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Title: Screening, Quantitation and Confirmation of Sulfonamides by Liquid Chromatography - Tandem Mass Spectrometry (LC-MS-MS)		
Revision: 05	Replaces: CLG-SUL4.04	Effective: 02/01/21

Contents

A. INTRODUCTION

1. Background / Summary of Procedure

Samples are extracted in ethyl acetate, then cleaned, dried, and reconstituted in methanol and mobile phase. The extracts are injected into an instrument for screening, quantitation, and/or confirmation by tandem mass spectrometry.

Quantitation is achieved by measuring the peak area 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 is suitable for the screening, quantification and/or confirmation of the following sulfonamides in muscle and liver tissues of porcine, bovine, ovine, caprine and avian species, processed products and fish of the order Siluriformes (catfish) at levels ≥ 0.05 ppm with the exception of Sulfaquinoxaline which quantitates at levels ≥ 0.10 ppm.

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

Note: Refer to 21CFR for tolerance values set by FDA and 40CFR for tolerance values set by EPA.

Note: This method may be performed using standards/solutions that contain fewer analytes than the method is applicable for, if the excluded analytes will not be included in the reported results

B. EQUIPMENT

Note: Equivalent equipment may be substituted.

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. Centrifuge-Durafuge 300, Thermo.

g. Polypropylene centrifuge tubes - 50 mL, Falcon Blue Max, Cat. No. 2098, and 15 mL, Falcon Blue Max, Cat. No. 2097, Falcon.

h. Micropipettor-Rainin EDP3.

i. Shaker – Reciprocating, Catalog No. 6010, Eberbach.

j. 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 / solutions may be substituted.

Note: All solvents are HPLC grade unless otherwise specified.

1. Reagents

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 ($K_2HPO_4 \cdot 3\ H_2O$) 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. STANDARD(S)

Note: Equivalent standards / solutions may be substituted. Purity and counterions are to be taken into account when calculating standard concentrations. In-house prepared standards shall be assigned an expiration date that is no later than the stability stated in the method.

1. Standard Information

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 of Standard Solution(s)

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

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

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

         [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. Preparation of Recovery Calibration Curve

   a. Weigh three (3) 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

E. SAMPLE RECEIPT AND 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 Controls and Samples.

   a. Screening - Weigh 3 blank tissue portions of 2.5 ± 0.1g each. One portion for a blank (negative control), the other 2 portions fortified with 25 µL (equivalent of 0.05 ppm of tissue) of the standard each (for Decision Level sample and the analyst recovery). Prepare additional control matrix for use as check sample when needed.

   b. Quantitation and Confirmation - In addition to the Recovery curve (D.3), weigh 2 blank tissue portions of 2.5 ± 0.1g each. One portion for a blank (negative control), the other portion fortify with 50 µL (equivalent of 0.10 ppm of tissue) of the standard to serve as the analyst recovery. Prepare additional control matrix for use as check sample when needed.

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

   d. Add 100 µL of Internal Standard Solution (2.5 µg/mL) to all tubes for a 0.1 ppm level fortification. Internal Standard Solution is added to all standards, recoveries, blanks, samples and internal checks.

      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.

2. Extraction Procedure

   a. Add 6.0 mL ethyl acetate to standards and samples. 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. 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. 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. 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. Extract is stable for one week prior to adding mobile phase A. 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.

3. LC Instrument Settings

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

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: 0.55 mL/min

Gradient Program:

Time (min)	Flow Rate (mL/min)	A (%)	B (%)
0	0.55	95	5
2	0.55	95	5
5	0.55	0	100
5.1	0.55	95	5
10	0.55	95	5

4. 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.

PolarityES+	
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 92.1	8 26	108	21
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

Analyte	Retention Time[1]	Filtered Parent Ion	Frag (V)[2]	Product Ion[3]	CE (V)[4]	Aux. Ions[3]	CE (V)
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.

5. Sample Set

   a. For Screening of Samples for Sulfonamides

      i. Recovery

      ii. Decision Level

      iii. Blank

      iv. Samples, up to a maximum of 13

      v. 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.

   b. For Quantitation and Confirmation of Samples for Sulfonamides

      i. Recovery curve

      ii. Recovery

      iii. Blank

      iv. Samples, up to a maximum of 11

      v. 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. DECISION CRITERIA / CALCULATIONS

1. For Screening of each compound of interest
   a. Review the retention time of the Chromatograph for each sulfonamide to verify that it is within ± 5% of the recovery sample.
   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. Screened positive sample must exhibit a response of more 50% of the Decision Level recovery used contemporaneously in the set.
   d. Blank must exhibit a response of less than 10% of the Decision Level recovery used contemporaneously in the set.

2. For Quantitation of each compound of interest:
   a. Review the retention time of the Chromatograph for each sulfonamide to verify that it is within ± 5% of the recovery sample
   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. If the compound of interest is reported with a quantitative value, the associated 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.

