0.1
Tulathromycin	1.0	1.0	1.0

J. WORKSHEET

The following worksheet is an example.



MACROLIDE/LINCOSAMIDE CONFIRMATION WORKSHEET

Analyst: _____ Sample FRZ- _____ to Holding FRZ- _____

Date Started: _____ Holding FRZ- _____ to FRZ- _____

Date Completed: _____

Set Number: _____ Extract Storage (REF/FRZ) Location/Dates: _____

Reviewed By (Init. And Date): _____ Turbovap Temperature (~ 40 C): _____

MWL ID # MWL ID #

MACLINC Ext. Standard(s): _____ Micropipettor(s): _____

MACLINC Working Standard(s): _____ Balance: _____

Oleandomycin Working Std.: _____ Centrifuge(s): _____

EtOAc pH meter: _____

50/50 meoh/H20 Waterbath: _____

2 M K_2CO_3 HPLC/MS: _____

0.2 M KH_2PO_4 _____

1:1 EtOAc/Hexane Method File Name: _____

Mobile Phase Injection Volume (uL): _____

A. 5/95 acetonitrile/water+0.1% fa _____

B. 95/5 acetonitrile/water+0.1% fa _____

C. 60/40 acetonitrile/water _____

1:20 Phosphoric Acid/Water _____

Sample Analysis Data

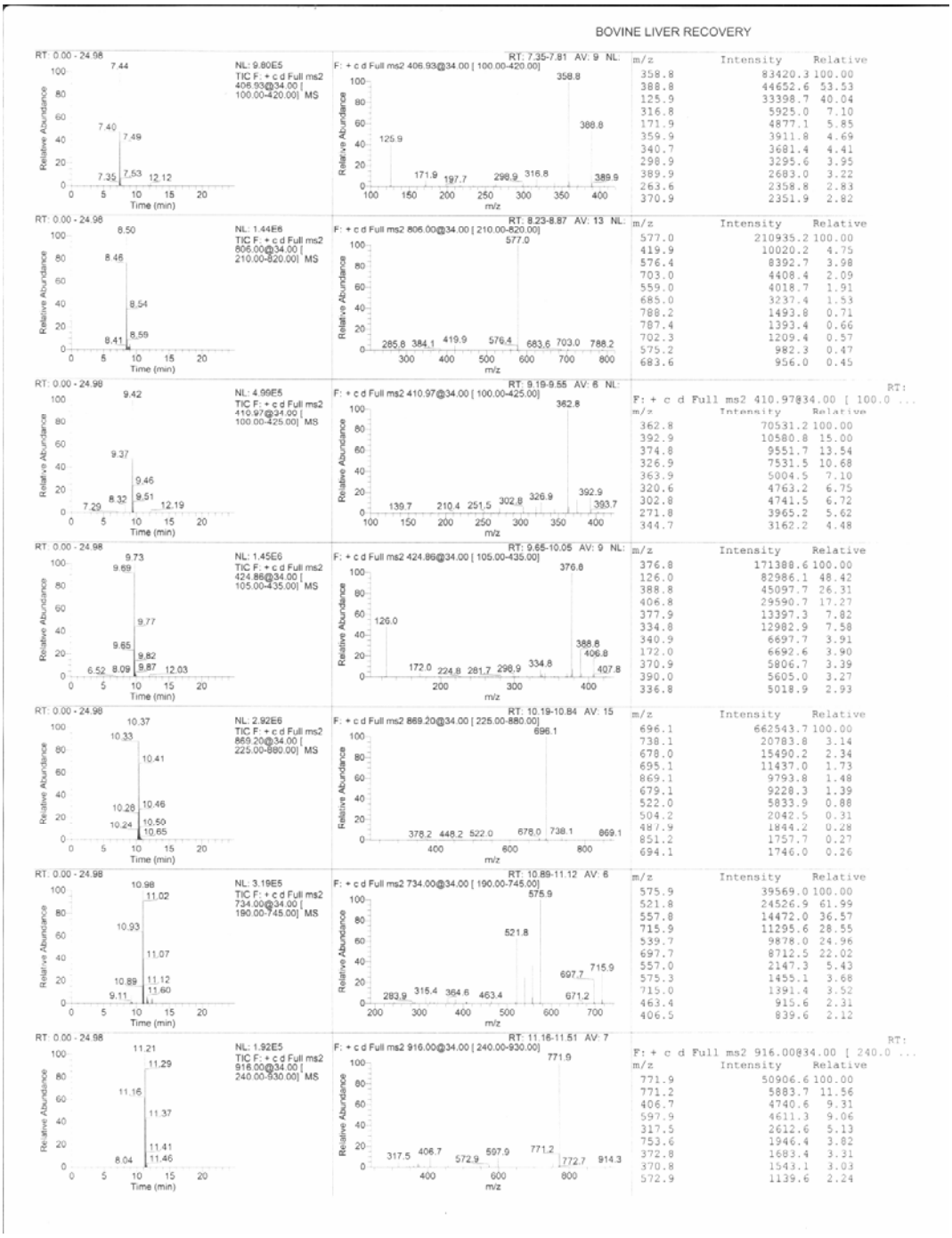
Sample No.	Lab No.	Form No.	Species	Tissue Type	Sample Wt. (5.00 ± 0.10 g)	Sample pH (9.8-10.2) E.1	Sample pH (9.8-10.2) E.6	MACLINC Ret. Time (X.XX min.)	MACLINC Ret. Time (with ± 0.2 min. of Fort. Extract(s))?	MACLINC TIC> 3X Noise Level?	MACLINC Qualifying/ Base Ion(s) Present?	MACLINC Visual Match (w/ Ext. Std.)?	MACLINC Ret. Time (X.XX min.)	MACLINC Ret. Time (with ± 0.2 min. of Fort. Extract(s))?	MACLINC TIC> 3X Noise Level?	MACLINC Qualifying/ Base Ion(s) Present?	MACLINC Visual Match (w/ Ext. Std.)?
1																	
2																	
3																	
4																	
5																	
6																	
7																	
8																	
9																	
10																	
11																	
12																	

