CLG-SUL.05		Page 1 of 38
Title: Determination and Confirmation of Sulfonamides		
Revision: 05	Replaces: CLG-SUL.04	Effective: 09/25/2009

Contents

A. INTRODUCTION

1. Theory

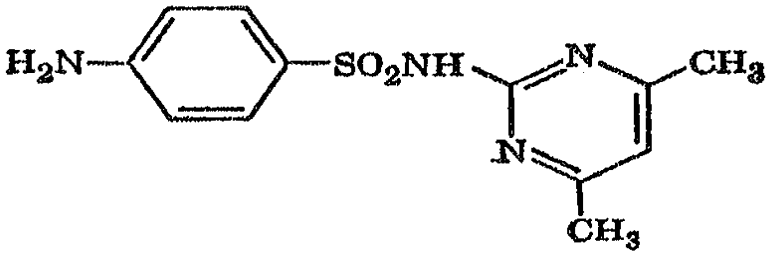
This tissue residue assay for sulfonamides uses thin layer chromatography with fluorometric scanning densitometry for quantitation. After addition of an internal standard, the tissue is extracted with ethyl acetate. The sulfonamides are then partitioned into glycine buffer. After pH adjustment, the aqueous phase is extracted with methylene chloride. Separation of the drugs from coextractives is carried out on a silica gel plate containing a preadsorbent spotting area. Visualization is accomplished using UV light after dipping in fluorescamine solution.

2. Applicability

This method is applicable to sulfonamides listed below.

Sulfonamides	Acronym	Species/Tissues	RR_f*
Sulfapyridine (Int. Std.)	SPY	All : liver, muscle	1.00
Sulfathiazole	STZ	Red meat : liver, muscle	0.88
Sulfadiazine	SDZ	Red meat : liver, muscle	1.12
Sulfamerazine	SRZ	Red meat : liver, muscle	1.21
Sulfachloropyridazine	SCP	Red meat : liver, muscle	1.24
Sulfamethoxypyridazine	SMP	Red meat : liver, muscle	1.25
Sulfamethazine	SMZ	All : liver, muscle	1.27
Sulfamethoxazole	SMO	Red Meat: liver, muscle	1.27
Sulfisoxazole	SFX	Red meat : liver, muscle	1.32
Sulfaquinoxaline	SQX	Poultry : liver, muscle	1.34
Sulfaethoxypyridazine	SEP	Red meat : liver, muscle	1.36
Sulfatroxazole	STX	Red meat : liver, muscle	1.38
Sulfaphenazole	SPZ	Red meat : liver, muscle	1.39
Sulfadimethoxine	SDM	Red meat : liver, muscle	1.43
Sulfadoxine	SDX	Red meat : liver, muscle	1.48

*RR_f = Relative retention factor with respect to Sulfapyridine.



Chemical Structure of Sulfamethazine

Note: Structures of functional groups of the above sulfonamides attached to sulfanilic acid through $–SO_2NH–$ bond are shown in Appendix, Section K.1.

B. EQUIPMENT

Note: An equivalent can be substituted for the following apparatus and instrumentation if necessary.

1. Apparatus
   a. PR 6000 centrifuge - Model number is HNS-2, Damon IEC.
   b. Temperature-controlled heating strip - Capable of 85 °C, AIS multispotter.
   c. Whatman LK6D silica gel plates - 20 x 20 cm. Plates have to be prewashed in methanol before use.
   d. pH meter.
   e. Vacuum aspirator with trap employing disposable Pasteur pipet.
   f. 50 mL screw-cap polypropylene centrifuge tubes - Cat. No. 5330, Corning, Cat. No. C3973-50, Scientific Products.
   g. Mechanical shaker, horizontal.
   h. Forced draft oven capable of 100 °C.
   i. TLC developing tank (2).
   j. Stainless steel dipping tank.
   k. Organomation N-Evap for evaporation by nitrogen and heat.
   l. Vortex test tube mixer.
   m. Syringes (10 to 100 µL).
   n. UV light box.
   o. Styrofoam ice chest or incubator set at 25 - 30 °C for insulating TLC tank during development.
   p. Chromatography paper (Whatman No. 1) or filter paper (Whatman No. 3). Use to

line the developing tank to ensure adequate vapor equilibration.

q. Micropipets, fixed or variable.

r. Blender - Waring blender.

s. Mixer - Robot Coupe or food grinder.

2. Instrumentation

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

C. REAGENTS AND SOLUTIONS

Note: An equivalent can be substituted for the following reagents and solutions if necessary.

1. Reagents

a. Ethyl acetate - HPLC or GC grade.

b. Methylene chloride - HPLC or GC grade.

c. Chloroform - distilled in glass with no ethanol preservative added.

d. Tert-butanol - reagent grade.

e. Methanol - HPLC or GC grade.

f. Acetone - HPLC or GC grade.

g. Hydrochloric acid (HCl) - concentrated: reagent grade.

h. Glycine - Reagent grade.

i. Hexane - HPLC or GC grade.

j. Sodium Hydroxide - Reagent grade.

k. Potassium hydrogen phosphate dibasic ($K_2HPO_4 \cdot 3H_2O$) - Reagent grade.

l. Potassium dihydrogen phosphate monobasic (KH_2PO_4) - Reagent grade.

m. Fluorescamine - Cat. No. 2-1650, Aldrich.

2. Solutions

a. Sodium hydroxide (10 N):

From reagent grade chemical: Dissolve 400 g of NaOH pellets to a total volume of 1 L with distilled water. (Caution - A great deal of heat will be generated. Add some NaOH and allow to cool before adding the remainder.)

b. Fluorescamine dipping solution:

Dissolve 120 mg fluorescamine (Pierce Chemical Co.) in 1000 mL acetone. Solution is stable for 1 month when stored in the dark.

c. TLC solvent system, Chloroform:tert-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**:** Sulfathiazole can be distinguished from Sulfapyridine using the chloroform:tert-butanol system, but unless conditions are optimal, densitometric resolution is incomplete. For this reason, the chloroform:tert-butanol solvent system (80:20) should be washed with water prior to use. The increase in polarity from the trace water absorbed by the organic phase will substantially increase the separation between Sulfathiazole, Sulfapyridine, and Sulfamethazine. However, the resolution between Sulfamethazine, Sulfaquinoxaline, and Sulfadimethoxine will decrease.

d. Glycine buffer (0.2 M):

Dissolve 30 g of glycine in 2 L of distilled water. Adjust with 10 N NaOH or NaOH pellets to pH 12.25 ± 0.05. If laboratory temperature drops below 20 °C, it may be necessary to warm this reagent prior to use.

e. 2 M phosphate buffer:

i. 2 M potassium hydrogen phosphate dibasic ($K_2HPO_4 \cdot 3H_2O$): Dissolve 45.6 g in 100 mL of distilled water.

ii. 2 M potassium dihydrogen phosphate monobasic (KH_2PO_4): Dissolve 272.2 g in 1 L of distilled water. Warm, if necessary to dissolve the KH_2PO_4.

iii. Add dibasic to the monobasic until the pH reaches 5.25.

f. 1.7 M HCl:

Add approximately 500 mL deionized water to a 1000 mL volumetric flask. Add 142 mL concentrated HCl. Dilute to volume with deionized water.

g. 1.7 M HCl /2 M phosphate buffer:

Prepare 1:1 v/v and adjust pH to 1.65.

h. 0.2 M phosphate buffer:

i. Weigh 45.65 g potassium phosphate dibasic crystals ($K_2HPO_4 \cdot 3H_2O$) and dissolve in 1000 mL distilled water in a volumetric flask (Solution 1).

ii. Dissolve 27.22 g KH_2PO_4, (potassium phosphate monobasic) in 1000 mL distilled water in a volumetric flask (Solution 2).

iii. 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.)

i. 1 N HCl:

Add approximately 500 mL deionized water to a 1000 mL volumetric flask. Add 83 mL concentrated HCl. Dilute to volume with deionized water.

j. 1 N NaOH:

Dilute 10 mL of 10 N NaOH to 100 mL of distilled water or 40 g of NaOH pellets to 1 L of distilled water.

D. STANDARDS

1. Source

Sulfonamide standards are available from:

a. Sigma Chemical (SPY, SDZ, STZ, SMZ, SMO, SCP, SMP, SDM, SPZ, SFX, SRZ).

b. U. S. Pharmacopoeia (SPY, SDZ, STZ, SMZ, SMO, SCP, SDM, SFX, SRZ, SDX).

c. Pfaltz & Bauer (STX, SQX).

d. Cyanamid (SEP).

e. Merck (SQX).

2. Preparation of Standards

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.

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 (used for fortification):

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

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.

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.

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.

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

Pipet 50 mL of the 10 µg/mL standard of the sulfonamide or mixed sulfonamides of interest into a 100 mL volumetric flask and bring to volume.

iv. Solution B (2.50 µg/mL sulfonamide of interest):

Pipet 25 mL of solution C into a 50 mL volumetric flask and bring to volume.

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.

3. Storage Conditions

All standards can be stored in polyethylene bottles and refrigerated at 2 - 8 °C, except for stock solution (≤ -10 °C).

4. Shelf Life Stability

a. Stock solution: 6 months.

b. Working solutions: 3 months.

Storage stability is based upon the storage conditions mentioned in D.3.

E. SAMPLE PREPARATION

1. Liver/Kidney:

a. Sample should be cool and soft (fully thawed) before processing

b. Cut tissues from different parts of large organs enough to make required number of samples. Avoid fat tissue if present.

c. Cut and blend entire organ (except fat) on small species. Examples: goat, and lamb.

d. Blend tissue in Waring blender. Process just long enough to make a homogeneous mixture.

e. Freeze blended tissue if it in not to be analyzed immediately.

2. Muscle:

a. Sample should be cool and soft (fully thawed) before processing

b. Cut lean tissue from different parts of muscle, avoid fat and connective tissue as much as possible.

c. Cut just enough tissue to prepare approximately ¾ to 1 pound of sample.

d. Cut into small ½ to 1 inch cubes before processing. The tougher the tissue, the

smaller the cube. Blend and mix using a Robot Coupe or food grinder.

e. Freeze blended tissue if it in not to be analyzed immediately.

