CLG-SUL4.01		Page 1 of 17
Title: Quantitation and Confirmation of Sulfonamides by Liquid Chromatography - Tandem Mass Spectrometry (LC-MS-MS)		
Revision: 01	Replaces: CLG-SUL4.00	Effective: 05/11/2011

Contents

A. INTRODUCTION

Sample extracts containing sulfonamides from the extraction detailed in CLG-SUL2 are reconstituted in 80% 0.1% formic acid / 20% IPA. The extracts are injected into a reverse phase liquid chromatography system followed by simultaneous quantitation and confirmation by tandem mass spectrometry.

1. Theory

Quantitation is achieved by measuring the peak height of the first daughter ion fragment, and confirmation is made by filtering each sulfonamide on the parent ion (M+H) and then calculating the ratios of two or more daughter ions.

2. Applicability

This method quantitates and confirms the following sulfonamides in tissue (muscle and liver tissues of porcine, bovine, and avian species), processed products and catfish at levels ≥ 0.05 ppm with the exception of Sulfaquinoxaline which quantitates at levels ≥ 0.10 ppm.

Sulfapyridine (SPY) (internal standard)	Sulfaquinoxaline (SQX)
Sulfathiazole (STZ)	Sulfaethoxypyridazine (SEP)
Sulfadiazine (SDZ)	Sulfadimethoxine (SDM)
Sulfachloropyridazine (SCP)	Sulfadoxine (SDX)
Sulfamethazine (SMZ)	Sulfamerazine (SMRZ)
Sulfamethoxazole (SMX)	Sulfisoxazole (SSXZ)
Sulfamethoxypyridazine (SMP)	Sulfamethizole (SMZL)

Note that all tissues may be extracted using an automated robotic extraction system (Zymark Technologies). However, it is recommended that catfish be extracted manually since the catfish extracts tend to form an emulsion when extracted by the robotic system.

3. Structures

Structures of functional groups of the above sulfonamides are shown in Appendix, Section K.2.

B. EQUIPMENT

Note: Equivalent apparatus and instrumentation may be substituted for the following.

1. Apparatus
    a. Balance - PB3002-S, Mettler.
    b. Vortex mixer - Thermolyne Maxi Mix model M-16715, Thermolyne Corp.
    c. Evaporator - Turbo-Vap LV, (Zymark) Caliper Life Science.
    d. Micro Centrifuge - Galaxy, Cat. No. 14D, VWR.
    e. 0.2 µm Centrifugal Filters, Cat. No. 82031-356, VWR.
    f. Zymark Robotic System - Zymate Pytechnology System equipped with the following modules:
        i. Zymate System V Core System with System V Operating System Version 2.62
        ii. ZP-510 MLS station (2)
        iii. ZP-830 Power and event controller
        iv. ZP-900-1 General purpose gripping hand
        v. ZP-900-2 General purpose gripping hand
        vi. ZP-013-7 50 mL centrifuge tube rack (2)
        vii. ZP-013-3 16x 100 mm test tube rack
        viii. ZP-913-1 Syringe hand, 1 - 4 mL
        ix. ZP-620-6 Dilute and dissolve station (50 mL tubes)
        x. Custom nozzle parking station - fabricated in house
        xi. ZP-620-2 Dilute and dissolve station (15 mL tubes)
        xii. Custom rack for pouring station - fabricated in house
        xiii. Custom rack for sodium acetate solution - fabricated in house
        xiv. ZP-77413 1 mL syringe hand
    g. Centrifuge-Durafuge 300, Thermo
    h. Polypropylene centrifuge tubes - 50 mL, Falcon Blue Max, Cat. No. 2098, and 15 mL, Falcon Blue Max, Cat. No. 2097, Falcon.
    i. Micropipettor-Rainin EDP3
    j. Shaker –Eberbach Reciprocating, Catalog No. 6010
    k. Fast flow filter columns - CC-09-m - Whale Scientific Inc.

2. Instrumentation

   a. Agilent Technologies 6410 Triple Quad Mass spectrometer equipped with an electrospray LC interface coupled to an Agilent 1200 series High Pressure Liquid Chromatography (HPLC) system and autosampler.

   b. HPLC Column - Cat. No. Eclipse XDB-C18 RRHT 1.8 µm, 2.1 x 50 mm, Zorbax

   c. In-Line Filter - 2 mm diameter, 0.2 µm pore size (optional but recommended to extend the column life).

