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Title: Determination and Confirmation of Florfenicol		
Revision: 04	Replaces: CLG-FLOR1.03 and CLG-FLOR3.00	Effective: 10/12/2010

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PART I: Determinative Method

A. INTRODUCTION

1. Theory

Florfenicol and related metabolites in bovine and poultry liver and muscle and catfish muscle homogenate are converted to florfenicol amine (FA) salts by acid-catalyzed hydrolysis. The hydrolysate is partitioned with ethyl acetate to remove lipids and other neutral interferences, and then made strongly basic to convert the salts to free FA. This solution is then applied to a diatomaceous earth column and the FA is extracted from the absorbed liquid with ethyl acetate. The organic extract is evaporated to dryness and the residue dissolved in an aqueous buffer and analyzed using reverse phase high performance liquid chromatography (HPLC) with UV detection.

2. Applicability

This procedure is applicable for analysis of florfenicol (as florfenicol amine) in bovine liver at levels ≥ 0.3 ppm, bovine muscle at levels ≥ 0.2 ppm, poultry liver at levels ≥ 1.5 ppm, poultry muscle at levels ≥ 0.6 ppm, and catfish muscle at levels $\geq$ 0.5 ppm.

B. EQUIPMENT

Note: Equivalent equipment may be substituted for those listed in this method.

1. Apparatus

a. Balance - 0.1 mg sensitivity, Mettler AE163 Electrobalance.

b. Food Processor capable of processing tissues using liquid nitrogen or dry ice, Robot-Coupe RSI-2Y1.

c. Centrifuge - Sorval RT-6000 Tabletop, Dupont, Inc.

d. Shaking water bath - with cover, capable of maintaining at least 95 °C, Precision Scientific.

e. Round-bottom tubes - 50 mL Corex/Pyrex or Teflon®, with Teflon® lined screw caps.

f. Vortex mixer - Thermolyne Corporation.

g. Liquid/liquid extraction cartridge, 20 mL capacity - Chem Elut™ CE1020 Sorbent Column with #16 Stopcocks, Varian/Analytichem International.

h. 125 mL round-bottom flasks - Chem Glass.

i. Rotary evaporator - Buchi.

j. Ultrasonic bath - Branson.

k. Micropipettes - Rainin EDP variable volume micropipettes with Rainin pipette tips, Rainin Instruments, Inc.

l. Transfer pipettes - glass, disposable.

m. Volumetric flasks - Class A, various sizes, Corning, Inc.

n. Volumetric pipettes - Class A, various sizes, Corning, Inc.

o. Pyrex Glass beakers - Corning, Inc.

p. Silanized glass wool - Supelco Inc.

q. pH meter - Sentron 2001, Sentron Inc.

r. pH Strips - ColorpHast® Chem Strips pH 7.0 to 14.0, EM Science, Inc.

s. Filters - Acrodisc LC25 PVDF 0.2 µm filters, Gelman Sciences.

t. Syringes - 3 cc Plastic, B-D.

u. Mobile phase degas and filtration unit with 0.45-µm aqueous and organic filters.

2. Instrumentation (HPLC equipment)

a. Detector - Spectroflow Model 783 UV detector at 220 nm.

b. Pump - Waters Model 600E pump (binary gradient solvent system at 1 mL/minute), preferably with on-line helium sparging capability.

c. Injector - Waters Model 715 (WISP) automatic sampler capable of delivering 100 µL on-column.

d. Column - MAC MOD Zorbax RX-C8 (4.6 mm i.d. x 250 mm, 5 µm particle size) column preceded by a Waters C8 Novapak cartridge precolumn. Note: Equivalent columns must meet the system suitability criteria stated in section K.2.

e. Column heater - If desired, a column heater may be used to control operating temperature.

f. Data System - Hewlett-Packard LC ChemStation.

C. REAGENTS AND SOLUTIONS

Note: Equivalent reagents and solutions may be substituted unless otherwise specified.

1. Reagents

Unless otherwise stated, only reagents of recognized analytical grade are used.

a. Water, HPLC - deionized, distilled, or of equal purity.

b. Ethyl acetate (EtOAc) - HPLC grade.

c. Acetonitrile (ACN) - HPLC grade.

d. Methanol (MeOH) - HPLC grade.

e. Triethylamine (TEA) - HPLC grade.

f. Hydrochloric acid (HCl) - concentrated (12N).

g. Sodium hydroxide (NaOH) - 50% (w/w) solution.

h. Potassium phosphate monobasic (KH_2PO_4) - reagent grade.

i. Phosphoric acid (H_3PO_4) - 85% (v/v), HPLC grade.

j. Methylene Chloride (CH_2Cl_2) - HPLC grade.

2. Solutions

a. 6N HCl:

Slowly add 500 mL of concentrated hydrochloric acid to 500 mL of deionized water and carefully mix. Store at room temperature. Solution is stable for one year.

b. 30% (w/w) NaOH:

Slowly add 600 mL of 50% (w/w) NaOH to 400 mL of deionized water and carefully mix. Store at room temperature in an inert plastic container. Solution is stable for one year.

c. Sample Buffer: 10 mM KH_2PO_4 (pH 4.0), 1% Acetonitrile:

Add 2.72 ± 0.2 g of KH_2PO_4 and 20 mL of acetonitrile to a 2 L volumetric flask containing approximately 1900 mL deionized water. Adjust pH of the solution, using a pH meter, to 4.0 ± 0.2 by the addition of approximately 60 µL of 85% H_3PO_4. Dilute to volume with deionized water, stopper, and mix. Solution is stable for 1 month when refrigerated.

d. HPLC Mobile Phases:

Note: If matrix interference is seen, variation in the % TEA of Mobile Phase A may be tried.

i. Mobile Phase A: 10 mM KH_2PO_4 (pH 4.0), 1% Acetonitrile, 0.01% TEA:

Add 2.72 ± 0.2 g of KH_2PO_4, 20 mL of ACN and 200 µL of TEA to a 2 L volumetric flask containing approximately 1900 mL of deionized water. Adjust the pH to 4.0 ± 0.2 by the addition (approximately 135 µL) of 85% H_3PO_4. Dilute to volume with deionized water, stopper, and mix. Filter the

solution through a 0.45 µm HPLC aqueous filter and degas with vacuum or helium sparge. Solution is stable for five days at room temperature.

ii. Mobile Phase B, 100% Acetonitrile:

Filter acetonitrile through a 0.45 µm HPLC organic filter and degas with vacuum or helium sparge.