F. ANALYTICAL PROCEDURE

1. Sample Extraction

a. Weigh 2.5 ± 0.1 g of frozen pre-ground tissue into a 50 mL polypropylene centrifuge tube. Allow to thaw.

b. Fortification standard curve:

Weigh four 2.5 ± 0.1 g of blank tissue as above and use as follows:

i. Blank tissue (1).

ii. Fortified tissues (3). Fortify them as follows:

Volume Added (µL)	Solution Used	µg Added	ppm*
100	A	0.125	0.05
100	B	0.250	0.10
100	C	0.500	0.20

*****ppm is based on 2.50 g as sample weight.

iii. Add 100 µL of standard solution D (2.50 µg/mL) to blank and fortified tissues. This will yield 0.10 ppm IS.

c. Samples:

i. Add 100 µL of standard solution D (2.50 µg/mL) to all low-level samples. This will yield 0.10 ppm of IS.

ii. Add 25 µL of the internal standard stock solution (1 mg/mL) to all high-level samples. This will yield 10 ppm of IS.

d. Allow samples to stand for 15 minutes.

e. Add 25 mL ethyl acetate via repipet.

f. Seal tubes with screw cap. Shake once or twice by hand to check for leakage.

g. Shake on horizontal shaker for 20 minutes at approximately 250 cycles/minute.

h. Centrifuge 5 minutes at 2500 rpm.

i. Decant supernatant into a clean 50 mL polypropylene centrifuge tube and add 10 mL of 0.20 M glycine buffer. Discard tissue residue and original tube.

j. Cap tube and shake on mechanical shaker for 5 minutes.

k. Centrifuge for 5 minutes at 2500 rpm.

l. Remove organic phase (upper layer) with Pasteur pipet attached to vacuum aspirator. Take care to remove any solid or emulsified material remaining at interface or clinging to tube wall.

m. Add 2 mL of 1.7 M HCl / 2 M phosphate buffer. Adjust, if necessary, to pH 5.25 ± 0.10 with buffer or 1 N NaOH or 1 N HCl.

n. Add 10 mL hexane. Cap and shake on mechanical shaker for 5 minutes.

o. Centrifuge for 5 minutes at 2500 rpm.

p. Remove organic phase (upper layer) with Pasteur pipet attached to vacuum aspirator. Use gentle vacuum to aspirate upper layer, especially if solids are present at the layer interface.

q. Add 10 mL methylene chloride. Cap and shake on mechanical shaker for 5 minutes.

r. Centrifuge 5 minutes at 2500 rpm.

s. Remove aqueous phase (upper layer) with Pasteur pipet attached to vacuum aspirator. Entire layer can be completely removed by tilting tube and keeping pipet aspirator at tube wall. Take care to remove any fat or particulates at the interface. If a gelatinous plug is present between the aqueous and organic phases, do not remove or aspirate it. Poor recovery will result.

Note: For sulfa levels fortified according to F.1.c.ii, skip steps t-w and proceed to section F.2.

t. Evaporate remaining organic phase under a stream of nitrogen on an N-Evap (40 ± 3 ºC). When the level has declined to 5 mL, rinse the sides of the tubes with an aliquot (ca. 2 mL) of methylene chloride. Repeat rinse when volume declines to approximately 2.5 mL and again at approximately 1.0 -1.5 mL.

u. Evaporate just to dryness. Do not allow residue to dry out.

v. Dissolve residue in 100 µL methanol and vortex samples for 30 seconds. Allow to stand for 5 minutes so that insoluble oils settle to the bottom of the tube.

w. Keep tubes tightly stoppered in case an additional analysis is required.

Note: Stopping points-within day. Any step may be used as a stopping point during the course of the day with two exceptions. It is not desirable to allow the sulfonamides to remain in the strongly basic glycine buffer for more than one hour. Likewise, the concentration to dryness step should not be allowed to go unattended. Significant losses may occur if the residue is allowed to evaporate beyond the "just to dryness" stage and remain that way for any length of time while heated.

2. Thin Layer Chromatography

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

a. Spot 20 uL (oil free) of each sample on the pre-absorbent layer of a LK6D thin layer plate. Spotting is facilitated by heating the pre-absorbent 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.

b. Develop the plate with methanol to approximately 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 for one more time.

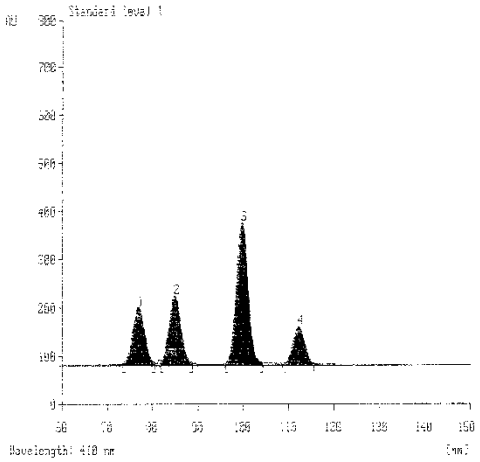
c. Develop the plate to approximately 6 cm from the interface in 80:20 chloroform:tert-butanol in a vapor-saturated tank contained in an insulated ice chest or incubator at 25 - 30 °C. Dry plate for 1 minute in an oven at approximately 100 °C. Redevelop plate in chloroform:tert-butanol to approximately 12 cm. Development must be carried out at 25 - 30 °C to ensure consistent Rf values across the plate.

d. Dry plate in an oven for 1 minute at approximately 100 °C, cool to room temperature and dip the plate in the fluorescamine solution. Keep plate in the solution only 1 - 2 seconds after fully submerged (longer immersions will result in high background).

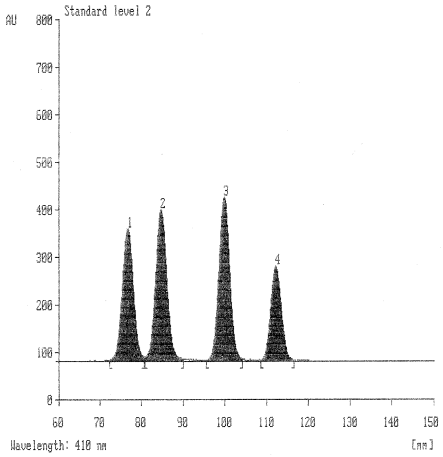
e. Allow plate to develop in the dark at room temperature for 15 - 30 minutes. View the plate under UV light to check for recoveries and very high sulfa levels.

f. Scan on densitometer, using a 410 ±10 nm excitation wavelength.

3. Chromatograms



0.05 ppm Standard



0.10 ppm Standard

1-Sulfadimethoxine (SDM), 2-Sulfamethazine (SMZ), 3-Sulfapyridine (SPY), 4-Sulfathiazole (STZ).

G. CALCULATIONS

1. For each plate developed, construct a standard curve from the fortified samples applied to that plate as follows:

   a. Measure the peak heights of the sulfonamides of interest and internal standard peaks of each fortified sample and calculate the peak height ratios.

$$\text{Peak height ratio} = \frac{\text{Peak ht. Sulfonamide of interest}}{\text{Peak ht. internal standard}}$$

   b. Using linear regression, construct a standard curve of sulfonamide concentration vs. peak height ratio.

      The equation is $y = mx + b$ where

      y = sulfonamide/IS peak height ratio
      x = sulfonamide concentration (as ppm in sample)
      m = slope
      b = y intercept

      The correlation coefficient (r) should be ≥ 0.995.

2. Using the regression slope and intercept, compute the sulfonamide concentration (x) for each incurred sample from the measured peak height ratio.

3. For high level samples, multiply the above result by 100.

H. HAZARD ANALYSIS

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

2. Procedure Steps

Section	*Hazard*	*Recommended Safe Procedures*
C. Reagents		
Ethyl Acetate Methylene Chloride Chloroform Tert-Butanol Methanol Acetone	These organic reagents are very flammable and corrosive and the vapors are extremely irritating to the skin, eyes, and respiratory tract.	These solvents should only be used in an efficient fume hood, away from any heat - generating device.
Concentrated HCl Sodium Hydroxide (10N)	HCl fumes are corrosive. Spattering may result in serious eye, skin, respiratory damage	Preparation and use of dilute HCl be performed easily in a fume hood.
F. Analytical Procedure		
Thin-Layer Chromatography	The hazards associated with the above solvents are increased since development cannot be done in a hood or other drafty environment.	The development tank can be set beside a hood so that plates can be removed and dried in the hood with minimum exposure to the analyst. A facemask may also be used.

3. Disposal Procedures

Organic solvent mixture	See Above	Segregate chlorinated from nonchlorinated solvents as much as possible. Hold in designated containers until disposed of by the waste disposal personnel. Final disposition will be by the contractor.

I. QUALITY ASSURANCE PLAN

1. Performance Standard

Analyte	*Analytical Range (ppm)*	*Acceptable Recovery (%)*	*Repeatability %CV*	*Reproducibility % (CV)*
All sulfonamides	0.05 – 0.2	80 - 120	12	15

2. Critical Control Points and Specifications

Record	*Acceptable Control*
a. Room Temperature	>20 °C (if not, warm up the glycine buffer solution.)
b. pH	5.25 ± 0.10

3. Readiness to Perform

a. Familiarization

i. Phase I

TLC, densitometric standard curve preparation: Four sulfonamides (Sulfamethazine, Sulfadimethoxine, Sulfaquinoxaline and Sulfathiazole) at four levels: 0, 0.05, 0.1, and 0.2 ppm.

The number of replicates is at the discretion of the supervisor.

ii. Phase II

Sample fortification (self-provided tissues) at four levels 0, 0.05, 0.10, and 0.20 ppm.

(a) Turkey liver and muscle -- Sulfaquinoxaline

(b) Turkey liver and muscle -- Sulfamethazine, and Sulfadimethoxine.

(c) Swine liver and muscle -- Sulfathiazole, Sulfadimethoxine, and Sulfamethazine.

(d) Beef liver and muscle -- Sulfadimethoxine, Sulfathiazole, and Sulfamethazine.

The number of replicates is at the discretion of the supervisor.

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

iii. Phase III: Check samples for analyst accreditation.

(a) Analyze 14 samples furnished by the Quality Assurance Manager (QAM) or Supervisor.

(b) Report analytical findings to the QAM and Supervisor.

Authorization from the QAM and Supervisor is required to commence official analysis.

b. Acceptability criteria.

See section I.1 above.

4. Intralaboratory Check Samples

a. System, minimum contents.

i. Frequency: minimum of 1 per week per analyst.

b. Acceptability criteria.

If unacceptable values are obtained, then:

i. Stop all official analyses by that analyst.

ii. Take corrective action.

5. Sample Acceptability and Stability

a. Matrices: Liver, kidney, muscle, processed products.

i. Sample receipt size, minimum weight: ~1 lb.

ii. Condition upon receipt: Chilled or frozen.

iii. Sample storage:

a. Time: Not more than 90 days (3 months).

b. Condition: Frozen (< -10 °C).

6. Sample Set

Each sample set must contain a tissue blank, a recovery, a standard curve of fortified tissues at 0.05, 0.10, and 0.20 ppm, and samples of the same tissue.

7. Sensitivity

Minimum proficiency level (MPL): 0.05 ppm.

J. WORKSHEET

[RESERVED]

II. Confirmatory Method

A. INTRODUCTION

1. Theory

Sulfonamides are extracted from tissue using the procedure described in Part I. The sulfonamides are first methylated with diazomethane and then acylated with pentafluoropropionic anhydride before detection and confirmation by GC/MS-El selected ion monitoring. Generally, the three ions selected for monitoring are the 238 ion ($C_2F_5CONHC_6H_4$), which is common to all, and the M-64 and M-65 ions, which are formed when the derivatized sulfonamide loses sulfur dioxide.

2. Applicability

Refer to Determinative Method Part I, section A.2.

B. EQUIPMENT

Note: An equivalent may be substituted for any apparatus or instrument listed below.

1. Apparatus

a. 5 mL disposable borosilicate glass centrifuge tube – Cat. No. 73785-5, Kimble; Cap, Cat. No. 73802-1341 5, Kimble.

b. N-Evap - Model 111, Organomation.

c. Diazomethane generator, Aldrich, Zl0, 025-0 - Distillation glassware, without ground glass joints, used to generate diazomethane from Diazald.

d. Vortex test tube mixer - Lab Line Instruments, Model No.1290, Super Mixer.

e. Nitrogen, high purity.

f. Syringes -10 µL gas-tight, Cat. No. 1701, Hamilton.

g. Repipetters - Eppendorf, 100 µL, 50 µL, and 25 µL.

h PR 6000 centrifuge - Model number is HNS-2, Damon IEC.

i. Disposable pipets, 5 ¾ inches, borosilicate glass - Cat. No. 72050, Kimble.