C. REAGENTS AND SOLUTIONS

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

1. Reagents

   Note: All solvents are HPLC grade unless otherwise specified.

   a. Isopropanol (2-Propanol),- Cat. No. A419-4, Fisher Scientific

   b. Methanol - Cat. No. 230-4, Burdick & Jackson,

   c. Formic Acid - 98% Pure, Cat. No. 06440, Fluka.

   d. Water - HPLC grade, or deionized water

   e. Potassium phosphate dibasic ($K_2HPO_4 \bullet 3\ H_2O$) - Molecular Biology Grade, Cat. No. 529567, Calbiochem.

   f. Potassium phosphate monobasic (KH_2PO_4) - 99.0% min. purity, Cat. No. 7100-12, Mallinckrodt Chemicals.

   g. Hexane-Cat. No. 296-1, EMD

   h. Ethyl Acetate-Cat. No.100-4, Burdick & Jackson

   i. Concentrated hydrochloric acid (HCl) - Reagent grade-Cat. No. 9535-4. JT Baker.

   j. Sodium Acetate - Anhydrous, Certified ACS Reagent grade-Cat. No. S210-2 Fisher Scientific.

   k. Acetone-Cat. No. A929-4, Fisher Scientific

2. Solutions

   a. HPLC Mobile Phase A (0.1% Formic Acid):

      Add 2 mL Formic Acid to approximately 500 mL of water in 2 L volumetric flask. Bring to volume with water.

b. HPLC Mobile Phase B (20% Isopropanol in 0.1% Formic Acid):

Add 800 mL HPLC Mobile Phase A (0.1% Formic Acid) to a 1 L HPLC mobile phase container. Add 200 mL Isopropanol to the same container. Mix thoroughly.

c. 0.2M Phosphate buffer:

Dissolve 45.65 g potassium phosphate dibasic crystals ($K_2HPO_4 \bullet 3\ H_2O$) with water in a volumetric flask and dilute to 1 L (Solution 1). Dissolve 27.22 g KH_2PO_4 (potassium phosphate monobasic) with water in a volumetric flask and dilute to 1 L (Solution 2). Adjust solution 1 to pH 7.55 ± 0.05 with Solution 2. (Use all of solution 1 and adjust with solution 2 at an approximately 80:20 ratio.)

d. 3.5M sodium acetate

Add 287.12 g to a 1000 mL volumetric flask. Dissolve with deionized water and bring to volume.

e. 3.2M HCl

Add approximately 500 mL deionized water to a 1000 mL volumetric flask. Add 277 mL conc. HCl. Dilute to volume with deionized water.

D. STANDARDS

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

1. Source

Reference standard materials are available from U.S. Pharmacopeia; Sigma Chemical Co.; Fluka Chemical Corp., and Pfaltz and Bauer.

Compound	Identifier	Compound	Identifier
Sulfathiazole	STZ	Sulfaethoxypyridazine	SEP
Sulfadiazine	SDZ	Sulfadimethoxine	SDM
Sulfachloropyridazine	SCP	Sulfadoxine	SDX
Sulfamethazine	SMZ	Sulfamerazine	SMRZ
Sulfamethoxazole	SMX	Sulfisoxazole	SSXZ
Sulfamethoxypyridazine	SMP	Sulfamethizole	SMZL
Sulfapyridine (internal standard)	SPY	Sulfaquinoxaline	SQX

2. Preparation

a. Stock solutions (1 mg/mL):

Weigh 100 ± 0.1 mg of the sulfonamide of interest including the internal standard (Sulfapyridine) into separate 100 mL volumetric flasks. Dissolve and bring to volume with acetone.

Note**:** If needed, a smaller amount of a sulfonamide stock solution may be made. If using a sodium salt of the sulfonamide then the weight must be corrected as needed. Dissolve the sodium salt of the sulfonamide with a few drops of distilled water and then bring to volume with acetone.

b. Recommended[1] Working standards (used for fortification):

i. Mixed standard solution (5.0 µg/mL) :

Pipet 0.5 mL of each stock sulfonamide solution (EXCEPT the internal standard, SPY) into a 100 mL volumetric flask. Bring to volume with phosphate buffer.

ii. Internal Standard Solution (IS) (2.50 µg/mL):

Pipet 0.5 mL of the 1 mg/mL Stock Solution (SPY) into a 200 mL volumetric flask. Bring to volume with phosphate buffer.