D. STANDARDS

Note: Different standard concentrations and volumes may be prepared for stock solutions and the standard curve.

1. Analytical Standard

a. Name: Florfenicol Amine - D-(threo)-1-(p-methylsulfonylphenyl)-2-amino-3-fluoro-1-propanol.

b. Molecular weight: 247.08.

c. Supplier: Schering-Plough (SCH 40458).

d. Purity: ≥ 98%.

The reference standard will be supplied with a certificate of analysis indicating exact purity.

e. Storage: Store at room temperature.

2. Preparation of Standard Solutions

a. FA Stock Standard I (100 ± 5 µg/mL):

Weigh amount of analytical standard (corrected for purity) equivalent to 10.0 ± 0.5 mg FA, and quantitatively transfer to a 100 mL volumetric flask. Add approximately 25 mL methanol, place flask in an ultrasonic bath, and swirl until all solids are dissolved. Dilute flask contents to volume with methanol, stopper, and invert to mix. Store in freezer at ≤ -10 °C. Solution is stable for five months.

b. FA Intermediate Standard (10 ± 0.5 µg/mL):

Pipet 5 mL of the FA stock solution (D.2.a.) to a 50 mL volumetric flask, dilute the content to volume with methanol, stopper the flask, and invert to mix. Store in freezer (≤ -10 °C). Solution is stable for five months.

3. Preparation of Standard Curve

Prepare a standard curve containing at least three concentration levels ranging from approximately 0.15 µg/mL to 10.0 µg/mL, depending on the range of concentrations to be quantified.

The following table lists some suggested concentrations. To prepare any concentration listed, transfer the amount of standard solution shown in column 2 to a 10 mL volumetric flask and dilute to volume with sample buffer. Stopper flasks and invert to mix.

Nominal Standard Curve Concentration, µg/mL	µL of FA Stock Standard Added	µL of FA Intermediate Standard Added
0.15		150
0.3	30	
0.6	60	
1.2	120	
2.0	200	
2.5	250	
5.0	500	
10.0	1000	

Note: Standard curve concentrations in µg/mL correspond to tissue concentrations in µg/g (ppm) when this analytical procedure is followed as specified. Standard curve solutions are stable for two weeks when stored under refrigeration.

E. SAMPLE PREPARATION

1. Preparation of Sample

   a. Prepare entire sample (or at least 450 g if sample weight exceeds 450 g). If sample is frozen, allow to thaw. Trim sample of extraneous connective tissue. Cut entire sample into thin slices or small cubes. If only a sub-sample is to be prepared, assure that the portions selected are representative of the entire sample.

   b. Using a food processor, thoroughly blend tissue to prepare a homogeneous sample mixture. Immediately transfer mixture to a suitable container and store in a freezer at ≤ -10 °C. Take care to avoid any unnecessary thaw/freeze cycles.

2. Preparation of Test Sample

   Allow sample to thaw. Analyze as soon as possible once thawed.

F. ANALYTICAL PROCEDURE

1. Sample Extraction and Cleanup

   a. Accurately weigh 2 ± 0.2 g of sample homogenate into a 50 mL round-bottom screw-cap tube. Record the weight to at least three significant figures.

i. Liver Controls

Prepare positive and negative muscle controls at this time. Weigh two portions of blank (shown to have no florfenicol) liver tissue. Fortify with FA stock standard as follows:

(a) For beef liver - Divide 7400 by the concentration of FA stock standard in µg/mL. Add this amount, in microliters, to one of the blanks to produce a 3.7 ppm positive control.

(b) For poultry liver - Divide 3000 by the concentration of the FA stock standard in µg/mL. Add this amount, in microliters, to one of the blanks to produce a 1.5 ppm positive control.

ii. Muscle controls

Prepare negative and positive muscle controls if needed. Weigh two portions of blank (shown to have no florfenicol) muscle tissue. Fortify with FA intermediate standard as follows:

(a) For beef muscle - Divide 600 by the concentration of the FA intermediate standard in µg/mL. Add this amount, in microliters, to one of the blanks to produce a 0.30 ppm positive control

(b) For poultry muscle - Divide 1200 by the concentration of the FA intermediate standard in µg/mL. Add this amount, in microliters, to one of the blanks to produce a 0.60 ppm positive control.

(c) For catfish muscle - Divide 1000 by the concentration of the FA intermediate standard in µg/mL. Add this amount, in microliters, to one of the blanks to produce a 0.50 ppm positive control.

b. Add 8 mL of 6N HCI to tube, cap tightly, and vortex mix for approximately 1 minute at a high setting.

c. Place tube in a shaking water bath set to maintain a temperature of 95 -100 °C, for at least 2 hours. The water bath must be covered to maintain this temperature. Briefly remove tube from the water bath and vortex mix at a high setting every 30 to 45 minutes during hydrolysis to ensure complete digestion.

Caution! Check to be sure the cap is tight. Wear necessary hand and eye protection.

d. After approximately 2 hours, remove tube and inspect its contents. The solution should be dark brown to black with only charred black flocculent material remaining. If pieces of undigested tissue remain, continue heating and mixing until digestion is complete. Allow tube to cool to room temperature.

Stopping Point: Digest may be held at room temperature for up to 3 hours.

e. Add approximately 20 mL of ethyl acetate to tube, cap, and shake vigorously by

hand or vortex for 2 minutes. Centrifuge tube for 5 minutes at approximately 2000 - 2500 rpm (~900 - 1400 g) to separate layers. Aspirate and discard the upper (ethyl acetate) layer. Do not disturb or transfer the black tarry interface.

Stopping point: Samples may be stored in a refrigerator overnight.

f. Adjust the pH of the hydrolyates to 12.5 or higher by slowly adding approximately 8 mL of 30% NaOH in small portions. After the addition of each portion, gently swirl tube to mix. When the 8 mL has been added, gently invert and/or vortex mix tube, and then check pH of contents with pH paper. If necessary, more 30% NaOH can be added in smaller portions and the pH re-checked until the specified value is achieved. If desired, the tubes can be immersed in an ice bath during and/or after the addition of NaOH.