2. Instrumentation

Hewlett Packard gas chromatograph 5890 with a Hewlett Packard mass selective detector 5970-electron impact (70 ev), or equivalent.

C. REAGENTS AND SOLUTIONS

Note: An equivalent may be substituted for the following reagents and solutions.

1. Reagents

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

   b. Ethyl acetate - Cat. No. E-196-4, Fisher Optima.

   c. Cyclohexane - Pesticide grade, Cat. No. C-553, Fisher.

   d. Diethylamine (DEA) - Reagent grade, Cat. No. D-46, Fisher.

   e. Pentafluoropropionic anhydride (PFPA) - Cat. No. 65193, Pierce.

   f. Diazomethane:

      Prepare using the generator manufacturer's instructions and the following reagents:

      Note: Diazomethane preparation must be carried out in a working fume hood behind a protective screen or shield. Operator should wear gloves to prevent skin contact with reagents or diazomethane. Caution must be observed when handling diazomethane as it is toxic and, under some conditions, explosive. Avoid using anything with sharp edges and ground glass in any generator being used. Freshly made diazomethane solution is golden yellow in color and can be used for a maximum of 1 week. Store in freezer.

      i. N-methyl-N-nitroso-p-toluenesulfonamide (PTS) - reagent grade. Handle with caution. Diazald, 99%, Cat. No. D2800-0, Aldrich.

      ii. 2-(2 Ethoxy ethoxy)-ethanol (Carbitol) - Reagent grade, Cat. No. EX 210 P5755, Matheson Coleman & Bell.

      iii. Potassium hydroxide (KOH) - 60% (W/V) aqueous, reagent grade.

      iv. Organic solvent: (for preparation/collection of diazomethane). Use either:

         (a) Ethyl ether - anhydrous reagent grade, peroxide free, Cat. No.106-4, Baxter, Burdick & Jackson.

         (b) Ethyl Acetate - Cat. No. E196-4, Fisher Optima.

         Note: Solvent must be anhydrous. Traces of water cause decolorization of diazomethane solution with accompanying poor derivatization. Diethyl ether may contain peroxides which can detonate when distilled or concentrated. If ether is used, it should be tested before using by shaking 1 mL of a freshly prepared saturated solution of potassium iodide with 9 mL of ether in a 25 mL glass-stoppered cylinder. Any yellow color indicates a concentration of peroxide greater than 0.005%, which is considered dangerous. Refer to CRC Handbook of Laboratory Safety, 2nd Edition, pp. 250 - 254, for additional information on this subject.

2. Solutions

   a. 1 M phosphate buffer (pH 6.8):

      Prepare by making a 1 M solution of Na_2HPO_4 and a 1 M solution of KH_2PO_4 and mixing 1:1 (v/v).

D. STANDARDS

1. Preparation of external standard

   a. Transfer 100 µL of 5 µg/mL of sulfonamide of interest (see Part I, Section D.2.b.iii) to a 5 mL disposable centrifuge tube. (This solution is equivalent to 0.10 ppm of sulfonamide of interest)

E. SAMPLE PREPARATION

Using the extract prepared as outlined in the Robotic or Determinative Method, section F, perform the following additional steps:

Note: In certain cases it may be necessary to extract a larger sample (5.0 g ± 0.1g), or eliminate or reduce the amount of the SPY internal standard, or both, Such cases include problems of co-elution, poor recovery, or poor response. One such case is STZ, which co-elutes with SPY. (In this case, use SDM as the internal standard instead.) STZ and several other sulfonamides have characteristic low recoveries. (For these sulfonamides with either a low recovery or low response, it helps to have a clean source. The best results are obtained when the relative abundance of mass/charge of 502 of PFTBA in the autotune is between 5 - 10%.)

1. Transfer the remaining methanol extracts of tissue blank, 0.1 ppm recovery and presumptive positive samples from the STLC-F analysis (approximately 100 µL) to a disposable 5 mL glass centrifuge tube.

2. Rinse the 50 mL centrifuge tube twice with 0.5 ± 0.1 mL portions of methanol and add the rinses to the extract in the 5 mL disposable glass centrifuge tube.

3. Evaporate the sample extract plus the two rinses to dryness under nitrogen at 40°C ± 3 °C.

   Note: Evaporate external standard of sulfonamide of interest (D.1.a) above to dryness.

4. If a diethyl ether solution of diazomethane is to be used, add 300 µL ± 30 µL ethyl acetate to the dried extract and external standard before adding diazomethane to tubes. Add 1.0 ± 0.1 mL diazomethane to the tube and mix. Let stand alone for at least 10 minutes to methylate the sulfonamides.

   Note: The diazomethane solution used should be a deep yellow. Do not use if the solution is pale yellow. During methylation, the solution must remain yellow to ensure that an excess of diazomethane is present. If color fades, add sufficient diazomethane to maintain a yellow color.

5. Reduce volume of the diazomethane solution to approximately 300 µl with nitrogen at room temperature.

6. Add 50 ± 5 µL 10 % PFPA in hexane. Mix. Add 25 ± 2.5 µL 5 % diethylamine in ethyl acetate. Let stand at least 30 minutes to acylate the sulfonamides.

7. Evaporate to near dryness under nitrogen at 40 °C ± 3 °C. Loss of the volatile derivatized sulfonamides is risked if it is evaporated to dryness. Add 100 - 200 µL of 10% ethyl acetate in cyclohexane. Mix.

   Note: Final volumes of the derivatized solutions must be the same for samples and external standard(s).

8. Add 300 ± 30 µL of a 1 M (pH 6.8) phosphate buffer. Mix by vortexing for 15 seconds. Centrifuge at 200 ± 20 g for 1 minute ± 30 sec. and quickly remove the aqueous bottom layer; otherwise, the acylation reaction will reverse at the aqueous interface. This step serves to remove the excess acylation reagents and the acylation byproduct pentafluoropropionic acid.

9. Add approximately 100 ± 10 mg sodium sulfate to remove any remaining water. Mix. Centrifuge at 200 *g* for 1 minute ± 30 sec.

10. Inject 2 - 10 µL from the organic layer for GC/MS analysis.

F. ANALYTICAL PROCEDURE

1. Data Acquisition

   a. GC parameters. (Example only. Analyst should optimize these parameters for the instrument and column being used.)

      i. Initial column temperature: 150 °C.

      ii. Time at temperature 1: 0 min.

      iii. Injector temperature: 260 °C.

      iv. Final temperature: 300 °C.

      v. Program rate: 30 °C/min.

      vi. Time at temperature 2: 10 min.

      vii. Total runtime: 15 min.

      viii. Helium linear velocity: 30 cm/sec.

      ix. Column:

         (a) HP 19091 A - 102, or equivalent.

         (b) Fused silica capillary column.

         (c) Film: Crosslinked methyl silicone (OV-1).

(d) Film thickness: 0.33 micron.

(e) Phase ratio: 150.

(f) Column length: 25 m.

(g) Column id: 0.20 mm.

b. MSD Parameters

i. Tune the MSD prior to analyzing.

ii. Examples of the MSD parameters currently being used. Analyst should optimize these parameters for the instrument being used.

(a) Transfer line: 300 °C.

(b) Electron multiplier: 600 added to the autotune value.

(c) Mode: Electron impact (70 ev). Selected ion monitoring.

(d) SIM dwell time: 100 msec.

(e) Program.

Time (min)	Event	Sulfa monitored
5.00	Mass spec. on.	
	Monitor ions:	
6.00 - 6.34	238 350 351	Sulfathiazole
	238 344 345	Sulfapyridine
	238 348 349	Sulfamethoxazole
6.35 - 6.89	238 373 374	Sulfamethazine
6.90 - 8.19	238 405 406	Sulfadoxine and Sulfadimethoxine
8.20 -15.00	238 395 396	Sulfaquinoxaline
15.00	Stop run.	

c. Inject 2 - 10 µL of the previously prepared external standard and plot the total ion chromatogram.

d. Inject 2 - 10 µL of the tissue blank and fortified tissue and plot the TIC.

e. Inject 2 - 10 µL of each sample and plot the TIC.

f. Determine the retention time and the peak heights of the selected ions for the sulfonamide(s) being confirmed.

2. Confirmation Criteria

   a. Compare the retention time of the sample with the retention time of the fortified tissue. The times should match ± 5 %. Compare the R_f of the sample with the R_f of the fortified tissue on the TLC plate. The R_f the sample must be indistinguishable from the R_f of the fortified tissue as recorded by the densitometer.

   b. The characteristic 238, M-64, and M-65 ions must be present.

   c. Compute a ratio of the M-64 and M-65 ions for the standard, fortified tissue, and sample. For successful confirmation, the ratio calculated for the candidate sample must agree within ± 20 % (relative) with the ratio calculated for the standard or fortified tissue. A list of the typical M-64/M-65 ratios is listed below.

Sulfa	M-64/M-65 ratio
Sulfathiazole	1.00
Sulfadoxine	0.40
Sulfadimethoxine	0.29
Sulfamethazine	0.24
Sulfaquinoxaline	0.23
Sulfapyridine	0.19
Sulfamethoxazole	0.18

3. Mass Spectra of derivatized analytes

   Note: See Appendix K.2 for mass spectra of derivatized sulfonamides.

G. CALCULATIONS

Not Applicable

H. HAZARD ANALYSIS

1. Required Protective Equipment - Safety glasses, plastic gloves, lab coat, shield, and efficient fume hood.

2. Hazards

Procedure Step	*Hazard*	*Recommended Safe Procedures*
II.C.1.f. Diazomethane generation	Explosion	Follow generator manufacturer's directions.
II.C.1.f.i. N-methyl-N-nitroso-p-toluenesulfonamide. (PTS)	Skin irritant	Avoid skin contact.
II.C.1.f.iv.(a). Ethyl ether	Detonation	Check for peroxides before using.
II.C.1.f.iii. Potassium hydroxide	Caustic	Avoid skin contact.
Diazomethane at 0 °C	Carcinogenic; toxic and explosive	Keep cold; use dilute solutions under fume hood and behind shield.
II.C.1.e. Pentafluoropropionic anhydride (PFPA)	Causes severe burns; harmful if swallowed	Avoid skin contact. Do not swallow.
II.C.1.d. Diethylamine	Flammable Skin irritant	Perform steps under an efficient hood. Keep from flame, sparks and heat. Avoid contact with skin and eyes.
II.C.1.a.,b.,c. Organic solvents	Flammable	Perform steps under an efficient hood. Keep from flame, sparks and heat.

3. Disposal Procedures

Procedure Step	*Hazard*	*Recommended Safe Procedures*
Organic solvents	See above	Hold in containers until disposed of by the contractor or the in-house specialist
Byproduct and excess reagents from diazomethane generation	See above	Neutralize and flush down, in-hood drain with large quantities of water. (Follow all Federal, State and Local regulations)

I. QUALITY ASSURANCE PLAN

1. Performance Standard
   a. No false positives from blank tissues
   b. No false negatives at or above 0.1 ppm

2. Critical Control Points and Specifications

Record	*Acceptable Control*
Step II.E.4	Diazomethane ≤1 week old, color a deep yellow
Step II.E.4	Methylation time ≥10 min.
Step II.E.6	Acylation time ≥ 30 min.