[1]Note: When quantitating large potential positives at levels above the routine curve, it may be necessary to make a more concentrated mixed standard solution (e.g. 50 µg/mL) to bracket the expected concentration(s) of analyte in the sample(s).

3. Standard Solution Stability

Place all working standards in polyethylene or polypropylene bottles and store refrigerated (2 - 8 °C). Shelf life is 3 months.

Place all stock solutions prepared in acetone in polyethylene or polypropylene bottles and store at < -10 °C. Shelf life is 6 months.

E. SAMPLE PREPARATION

Samples of liver and muscle are processed until homogeneous. All samples are stored refrigerated or frozen until analyzed.

F. ANALYTICAL PROCEDURE

1. Preparation of standard curve and recoveries.

a. Weigh a 2.5 ± 0.1 g portion of a blank control matrix for the blank.

b. Weigh four (4) additional 2.5 ± 0.1 g portions of a blank control matrix in 50 mL

polypropylene centrifuge tubes. Fortify each tube as follows:

Level of Fortification (ppm of tissue)	Fortification Solution (µg/mL)	Amount spiked (µL)
0.05	5.0	25
0.10	5.0	50
0.20	5.0	100
Recovery (0.10 ppm)	5.0	50

c. Add 100 µL of Internal Standard Solution (2.5 µg/mL) to all standards, recoveries, blanks and internal checks.

2. Preparation of samples to be analyzed.

a. Weigh 2.5 ± 0.1 g of each thawed sample into a 50 mL polypropylene centrifuge tube.

b. Add 100 µL of Internal Standard Solution (2.5 µg/mL) to all samples for a 0.1 ppm level fortification.

Note: Sample amounts less than 2.5 grams may be used when confirming suspected high positive values. Also, a higher concentration of the internal standard may be used to allow for sample extract dilution.

3. Extraction

a. Add 6.0 mL ethyl acetate to standards and samples and vortex for 2 minutes. Let samples stand for at least 10 minutes.

Manual – Vortex or shake by hand to break up sample. Place samples on horizontal shaker for approximately 10 minutes on high or vortex for 2 minutes. Let stand at least 10 minutes or centrifuge for 5 minutes at 2500 rpm.

b. Filter ethyl acetate through a fast flow filter column and collect filtrate into a clean 15-mL centrifuge tube.

c. Add 1.0 mL 3.2M HCl.

d. Vortex for 30 seconds and let stand for at least 5 minutes.

Manual – Shake approximately 5 minutes on high or vortex for 30 seconds. Let stand at least 5 minutes or centrifuge for 5 minutes at 2500 rpm.

e. Aspirate ethyl acetate to waste.

f. Add 5.0 mL hexane and vortex for 30 sec., let stand for 5 minutes.

Manual – Shake approximately 5 minutes on low or vortex for 30 sec., and let stand for at least 5 minutes or centrifuge for 5 minutes at 2500 rpm.

g. Aspirate hexane to waste.

h. Add 2.0 mL 3.5 M sodium acetate.

i. Add 3.0 mL ethyl acetate, vortex for 30 sec., let stand for at least 5 minutes.

Manual – Shake 5 minutes on low or vortex for 30 sec. and let stand for at least 5 minutes or centrifuge for 5 minutes at 2500 rpm.

j. Transfer ethyl acetate to a clean centrifuge tube.

k. Evaporate final extract to dryness under nitrogen with a water bath temperature set to 40 ± 5°C.

l. Add 100 µL of methanol to the residue. Vortex on high to dissolve. Dilute the samples and standards with 400 µL of mobile phase A. The volume of mobile phase A added must be consistent across all samples and standards. Vortex again on high to mix.

m. Transfer to centrifugal filter tubes.

n. Centrifuge at approximately 3000 rpm until sufficient volume of filtrate has been collected for HPLC analysis (approximately 5 to 10 minutes).

o. Transfer to LC autosampler vials.

Note: Extract stability for SSXZ & STZ is 24 hours for liver and muscle for quantitation when refrigerated. All other analytes are stable for ten days in liver extracts and for two days in muscle extracts when refrigerated.

4. LC Instrument Settings

Note: The instrument parameters listed here are examples that worked with the equipment listed in the method. The analyst should optimize parameters for the instrument used.