Caution! Reaction is highly exothermic. Take care to work in a hood and wear eye and hand protection.

g. Attach a stopcock to the sorbent column mounted above a 125 mL round bottom flask. When sample tube is cool to the touch, manually invert a few times and pour contents onto the top of the column (If addition displaces column packing, a glass wool plug may be placed on top of the column prior to adding sample solution). Once the solution passes just below the surface of the sorbent bed (may require application of pressure/vacuum), let the column stand 15 - 60 minutes to allow extract to thoroughly adsorb.

h. Add 20 mL of ethyl acetate or methylene chloride to sample tube, manually invert several times and then pour this solution into the corresponding sorbent column. The column eluent should drip slowly (approx. one drop per second). If the tube drips too fast, adjust flow using the stopcock at the end of the column. Collect eluent in a round bottom flask. This step should be performed in a hood.

Note: In cases where substantial interference is observed in the HPLC assay of the control liver tissue extracts, methylene chloride may be substituted for ethyl acetate to reduce the background interference.

Note: Routine use of methylene chloride is discouraged due to safe handling and disposal issues.

i. Repeat step h twice, adding each 20 mL portion of solvent as the previous eluent passes below the top of the column bed. Collect the combined eluents into the round bottom flask.

j. When the last portion of eluting solvent is added, allow the column to stand until the eluent flow completely stops. Do not force the remaining solvent through the column. Remove column from the flask and discard.

Stopping Point: Flask may be stoppered and stored overnight in a freezer, refrigerator, or at room temperature.

k. Gently evaporate the contents of the round-bottom flask to dryness under vacuum using a rotary evaporator at 45 - 50 °C. If solutions are prone to bumping or spattering, rinse rotary evaporator adaptor with ethyl acetate between samples. Remove flask from the rotary evaporator upon dryness. The total evaporation process should take 10 -15 minutes.

Stopping point: Flasks may be stoppered and stored in a freezer overnight.

l. Add 2 mL of sample buffer to the round bottom flask and dissolve the residue by mixing on a vortex mixer for about 30 seconds, then gently rotating the flask in an ultrasonic bath for about 30 seconds. During both vortexing and sonication, ensure that the buffer solution contacts all glass surfaces. Using a 3 cc plastic syringe, filter the solution through 0.2 µm Acrodisc filter into an injection vial for HPLC analysis.

Stopping point: Vials may be stored in a refrigerator up to 5 days. If there is any interruption in the HPLC analysis, the extracts are stable for 48 hours at room temperature and can be reinjected. Note: If solutions become cloudy on standing prior to HPLC analysis, they should be re-filtered to remove all particulates.

2. HPLC Analysis

a. HPLC Instrument Operating Conditions

Note: Analyst may need to make minor modifications to injection volume or gradient to optimize the performance of the instrument used.

i. UV absorbance: 220 nm.

ii. Injection Volume: 80 - 100 µL (Use a constant volume for all injections).

iii. Flow Rate: 1 mL/minute.

iv. Gradient:

Time (min.)	% A	%B	Action
0	100	0	(Initial conditions)
10	100	0	10 min. hold at initial conditions
20	20	80	10 min. linear gradient to flush column
25	20	80	5 min. hold (column flush)
35	100	0	10 min. Linear gradient to initial conditions
50	100	0	15 min. hold for equilibration

b. Determining initial System Suitability

Test for system suitability during initial HPLC setup. See "Determining Initial System Suitability in section K.2.

c. Injection Sequence for Samples, Controls and Standards

i. Equilibrate HPLC system with mobile phase A at 1.0 mL/min until a stable baseline is achieved prior to starting any series of injections.

ii. Inject standard curve series. Verify that FA elutes prior to start of the LC column flush gradient.

iii. Process samples and controls such that no more than 12 are injected between standards, and that standards are also injected at the end of the sequence.

Note: If system is stable, only a mid level standard need be re-injected during and after the sample injection sequence to verify instrumental response consistency.

d. Example Chromatograms: See Section K.1.

G. CALCULATIONS

1. Determine retention times and peak areas for all standards, controls, and samples injected.

2. Using linear regression analysis, construct a standard curve from external standard calibration values. Plot peak area (y coordinate) against standard concentration in µg/mL (x coordinate). Calculate slope (m), intercept (b), and correlation coefficient (r) of the regression line.

3. Samples may be quantified only if the correlation coefficient calculated for the standard curve equals or exceeds 0.99.

4. Identification of FA in a sample requires that its chromatogram show a peak eluting with a retention time within ± 5% of that determined for the FA peak in the positive control.

5. Calculate FA concentrations in samples using the equation:

$$\text{FA, ppm} = \frac{(y - b) \times V}{m \times W}$$

Where:

y = FA peak area of the sample extract

m = slope of the standard curve

b = intercept of the standard curve

W = sample weight, in grams

V = volume of the sample extract, in mL

Note: Samples showing florfenicol amine responses outside the range of the standard curve must be diluted with sufficient sample buffer to bring response within range for best quantitation. In such cases V must be adjusted to reflect this dilution.

H. SAFETY INFORMATION AND PRECAUTIONS

1. Required Personal Protective Equipment: Safety glasses, lab coat, protective gloves.

2. Hazards

Procedure Step	Hazard	Recommended Safe Procedures
Concentrated acids and bases HCl, H_3PO_4 NaOH, TEA	Corrosive. Contact with liquids can result in burns and severe skin, eye, and respiratory irritation (HCl, TEA).	Prepare solutions using these reagents with care in a well- ventilated area such as a fume hood. Wear protective eyewear, gloves, and clothing when handling.
Organic Solvents, EtOAc, MeOH, ACN, CH_2Cl_2.	Flammable, vapors are corrosive to the skin, eyes, and respiratory system. CH_2Cl_2 is a possible carcinogen.	Use only in an efficient fume hood, away from any electrical or heating devices.

