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Title: Screening of Sulfonamides by Automated Robotic Extraction and Thin Layer Chromatography (TLC)		
Revision: 02	Replaces: CLG-SUL3.01	Effective: 02/24/2012

Contents

A. INTRODUCTION

1. Summary of Procedure

   This automated screening procedure for sulfonamides uses thin layer chromatography with fluorometric scanning densitometry. After extraction, separation of the drugs in the extract is carried out on a silica gel plate containing a preadsorbent spotting area. Visualization is accomplished using UV light after dipping the plate in fluorescamine solution.

2. Applicability

   This method is suitable for the screening of sulfamethazine, sulfathiazole, and sulfadimethoxine in porcine, bovine, ovine, caprine, and avian muscle and liver tissue, processed products, eggs, and egg products at levels ≥ 0.05 ppm.

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

B. EQUIPMENT

Note: Equivalent equipment may be substituted.

1. Apparatus

   a. 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 I00 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

b. Thelco Model 6M incubator - GCA Corp.

c. Convection oven - Model 20, GCA Corp.

d. Balance - PB3002-S, Mettler.

e. N-EVAP - Model 112 solvent evaporation system, Organomation Associates, Inc.

f. TLC developing tank - glass with glass top, Cat. No. 7645, Alltech Associates, Inc.

g. Stainless steel dipping tank.

h. Vortex mixer - Thermolyne Maxi Mix model M-16715, Thermolyne Corp.

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

j. Saturation pads - 20 x 20-cm, Cat. No. 7623, Alltech Associates, Inc.

k. Open tip fast flow filter - Cat. No. CC-09-m, Whale Scientific Inc.

l. Pipet tip, 5 mL - Cat. No. ZA7306, Zymark Corp.

m. Blender - Waring Products Division.

n. Robot Coupe processor - Robot Coupe U.S.A., Inc.

o. Silica gel thin layer plates with pre-absorbant layer - Whatman LK6D, Cat. No. 4865821, Alltech Associates, Inc.

p. Micropipettor – Rainin EDP3

q. Centrifuge – Durafuge 300, Thermo

r. Shaker, Reciprocating - Cat. No. 6010, Eberbach

2. Instrumentation

a. CAMAG TLC Scanner III scanning densitometer with data system and printer.

b. Spectroline Model C-3F UV light box - Black Light Eastern Corp.

C. REAGENTS AND SOLUTIONS

Note: Equivalent reagents / solutions may be substituted. The stability time frame of the solution is dependant on the expiration date of the components used or the listed expiration date, whichever is soonest.

1. Reagents

   Note: All solvents are HPLC grade unless otherwise specified.

   a. Hexane - Optima, Cat. No. H303-4, Fisher Scientific

   b. Ethyl Acetate – Cat. No. E196-4, Fisher Scientific

   c. Chloroform with Amylene – Cat. No. C297-4, Fisher Scientific

   d. Methanol – Cat. No. C493-E16, Thomas Scientific

   e. Acetone – Cat No. A929-4, Fisher Scientific

   f. Tert-butanol - Cat. No A401-1, Fisher Scientific

   g. Concentrated hydrochloric acid (HCl) - Reagent grade, Cat. No A144S-500, Fisher Scientific

   h. Diethylamine - Reagent grade, Cat. No. 9216-01, Baker

   i. Sodium Acetate - Anhydrous, Certified ACS Reagent grade, Cat. No. 3472-01, J.T. Baker

   j. Deionized Water – Double de-ionized water

   k. Fluorescamine – Cat. No. F9015-1G, Sigma–Aldrich

   l. Potassium phosphate dibasic, crystals - Cat. No. 7080, Mallinckrodt

   m. Potassium phosphate monobasic – Cat. No. 7100-12, Mallinckrodt

2. Solutions

   a. Fluorescamine dipping solution

      Dissolve 30 mg in 250 mL acetone. Solution is stable for 1 month when stored in the dark.

   b. TLC solvent system: chloroform/t-butanol (80:20 v/v)

      Add 100 mL solvent (80 mL chloroform and 20 mL t-butanol) and 20 mL deionized water to a 250 mL separatory funnel. Equilibrate by shaking for 30 seconds and allow phases to separate.