Column Temperature: 70°C

Injection Volume: 4 µL

Initial Flow Rate: 0.65 mL/min

Gradient Program:

Time (min)	Flow Rate (mL/min)	A (%)	B (%)
0	0.65	95	5
2	0.65	95	5
5	0.65	0	100
5.1	0.65	95	5
10	0.65	95	5

5. Mass Spec/ LC interface Settings

Note: The instrument parameters listed here are examples that worked with the equipment listed in the method. Others setting may yield equivalent results. The analyst should optimize parameters for the instrument used.

Polarity ES+

Desolvation Gas Temp 350°C

Capillary Voltage 4000 V

Drying gas flow 10.5 L/min

Nebulizer gas pressure 53 psi

Mass Spectrometer Programming:

Analyte	Retention Time[1]	Filtered Parent Ion	Frag (V)[2]	Product Ion[3]	CE (V)[4]	Aux. Ions[3]	CE (V)
SDZ	0.65	251.1	107	156 108	11 22	92.1 185.1 96.1	25 10 15
STZ	0.78	256	103	156 108	10 21	92.1	25
SPY	0.97	250.1	110	156 108	12 23	92.1 184.1 95.1	25 10 15
SMRZ	1.194	265.1	100	156 92.1	13 28	108	24
SMZL	1.71	271	98	156	8	108	21

				92.1	26		
SMP	2.321	281.1	115	126.1 188	12 17	156 108 92.1	13 24 29
SMZ	2.40	279.1	125	186 124.1	13 24	156 108 92.1	15 20 25
SCP	2.48	285	100	156 108	10 23	92.1	28
SMX	2.74	254.1	102	156 108	11 23	92.1 188.1	25 10
SSXZ	4.05	268.1	100	156 113.1	8 12	108 92.1	23 27
SDX	4.35	311.1	120	156 108	14 27	92.1 245.1	25 10
SEP	4.71	295.1	124	156 92.1	14 29	108	24
SDM	5.08	311.1	127	156 108	18 29	92.1 245.1 218	30 15 15
SQX	5.29	301.1	130	156 108	11 29	92.1	35

(1) **Very Important** - If the parent and daughter ions for two analytes are not completely resolved by mass, then the analytes must be completely resolved chromatographically (baseline resolution). For example, SDM and SDX must be completely resolved chromatographically; so must SDZ and SPY.

(2) Fragmentation Energies - The fragmentation energies are instrument specific and should be optimized on each instrument for each parent ion.

(3) Product Ions – The first product ion listed is the recommended quantitation ion, though the other ions may be used in case of unusual interferences or changes in instrument conditions. The auxiliary ions are at much lower abundances but can be used to help identify a compound if an unusual interference is present

(4) Collision Energies (CE) - The CE settings are instrument specific and should be optimized on each instrument for each product ion.

6. Injection Sequence
   a. Inject calibration standard(s)
   b. Inject the recovery sample
   c. Inject the blank sample and verify the absence of analytes above 5% of the recovery or sample concentration(s).
   d. Inject sample extract(s).
   e. Re-inject the calibration standard at the appropriate level. At least every 20 injections and at the end of the run to verify instrument response.

      Note: If significant carryover is detected, inject wash solution as needed until it is reduced to an acceptable level.

G. CALCULATIONS

1. For Quantitation of each compound of interest:
   a. Review the chromatograms to verify that the analyte peaks are within the retention time windows and that the peaks are integrated correctly.
   b. Calculate the normalized peak for each component of interest by dividing the component response by the internal standard response:

$$\text{Normalized Response Component 1} = \frac{\text{Response of Component 1}}{\text{Response of Internal Standard}}$$

   c. Generate a linear curve fit to each analyte in standard curve using normalized response to concentration in tissue (µg/g or ppm).
   d. Standard curve must have a correlation coefficient greater than or equal to 0.995.
   e. Blank must exhibit a response of less than 5% of the recovery used contemporaneously in the set.

2. For Confirmation:
   a. Choose a standard or recovery containing the analyte of interest.
   b. Identify 2 product ion peaks in the sample and verify that their peaks are present with a signal to noise ratio ≥ 3. Auxiliary ions may be used if necessary.
   c. Identify the retention time of the two product ion peaks in the standard or recovery and in the sample of interest. The sample peak retention times must be within ± 5% of the standard or recovery retention times.

d. Calculate the ratio of the response of product ion #2 to product ion #1 in the standard or recovery for the analyte of interest:

Ratio = Product ion#2/ Product Ion #1

Note: Ion ratio should be less than 1. If not, then invert the ratio.

e. Calculate the same ratio for the analyte in sample of interest. The sample ratios must match within ± 10% absolute difference of the standard ratio.