3. Disposal Procedures

Procedure Step	Hazard	Recommended Safe Procedures
Concentrated acids and bases (see above)	See Hazards, above	Neutralize solutions to meet local, state, and federal guidelines.
Organic Solvents (see above)	See Hazards, above	Collect waste in tightly sealed container and store away from non-compatibles in a cool, well ventilated, flammable liquid storage area/cabinet for disposal in accordance with local, state, and Federal regulations.

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I. QUALITY ASSURANCE PLAN

1. Performance Standard

	Analytical Range			
Analyte	*Bovine*	*Poultry*	*Catfish*	*Acceptable Recovery*
Florfenicol amine	≥ 0.3 ppm (liver) ≥ 0.2 ppm (muscle)	≥ 1.5 ppm (liver) ≥ 0.6 ppm (muscle)	*≥ 0.5 ppm (muscle)*	70 - 110%

2. Critical Control Points and Specifications

Step	*Record*	*Acceptable Control*
F.1.a.	Sample Weight	2.0 ± 0.2 g
F.1.d.	Acid Digestion	Ensure that the samples are fully digested with only charred black flocculent material remaining.
F.1.e.	Phase Transfer	Do not disturb or transfer the black tarry interface.
F.1.h.	Column Elution	Eluate must drip at approximately one drop/second from the column.
F.1.k.	Concentrate to Dryness	Allow no solvent to remain and remove upon dryness.
F.1.l.	Dissolving the residue	Ensure that the buffer contacts all glass surfaces.

3. Readiness To Perform

   a. Familiarization

      i. Phase I: Standards.

         Duplicate standard curves containing FA levels of 0.15, 0.3, and 2.0 µg/mL on each of 3 different days.

      ii. Phase II: Fortified samples.

         Three sets on three different days.

(a) Set 1: Duplicate beef muscle at levels of 0, 0.2, 0.4, and 0.8 ppm

(a) Set 2: Duplicate beef liver at levels of 0, 0.3, 0.6, and 1.2 ppm.

(b) Set 3: Duplicate poultry muscle at levels of 0, 0.6, 1.0, and 1.5 ppm or duplicate catfish muscle at levels of 0, 0.5, 1.0, and 2.0 ppm.

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

iii. Phase III: Check samples for analyst accreditation.

(a) A minimum of 6 samples, blind to the analyst, prepared from bovine liver, one of which must be blank and the remainder fortified between 0.3 to 1.2 ppm.

(a) Authorization from Quality Assurance Manager (QAM) and Supervisor is required to commence official analysis.

b. Acceptability criteria

Refer to section I.1 above.

4. Intralaboratory Check Samples

a. System, minimum contents.

i. Frequency: Once per week per analyst, when analyses are performed.

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 for this method.

ii. Take corrective action.

5. Sample Acceptability and Stability

a. Matrix: Bovine and poultry liver and muscle, and catfish muscle.

b. Minimum Sample Size: 250 g.

c. Condition upon Receipt: Frozen or with ice crystals.

d. Sample storage:

i. Time:

(a) Bovine and poultry liver and muscle: One year

(b) Catfish muscle: 4.5 months

i. Condition: Frozen

6. Sample Set

For each batch of up to 20 samples, include:

a. Negative control (tissue blank).

b. Positive control (fortified blank).

c. Test samples to be analyzed.

7. Analyst Capability

a. Minimum Level of Applicability (MLA):

i. Bovine Liver: 0.3 ppm; Poultry Liver: 1.5 ppm*;*

ii. Bovine Muscle: 0.2 ppm; Poultry Muscle: 0.6 ppm; Catfish Muscle: 0.5 ppm

J. WORKSHEET

The following is an example of a worksheet.

Analyst:	
Date Started:	
Date Completed:	

Fill in grey areas only

Critical Control Points:	
$r^2 \geq 0.98$	for std. curve
Sample wt.	2.0 ± 0.2 g
Recovery	70-110%
RT	8-10 minutes
Std ,sample RT	NMT ±30 sec from Avg.

Calibration Data (y=mx+b)					
Standards ILN	Concentration (ug/mL)	FFA Pk. Area	Linear Regression Analysis		RT (min)
			m		
			b		
			r^2		
			S		

Instrumental Conditions	
HPLC:	Agilent 1100
Column:	Zorbax RX-C8 5µm 4.6 x 250mm
Flow Rate	1mL/min
Detector	DAD
Wavelength (nm)	220
Inj. Volume (µL)	80

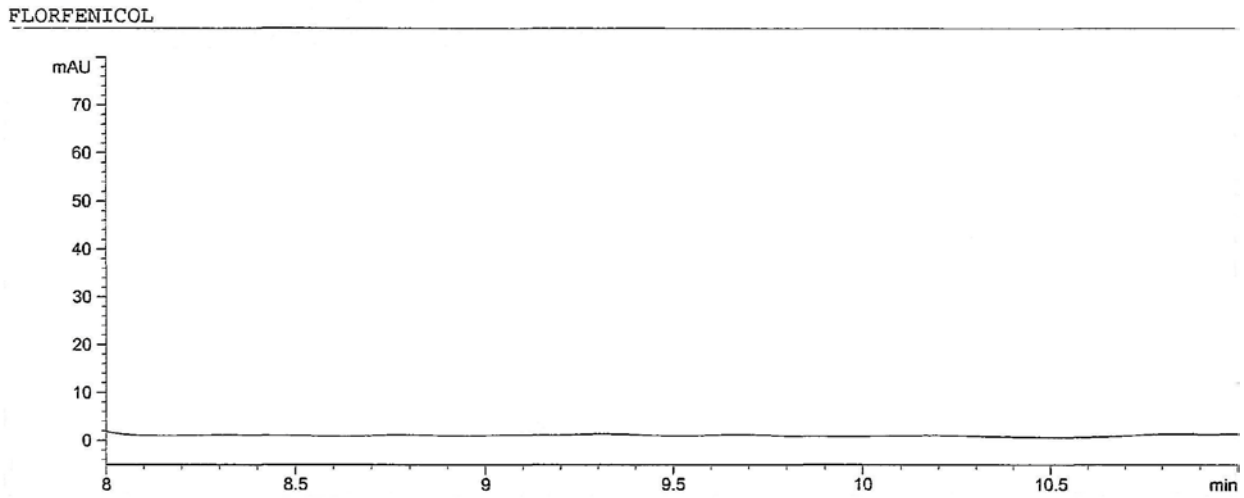
Spike std conc'n(µg/mL)	