3. Readiness To Perform
   a. Familiarization
      i. Phase I:

      On two separate days, analyze by selected ion monitoring a 0.1 ppm external standard containing the four sulfonamides normally confirmed (SMZ, SDM, STZ, and SQX. Monitor 238, M-64, and M-65 ions for each sulfonamide. Determine the retention time and the M-64/M-65 ratio for each sulfonamide.

      ii. Phase II:

      Analyst fortified samples. Conduct replicate analyses of control tissue fortified with 0.10 ppm of the appropriate sulfonamide. It is suggested that liver and muscle tissue from swine, turkey, and bovine species be confirmed with particular emphasis on:

      (a) Sulfaquinoxaline (SQX), sulfadimethoxine (SDM), and sulfamethazine (SMZ) in turkey.
      (b) Sulfamethazine, sulfathiazole (STZ), and sulfadimethoxine in swine.
      (c) Sulfamethazine and sulfathiazole in beef.

      It is suggested that the above combinations be run twice in turkey liver and muscle, swine liver and muscle, and beef liver and muscle omitting sulfapyridine as internal standard when sulfathiazole is being confirmed. (Total number of fortified samples is twelve.) Also include at least 1 blank for each tissue/species. (Total of six blanks.) Must be able to meet criteria for confirmation in section F.2.

      Note: Phases I and II may be performed concurrently.

      iii. Phase III:

      Check samples for analyst accreditation.

(a) Each analyst will be required to analyze a minimum of six blind samples. One of these samples should be at the none detected level, the other five will be at the violative level. These samples may be prepared in-house from incurred or fortified samples. If analyses for Phase III fail to meet the acceptance criteria described in Table 1 or satisfy acceptance criteria specified in the method then a corrective action must be completed and Phase III restarted.

(b) See phase II for sulfa drugs to use.

(c) Report analytical findings QAM and supervisor or designee.

(d) Authorization from QAM and Supervisor is required to commence official analysis.

b. Acceptability criteria.

Refer to section J.1 above.

4. Intralaboratory Check Samples

a. System, minimum contents.

i. Frequency: 1 per week as samples analyzed.

b. Acceptability criteria.

If unacceptable values are obtained, then:

i. Stop all official analyses by that analyst.

ii. Take corrective action.

5. Sample Acceptability and Stability

See Determinative Method, section J.5.

6. Sample Set

a. Standards

b. Tissue blank

c. Blank tissue fortified with sulfonamide of interest.

d. Samples

7. Sensitivity

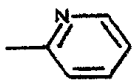
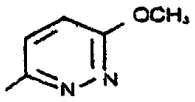
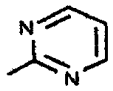
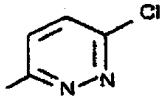
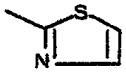
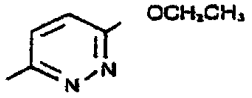
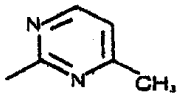
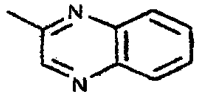
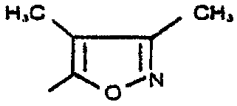
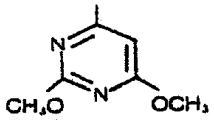
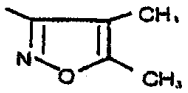
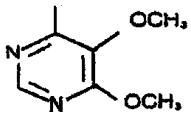
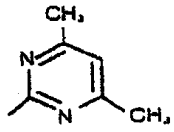
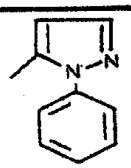
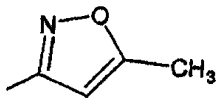
a. Lowest reliable confirmation (LRC): 0.1 ppm.

J. WORKSHEET

[RESERVED]

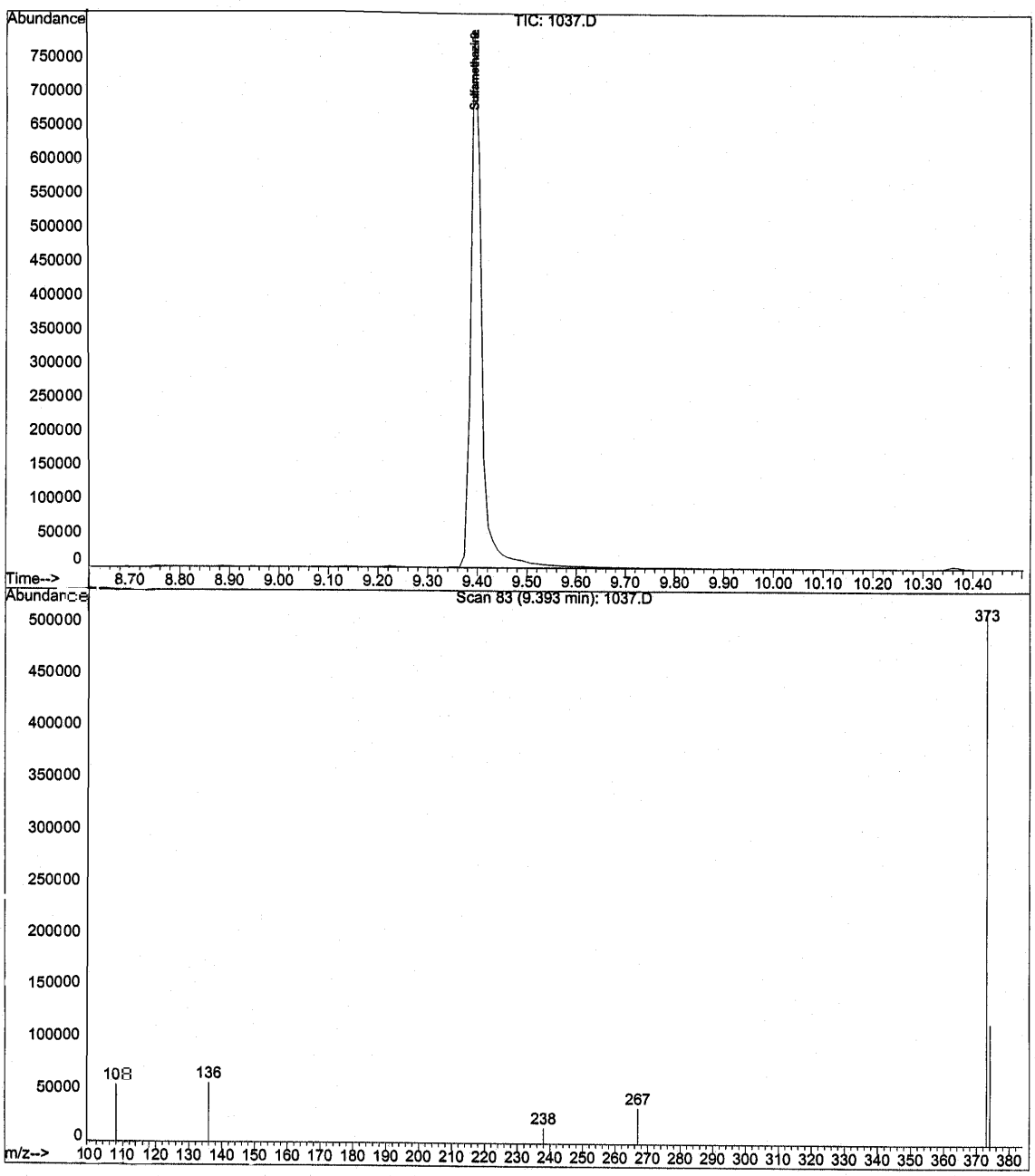
K. APPENDIX

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

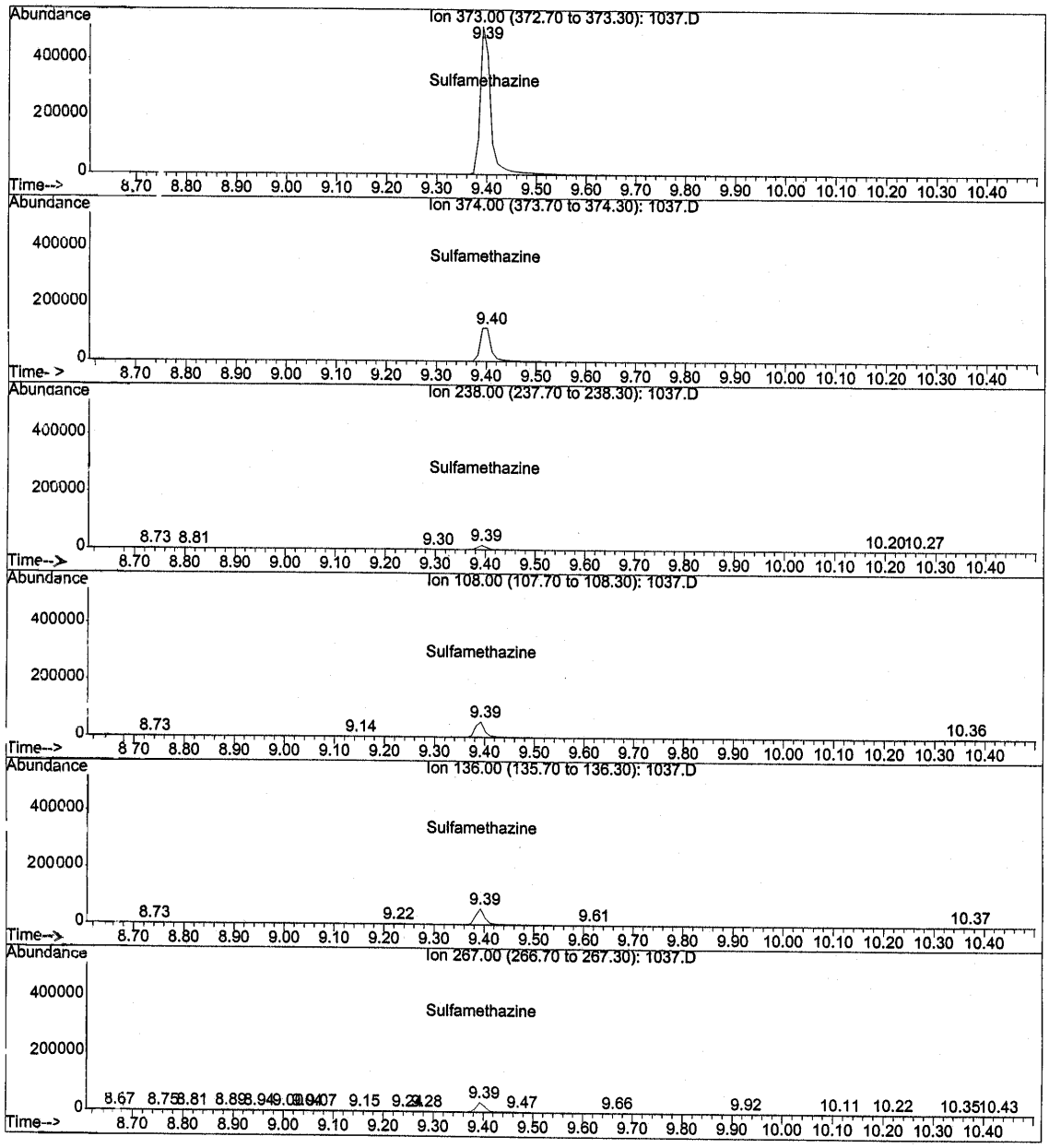
Sulfapyridine $C_{11}H_{11}N_3O_2S$ 249.3 	**Sulfamethoxypyridazine** $C_{11}H_{12}N_4O_3S$ 280.3 
Sulfadiazine $C_{10}H_{10}N_4O_2S$ 250.3 	**Sulfachlorpyridazine** $C_{10}H_9ClN_4O_2S$ 284.7 
Sulfathiazole $C_9H_9N_3O_2S$ 255.3 	**Sulfethoxypyridazine** $C_{12}H_{14}N_4O_3S$ 294.3 
Sulfamerazine $C_{11}H_{12}N_4O_2S$ 264.3 	**Sulfaquinoxaline** $C_{14}H_{12}N_4O_2S$ 300.3 
Sulfisoxazole $C_{11}H_{12}N_3O_3S$ 267.3 	**Sulfadimethoxine** $C_{12}H_{14}N_4O_4S$ 310.3 
Sulfatroxazole $C_{11}H_{13}N_3O_3S$ 267.3 	**Sulfadoxine** $C_{12}H_{14}N_4O_4S$ 310.3 
Sulfamethazine $C_{12}H_{14}N_4O_2S$ 278.3 	**Sulfaphenazole** $C_{15}H_{14}N_4O_2S$ 314.35 
Sulfamethoxazole $C_{10}H_{11}1N_3O_3S$ 253.28 	