Note: See Table in section F.5.

H. HAZARD ANALYSIS

1. Required Protective Equipment - Eye protection, non-permeable gloves, and lab coats.

2. Hazards

Reagent	*Hazard*	*Recommended Safe Procedures*
Ethyl acetate, hexane, diethylamine, chloroform, t-butanol, methanol, acetone	These solvents may be flammable and may produce toxic effects to skin, eyes, and the respiratory system.	Use reagents in an efficient fume hood away from all electrical devices and open flames. Use approved gloves and protect skin from exposure.

3. Disposal Procedures

Procedure Step	*Hazard*	*Recommended Safe Procedures*
Waste Organic Solvents	See above	Gather and store in an approved collection container until disposed of by a contractor or an in house specialist.

I. QUALITY ASSURANCE PLAN

1. Performance Standard

Note: Performance standards are applicable for analyte of interest.

a. For quantitation:

Analyte	*Analytical Range (ppm)*	*Acceptable Recovery (%)*
All sulfonamides	0.05 - 0.2	80 - 120

Regression coefficient (r) ≥ 0.995

Note: The instrument's linearity was demonstrated to 0.6 ppm.

b. For confirmation:

Refer to Section G.

2. Critical Control Points and Specifications:

	Record	*Acceptable Control*
a.	Analyte peaks in standard	Must fall completely within their respective retention time windows.
b.	Any analyte with parent and daughter masses that are not completely resolved by mass	Analytes must be resolved chromatographically.

3. Readiness to Perform

a. Familiarization in Meat and Meat Products

i. Phase I: NA

ii. Phase II: Recovery curve and fortified samples at levels, 0, 0.05, 0.10, and 0.2 ppm. Repeat set over at least 3 days for a total of 12 samples. The number of replicates is at the discretion of the supervisor.

iii. Phase III: Check samples for analyst accreditation.

(a) Analyze a minimum of 8 unknown samples furnished by Quality Assurance Manager (QAM) or Supervisor ranging from 0.05 - 0.2 ppm in liver or muscle. At least 1, but not more than 2 of the samples should be blank.

(b) Authorization from the QAM and supervisor is required to commence official analysis.

b. Acceptability criteria.

Refer to I. 1.

4. Intralaboratory Check Samples
   a. System, minimum contents:
      i. Frequency: minimum of 1 per week per analyst when samples are analyzed.
      ii. Records are to be maintained by analyst and reviewed by the supervisor and Laboratory Quality Manager, Chemistry.
   b. Acceptability criteria.

      Refer to 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. Matrices: Liver, muscle, processed products and catfish.
   b. Species: Avian, bovine, porcine
   c. Sample receipt size: Meat, approximately 1 lb.
   d. Condition upon receipt: Chilled or frozen.
   e. Sample storage:
      i. Time: Not more than 90 days (3 months).
      ii. Condition: Frozen

6. Sample Set

   Each sample set must contain:
   a. Blank
   b. Recovery
   c. Samples

7. Minimum Level of Applicability (MLA):
   a. Quantitation: 0.05 ppm for all analytes except SQX which is 0.1 ppm
   b. Confirmation: 0.05 ppm