Sample Data									
Sample ILN	Sample Wt. (g)	Final Volume (mL)	FFA Pk. Area	FFA Conc'n (µg/mL)	FFA Conc'n (µg/g)	Spike (µL)	Spike (µg/g)	Recovery %	RT (min)
				0.00	0.00				
				0.00	0.00				
				0.00	0.00				
				0.00	0.00				
				0.00	0.00				
				0.00	0.00				
				0.00	0.00				
				0.00	0.00				
				0.00	0.00				
				0.00	0.00				
				0.00	0.00				
				0.00	0.00				
				0.00	0.00				
				0.00	0.00				
				0.00	0.00				
				0.00	0.00				
				0.00	0.00				

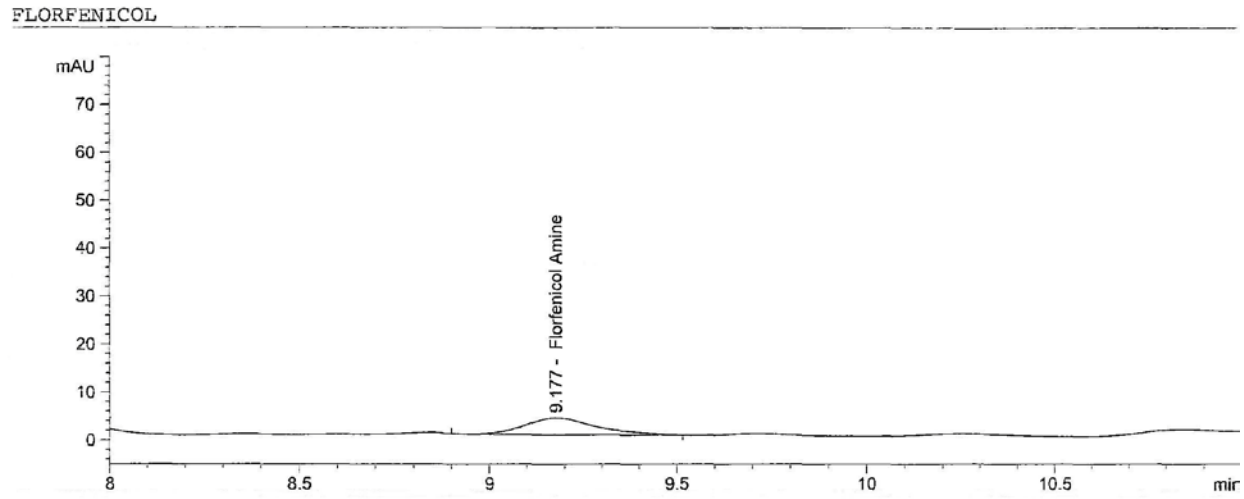
K. APPENDIX

1. Chromatograms

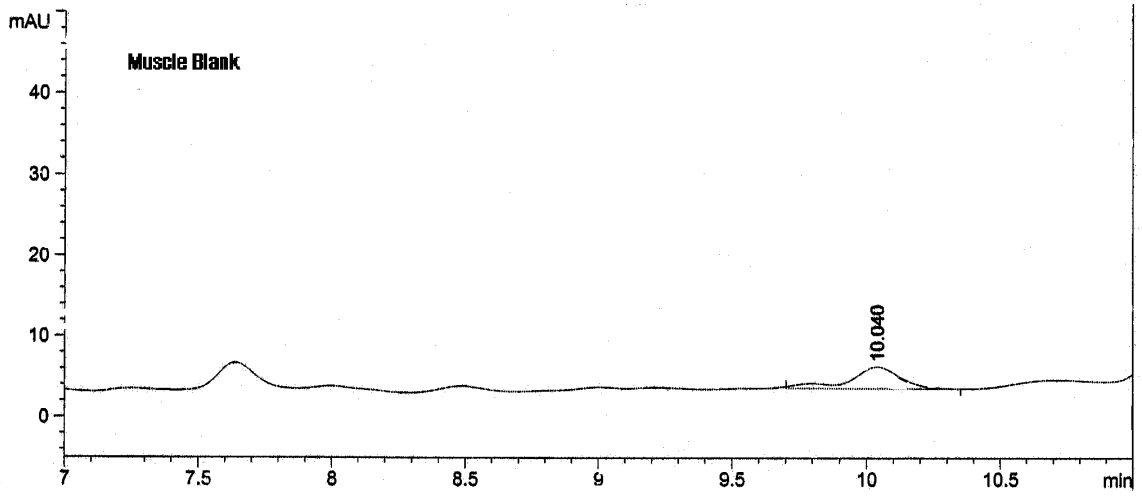
   a. Bovine Liver Blank



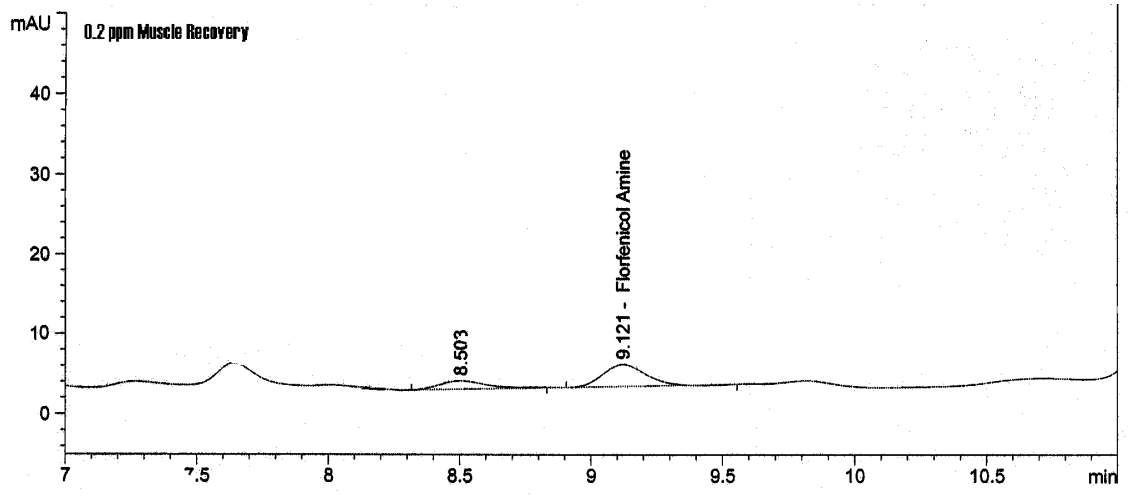
   b. Bovine Liver Recovery, 0.3 ppm



c. Bovine Muscle Blank



d. Bovine Muscle Recovery, 0.2 ppm



2. Determining System Suitability

Perform the following system suitability at initial HPLC setup:

a. Inject (at least three replicates) of a middle range standard. The percent relative standard deviation of the peak area response should be < 5%.

b. Peak asymmetry (AS) of a middle range standard should be < 2. Calculate AS as follows:

AS = CB/AC, where:

AC = peak width from start of peak to center at 10% peak height.

