Target Analyte: Fenbendazole Sulfone	MERCK Animal Health

METHOD TITLE: Determinative and Confirmatory Procedures for Fenbendazole Sulfone in Liver Tissues of Broiler Chicken Using LC-MS/MS, Version 6.0

APPROVAL SIGNATURES:

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1 GLOSSARY OF ABBREVIATIONS AND DEFINITION OF TERMS

This section provides abbreviations and definitions of terms and concepts commonly used throughout this method.

ACN	Acetonitrile
AHR	Animal Health Residue Study
amu	Atomic Mass Unit
BA	Bioanalytical
CV	Coefficient of Variation
DMSO	Dimethyl Sulfoxide
FBZ-SO_2	Fenbendazole Sulfone
FBZ-SO_2-D_3	Fenbendazole Sulfone-D_3
HDPE	High-Density Polyethylene
HPLC	High Performance Liquid Chromatography
LC-MS/MS	High Performance Liquid Chromatography – Tandem Mass Spectrometry
IS	Internal Standard
LC-MS	Liquid Chromatography – Mass Spectrometry
LOQ	Limit of Quantitation
LLOQ	Lower Limit of Quantitation
MAH	Merck Animal Health
MSDS	Material Safety Data Sheet
n	Number of Samples
NA	Not Applicable
ppm	Parts per Million (µg/g)
psi	Pounds per Square Inch
MilliQ water	Water purified by a Millipore Synthesis A10
pw	Peak Width
QC	Quality Control (fortified tissue)
Control Blank	Blank matrix sample, fortified with IS only
Double Blank	Double Blank matrix sample, not fortified with IS or analyte
PAR	Peak Area Ratio
RCF	Relative Centrifugal Force (x g)
rpm	Rotations per Minute
s	Second
Solvent Blank	Methanol (MeOH) Sample
SL	Solvent Level
SST	System Suitability Test
STD	Standard Calibrator
ULOQ	Upper Limit of Quantitation

v/v	Volume per Volume
v/v/v	Volume per Volume per Volume
WS	Working Solution

2 SCOPE AND FIELD OF APPLICATION

Fenbendazole sulfone is a metabolite of fenbendazole. Fenbendazole is a broad spectrum benzimidazole anthelmintic used against gastrointestinal parasites and intended for use as a veterinary drug in broiler chickens. This procedure describes the determinative and confirmatory SOP for the quantitation and identification of fenbendazole sulfone (FBZ-SO_2) in broiler chicken liver tissue for the proposed tolerance of 5.3 ppm. The determinative method consists of a sample solvent extraction followed by LC-MS/MS detection. The calibration curve range for the marker compound is 2.5 – 30 ng/mL (1 – 12 ppm tissue equivalents) in the chicken liver tissue.

The current method was validated in compliance with the following regulations and guidance documents:

- Food and Drug Administration/Center for Veterinary Medicine's (FDA/CVM's) Guidance for Industry 3, 2006: General Principles for Evaluating the Safety of Compounds Used in Food-producing Animals; V. Guidance for Approval of a Method of Analysis for Residues.
- FDA/CVM's Guidance for Industry 118, 2003: Mass Spectrometry for Confirmation of the Identity of Animal Drug Residues.
- FDA/CVM's Guidance for Industry 208, 2011**:** Studies to Evaluate the Metabolism and Residue Kinetics of Veterinary Drugs in Food-Producing Animals: Validation of Analytical Methods Used in Residue Depletion Studies.

Table 2-1: Fortification Concentrations and Calibration Curve Range

Species	Tissue	Marker Residue	Tissue Concentration Range ppm (µg/g or mg/kg)	Analytical Curve Range (Tissue Equivalent) ppm (µg/g or mg/kg)
Chicken	liver (target tissue)	Fenbendazole Sulfone	1.6 to 10.6	1.0 to 12.0

The compounds listed in Table 2-2 are other veterinary drugs registered for use in chicken in the U.S. They were tested and have shown not to interfere with the method.

Table 2-2: Compounds (Drugs) Tested for Interference

Bacitracin Zinc Salt	Narasin
Lasalocid	Salinomycin
Tylosin	Virginiamycin
Robenidine Hydrochloride	Fenbendazole
Bambermycin	Nicarbazin
Monensin	Diclazuril

3 PRINCIPLE

Approximately one gram of homogenized chicken liver is fortified with internal standard (FBZ-SO_2-D_3) and then extracted twice with methanol in two extraction steps. The sample extract is diluted to 20 mL with methanol. An aliquot of the methanol extract is diluted 20x with MilliQ Water/Acetonitrile (70/30, v/v). The resulting solution is quantitatively analyzed using gradient reverse phase liquid chromatography with mass-spectrometric detection (LC-MS/MS) using a positive ion multiple-reaction monitoring (MRM) with ion transition of *m/z* 332 → *m/z* 300 for fenbendazole sulfone (FBZ-SO_2) and *m/z* 335 →*m/z* 300 for FBZ-SO_2-D_3. These two transitions will be used for the determinative method for all tests within a study.

Additional ion transitions from FBZ-SO_2, m/z 332 → *m/z* 159 as qualifier 1 and *m/z* 332 → *m/z* 104 as qualifier 2 are monitored along with m/z 300 used for determinative method, for the confirmatory procedure

4 WARNINGS AND SAFETY PRECAUTIONS

Take safety precautions common in the laboratory, *e.g.* wear lab coat, goggles and gloves if necessary. The MSDS of fenbendazole sulfone and fenbendazole sulfone-D_3 are presented in Section 21 in this SOP.

5 REAGENTS AND MATERIALS

5.1 Reagent/Chemical

During the analysis, unless otherwise stated, use only reagents of recognized analytical grade and water of equivalent purity. Chemical formulas are in parenthesis. Alternate suppliers may be used.