3. For Confirmation of each compound of interest:
   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. SAFETY INFORMATION AND PRECAUTIONS

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

2. Hazards

Consult all Safety Data Sheets (SDS) associated with the method.

3. Disposal Procedures

Follow federal, state and local regulations

I. QUALITY ASSURANCE PLAN

1. Performance Standard

a. Screening

For a screening set acceptance of fortified quality control samples, 12 of 13 sulfonamide analytes must be present and must exhibit a response of more 50% of the Decision Level used contemporaneously in the set.

b. Quantitation and Confirmation

For a Quantitation/Confirmation set acceptance of fortified quality control samples when all sulfonamide analytes are present, 12 of 13 analytes must meet confirmation criteria. In addition if analytes of interest with tolerance are present, the analyte must meet quantitation criteria.

Note: Performance standards are applicable for analyte of interest.

c. For quantitation:

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

Correlation coefficient (r) ≥ 0.995

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

d. For confirmation: Refer to Section G.

2. Intralaboratory Check Samples

a. Acceptability criteria.

Refer to I. 1.

If unacceptable values are obtained, then:

i. Investigate following established procedures.

ii. Take corrective action as warranted.

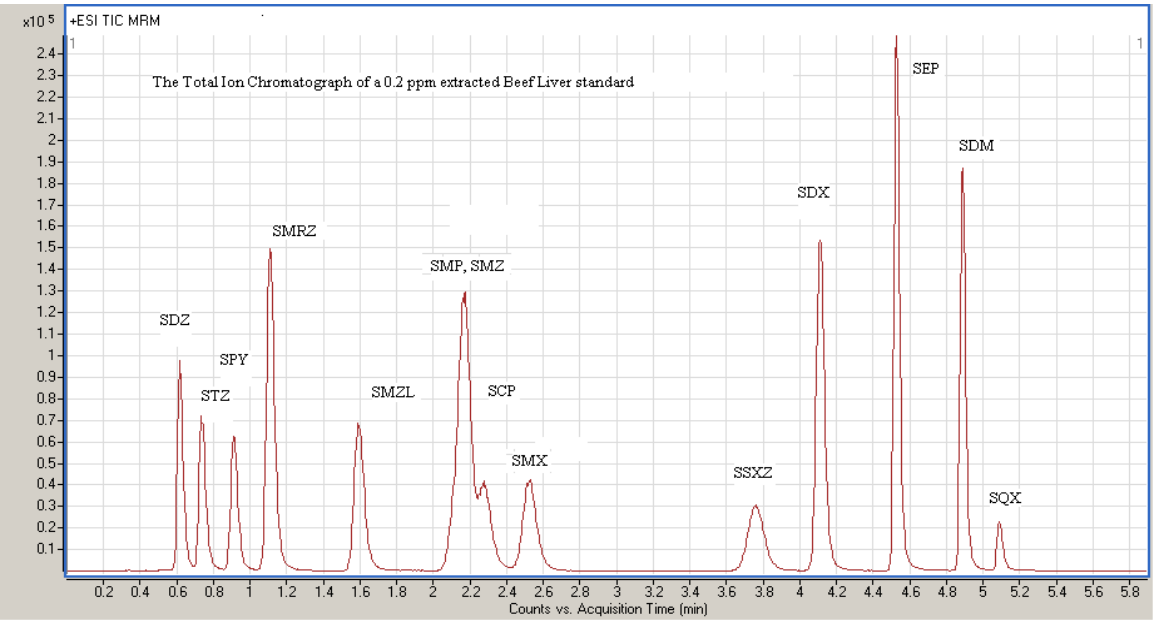
J. APPENDIX

1. References

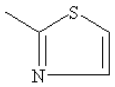
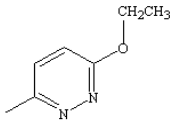
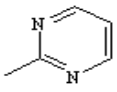
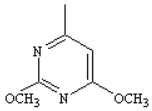
Reserved

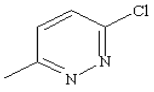
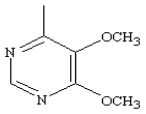
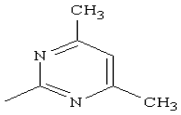
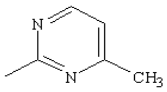
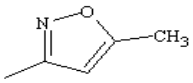
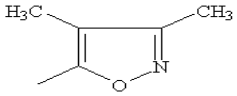
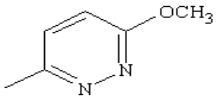
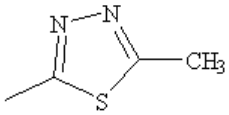
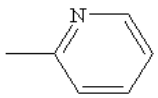
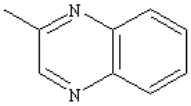
2. Sample Chromatograms

a. TIC chromatogram of 0.2ppm Beef Liver standard



3. 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** 

K. APPROVALS AND AUTHORITIES

1. Approvals on file.

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