CB = peak width from center to end of peak at 10% peak height.

c. The florfenicol amine peak should elute prior to beginning of the column flush gradient (approximately 8 - 10 minutes). For one day's analysis the retention time for florfenicol amine should not vary more than ± 30 seconds from the mean.

The UV Detector should be capable of providing a 10:1 signal to noise ratio when 10 ng florfenicol amine (e.g. 20 µL injection of the 0.5 µg/mL standard) is injected on-column

3. References:

"High Performance Liquid Chromatography Method for the Determination of Florfenicol Amine in Cattle Liver". (Revision Date: March 14, 1996). Alice M. Bova, Schering-Plough Research Institute, Lafayette, New Jersey.

"Florfenicol Amine – Method for the Determination of Residues in Poultry Tissues using HPLC with UV Detection version 1.1". Schering-Plough Research Institute.

"Determination of Florfenicol Amine Residues in Aquaculture Species by HPLC with UV Detection (v. 2.0)". (Revision Date: May 31, 2007). Mohammad Mushtaq, Schering-Plough Research Institute, Lafayette, New Jersey.

PART II: Confirmatory Method

A. INTRODUCTION

1. Theory

   Florfenicol is extracted from tissue using the procedure described in Part I:Determinative Method. The extract is injected into the HPLC/MS/MS and confirmation is accomplished using positive ion chemical ionization.

2. Applicability

   This procedure is applicable for analysis of florfenicol (as florfenicol amine) in bovine liver and muscle at levels of $\geq$ 0.3 ppm, poultry liver at levels of $\geq$ 1.5 ppm, poultry muscle at levels of $\geq$ 0.6 ppm, and catfish muscle at levels of $\geq$ 0.5 ppm.

B. EQUIPMENT

Note: Equivalent equipment may be substituted for those listed below.

1. Apparatus

   a. Refer to Part I: Determinative Method, section B1.

2. Instrumentation (HPLC-MS/MS equipment)

   a. HPLC/Mass Spectrometer - Micromass Quattro Micro API equipped with APCI LC interface coupled to a Waters HPLC and autosampler.

   b. LC column - Hewlitt Packard, Zorbax Rx, C8, 2.1 x 150 mm, 5 μm packing.

   c. Guard column - Agilent XDB-CN, 2.1 x 12.5 mm, 5 μm packing.

   d. Data System - MassLynx™ Software.

C. REAGENTS AND SOLUTIONS

Note: Equivalent reagents and solutions may be substituted for those listed below.

1. Reagents

   a. Refer to Part I: Determinative Method, Section C.1.

   b. Ammonium Acetate (CH_3COONH_4) - Spectrum Chemical MFG Corp

2. Solutions

   a. Refer to Part I: Determinative Method, Section C.2.

b. HPLC Mobile Phases:

i. Mobile Phase A: 0.01 M (CH_3COONH_4)

Add 0.8 ± 0.02 g of CH_3COONH_4 to a 1 L volumetric flask and dilute to volume with deionized water, stopper, and mix. Filter the solution through a 0.45 µm HPLC aqueous filter and degas with vacuum or helium sparge. Solution is stable for 1 week at room temperature.

ii. Mobile Phase B, 100% Methanol:

Filter methanol through a 0.45 µm HPLC organic filter and degas with vacuum or helium sparge.

D. STANDARDS

Refer to Part I: Determinative Method, Section D.

E. SAMPLE PREPARATION

1. Preparation of Sample

Refer to Part I: Determinative Method, Section E.

F. ANALYTICAL PROCEDURE

1. Sample Extraction

Refer to Part I: Determinative Method, Section F.1.

Inject the extract generated from step F.1.l from Part I: Determinative Method.

2. HPLC-MS/MS Analysis

a. HPLC Instrument Operating Conditions

Note: Analyst may need to make minor modifications to system parameters to optimize the performance of the instrument used.

i. Flow rate: 0.3 mL/min

ii. Injection volume: 50 µL

iii. Gradient:

Time (min.)	% A	%B	Action
0.00	100	0	(Initial conditions)
1.00	100	0	1 min. hold at initial conditions
6.00	80	20	concave gradient
11.00	80	20	hold
Time (min.)	% A	% B	Action
21.00	0	100	convex gradient
23.00	0	100	hold column flush
28.00	100	0	convex gradient
32.50	100	0	hold at initial conditions

Inject standard. Verify that all monitored product ions are present.

iv. Inject the recovery and blank. Verify the absence of analyte carryover in the blank. If significant carryover is detected, inject solvent or blank until reduced to an acceptable level.

b. MS Tuning parameters

Note: Table contains recommended values. Instrumental settings may be adjusted if necessary to optimize performance.

Parameter	Setting
Ion Source	APCI (positive mode)
Corona	2 µA
Cone	25 v
Extractor	5 v
RF Lens	0.2 v
Source Temperature	130 °C
APCI Probe Temperature	500 °C
Desolvation Gas Flow	300 L/hr
Cone Gas Flow	100 L/hr

c. Fragment ions Monitored:

230 m/z

197 m/z

151 m/z

130 m/z

d. Chromatograms: Refer to section K.2.

e. Confirmation Criteria

i. The retention time of the sample must be within 5% of that for the florfenicol amine standard or the recovery.

ii. The observation of all four ions is needed to identify florfenicol amine.

iii. The relative ion abundance ratios of at least two of the ions (m/z 151, m/z 197 and m/z 230) to that of m/z 130 must be within 10% of that of the reference. If all three ions are used to determine ratios the criteria is extended to 20% that of the reference.

iv. The signal to noise ratio of all ion chromatogram peaks used to calculate abundance ratios in the reference and the sample is at least 3:1.