      Note: TLC developing solvent is equilibrated with water prior to use. Discard chloroform/tert-butanol in the developing chamber daily.

   c. 0.2 M phosphate buffer

      Weigh 45.65 g potassium phosphate dibasic crystals ($K_2HPO_4 \bullet 3\ H_2O$) and dissolve in 1000 mL distilled water in a volumetric flask (Solution 1). Dissolve 27.22 g KH_2PO_4, (potassium phosphate monobasic) in 1000 mL distilled water in a volumetric flask (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. 3.5 M sodium acetate

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

e. 3.2 M HCl

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

f. 25% Diethylamine solution

Add 25 mL diethylamine to a 100 mL volumetric flask. Dilute to volume with ethyl acetate.

D. STANDARD(S)

Note: Equivalent standards / solutions may be substituted. Purity and counter ions are to be taken into account when calculating standard concentrations. The stability time frame of the solution is dependant on the expiration date of the components used. In-house prepared standards shall be assigned an expiration date that is no later than the expiration date of the earliest expiring component or no later than the stability stated in the method, whichever ends soonest.

1. Standard Information

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

Compound	Cat. No.		Identifier
Sulfamethazine	# S-6256	Sigma	(SMZ)
Sulfapyridine	# S-6252	Sigma	(SPY Internal Standard - IS)
Sulfadimethoxine	# S-7007	Sigma	(SDM)
Sulfathiazole	# S-9876	Sigma	(STZ)

2. Preparation of Standard Solution(s)

a. Stock solution (1 mg/mL):

All sulfonamide standards, including the internal standard (Sulfapyridine) are prepared as follows:

Weigh 100 ± 0.1 mg of each sulfonamide into separate 100 mL volumetric flasks. Dissolve and bring to volume with acetone. Solutions are stable for 6 months when stored in polyethylene bottles at < -10 °C.

Note**:** 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. Working standards (10 µg/mL used for fortification):

All working standards are diluted with 0.2 M phosphate buffer (C.2.c).

i. 10 µg/mL standards:

(a) Sulfonamide mixed standard solution.

Pipet 1 mL of stock solution of each sulfonamide of interest into a 100 mL volumetric flask. Do not add internal standard. Bring to volume. The solution is stable for 3 months when stored in polyethylene bottles at 2 - 8 °C.

(b) Internal standard (IS) solution.

Pipet 1 mL of the 1 mg/mL internal standard (IS) solution into a 100 mL volumetric flask. Bring to volume. The solution is stable for 3 months when stored in polyethylene bottles at 2 - 8 °C.

ii. Solution D (2.50 µg/mL IS):

Pipet 25 mL of the 10 µg/mL IS solution into a 100 mL volumetric flask and bring to volume. The solution is stable for 3 months when stored in polyethylene bottles at 2 - 8 °C.

iii. Solution C (5.00 µg/mL sulfonamide of interest):

Pipet 50 mL of the 10 µg/mL sulfonamide mixed standard solution into a 100 mL volumetric flask and bring to volume. The solution is stable for 3 months when stored in polyethylene bottles at 2 - 8 °C.

iv. Solution B (2.5 µg/mL sulfonamide of interest):

Pipet 25 mL of solution C into a 50 mL volumetric flask and bring to volume. The solution is stable for 3 months when stored in polyethylene bottles at 2 - 8 °C.

v. Solution A (1.25 µg/mL sulfonamide of interest):

Pipet 25 mL of solution B into a 50 mL volumetric flask and bring to volume. The solution is stable for 3 months when stored in polyethylene bottles at 2 - 8 °C.

Note: Standard solutions C, B, and A may be used to prepare check samples.

E. SAMPLE PREPARATION

Samples of liver are blended until homogeneous. Muscle or processed product samples are processed until homogeneous, if necessary. Egg and egg products should be mixed

or shaken before weighing to ensure homogeneity. All samples are stored refrigerated or frozen until analyzed.