Table 5-1: Reagent/Chemicals to be used in this test procedure

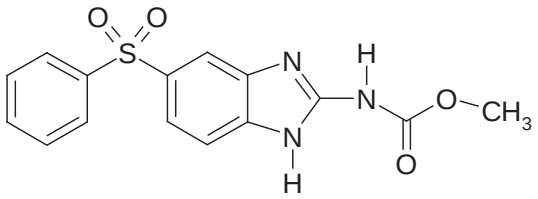
Chemical	Quality or purity	Supplier/Catalog Number
Dry Ice	NA	NA
Methanol (MeOH)	Optima or HPLC	Fisher/A454-4
Acetonitrile (ACN)	Optima	Fisher/A996-4
Acetonitrile + 0.1% formic acid	HPLC	Fisher/HB9823-4
Formic Acid	Certified ACS or HPLC	Fisher/A118P-500
Dimethyl sulfoxide (DMSO)	HPLC or Certified ACS	Fisher/D128-4
0.1% formic acid in Water	HPLC	Fisher/LS118-4
Water (H_2O)	18 mΩ/cm	Millipore or equivalent

Table 5-2: Reagents to be Used in this Test Procedure	
Solution	**Preparation and Storage**
HPLC – Mobile Phase A Mobile Phase A: 0.1% Formic Acid in Water, v/v	Use commercially available pre-made 0.1% formic acid in water. Alternately, it can be made in the lab by adding 1000 mL of MilliQ water to a glass reagent bottle using a graduated cylinder and then adding 1 mL of formic acid (88%, certified ACS or HPLC grade) using a pipette. .Mobile phase is stable for 2 weeks at room temperature once transferred into reagent reservoir.
HPLC – Mobile Phase B Mobile Phase B: 0.1% Formic Acid in Acetonitrile, v/v	Use commercially available pre-made 0.1% formic acid in acetonitrile. Alternately, it can be made by adding 1000 mL of acetonitrile to a glass reagent bottle using a graduated cylinder and adding 1 mL of formic acid (88%, certified ACS or HPLC grade) using a pipette. Mobile phase is stable for 2 weeks at room temperature once transferred into reagent reservoir.
Dilution Solution: MilliQ Water/Acetonitrile, 70/30, v/v	Add 700 mL of water using a graduated cylinder and 300 mL of acetonitrile using a graduated cylinder into a glass reagent bottle. Mix well. Store at room temperature and stable for 1 month.
Autosampler Wash 1 Solution: Mobile Phase A/Mobile Phase B 70/30, v/v	Add 700 mL of mobile phase A (water +0.1% formic acid) and Add 300 mL of mobile phase B (acetonitrile +0.1% formic acid) using a graduated cylinder into a glass reagent bottle. Mix well. Store at room temperature. Store at room temperature and stable for 2 weeks.
Autosampler Wash 2 Solution: 100% Acetonitrile **(TSQ Quantum Ultra-Accela Autosampler-Wash Solution)**	Acetonitrile. Stable for 1 month at room temperature.

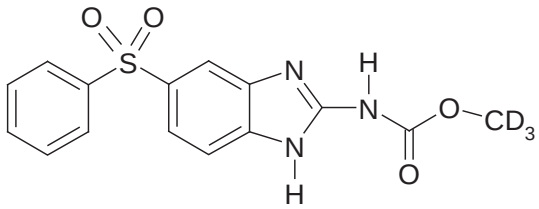
NOTE: Only one autosampler wash (acetonitrile, wash solution 2) was used for the Accela Autosampler. If autosampler can only accommodate one wash and if the use of acetonitrile causes a problem with chromatography, wash solution 1 may be substituted.

5.3 Reference Compound

5.3.1 Reference Compound FBZ-SO_2

Name:	FBZ-SO_2 (Fenbendazole Sulfone)
Compound Number	AH 247250
CAS Number	54029-20-8
Chemical name:	(5-Benzenesulfonyl-1*H*-benzoimidazol-2-yl)-carbamic acid methyl ester
Formula:	$C_{15}H_{13}N_3O_4S$
Molecular weight:	331.35 g/mol
Appearance / colour:	Solid white powder
Storage conditions:	- 25 °C ±10ºC, protect from light
Supplier:	Australian Government National Measurement Institute (Pymble NSW, Australia)
Structural formula:	

5.3.2 FBZ-SO_2-D_3 (Used as Internal Standard)

Name:	Fenbendazole Sulfone-D_3
CAS-No.:	1228182-49-7
Chemical Name:	(5- Benzenesulfonyl-1-H-benzoimidazol-2-yl)-carbamic acid methyl-D_3 ester
Formula:	$C_{15}H_{10}D_3N_3O_4S$
Molecular Weight:	334.35 g/mol
Appearance/Color:	solid white powder
Storage Conditions:	2-8 °C, protect from light
Supplier	Witega (Berlin, Germany)
Structural formula:	

6 APPARATUS AND EQUIPMENT

6.1 General Apparatus

Equivalent apparatus may be substituted if acceptable performance is demonstrated, except where indicated. Manufacturers, model numbers, and part numbers specified here were used during method development and validation.

Table 6-1: Device list
Balance - analytical, with a precision of at least 0.1 mg
Balance - capable of weighing 1 g accurately (at least ±0.01 g)
Centrifuge, refrigerated – capable of attaining ~ 2400 x g (4000 rpm for Sorvall Legend XTR)
Cylinders - graduated – 100, 250, 500, 1000 and 2000 mL
Flasks - volumetric with glass stopper – 10, 25, 50 mL
Freezers – temperatures ≤ -65 and set at -20°C
Refrigerator - capable of maintaining temperatures 2-8°C
Millipore Water System
Rainin EDP3 Pipettes and tips
Robot Coupe®, commercially available cryogenic meat grinder or food blender such as Waring Commercial Laboratory Blender
Vortex mixer – Vortex-Genie 2
Multitube Vortex

6.2 Supplies

The following supplies are listed as examples, unless otherwise stated. Supplies of equivalent quality and abilities provided by other vendors may be used.

Table 6-2: Supplies
15-mL polypropylene graduated centrifuge tubes with screw cap - Fisher brand
50-mL polypropylene graduated centrifuge tubes with screw cap - Fisher brand
2 mL 96-well plates and cap mats - Analytical Sales and Services
2 mL glass autosampler vials

6.3 LC-MS Equipment

Equivalent apparatus and software may be substituted if acceptable performance is demonstrated as suggested in Section 11. Manufacturers and model numbers specified here were used during method development and validation.

Table 6-3: LC-MS List
Perkin Elmer Flexar UHPLC Pump, CTC PAL autosampler
Primary HPLC Column: MacMod Ace 3 C18, 2.1 x 50 mm, Part Number ACE-111-0502 Alternate HPLC Column (used for ruggedness test): Acclaim 120 C18, 3 µm, 2.1 x 150 mm, product # 059130
MS spectrometer– Applied Biosystems, API 4000, Triple Quadrupole
LC/MS Data acquisition system – Applied Biosystems, Analyst, Version 1.4.2
MS spectrometer– Thermo TSQ Vantage, Triple Quadrupole
LC/MS Data acquisition system – LC Quan , version 2.6
Data calculation software – Thermo Fisher Scientific, Watson LIMS, Version 7.3.0.01 or newer, and Microsoft Excel

7 PREPARATION OF STANDARD SOLUTIONS

Different volumes with the same concentrations can be prepared and it is not considered to be a method deviation. All solutions should be mixed well before transfer or use. The exact concentrations should be reported and used throughout all calculations. The following solutions should be stored in a freezer set at -20°C. Return solutions to freezer immediately after use.