G. CALCULATIONS

1. The florfenicol amine fragment ions of m/z 151, 197 and 230 are expressed as a percentage of the base fragment ion of m/z 130.

2. Calculate the relative percentages of the florfenicol amine fragment ions as follows:

$$\frac{Pn}{Pb} \times 100 = \% \text{ relative abundance}$$

Where:

Pn = Peak area or height of fragment ion at m/z = 151, 197 or 230

Pb = Peak area or height of the base fragment ion at m/z = 130

H. SAFETY INFORMATION AND PRECAUTIONS

1. Required Personal Protective Equipment: Safety glasses, lab coat, protective gloves.

2. Hazards

Refer to Part I: Determinative Method, Section H.

I. QUALITY ASSURANCE PLAN

1. Performance Standard

	Analytical Range			*False Positive Rate*	*False Negative Rate*
Analyte	*Bovine*	*Poultry*	*Catfish*		
Florfenicol amine	≥ 0.3 ppm (muscle and liver)	≥ 1.5ppm (liver) ≥ 0.6ppm (muscle)	≥ 0.5 ppm (muscle)	0%	0%

2. Critical Control Points and Specifications

   Refer to Part I: Determinative Method, Section I.

3. Readiness To Perform

   a. Familiarization

      i. Phase I: Standards - Analyze at least one external standard on each of three different days.

      ii. Phase II: Fortified samples –

         (a) Beef liver fortified at 0 and 0.3 ppm.

         (b) Beef muscle fortified at 0 and 0.3 ppm.

         (c) Poultry liver fortified at 0 and 1.5 ppm.

         (d) Poultry muscle fortified at 0 and 0.6 ppm.

         (e) Catfish muscle fortified at 0 and 0.5 ppm.

         Sets must include required method controls in addition to the fortified samples.

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

      iii. Phase III: Check samples for analyst accreditation.

         (a) A minimum of 6 samples, blind to the analyst, prepared from bovine liver. At least one of the unknowns must be blank and the remainder fortified at 0.3 ppm.

(b) Authorization from the Quality Assurance Manager (QAM) and Supervisor is required to commence official analysis.

b. Acceptability criteria

Refer to Part II Section I.1 above.

4. Intralaboratory Check Samples

a. System, minimum contents.

i. Frequency: Once per week per analyst, when analyses are performed.

b. Acceptability criteria

Refer to Part II, Section I.1

If unacceptable values are obtained, then:

i. Stop all official analyses by that analyst for this method.

ii. Take corrective action..

5. Sample Acceptability and Stability

a. Matrix: Bovine liver and muscle, poultry liver and muscle, and catfish muscle.

b. Minimum Sample Size: 1 pound.

c. Condition upon Receipt: Frozen or with ice crystals.

d. Sample storage:

i. Time:

Bovine: One year

Poultry: One year

Catfish: 4.5 months

ii. Condition: Frozen

6. Sample Set

For each batch of up to 20 samples, include:

a. Negative control (tissue blank).

b. Positive control (fortified blank).

c. Test samples to be analyzed.

7. Analyst Capability

a. Minimum Level of Applicability (MLA):

i. Bovine Liver: 0.3 ppm; Poultry Liver: 1.5 ppm

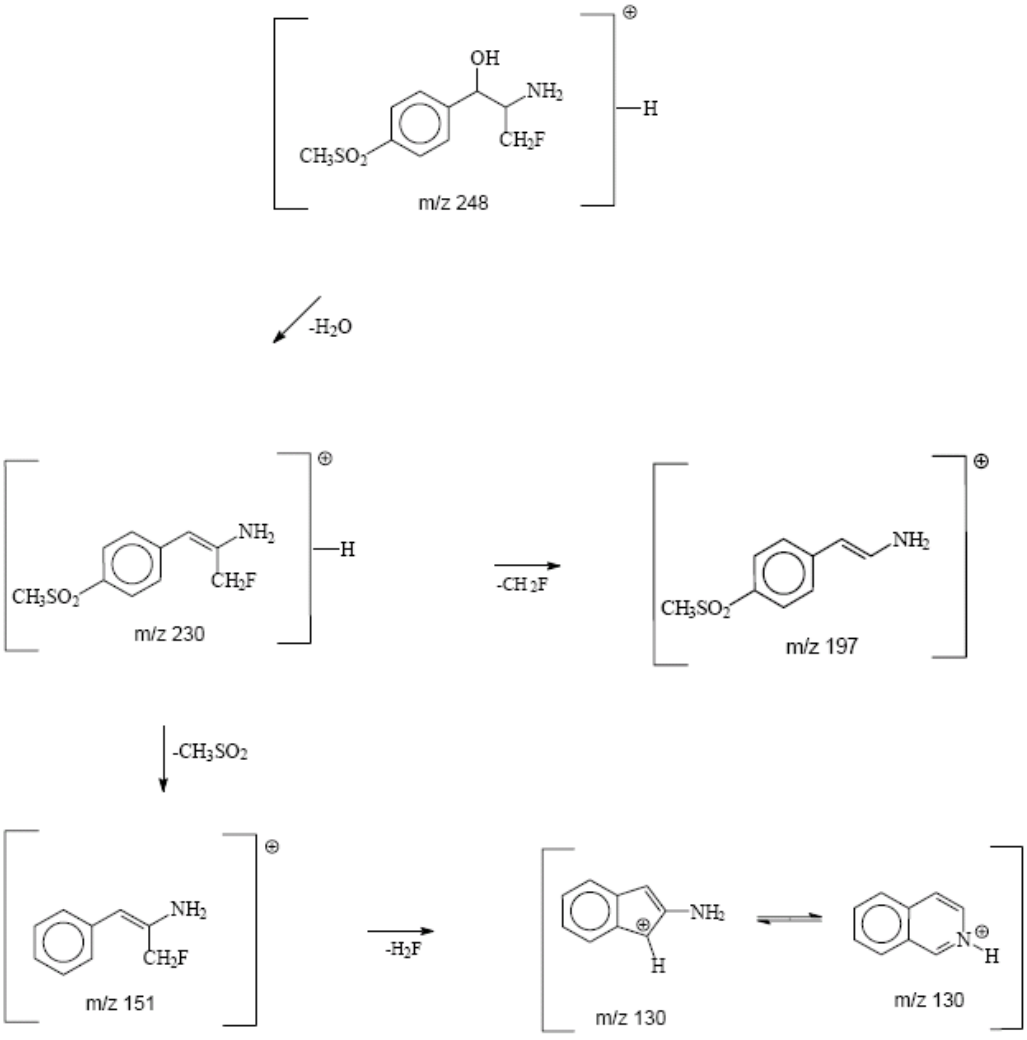
ii. Bovine Muscle: 0.3 ppm; Poultry Muscle: 0.6 ppm; Catfish Muscle: 0.5 ppm