2. Mass spectra of derivatized sulfonamides are shown on the following pages.

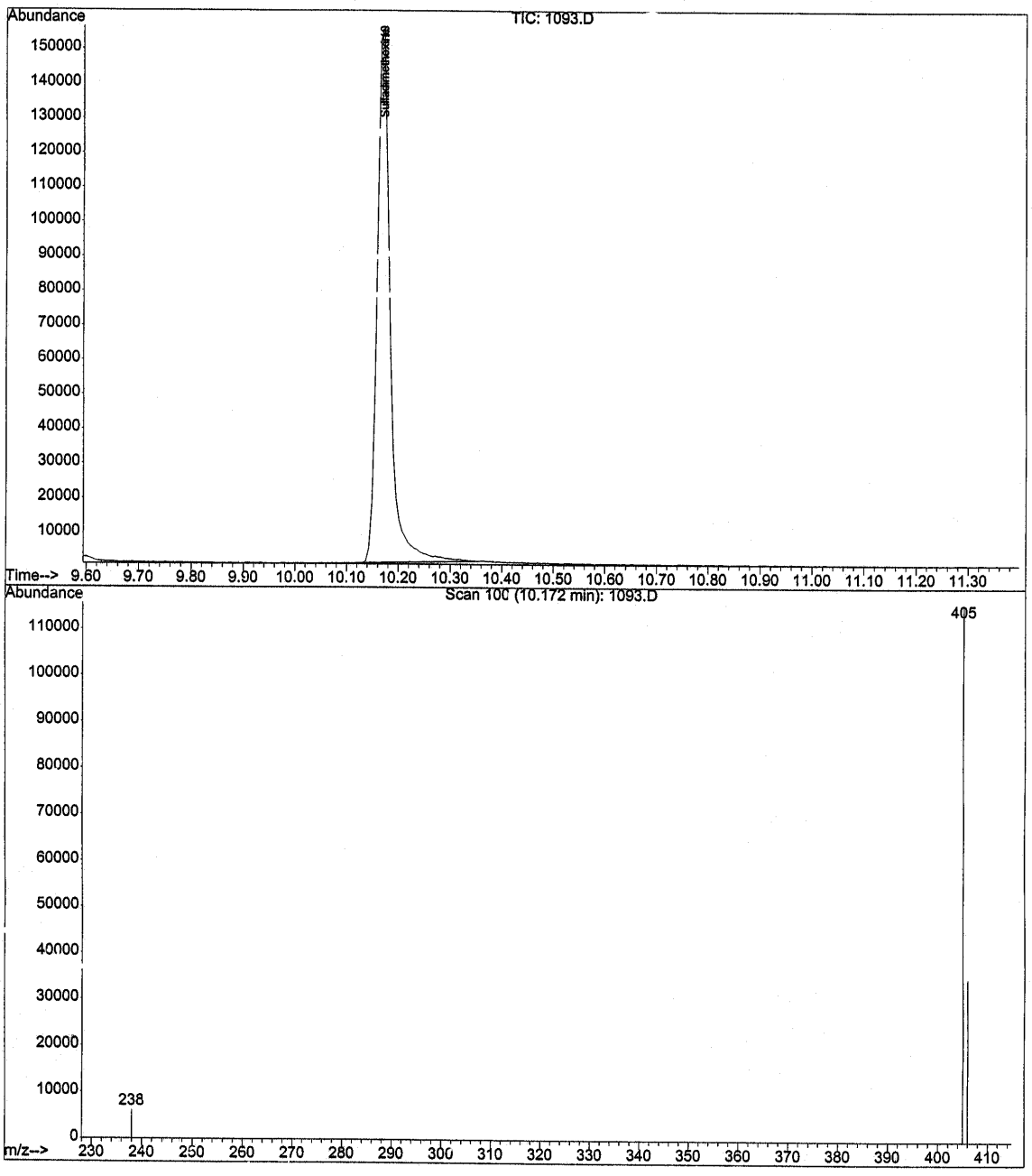
   a. SMZ External Standard (0.1 ppm): Total Ion Chromatogram (TIC).



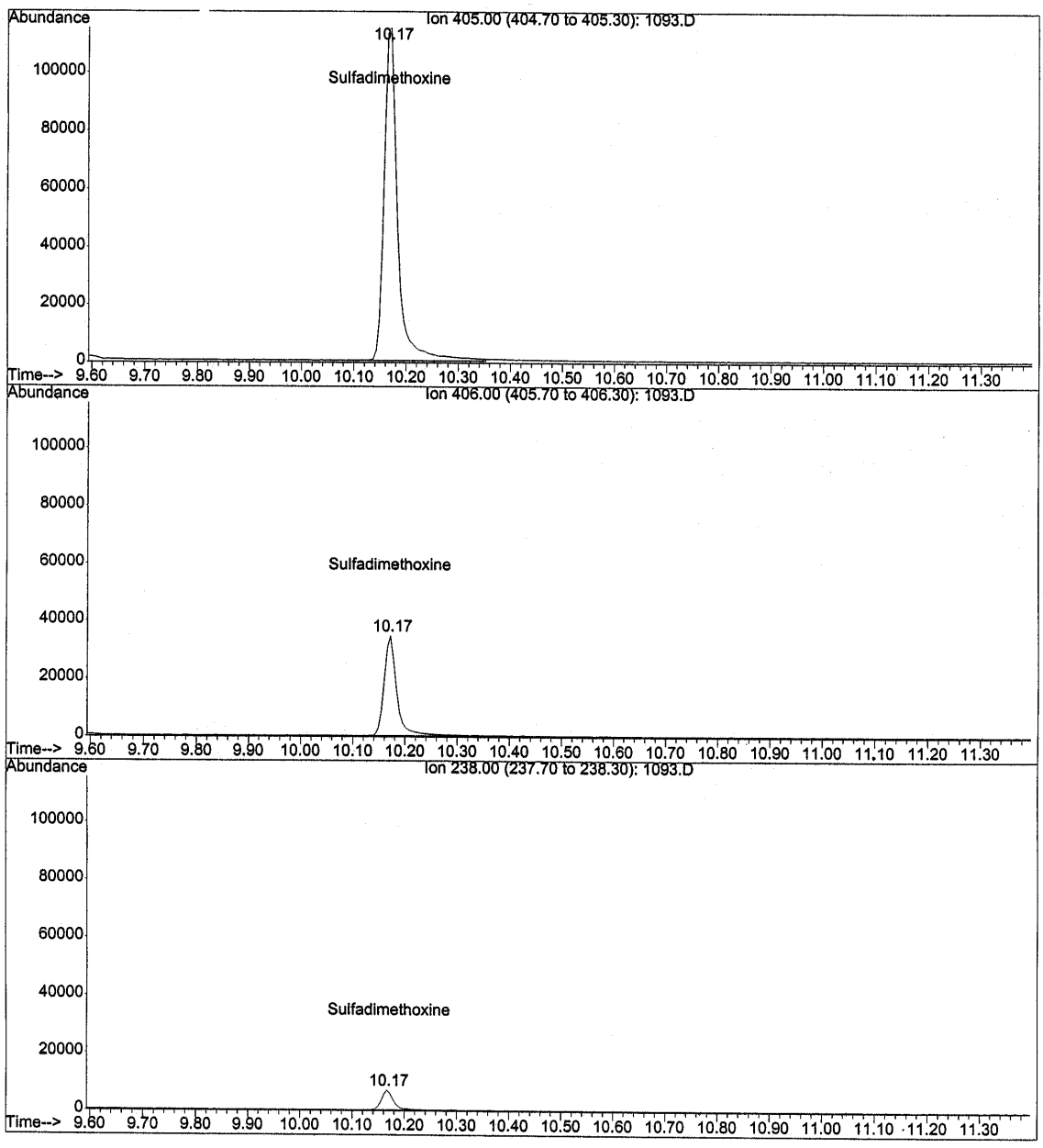
SMZ Reconstructed Ion Chromatogram (RIC)