7.1 FBZ-SO_2 and FBZ-SO_2-D_3 DMSO Stock Solution

All stock solutions of FBZ-SO_2 and FBZ-SO_2-D_3 are prepared in dimethyl sulfoxide (DMSO). The FBZ-SO_2 and FBZ-SO_2-D_3 DMSO stock solutions are stored in a -20°C freezer.

7.1.1 Preparation of FBZ-SO_2 STD DMSO Stock Solution at 2,000 µg/mL (FBZ-SO_2 DMSO Stock 1)

Weigh approximately 20 mg of reference standard directly into an appropriate container and record the exact weight to the nearest 0.1 mg. Using a calibrated pipette, add an appropriate amount of DMSO to yield a concentration of 2000 µg/mL, after correction for purity, and dissolve (vortex to mix) the standard. The exact concentration, rounded to 3 significant figures, should be reported and used throughout all calculations. This solution is used for the preparation of standard curve working solutions. The stability of this stock solution is 83 days.

Critical Note: the material tends to stick to a metal spatula and a flat spatula works better than one with a groove or indent. Also an anti-static gun can be used if there is still difficulty getting the standard off of the spatula.

7.1.2 Preparation of FBZ-SO_2 Quality Control DMSO Stock Solution at 2,000 µg/mL (FBZ-SO_2 DMSO Stock 2)

This solution is prepared from a second independent weighing procedure (according to Section 7.1.1). It is applied for preparation of the quality control (QC) solutions and spiking of the QC samples.

7.1.3 Comparison of Stock Solutions

A stock solution comparison is required when new stock solutions are prepared. Two stock solutions are prepared. One is used for the preparation of standard curve working solutions. The other stock solution is used to prepare QC working solutions. In addition, the stock comparison solutions are prepared to evaluate the stability of the stock solution for fenbendazole sulfone. One old stock solution is compared to a freshly prepared stock solution.

Each of the two stock solutions needs to be properly diluted with MilliQ Water: Acetonitrile (70:30, v/v) according to following schemes. The suggested concentrations are 10 ng/mL for FBZ-SO_2 and 12 ng/mL for IS.

Table 7-1-1: Preparation of Intermediate Solutions for Stock Comparison

Intermediate Solution ID	Conc. (ng/mL)	FBZ-SO_2 DMSO Stock Solution		Volumetric Flask (mL)
		Conc. (µg/mL)	Volume (µL)	
FBZ-SO_2 STD Stock Inter Solution	5,000	2,000 (Stock Solution)	125	50
FBZ-SO_2 QC Stock Inter Solution	5,000	2,000 (QC Stock Solution)	125	50

Table 7-1-2: Preparation of Final Dilutions for Stock Comparison

Final Dilution Solution ID	Conc. (ng/mL) (FBZ-SO_2/IS)	FBZ-SO_2 Stock Inter Solution		IS Fort. Solution (Section 7.4)		Volumetric Flask (mL)
		Conc. (ng/mL)	Volume (µL)	Conc. µg/mL)	Volume (µL)	
FBZ-SO_2-STD-Stock Final Dilution	10/12	5,000	200	30	40	100
FBZ-SO_2-QC-Stock Final Dilution	10/12	5,000	200	30	40	100

The two final dilution solutions will be analyzed using LC/MS-MS (n=6 injections of each stock solution in alteration) and the results compared for equivalence. If the mean percent difference of the peak area ratio (PAR) are within ± 5% and precision of the replicates are ≤5%, they will be considered equivalent. If the mean percent difference and/or precision are not within ± 5%, the solutions are not considered equivalent and fresh solutions (stock and/or intermediate) will be prepared and compared.

$$\% \text{ Difference} = 100 \times \frac{\text{(mean of PAR of stock A – mean of PAR of stock B)}}{\text{((mean of PAR of stock A + mean of PAR of stock B)/2)}}$$

The final dilution solutions are to be used freshly and discarded after use.

7.1.4 Preparation of FBZ-SO_2-D_3 DMSO Internal Standard Stock Solution at 1,000 µg/mL (FBZ-SO_2-D_3 DMSO Stock 1)

Weigh at least 10 mg of FBZ-SO_2-D_3 reference standard directly in an appropriate container and record the exact weight to the nearest 0.1 mg. Using a calibrated pipette, add an appropriate amount of DMSO to yield a concentration of 1000 µg/mL, after correction for purity, and dissolve (vortex to mix) the standard. The stability of this stock solution is 83 days.

7.2 Working Solution for FBZ-SO_2 Calibration Standards for Liver (SL 9 Liver – SL 1 Liver)

Transfer aliquots of the FBZ-SO_2 STD DMSO stock solution 1 (7.1.1) into appropriate flasks and dilute with methanol according to the following scheme (Table 7-2-1). All working solutions are stored in a -20°C freezer and stable for 78 days.

Table 7-2-1: Working solution for FBZ-SO_2 calibration standards (liver) – scheme for aliquot transfers of solutions

Working Solution	Concentration [µg/mL]	Volume of Solution	Volumetric Flask [mL]
SL 9 Liver	1000	5.0 mL of stock solution (Volume is dependent upon stock solution concentration)	10
SL 8 Liver	120	1.2 mL of SL 9 Liver	10
SL 7 Liver	100	1.0 mL of SL 9 Liver	10
SL 6 Liver	80	2.0 mL of SL 9 Liver	25
SL 5 Liver	70	700 µL of SL 9 Liver	10
SL 4 Liver	50	500 µL of SL 9 Liver	10
SL 3 Liver	30	3.75 mL of SL 6 Liver	10
SL 2 Liver	20	2.5 mL of SL 6 Liver	10
SL 1 Liver	10	1.25 mL of SL 6 Liver	10

7.3 $FBZ\text{-}SO_2$ Quality Control Fortification Solutions for Liver

Transfer aliquots of the $FBZ\text{-}SO_2$ QC DMSO stock solution 2 (7.1.2) into appropriate flasks and dilute with methanol according to the following scheme (Table 7-3-1). All QC fortification solutions are stored in a -20°C freezer and stable for 78 days.