REMARKS: Note: Place a check mark where applicable to indicate a positive response. A checkmark in each of the four columns represents a positive confirmation

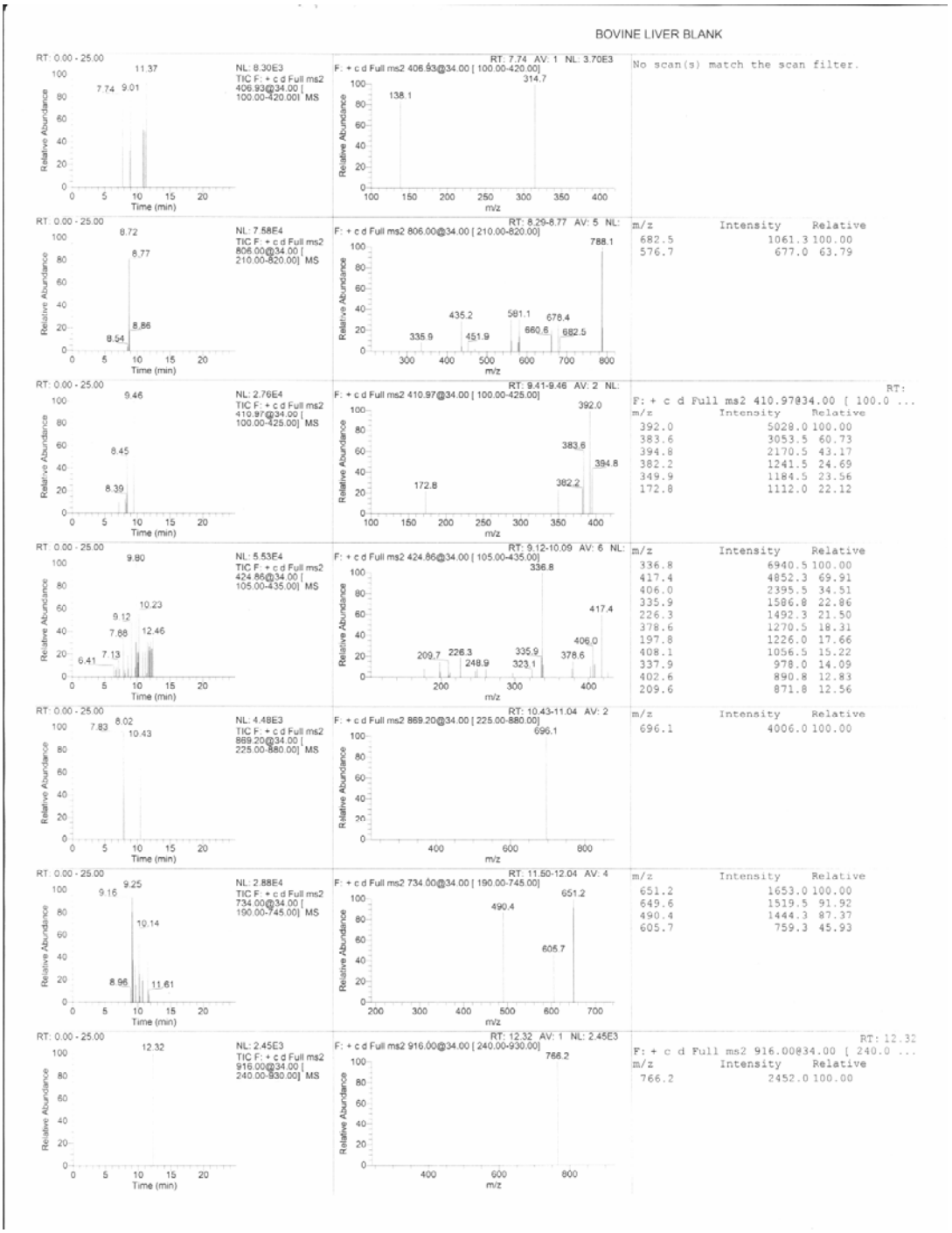
K. APPENDIX

1. Mass spectra for Macrolide/Lincosamide