F. ANALYTICAL PROCEDURE

1. Preparation of Controls

   a. Tissue, Processed Products, and Liquid Eggs Recovery Standards and/or Negative Control Blank

      i. Weigh three 2.5 ± 0.1 g of blank control matrix. Fortify in duplicate two of the blanks each with 100 ± 1 µL fortification standard Solution A. Assign one of these standards to be used as the Decision Level standard and the other the analyst recovery. Use the third control as a blank.

         Note: The blank is optional if using previously tested tissue.

      ii. Prepare additional control matrix for use as check sample when needed.

      iii. Add 100 µL of standard solution D (2.50 µg/mL) to the blank, the analyst recovery, and the Decision Level standard, as well as the internal check sample, when used. This will yield 0.10 ppm IS.

   b. Powdered Eggs Recovery Standards and/or Negative Control Blank

      i. Weigh three 1.0 ± 0.1 g of blank control matrix. Add 1 mL distilled H2O. Fortify in duplicate two of the blanks each with 40 ± 1 µL fortification standard Solution A. Assign one of these standards to be used as the Decision Level standard and the other the analyst recovery. Use the third control as a blank.

         Note: The blank is optional if using previously tested tissue.

      ii. Prepare additional control matrix for use as check sample when needed.

      iii. Add 40 µL of standard solution D (2.50 µg/mL) to the blank, the analyst recovery, and the Decision Level standard, as well as the internal check sample, when used. This will yield 0.10 ppm IS.

2. Extraction Procedure

   a. Tissue, Processed Products, and liquid egg samples

      Weigh 2.5 ± 0.1 g of samples into a 50 mL polypropylene centrifuge tube. Allow to thaw, if necessary. Add 100 µL of standard solution D (2.50 µg/mL). This will yield 0.10 ppm IS.

   b. Powdered egg samples

      Weigh 1.0 ± 0.1 g of samples into a 50 mL polypropylene centrifuge tube. Allow to thaw, if necessary. Add 1 mL distilled H2O. Add 40 µL of standard solution D

(2.50 µg/mL). This will yield 0.10 ppm IS.

Note: The following steps are performed manually or automatically by the robotic system.

c. Samples and controls continue here.

d. Addition of extraction solvent

i. Tissue, Processed Products, and Powdered Eggs

Add 6.0 mL ethyl acetate to tissue 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.

ii. Liquid Eggs

Add 10.0 mL ethyl acetate to the liquid egg 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.

Note: Liquid egg samples sometimes form emulsions after shaking. Vortex the sample on the highest vortexer setting until the emulsion breaks up.

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

Note: Because the fast flow filters do not stop liquid egg from passing into the 15 mL centrifuge tube, care must be taken when pouring the samples through the filters. Liquid egg mixed into the ethyl acetate extract will cause emulsion problems later in the method.

f. Add 1.0 mL 3.2 M HCl.

g. 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.

h. Aspirate ethyl acetate to waste.

i. Add 5.0 mL hexane and vortex for 30 seconds. Let stand for 5 minutes.

Manual – Shake approximately 5 minutes on low or vortex for 30 seconds. Let

stand for at least 5 minutes or centrifuge for 5 minutes at 2500 rpm.

j. Aspirate hexane to waste.

k. Add 2.0 mL 3.5 M sodium acetate.

l. Add 3.0 mL ethyl acetate and vortex for 30 seconds. Let stand for at least 5 minutes.

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

m. Transfer ethyl acetate to a clean centrifuge tube.

3. Development Procedure (continued, for all matrices):

Note: The spotting and developing steps may be optimized to account for changes in plate manufacture.

a. To each sample tube, either add 10 µL manually or add an equivalent amount of diethylamine solution (C.2.f) using the robot.