Table 7-3-1: Working Solution for $FBZ\text{-}SO_2$ Quality Control Standards (Liver) – Scheme for Aliquot Transfers of Solutions

Working Solution	Concentration [µg/mL]	Volume of Solution	Volumetric Flask [mL]
QC SL 5 Liver	500	5.0 mL of QC stock solution	20
QC SL 4 Liver	106	5.3 mL of QC SL 5 Liver	25
QC SL 3 Liver	64	3.2 mL of QC SL 5 Liver	25
QC SL 2 Liver	32	1.6 mL of QC SL 5 Liver	25
QC SL 1 Liver	16	800 µL of QC SL 5 Liver	25

7.4 $FBZ\text{-}SO_2\text{-}D_3$ Internal Standard Fortification Solution for Liver

Transfer aliquot of $FBZ\text{-}SO_2\text{-}D_3$ DMSO internal standard stock solution (7.1.4) into appropriate flask and dilute with methanol according to the following scheme (Table 7-4-1). The IS fortification solution is stored in a -20°C freezer and stable for 78 days.

Table 7-4-1: $FBZ\text{-}SO_2\text{-}D_3$ Internal Standard Fortification Solution for Liver – Scheme for Aliquot Transfers of Solutions

Working Solution	Concentration [µg/mL]	Volume of Solution	Volumetric Flask [mL]
$FBZ\text{-}SO_2\text{-}D_3$ IS Fortification	30	1.5 mL of $FBZ\text{-}SO_2$ D_3 DMSO stock solution (Volume is dependent from stock solution concentration)	50

7.5 Solvent Calibration Curve for Liver

For preparation of the solvent calibration curve: add 100 µL of the respective working solutions (7.2), 100 µL of the IS ($FBZ\text{-}SO_2\text{-}D_3$) fortification solution (7.4) (30 µg/mL) to a 20 mL vial or 20 mL volumetric flask. Pipette 19.8 mL of methanol to the vial or fill the volumetric flask to volume with methanol and then mix well to give W-Mix-Stds (see Table 7-5-1). Mix 0.5 mL of each W-Mix-Stds solution with 9.5 mL of MilliQ Water/Acetonitrile (70/30, v/v) and vortexed well to give Liver-Stds (see Table 7-5-2). Transfer 1.0 mL of Liver-Stds to a 96-well plate fresh daily for LC-MS/MS analysis. The standard solution concentrations (ng/mL) and the corresponding tissue concentrations (ppm) are specified in Table 7-5-2. Store all W-Mix-Stds in freezer set at -20°C. The W-Mix-Stds solution is stable for 78 days. The calibration standards are prepared fresh daily.

Solvent calibration standards are prepared at concentrations in ng/mL corresponding to the final tissue extract concentrations in µg/g or ppm. The correlation between solvent calibration standard concentrations and tissue equivalent concentrations are presented in Table 7-5-2. Correlation function between solvent calibration (ng/mL) and respective tissue equivalent concentration (ppm) is:

Concentration in liver (ppm) = determined concentration (ng/mL) x 0.4 (conversion factor), where the conversion factor of 0.4 was calculated as following:

ng/mL x extraction volume (mL) x dilution factor/liver sample weight (g) = ng/mL x 20 mL x 20/1 g = 400 ng/g (ppb) = 0.4 µg/g (ppm). For example, extract concentration of 7.5 ng/mL is equal to 3 ppm tissue equivalent concentration.

Refer to extraction steps 9.2i, 9.2j, and 9.1a for extraction volume, dilution factor, and liver sample weight.

Table 7-5-1: Preparation of W-Mix STD Solutions

W-Mix-Stds: Mix 100 µL of FBZ-SO_2-D_3 fortification solution (7.4**) with 100 µL of SL-1-8 liver (7.2) and dilute to volume with methanol**

Solution ID	Vol. of SL 1-8 liver	Vol. of IS solution	Final Vol (mL)	Conc. (ng/mL)
W-Mix-Std-8	100 µL of SL 8 liver	100 µL	20	600
W-Mix-Std-7	100 µL of SL 7 Liver	100 µL	20	500
W-Mix-Std-6	100 µL of SL 6 Liver	100 µL	20	400
W-Mix-Std-5	100 µL of SL 5 liver	100 µL	20	350
W-Mix-Std-4	100 µL of SL 4 liver	100 µL	20	250
W-Mix-Std-3	100 µL of SL 3 liver	100 µL	20	150
W-Mix-Std-2	100 µL of SL 2 liver	100 µL	20	100
W-Mix-Std-1	100 µL of SL 1 liver	100 µL	20	50

Note: the nominal concentration of internal standard in W-Mix-Stds is 150 ng/mL for 30 µg/mL IS-fortification solution.

Table 7-5-2: Preparation of Solvent Calibration Curve (Liver)			
Calibration Curve: Mix 0.5 mL of W-Mix-Stds with 9.5 mL dilution solution (MilliQ Water/Acetonitrile, 70/30, v/v) in 20 mL vial			
Std-ID	**W-Mix-STD Solution ID**	**Conc. (ng/mL)**	**Liver Equivalent Conc. (ppm)**
Liver-Std-8	W-Mix-Std-8	30.0	12.0
Liver-Std-7	W-Mix-Std-7	25.0	10.0
Liver-Std-6	W-Mix-Std-6	20.0	8.0
Liver-Std-5	W-Mix-Std-5	17.5	7.0
Liver-Std-4	W-Mix-Std-4	12.5	5.0
Liver-Std-3	W-Mix-Std-3	7.5	3.0
Liver-Std-2	W-Mix-Std-2	5.0	2.0
Liver-Std-1	W-Mix-Std-1	2.5	1.0

Note: the nominal concentration of internal standard in Liver-Stds is 7.5 ng/mL for 30 µg/mL IS-fortification solution.

7.6 Preparation of Quality Control Samples for Liver

For routine use, a minimum of one Double Blank, one Control Blank, and two liver QC samples at tolerance are required for each sample analysis set.

For preparation of the QC samples, 100 µL of the respective QC fortification solutions (7.3) and 100 µL of the IS fortification solution (7.4) (30 µg/mL) are spiked into 1 g of blank liver (see Table 7-6-1). For routine sample analysis, QC samples are prepared fresh daily.