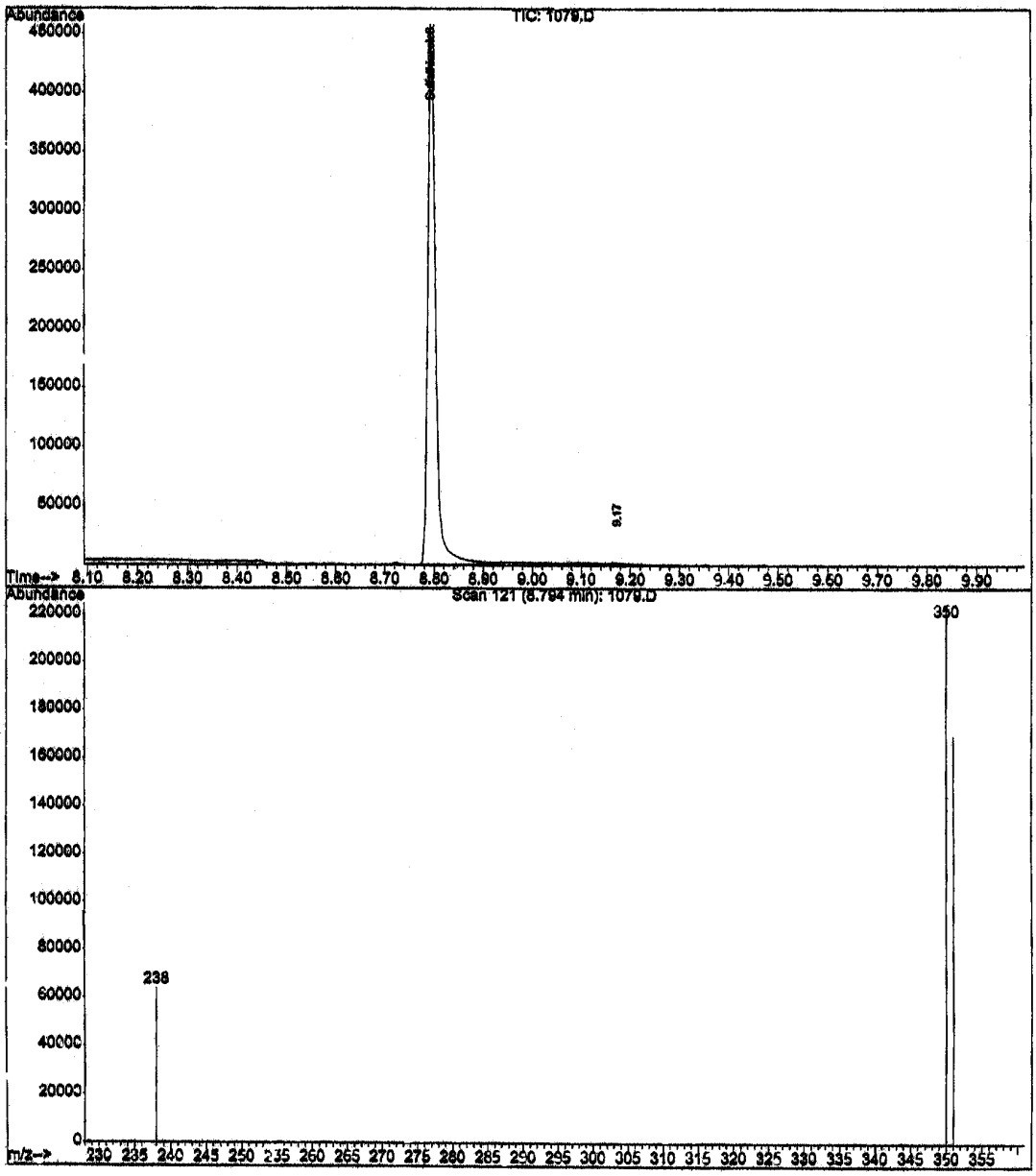
b. SDM External Standard (0.1 ppm) : Total Ion Chromatogram (TIC)



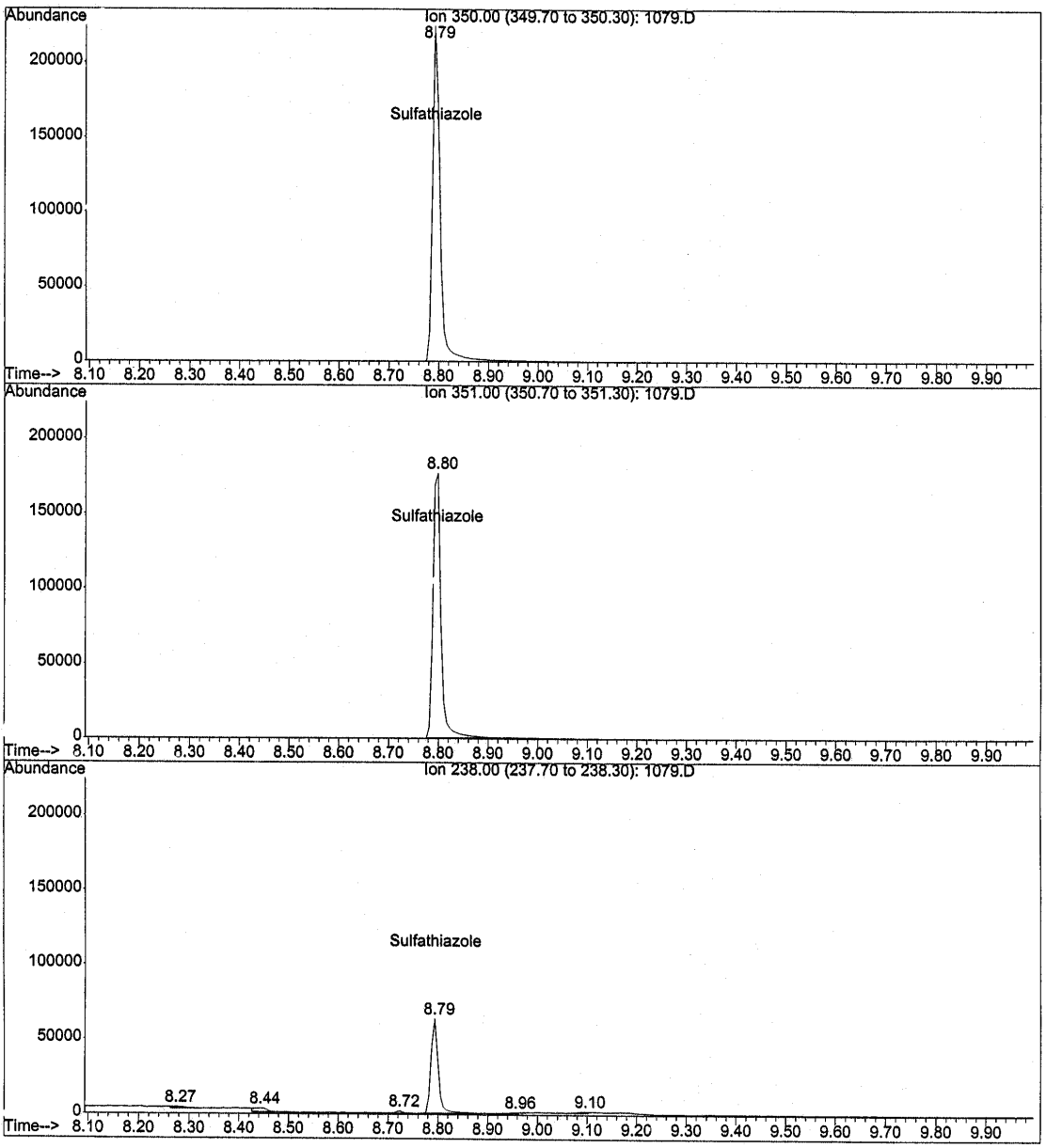
SDM Reconstructed Ion Chromatogram (RIC)



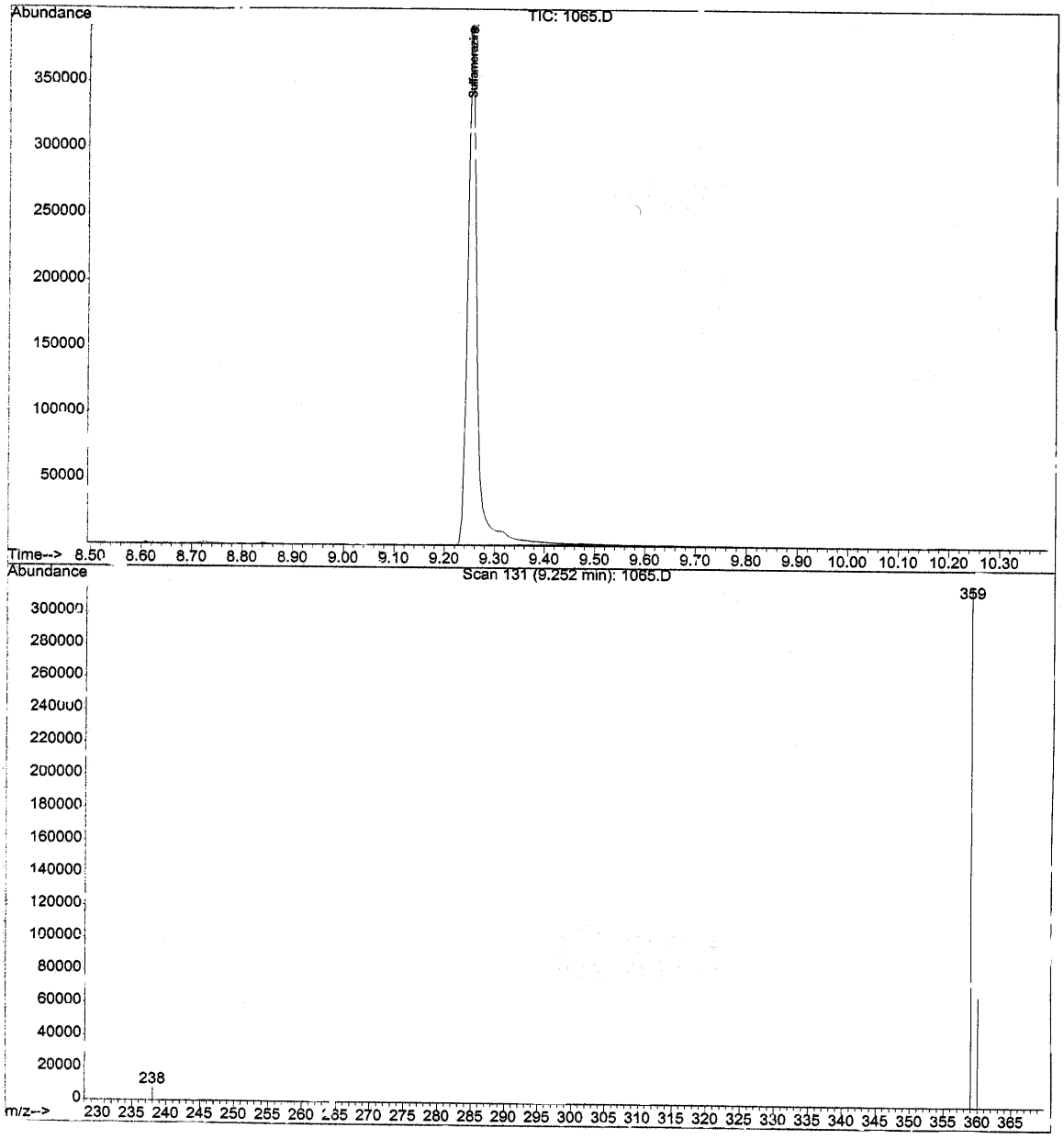
c. STZ External Standard (0.1 ppm) : Total Ion Chromatogram (TIC)