Note: The remaining steps are carried out manually.

b. Evaporate to dryness under nitrogen using an N-Evap with water bath temperature set to 40 ± 5 °C.

c. Add 100 µL methanol to each sample tube and re-dissolve residue by mixing briefly using a vortex mixer.

d. Spot 20 µL of each sample on the pre-absorbant layer of a LK6D thin layer plate. Spotting is facilitated by heating the pre-absorbant layer using a small hot plate or heat strip. The heat strip or hot plate should be hot enough to evaporate the spot within a few seconds. Position the sample spot approximately 10 - 15 mm from the silica gel interface. Do not use the outermost lanes. Accurate densitometry depends on careful spotting technique to achieve an even band. After spotting, dry plate for at least 10 minutes in a forced air drying oven set to 100 °C. This drying step is helpful in preventing band broadening when plate is developed.

e. Develop the plate with methanol to a distance of 1 cm above the silica gel interface. Remove the plate and dry for at least 1 minute in a convection oven at 100 °C. Repeat this development cycle if necessary.

f. Develop the plate to a distance of 6 cm above the silica gel interface in chloroform/t-butanol (80:20) in a saturated developing tank. Dry plate for at least 1 minute in an oven at 100 °C. Redevelop the plate in chloroform/t-butanol (80:20) to a distance of 12 cm above the silica gel interface. (Development should be carried out at 25 - 30 °C to insure consistent RR1 values.)

g. Oven dry plate for at least 1 minute at 100 °C, cool to room temperature and dip

in fluorescamine solution. Keep plate in solution 1 - 2 seconds after fully submerged (longer immersions will result in high background).

h. Place plate in the dark for at least 15 - 30 minutes to allow for development.

i. Scan plate using densitometer using 410 ± 10 nm excitation.

4. Instrumental Settings

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

Set up Scanning Densitometer according to manufacturer instructions. The following are conditions for a CAMAG Scanning Densitometer and should be used for reference only.

Scanner settings:

Plate size (width x height)	20 x 20 cm
Application position Y	10.0 mm
Position of solvent front	145.0 mm
Scan start position Y	60 mm
Scan end position Y	145.0 mm
Scan start position X	20.0 mm
Distance between tracks	10.0 mm
Number of tracks	18
Lamp	Mercury
Monochromator bandwidth	20 nm
Wavelength	410 nm
Slit dimension	6.0 x 0.20 mm
Data step resolution	100 fm
Display scaling	700 AU
Measurement mode	Fluorescence / Reflection
Scanning speed	10.0 mm/sec.
Optical filter	460 nm filter
Zeroing mode	Automatic
Y-position for 0 adjustment	60.0 mm, Track: 1
Quick scan from	60.0 mm to 145 mm, all tracks
Offset	10%
Sensitivity	Automatic
High voltage of PM	257 V

Integration Parameters

Video integration	no
Baseline correction	yes
Peak thresh. height	10 AU
Peak thresh. area	300
Peak thresh. slope	3

Filter factor	3
Data selection factor	1
Track start position	88.1 mm
Track end position	136.8 mm

5. Sample Set
   a. Blank (optional)
   b. Decision Level Standard
   c. Recovery
   d. Check Sample (if necessary)
   e. Up to 22 Samples

G. CALCULATIONS / IDENTIFICATION

For each plate:

1. Measurement

   Measure the peak heights of all sulfonamides of interest and internal standard peak for each track. Calculate peak height ratio by dividing sulfonamide peak height by internal standard peak height.

$$\text{Peak Height Ratio} = \frac{\text{Sulfonamide peak height}}{\text{Internal Standard Peak Height}}$$

2. Decision Level

   Establish a decision level for each sulfonamide using the first of the fortified samples prepared as follows:

   Decision Level = 0.75 X Peak Height Ratio

3. Assessment

   Samples and/or quality control standards are considered screened positive for each sulfonamide when their calculated peak height ratio is greater than or equal to the decision level.

   Note: This method will additionally screen for other sulfonamides. Unidentified analytical responses (see Section K.3) may be forwarded for additional determinative and/or confirmation analysis as needed.

   Samples and/or quality control standards are considered screened negative for each sulfonamide when their calculated peak height ratio is less than the decision level.

Note: For processed products the internal standard peak heights may need to be monitored to ensure successful analyses.

H. SAFETY INFORMATION AND PRECAUTIONS

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

2. Hazards

Procedure Step	*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

Follow local, state and federal guidelines for disposal.

I. QUALITY ASSURANCE PLAN

1. Performance Standard
   a. No false negatives in quality assurance samples fortified at the ≥ 0.05 ppm level.
   b. No false positives for blank control samples.