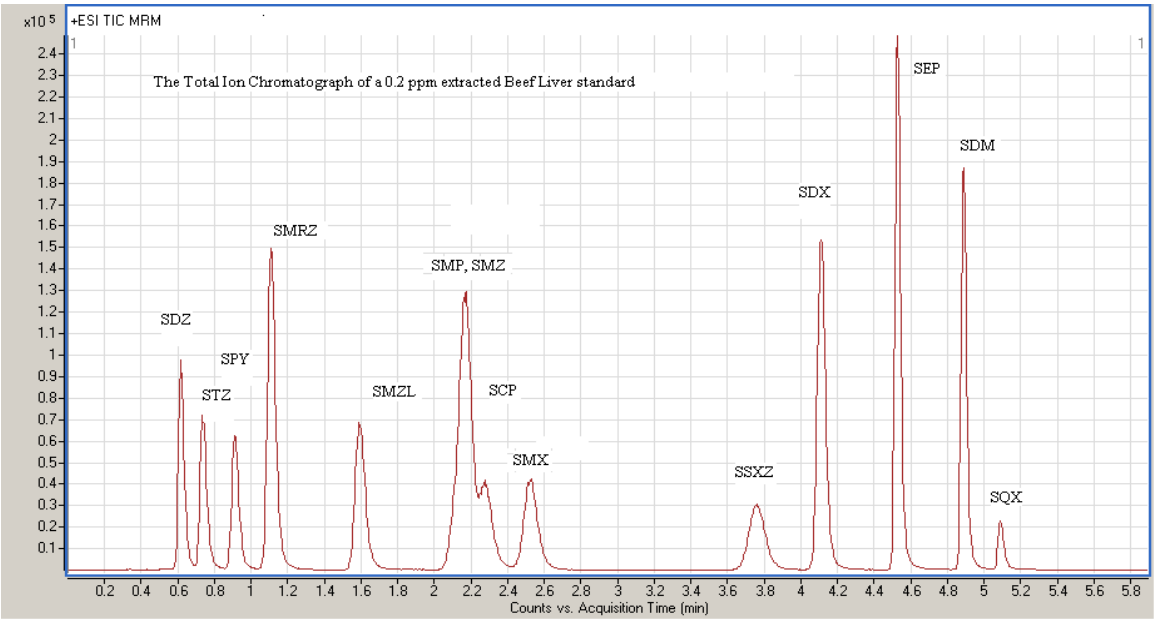
J. WORKSHEET

[RESERVED]

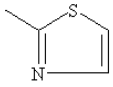
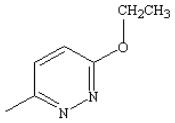
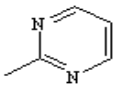
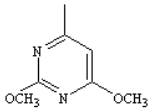
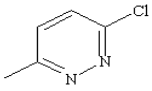
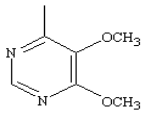
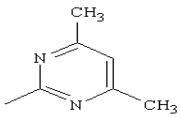
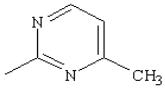
K. APPENDIX

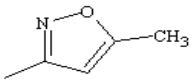
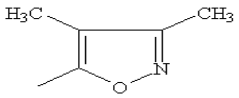
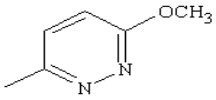
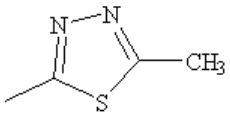
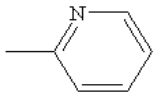
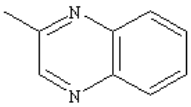
1. Sample Chromatograms

   a. TIC chromatogram of 0.2ppm Beef Liver standard



2. Structures of functional groups attached to sulfanilic acid through $–SO_2NH–$ bond

Sulfathiazole **$C_9H_9N_3O_2S_2$** **255.3** 	**Sulfaethoxypyridazine** **$C_{12}H_{14}N_4O_3S$** **294.3** 
Sulfadiazine **$C_{10}H_{10}N_4O_2S$** **250.3** 	**Sulfadimethoxine** **$C_{12}H_{14}N_4O_4S$** **310.3** 
Sulfachloropyridazine **$C_{10}H_9ClN_4O_2S$** **284.7** 	**Sulfadoxine** **$C_{12}H_{14}N_4O_4S$** **310.3** 
Sulfamethazine **$C_{12}H_{14}N_4O_2S$** **278.3** 	**Sulfamerazine** **$C_{11}H_{12}N_4O_2S$** **264.3** 

Sulfamethoxazole **$C_{10}H_{11}N_3O_3S$** **253.2** 	**Sulfisoxazole** **$C_{11}H_{13}N_3O_3S$** **267.3** 
Sulfamethoxypyridazine **$C_{11}H_{12}N_4O_3S$** **280.3** 	**Sulfamethizole** **$C_9H_{10}N_4O_2S_2$** **270.3** 
Sulfapyridine **$C_{11}H_{11}N_3O_2S$** **249.3** 	**Sulfaquinoxaline** **$C_{14}H_{12}N_4O_2S$** **300.3** 

L. APPROVALS AND AUTHORITIES

1. Approvals on file.

2. Issuing Authority: Director, Laboratory Quality Assurance Division.