Table 7-6-1:Quality Control Samples (Liver)			
QC Sample ID	**Concentration of quality control samples [ppm]**	**Spiking volume of internal standard solution**	**Spike volume of working solution**
QC4	10.6	100 µL	100 µL of QC SL 4 Liver
QC3	6.4	100 µL	100 µL of QC SL 3 Liver
QC2	3.2	100 µL	100 µL of QC SL 2 Liver
QC1	1.6	100 µL	100 µL of QC SL 1 Liver

Further sample preparation is described in Section 9.1.

8 SAMPLE HANDLING AND SAMPLING

8.1 Homogenize Tissue Sample

A Robot Coupe®, a meat grinder, or a food blender may be used to process tissues. Tissue sample is chopped into small pieces to facilitate the grinding process. If it is frozen intact, the tissue may need to be partially thawed before chopping into the small pieces that will fit into the grinding apparatus.

Chopped tissue is mixed with a sufficient amount of dry ice and ground with a Robot Coupe® or other food processor until it becomes a uniform powder. The powdered tissue (containing dry ice) is transferred into a suitable container. The container is loosely sealed or caped and stored in freezer (-20°C) overnight or longer to allow the dry ice sublime. After all the dry ice has been sublimed, the container is sealed and stored at ≤-65°C in a freezer for longer term storage.

Mixed incurred samples (ground or unground) can also be processed with above procedure.

8.2 Sample storage

Control and incurred samples are stored in suitable container in a freezer at ≤-65°C. It is recommended to keep tissue in frozen powdered form until analysis. Samples are stable for 6 months (182 days) stored at -20°C and – 80°C. Stability of fenbendazole sulfone in chicken liver after 4 freeze-thaw cycles and 24-hour stability in chicken liver at room temperature has been demonstrated.

9 PROCEDURE FOR DETERMINATION OF FBZ-SO_2 IN CHICKEN LIVER

It is also suggested to have sample labels (four sets of labels per sample) and necessary containers ready before performing the procedure.

9.1 Preparation of incurred, quality control, control, and double blank samples

9.1a Accurately weigh 1.00 g (±0.05 g) of control or incurred sample into a 15-mL polypropylene tube. Record or print the exact weight as shown on the balance. Centrifuge the sample at 1000 rpm (200x g) for approximately 1 min. Completely thaw tissues prior to the fortification step.

Note: Tissue samples can be weighed out on a different day to facilitate the process. Samples and spatulas should be kept on dry ice during weighing. Store the weighed samples at ≤ -65 ℃ until ready for use.

9.1b Add 200 µL methanol for the double blank sample. Add 100 µL methanol and 100 µL of internal standard fortification solution (7.4) for control and incurred sample. Add 100 µL QC fortification solution (7.3) and 100 µL of internal fortification standard (7.4) for QC samples. Briefly vortex and leave the sample on the bench for no more than 10 min before extraction.

9.2 Extraction of tissue sample

9.2a Add 8 mL of methanol into the 15-mL polypropylene tube containing the sample using a pipette.

9.2b Vortex the sample for *ca.* 10 min. at high speed (setting at 7-9) using a multitube vortexer. Visually inspect all tubes to ensure tissue is swirling up and thoroughly mixed. If any sample did not swirl up during the initial vortex, vortex the individual tube on a regular vortex mixer for up to 10 seconds so that the tissue solid can be mixed well with the extraction solvent, then put the individual sample back onto the multitube vortexer for 10 more minutes.

9.2c Centrifuge the sample at 2400x g (4000 rpm for Sorvall Legend XTR) for *ca.* 10 min. at *ca.* 10°C.

9.2d Transfer the supernatant to a clean pre-labeled 50-mL polypropylene tube.

9.2e Add 8 mL of methanol into the 15-mL polypropylene tube containing the pellet using a pipette.

Critical Step: Pellet may be difficult to re-suspend. Allow the pellet to sit for *ca.* 10 minutes before vortexing. Vortex each sample individually prior to placing the samples on the multitube vortexer (9.2f). If the pellet is difficult to re-suspend, a clean spatula may be used to break the pellet or the tube can be tapped against the bench top.

9.2f Vortex the sample for *ca.* 10 min. at high speed (setting at 7-9) using a multitube vortexer. Visually inspect all tubes to ensure tissue is swirling up and thoroughly mixed. If any sample did not swirl up during the initial vortex, vortex the individual tube on a regular vortex mixer for up to 10 seconds so that the tissue solid can be mixed well with the extraction solvent, then put the individual sample back onto the multitube vortexer for 10 more minutes.

9.2g Centrifuge the sample at 2400x g (4000 rpm for Sorvall Legend XTR) for *ca.* 10 min. at *ca.* 10°C.

9.2h Transfer the supernatant to the same pre-labeled 50-mL polypropylene tube (9.2d).

9.2i Adjust the volume to 20 mL mark with methanol. Vortex and mix well.

9.2j Pipette 50 µL of the methanol liver tissue extract into the appropriate wells of a 2 mL 96-well plate or 2 mL autosampler vial and mix with 950 µL of dilution solvent, MilliQ Water/Acetonitrile (70/30, v/v). Vortex and mix well for LC-MS/MS analysis. Store remaining methanol extract in refrigerator for possible re-assay. The methanol extract is stable for 31 days at refrigerated storage. Extracted samples in 96-well plates or autosampler vials, stored at room temperature , are stable for up to 25 days.

10 METHOD FLOW CHART

Transfer 1.00 ±0.05 g of the frozen homogenate into a 15-mL polypropylene centrifuge tube. Centrifuge the aliquots at 1000 rpm (200x g) for 1 minute. Completely thaw the samples prior to fortification.

↓

Add 200 µL methanol for the double blank sample. Add 100 µL methanol and 100 µL of internal standard fortification solution for control and incurred sample. Add 100 µL QC fortification solution and 100 µL of internal standard fortification solution for QC samples. Briefly vortex. Leave the sample on the bench for no more than 10 min before extraction.

↓

Add 8 mL methanol and vortex for approximately 10 min.

↓

Centrifuge at 4000 rpm (2400x g for Sorvall Legend XTR) for *ca.* 10 min at *ca.* 10 °C.

↓

Transfer the supernatant to a clean pre-labeled 50-mL polypropylene tube

↓

Add 8 mL methanol and vortex for approximately 10 min.

Critical Step: Pellet may be difficult to re-suspend. Allow the pellet to sit for *ca*. 10 minutes before vortexing. Vortex each sample individually prior to placing the samples on the multitube vortexer (9.2f). If the pellet is difficult to re-suspend, a spatula may be used to break the pellet or the tube can be tapped against the bench top.