J. WORKSHEET

[RESERVED]

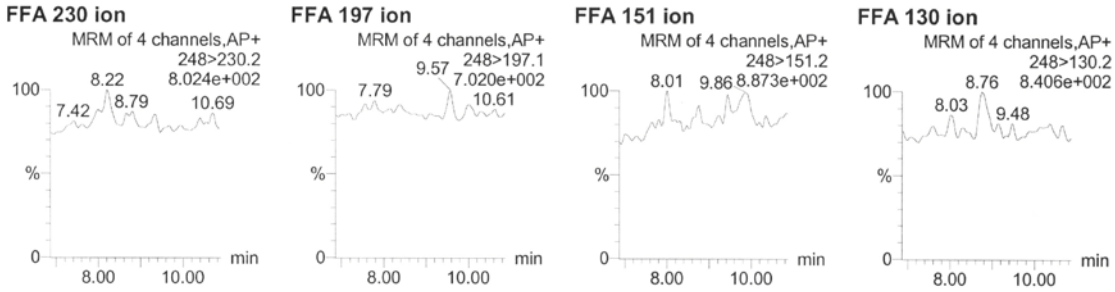
K. APPENDIX

1. Proposed Daughter Ion Fragmentation Pathway for Florfenicol Amine

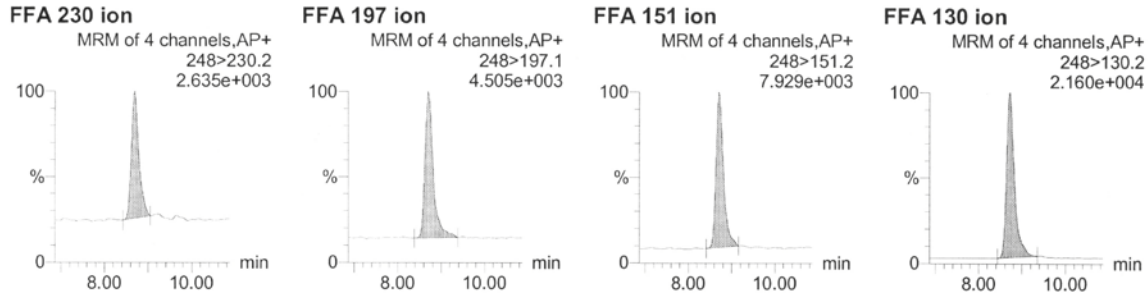


2. Chromatograms

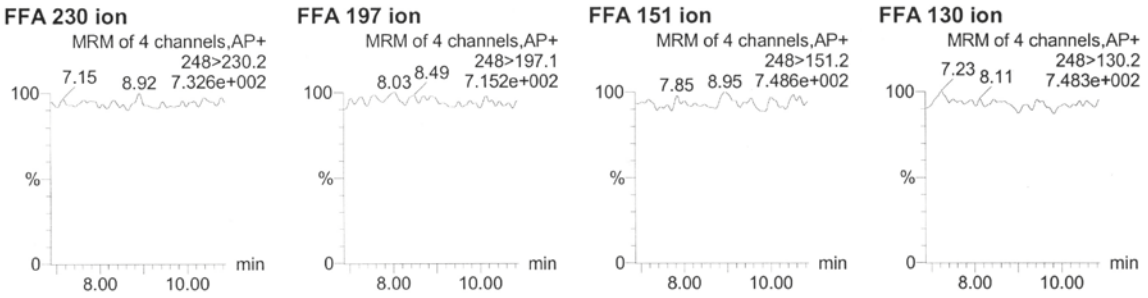
a. Bovine liver blank



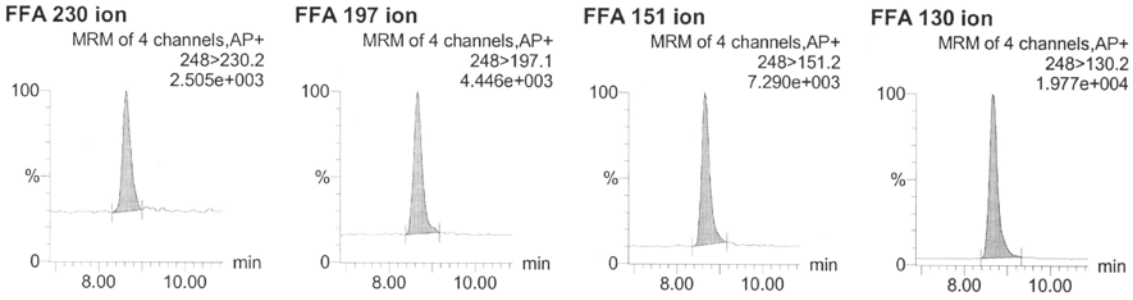
b. Bovine liver recovery, 0.3 ppm



c. Bovine muscle blank



d. Bovine muscle recovery, 0.3 ppm,



3. Reference:

Florfenicol Amine-Method for the Confirmation of Residues in Swine Liver Using HPLC and MS/MS Detection-version 1.0. Schering-Plough Research Institute, Lafayette, New Jersey.

L. APPROVALS AND AUTHORITIES

1. Approvals are on file.

2. Issuing Authority: Director, Laboratory Quality Assurance Division (LQAD).