2. Critical Control Points and Specifications

Record	*Acceptable Control*
Sample weight	2.5 g ± 0.10 g
Sample weight, powdered eggs	1.0 g ± 0.10 g

3. Intralaboratory Check Samples
   a. System, minimum contents.

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

ii. Records are to be maintained.

b. Acceptability criteria.

Refer to I. 1.

If unacceptable values are obtained, then:

i. Investigate following established procedures.

ii. Take corrective action as warranted.

4. Sample Condition upon Receipt - Chilled or frozen

J. APPENDIX

1. References

a. Chemistry Chemical Guidebook method 5.018 (Revised June, 1987).

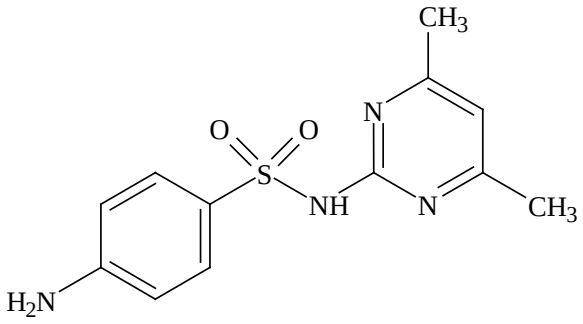
b. N. Takeda and Y. Akiyama, Journal of Chromatography, 558,175-180 (1991).

2. Chromatograms/spectra

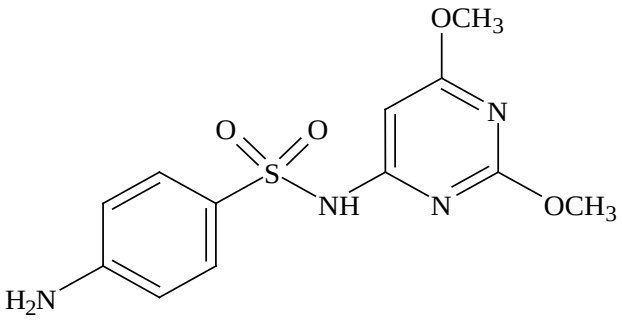
Reserved

3. Structures

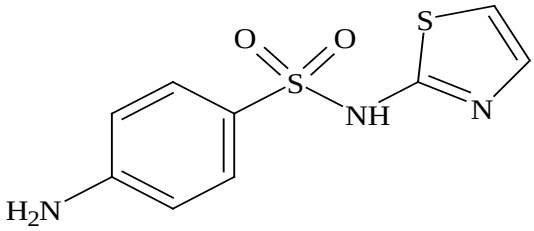
a. Sulfamethazine



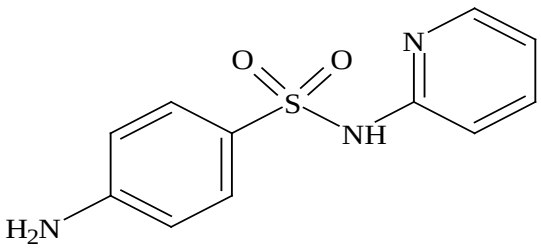
b. Sulfadimethoxine



c. Sulfathiazole



d. Sulfapyridine (internal standard)



4. Relative Retention Factor (Rf) Values for Sulfonamides of Interest

Sulfonamides	Identifier	RR_1 value
Sulfapyridine	SPY	1.00
Sulfadiazine	SDZ	1.12
Sulfathiazole	STZ	0.88
Sulfamethazine	SMZ	1.27
Sulfachloropyridazine	SCP	1.24
Sulfamethoxypyridazine	SMP	1.25
Sulfaquinoxaline	SQX	1.34
Sulfadimethoxine	SDM	1.43
Sulfaethoxypyridazine	SEP	1.36
Sulfaphenazole	SPZ	1.39
Sulfatroxazole	STX	1.38
Sulfisoxazole	SFX	1.32
Sulfadoxine	SDX	1.48

Note: RR_1 = Relative retention factor normalized to, the internal standard, sulfapyridine.

K. APPROVALS AND AUTHORITIES

1. Approvals on file.

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