Centrifuge at 2400x g (4000 rpm for Sorvall Legend XTR) for *ca.* 10 min at *ca.* 10 °C.

↓

Combine the methanol extracts and adjust the volume to 20 mL mark with methanol. Vortex and mix well

↓

Pipette 50 µL of the methanol liver tissue extract into the appropriate wells of a 2 mL 96-well plate or autosampler vial and mix with 950 µL of dilution solvent, MilliQ Water/Acetonitrile (70/30, v/v), for LC-MS/MS analysis.

11 LC-MS/MS ANALYSIS

Equivalent apparatus may be substituted if acceptable performance is demonstrated. Manufacturers and model numbers specified here were used during method development and validation.

On occasions it may be necessary to adjust the LC and MS conditions slightly to achieve acceptable peak shape and sensitivity. The LC and MS conditions should be adjusted such that acceptable performance of the LC-MS/MS system is met (Section 13.1).

11.1 HPLC Conditions

Settings may depend on the HPLC system used and are for example only. Alternate column, mobile phase composition, and LC-MS/MS platform can be used per ruggedness tests. Refer to Section 22.2 for details.

HPLC System:	Perkin Elmer Flexar-FX 15 UHPLC Pump, LEAP HTC PAL Autosampler
Column:	MacMod Ace 3 C18, 2.1 x 50 mm Part Number ACE-111-0502
Column Temperature:	Ambient
Autosampler Temperature:	Ambient
Mobile Phase A:	0.1% Formic Acid in Water (v/v)
Mobile Phase B:	0.1% Formic Acid in Acetonitrile (v/v)
Injection Volume:	10 µL (may vary)
Run Time:	5.2 min/inj. (may vary to allow column to re-equilibrate between injections)
Retention Time:	*ca.* 1.5 min

Gradient Table:

Time (min)	Flow (µL/min)	Mobile Phase A (%)	Mobile Phase B (%)
Initial (0.1)*	400	70	30
0.3	400	70	30
2.0	400	25	75
2.1	400	0	100
3.1	400	0	100
3.2	400	70	30
5.2	400	70	30

*PE Pump starts from 0.1, Thermo Pump starts from 0.0

11.2 MS Conditions

11.2.1 Tuning of Mass Spectrometer and MS Full Scan

The MS response of FBZ-SO_2 and FBZ-SO_2-D_3 can be tuned by infusion of appropriate solutions. Typically, the tuning is done by infusing a solution of the analyte of interest diluted in mobile phase using a tee connector prior to introduction into the MS. The conditions should be optimized in full scan mode for adequate detection of FBZ-SO_2 and FBZ-SO_2-D_3 parent ions (*m/z* 332, *m/z* 335, respectively). The MS conditions should then be optimized in MS/MS mode for adequate detection of product ion at *m/z* 300 for both FBZ-SO_2 and FBZ-SO_2-D_3. The resultant MS parameter should be used for all analyses, although the operator may vary conditions for adequate sensitivity. The structure and proposed fragmentation pattern of FBZ-SO_2 is shown in section 20. The MS spectra of FBZ-SO_2 at various collision energy will be listed after relevant tests.

11.2.2 MS Conditions

The MS should be tuned as in Step 11.2.1. The suggested MS parameters and peak mass centers are as follows. Settings may depend on the MS system used and are for example only. The actual tune file has to be documented.

Table 11.2.2-1: System Parameter API 4000/ API 4000 QTRAP

Ionization interface	Turbo Ion Spray
Ionization mode	Positive
Approximate MS run time [min]	2.5
Source (TEM) Temperature [°C]	600
Curtain (CUR) gas [psi]	10
Collision (CAD) gas [psi]	4
Ion source gas (GS1) 1 [psi]	40
Ion source gas (GS2) 2 [psi]	60
Ion (IS) Spray [V]	4000
Entrance (EP) potential [V]	10

MRM MS/MS transition parameters (API-4000) as follows

Table 11.2.2-2: MS/MS Transition Parameter

Reference compound	Precursor ion Q1 mass [amu]	Collision energy [V]	Q3 mass [amu]	Dwell time [ms]
FBZ-SO2 [a]	332	38	300 (quantifier)	150
FBZ-SO2-Qual_1 [b]	332	55	159 (qualifier)	150
FBZ-SO2-Qual_2 [b]	332	78	104(qualifier)	150
FBZ-SO2-D3 [a]	335	38	300	150

a: quantitation purposes

b: qualifier transition used with confirmatory method, not used for quantitative purposes

The MS parameters should be established by tuning of the instrument to be used and its calibration. Differences from the above parameters are not considered a method deviation.

The mass spectrometer should be optimized for the confirmatory procedure using a standard solution with concentration equivalent to the proposed fenbendazole sulfone tolerance (5.3 ppm), to obtain a signal to noise ratio ($R_{S/N}$) ≥ 100.

11.3 System Suitability Test and Sample Injection Sequence

The LC-MS system should be conditioned first with approximately 5 injections of FBZ – SO_2 standard at lowest level (standard 1).

11.3.1 System Suitability Test (SST)

Once the system is stabilized, system suitability should be performed by injection of the lowest standard 1 for at least 5 times to assess reproducibility and sensitivity of MS response. Refer to Section 13.1 for system suitability acceptance criteria.

11.3.2 Carry Over Test

System carry over is assessed by injecting one highest standard (Std-8) immediately followed by a solvent blank (MilliQ Water/Acetonitrile 70:30 , v/v).

11.3.3 Bracket Standard

All 8 standards are run before extracted samples including control samples, double blank, QC, and incurred samples. The extracted samples are followed (bracketed) by all 8 standards.

11.3.4 Analysis Sequence

A possible sequence order consisting of system suitability test (SST) samples, solvent calibration, and QC samples within a series is presented below. The SST solutions (Section 11.3.1) are used to check the LC-MS system.

System Suitability Test SSTL (Std-1)	n ≥5 injections (SSTL reproducibility)
System Suitability Test SSTH (Std-8)	1 injection (SSTH)
Solvent blank (MilliQ Water/Acetonitrile, 70:30, v/v)	1-2 injections (1st injection used for carry over test)
Std-1 to Std-8	1 injection each
Solvent blank	1 injection
Followed by tissue samples, including double blank, control, QCs, and study samples.	1 injection each
Solvent blank	1 injection
Std-1 to Std-8	1 injection each (from same vial or well)

12 CALCULATION AND REPORTING OF RESULTS

12.1 Method of Calculation

Quantitation of FBZ-SO_2 is accomplished using an internal standard calibration method with a FBZ-SO_2 standard concentration range of 1.0 ppm to 12.0 ppm for liver. A standard calibration curve is generated from a weighted (1/x) linear regression analysis of peak area ratio versus concentration (ppm) of FBZ-SO_2. The standard curve plots peak area ratio of FBZ-SO_2/FBZ-SO_2-D_3 versus concentration of FBZ-SO_2 from calibration standards.