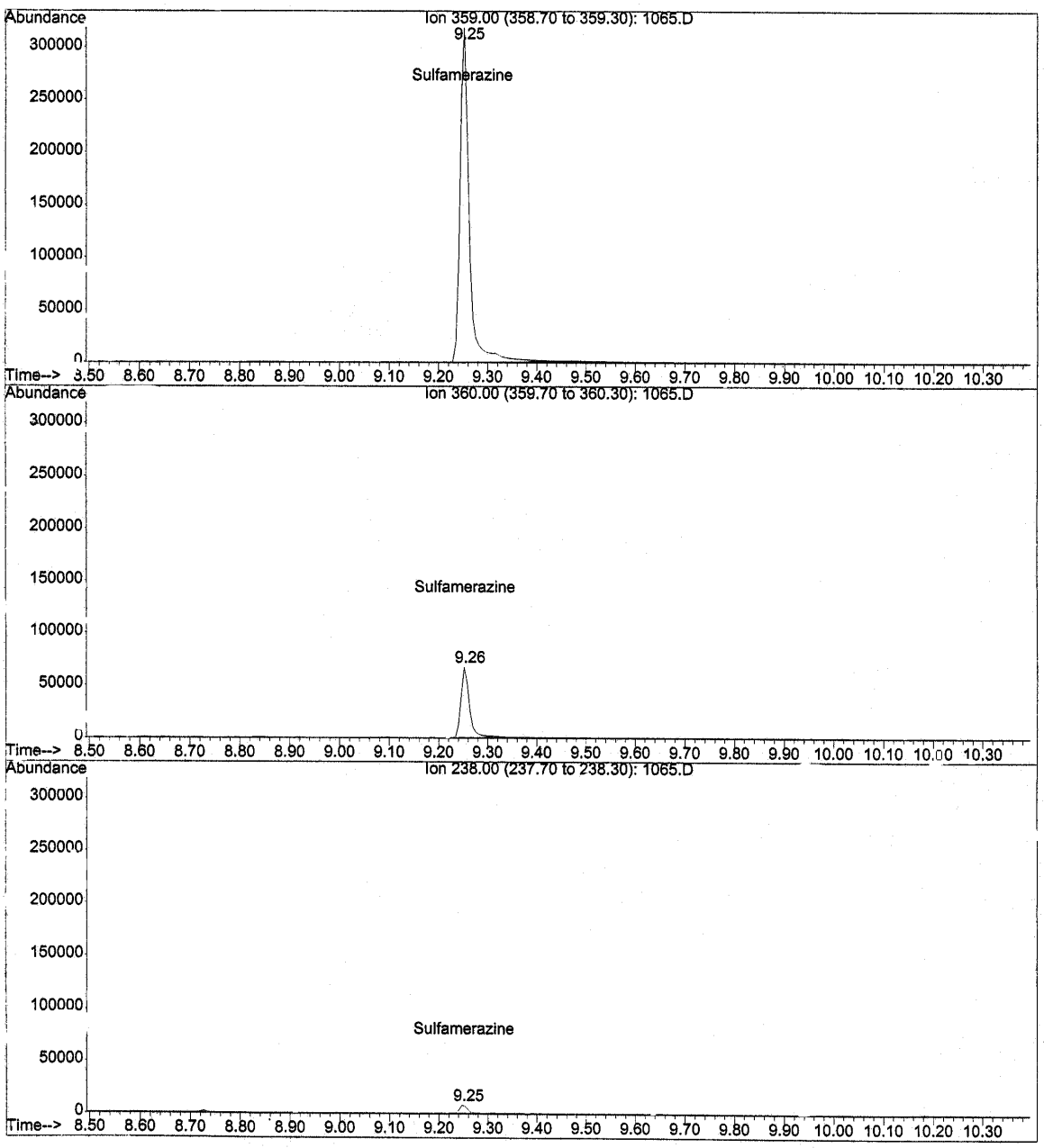
STZ Reconstructed Ion Chromatogram (RIC)



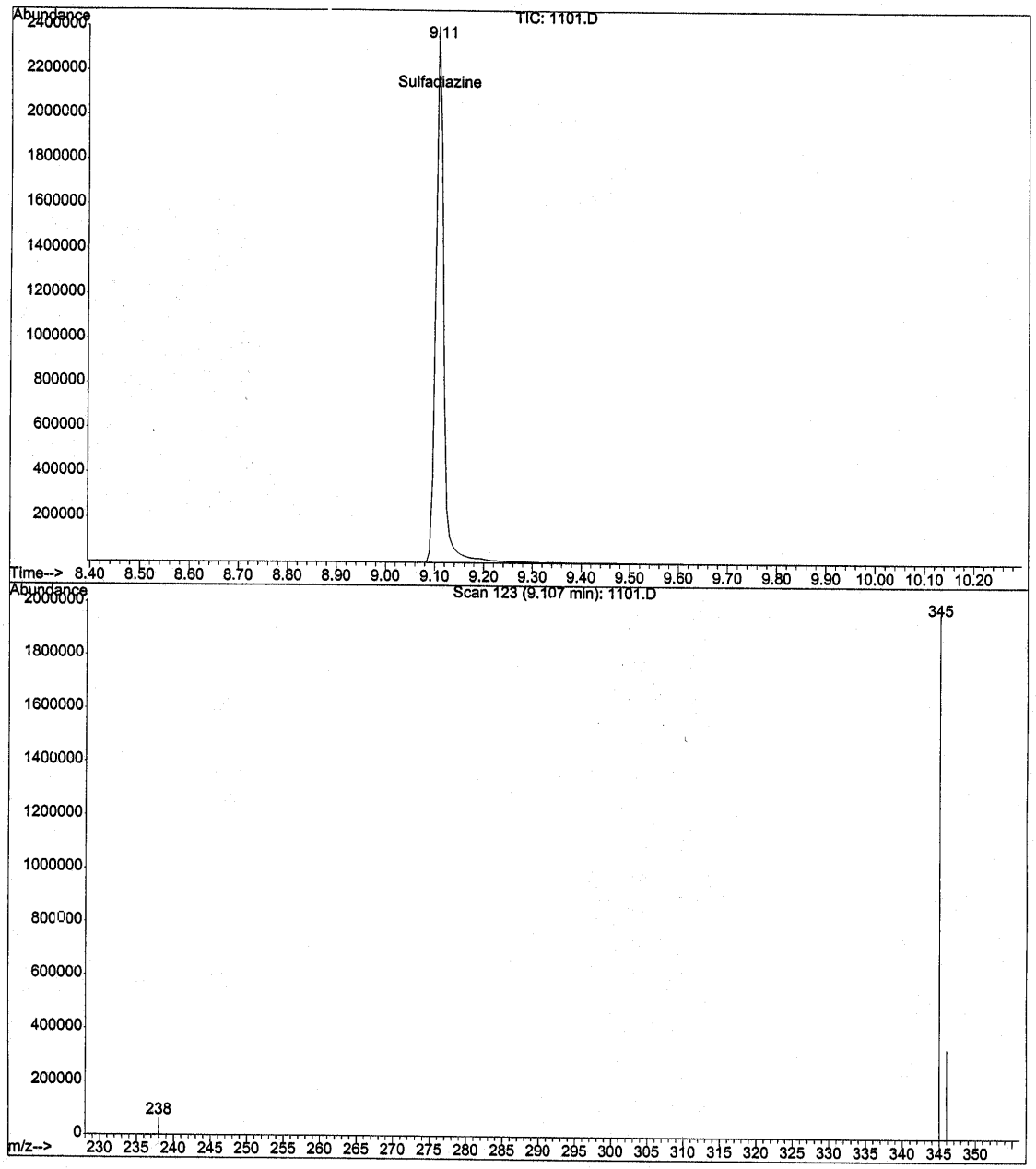
d. SRZ External Standard (0.1 ppm) : Total Ion Chromatogram (TIC)



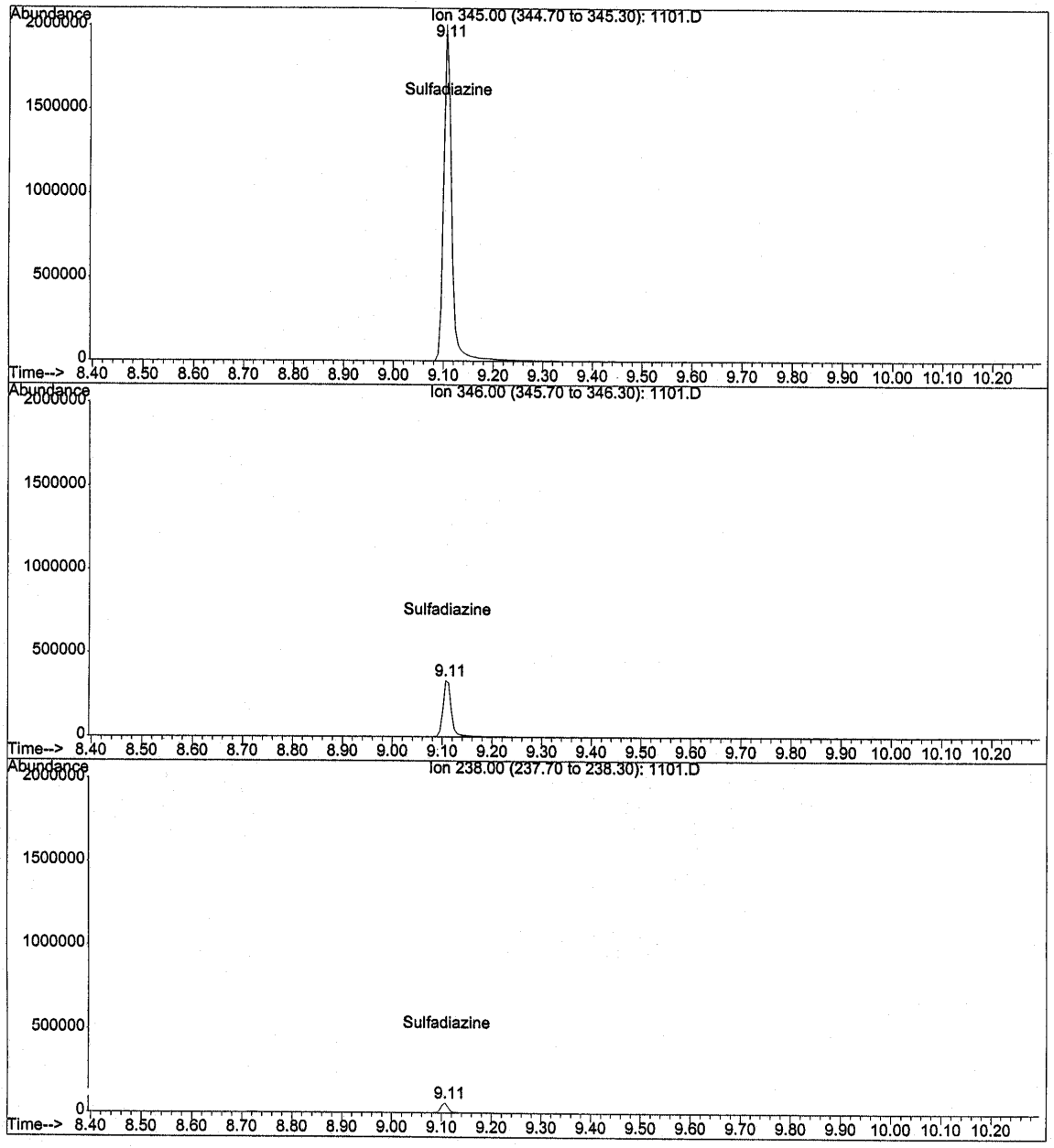
SRZ Reconstructed Ion Chromatogram (RIC)