Antibiotics are shown on the following pages.

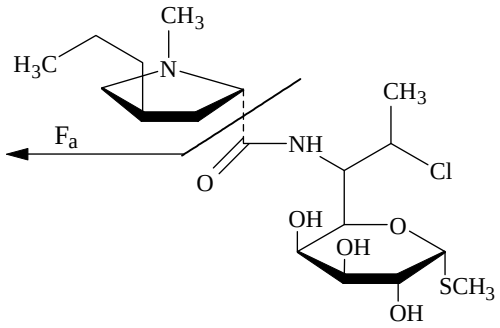
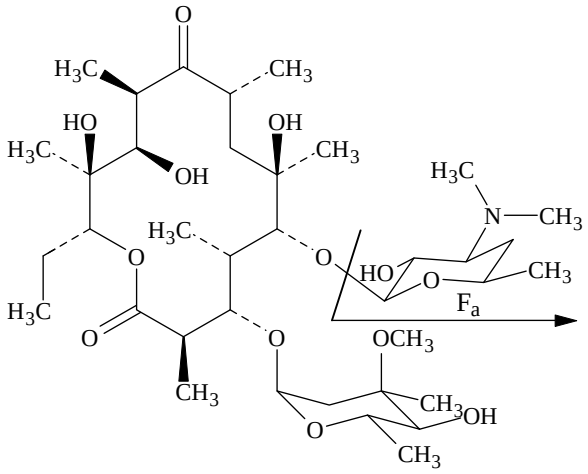
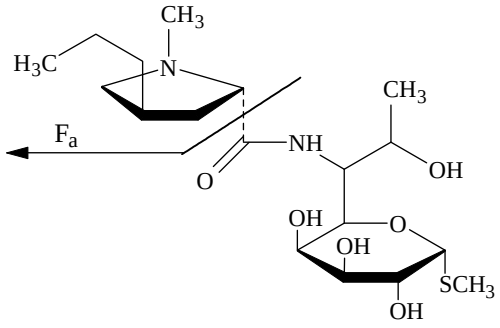
a. Bovine Liver Recovery, 1.0 ppm tulathromycin, 0.1 ppm all other analytes (positive control)