A linear regression curve fit equation for the standard curve will determine the concentration of the sample solutions injected using the following equation:

$y = mx+b$

The concentration of each sample is calculated using the formula:

$$x = \frac{y - b}{m}$$

Where, y = MS detector calculated response using the ratio of analyte to IS
x = sample concentration (ppm)
m = slope
b = y-intercept

Typically, FBZ-SO_2 concentrations of standard curve point are expressed as ppm tissue residue equivalent.

The regression equation is then used to calculate the concentration of FBZ-SO_2 in the bracketed samples. If the regression obtained in an analytical set yields an acceptable coefficient of determination and meets the stated criteria (Section 13.3), the regression equation can be used to determine the concentration of each sample in the set. If the regression does not meet acceptability criteria, the set is deemed not acceptable and has to be repeated by re-injecting the standards and samples or by preparing new standards and/or new sample extracts for re-analysis.

12.2 Calculation of Sample Concentrations

The exact concentration, rounded to 3 significant figures, should be reported and used throughout all of the calculations.

The following equation will calculate the concentration in ppm:

$$C_T = \frac{(C_I)}{Sw}$$

Where:
C_T is the concentration of FBZ-SO_2 in ppm in the sample,
C_I is the calculated concentration of FBZ-SO_2 in ppm from the standard curve where the nominal concentrations of standards are in ppm and are based on 1.0 gram sample size.
S_W is the sample weight in g of the incurred samples (nominal weight of 1 g is used for fortified samples and exact weight is used for incurred samples).

An example of a concentration calculation for an incurred sample is given below:

C_I = 4.21 ppm S_W = 1.06 g

$$C_T = \frac{4.21}{1.06} = 3.97 \text{ ppm}$$

Recoveries (a measure of accuracy) are calculated from fortified QC samples using the equation:

$$\%\text{Recovery} = \left(\frac{C_T}{C_F}\right) \times 100$$

Where:
C_T is the measured concentration of FBZ in ppm in the sample,
C_F is the tissue fortification level in ppm.

An example of recovery calculation is given below:

C_T=6.00 ppm C_F=6.40 ppm

$$\%\text{Recovery} = \left(\frac{6.00}{6.40}\right) \times 100 = 93.8\%$$

12.3 Automation of Calculations

The chromatographic software may be used to integrate chromatograms, calculate results, and print and save reports. Use the same integration parameters to integrate all chromatograms within an entire batch. Verify that all chromatograms are correctly integrated. Resultant reports may then be generated and printed. The generated results can be imported to Thermo Watson LIMS for further data calculation and summary.

13 ACCEPTABILITY CRITERIA

Analytical data must meet the following criteria to establish adequate performance of the method.

13.1 System Suitability Test: Reproducibility and System Carry-over

To demonstrate acceptable performance of the LC-MS/MS system, the system suitability injections of a standard at the lowest calibration level (SSTL, standard 1) should be performed prior to injection of a sample set (Section 11.3.1).

A minimum signal-to-noise ratio of 10:1 and reproducible FBZ-SO_2/FBZ-SO_2-D_3 peak area ratio and FBZ-SO_2 retention times with CV ≤ 5% must be met for at least five consecutive injections of standard 1.

The system carry over (solvent blank) after injection of SSTH (standard 8) has to be <20.0% of the average FBZ-SO_2 area of SSTL (standard 1).

The raw data and calculated results from the consecutive injections are documented with each injection set.

If the MS detector sensitivity is low and gives poor precision at the LOQ, tuning the instrument may improve the sensitivity. If the sensitivity remains low, instrument calibration, cleaning, and/or repair should be performed.

If the MS detector sensitivity is too high and gives a non-linear standard curve, the instrument parameters may be changed to decrease the response.

13.2 Accuracy and Precision: Quality Control Sample Acceptance Criteria

The results of the QC samples will provide the basis for accepting or rejecting the analytical run. The acceptance criteria of the mean accuracy of QC samples are 80% to 110% for levels ≥0.1 ppm. Acceptance criteria for precision expressed as coefficient of variation (% CV) were set to ≤10% for levels ≥0.1 ppm.

13.3 Standard Calibration Curve

The linear regression (1/x weighting) should have a coefficient of determination (r^2) ≥0.990 or correlation coefficient (r) ≥0.995 for a standard curve of FBZ-SO_2 ranging from 1.0 ppm to 12.0 ppm for liver. The nominal concentration of internal standard in the standard calibration solutions is 3.0 ppm for chicken liver.

Back-calculated accuracy should be within ±10% of the nominal, except the lowest standard, which should be within ±15% of the nominal. A maximum of two standard replicates can be excluded if they cannot meet the above accuracy criteria, but not from the same concentration level. A standard can also be excluded if an instrument problem or injection error occurs during the analysis of that standard.

13.4 Selectivity

Control tissues should not contain endogenous or exogenous substances that may interfere at the retention time of FBZ-SO_2. Typically, any interference less than 10% of FBZ-SO_2 peak area at 0.5x of the proposed tolerance (5.3 ppm for liver) is considered to be acceptable.

14 LIMIT OF QUANTITATION

The limit of quantitation (LOQ) is the lowest level where the acceptable accuracy and reproducibility have been obtained. An LOQ of 1.0 ppm (equivalent to lowest standard) for liver was established.

Quantitative information below the LOQ should be reported and footnoted as BLOQ. The analyst should note this result with appropriate annotations and footnotes in the analytical results.

The upper limit of quantitation (ULOQ) is set at the highest concentration of FBZ-SO_2 in the calibration standard curve. Accordingly, for fortified and incurred samples, the ULOQ is 12.0 ppm for liver.

15 DILUTION

Quantitative results for incurred samples and fortified QC samples should only be reported within the concentration range for which the standard curve demonstrates acceptable linear regression. When a quantitative result is above the standard curve range, it should be marked (suggested "ALQ"). Aliquots of the methanol extract can be diluted with control blank extract and re-analyzed.