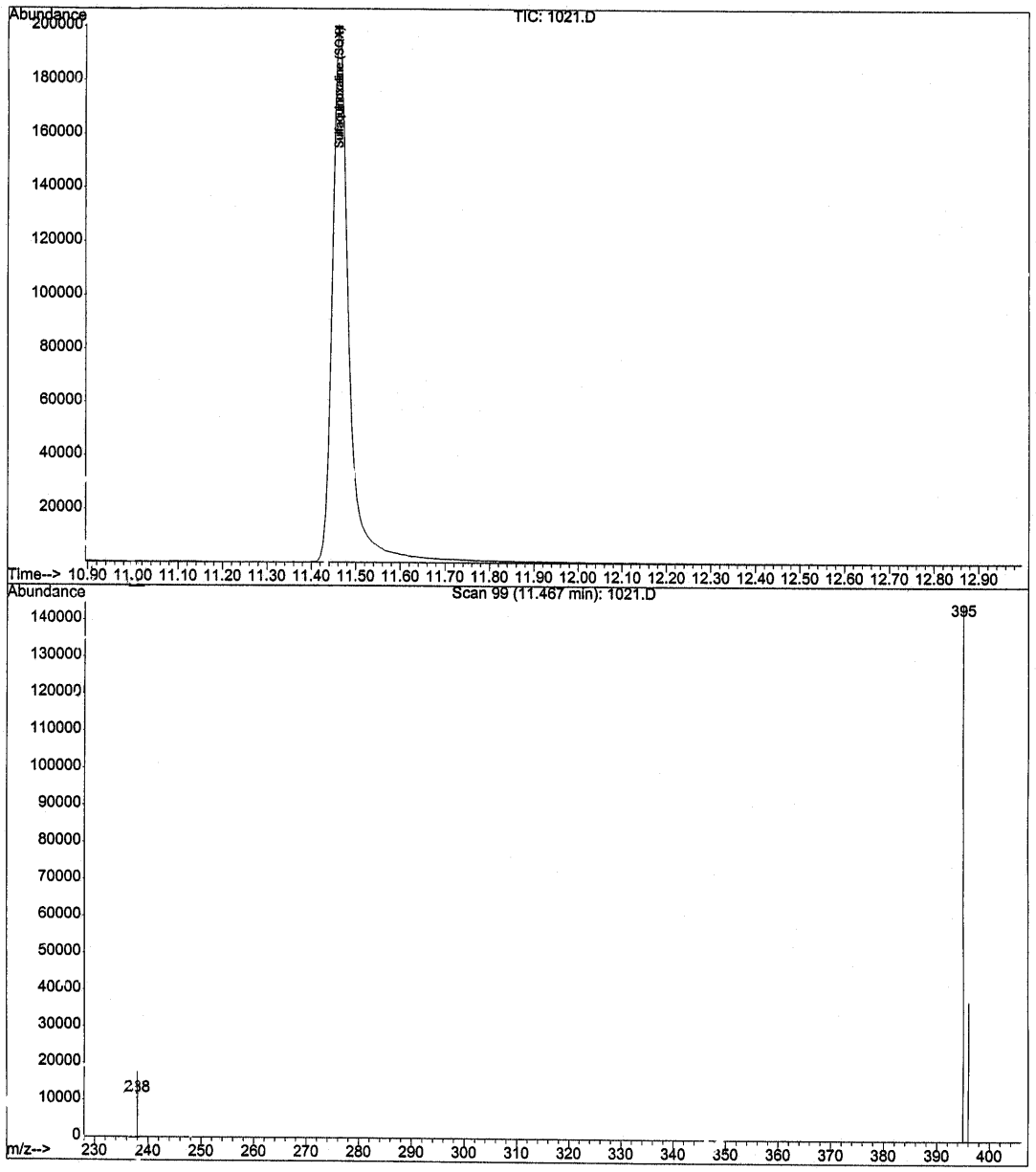
e. SDZ External Standard (0.1 ppm): Total Ion Chromatogram (TIC)



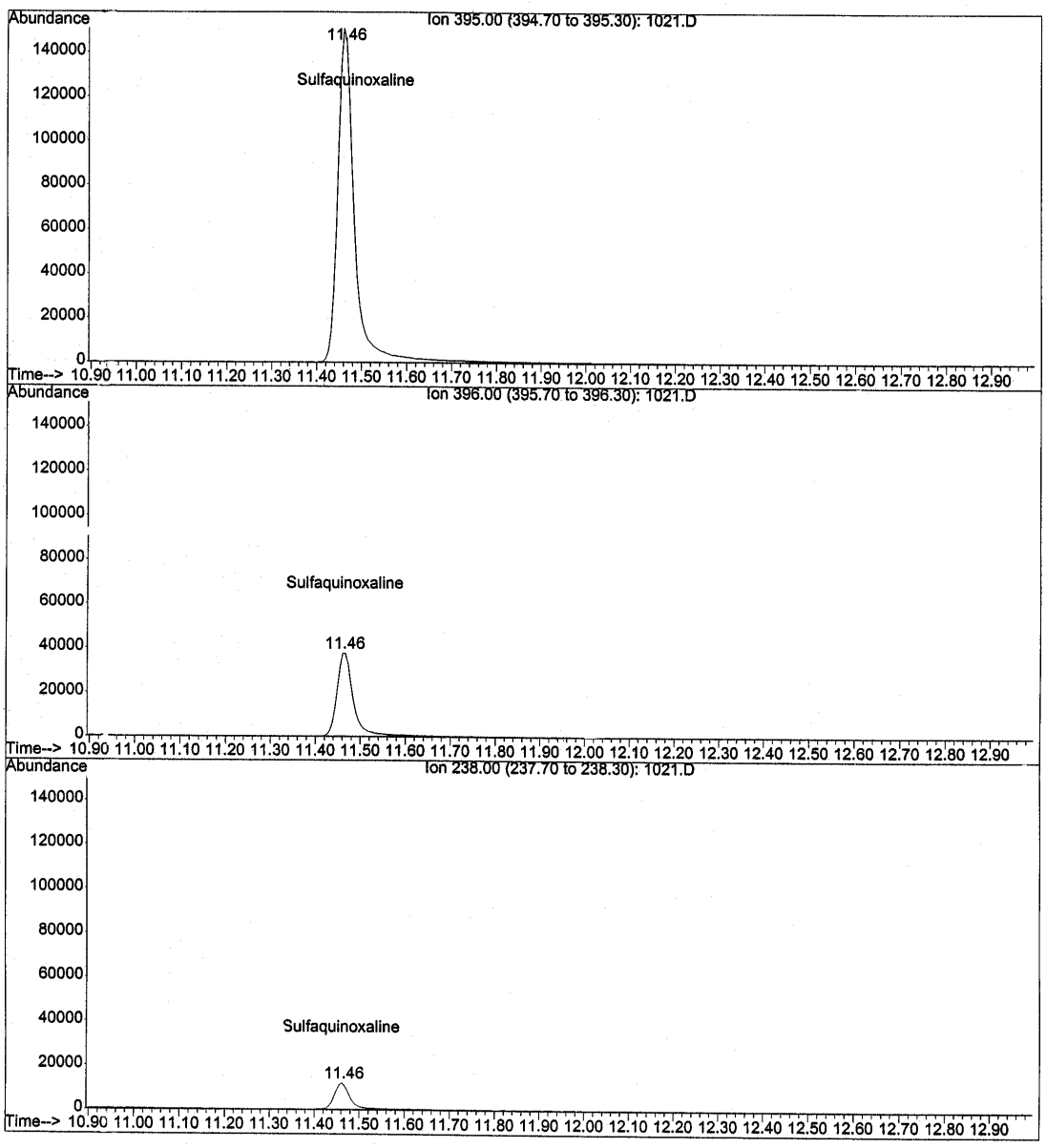
SDZ Reconstructed Ion Chromatogram (RIC)



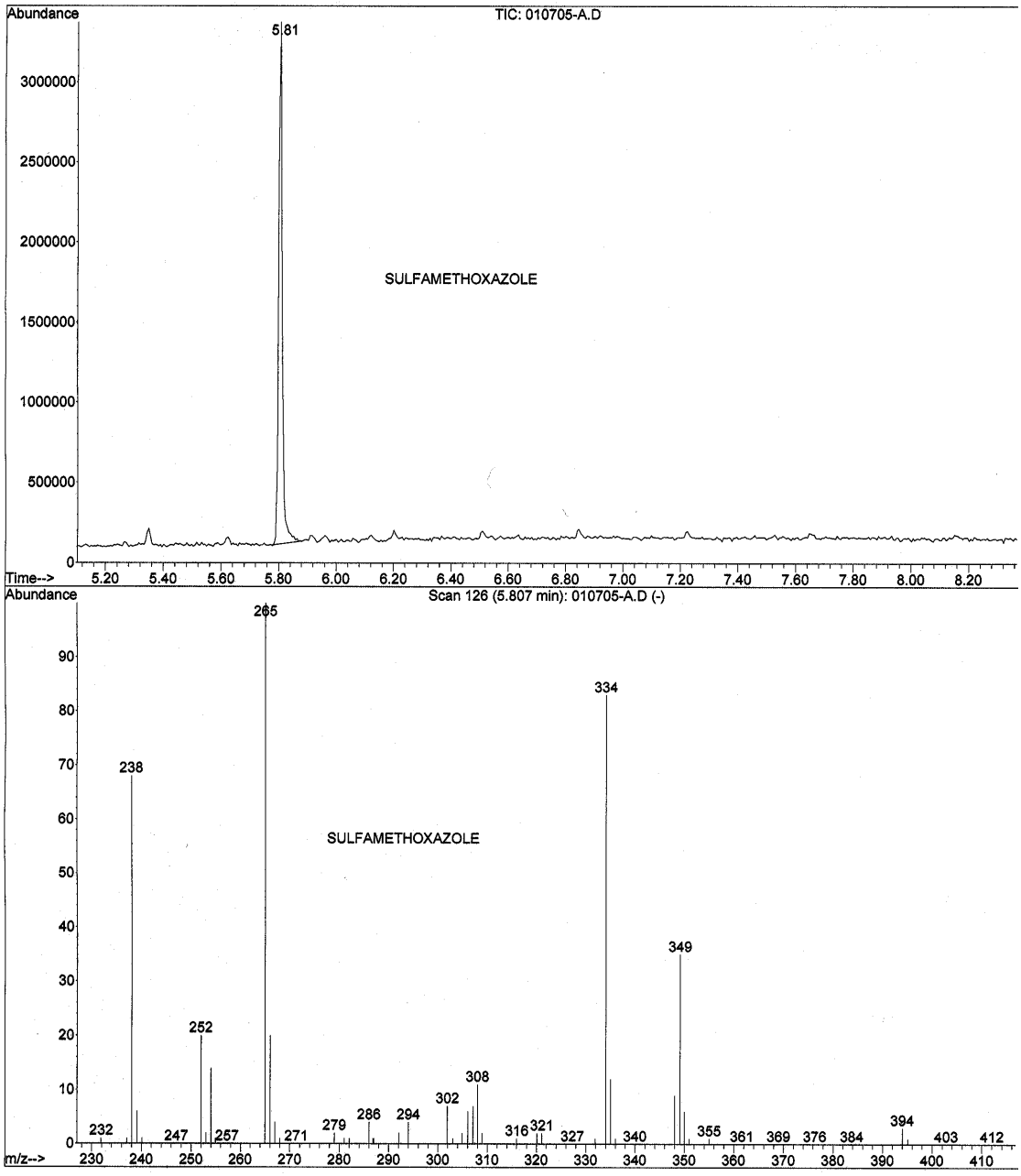
f. SQX External Standard (0.1 ppm) : Total Ion Chromatogram (TIC)



SQX Reconstructed Ion Chromatogram (RIC)



g. SMO (Sulfamethoxazole) External Standard (0.1 ppm): Top spectrum -Total Ion Chromatogram (TIC) and Bottom spectrum - Reconstructed Chromatogram (RIC)



L. APPROVALS AND AUTHORITIES

1. Approvals on file.

2. Issuing Authority: Director, Laboratory Quality Assurance Division