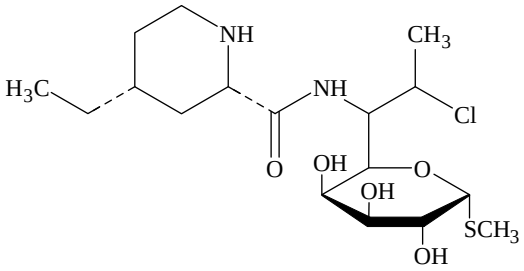
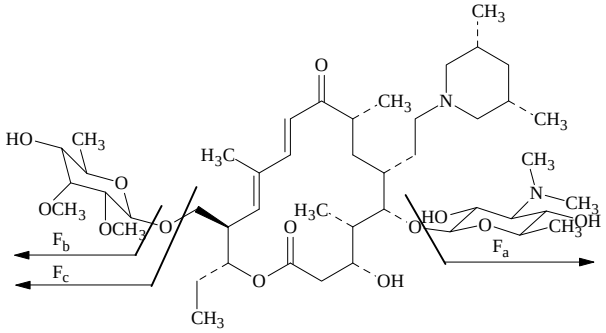
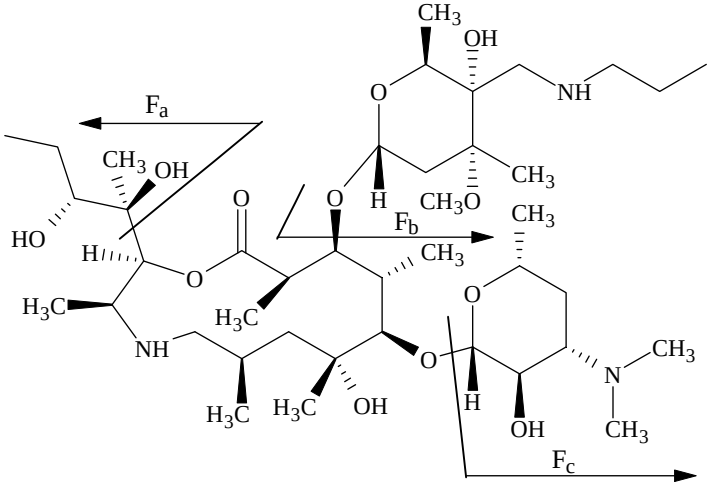
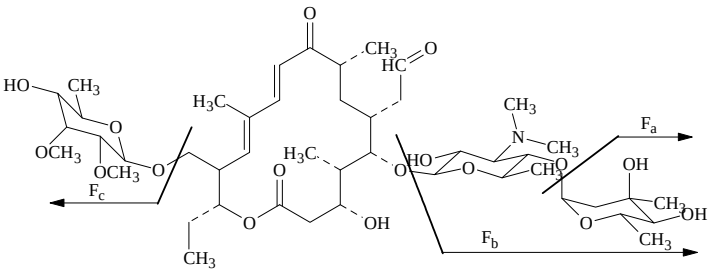


b. Bovine Liver Blank (negative control)



2. Proposed MS fragmentation patterns

Compound	Structure	Mass	Fragment
Clindamycin		425 389 377 126	$[M+H]^+$ $[M+H-2H_2O]^+$ $[M-SCH_3]^+$ $[F_a]^+$
Erythromycin		734 576 558 522	$[M+H]^+$ $[M+H-F_a]^+$ $[M+H-F_a-H_2O]^+$ $[M+H-F_a-3H_2O]^+$
Lincomycin		407 389 359 126	$[M+H]^+$ $[M+H-H_2O]^+$ $[M-SCH_3]^+$ $[F_a]^+$

Pirlimycin		411 393 363 327	$[M+H]^+$ $[M+H-H_2O]^+$ $[M-SCH_3]^+$ $[M-SCH_3-2H_2O]^+$
Tilmicosin		870 696 678 522	$[M+H]^+$ $[M+H-F_a]^+$ $[M-F_c]^+$ $[M+H-F_a-F_b]^+$
Tulathromycin		806 703 577 420	$[M+H]^+$ $[M+H-F_a]^+$ $[M-F_b+H_2O]^+$ $[M+H-F_b-F_c+H_2O]^+$
Tylosin		916 772 598 407	$[M+H]^+$ $[M+H-F_a]^+$ $[M+H-F_b]^+$ $[M+H-F_b-F_c]^+$

L. APPROVALS AND AUTHORITIES

Approvals on file.

Issuing Authority: Laboratory Quality Assurance Division (LQAD).