16 STABILITY

16.1 Stability of FBZ-SO_2 and FBZ-SO_2-D_3 Stock Standard Solutions or Working Standard Solutions

All stock standard solutions (Section 7) stored at -20 °C are stable for 83 days. All working standard solutions (Section 7) stored at -20 °C are stable for 78 days.

16.2 Stability of Tissue Extract

Tissue methanol extract is stable for up to 31 days at refrigerated storage.

16.3 Stability of Final Extracts in Dilution Solution

Final extracts in dilution solution in 96-well plates or autosampler vials stored at room temperature are stable for up to 25 days.

16.4 Stability of Samples after 4 freeze-thaw cycles

Samples are stable after 4 freeze-thaw cycles.

16.5 Long Term Freezer Storage Stability

Samples are stable for 6 months (182 days) stored at -20 ^{o}C and – 80^{o}C, based on Intervet study N09-070-01. Refer to reference section 24.2.

17 NOTES TO ANALYSTS

17.1 Minimization of Carryover

To minimize possible carryover of FBZ-SO_2, it is recommended to inject solvent blank (MilliQ Water/Acetonitrile, 70/30, v/v) after injection of a high concentration calibration standards or sample.

17.2 IS Monitoring and LC-MS/MS System Cleanness

Monitor IS performance by matric plot to ensure there is no major variability. Otherwise, troubleshoot the system. When instrument responses are decreased overtime, the analytical HPLC column may be changed or the Mass Spec ion source may be cleaned.

18 CONFIRMATORY METHOD

18.1 Confirmatory analysis

Confirmatory analysis is to be done by reinjection of relevant batches. Additional ion transitions from FBZ-SO2, m/z 332 → m/z 159 as qualifier 1 and m/z 332 → m/z 104 as qualifier 2 are monitored along with 300 used for determinative method, for the confirmatory method. The MS/MS transition parameters for confirmatory analysis are listed in Table 11.2.2-2.

18.2 Acceptance Criteria

Acceptance criteria for confirmatory analysis are listed as:
Relative abundance ratio (RAR) in QC and incurred samples should match the average RAR in solvent standards within ± 10%. The retention time of the confirmatory peaks in QC and incurred samples should match the retention time of the quantitative peak in solvent standards within ± 5%. Signal to noise ratio ($R_{S/N}$) should be ≥ 100.

18.3 Summary Confirmatory Results for Standards

Run ID	Sample ID	m/z 300 Peak Area	m/z 159 Peak Area	m/z 104 Peak Area	RAPAR* m/z 159 Individual	RAPAR* m/z 104 Individual	Retention Time (min) m/z 300 Individual	Retention Time (min) m/z 159 Individual	Retention Time (min) m/z 104 Individual	S/N Ratio (> 50) m/z 159 Individual	S/N Ratio (> 50) m/z 104 Individual
2	2 009 S13264-00 Liver-Std-1 1 1	290000	247000	78300	85.2	27.0	1.57	1.57	1.57	1900	297
	2 010 S13264-00 Liver-Std-2 1 1	424000	368000	111000	86.8	26.2	1.57	1.57	1.57	3070	450
	2 011 S13264-00 Liver-Std-3 1 1	601000	515000	164000	85.7	27.3	1.56	1.57	1.55	6070	833
	2 012 S13264-00 Liver-Std-4 1 1	951000	824000	265000	86.6	27.9	1.57	1.56	1.57	10700	885
	2 013 S13264-00 Liver-Std-5 1 1	1350000	1130000	380000	83.7	28.1	1.57	1.57	1.57	6700	1360
	2 014 S13264-00 Liver-Std-6 1 1	1470000	1220000	403000	83.0	27.4	1.57	1.57	1.57	9270	1330
	2 015 S13264-00 Liver-Std-7 1 1	1750000	1490000	498000	85.1	28.5	1.58	1.57	1.57	13600	1730
	2 016 S13264-00 Liver-Std-8 1 1	2050000	1760000	600000	85.9	29.3	1.57	1.57	1.57	15000	1650
	2 037 S13264-00 Liver-Std-1 2 1	302000	262000	80100	86.8	26.5	1.57	1.57	1.57	2020	278
	2 038 S13264-00 Liver-Std-2 2 1	412000	359000	111000	87.1	26.9	1.57	1.57	1.57	3250	372
	2 039 S13264-00 Liver-Std-3 2 1	586000	504000	165000	86.0	28.2	1.57	1.57	1.57	4090	620
	2 040 S13264-00 Liver-Std-4 2 1	958000	845000	269000	88.2	28.1	1.57	1.57	1.56	9590	1080
	2 041 S13264-00 Liver-Std-5 2 1	1350000	1150000	376000	85.2	27.9	1.57	1.57	1.56	9310	1620
	2 042 S13264-00 Liver-Std-6 2 1	1430000	1190000	391000	83.2	27.3	1.57	1.57	1.57	11500	1520
	2 043 S13264-00 Liver-Std-7 2 1	1700000	1480000	495000	87.1	29.1	1.57	1.57	1.56	14700	2020
	2 044 S13264-00 Liver-Std-8 2 1	2040000	1770000	585000	86.8	28.7	1.57	1.56	1.57	16900	2320
				Average:	85.8	27.8	1.57	1.57	1.57	NA	
3	3 009 S13264-00 Liver-Std-1 1 1	114000	94200	29400	82.6	25.8	1.58	1.58	1.57	790	124
	3 010 S13264-00 Liver-Std-2 1 1	205000	171000	54300	83.4	26.5	1.58	1.58	1.58	1400	236
	3 011 S13264-00 Liver-Std-3 1 1	287000	236000	75800	82.2	26.4	1.58	1.58	1.58	1830	406
	3 012 S13264-00 Liver-Std-4 1 1	472000	392000	127000	83.1	26.9	1.57	1.57	1.57	3140	642
	3 013 S13264-00 Liver-Std-5 1 1	653000	543000	172000	83.2	26.3	1.59	1.58	1.58	4550	1160
	3 014 S13264-00 Liver-Std-6 1 1	728000	605000	194000	83.1	26.6	1.58	1.58	1.57	5390	1450
	3 015 S13264-00 Liver-Std-7 1 1	895000	749000	247000	83.7	27.6	1.58	1.58	1.57	7130	1130
	3 016 S13264-00 Liver-Std-8 1 1	991000	839000	269000	84.7	27.1	1.57	1.57	1.57	6330	1370
	3 037 S13264-00 Liver-Std-1 2 1	118000	94500	27900	80.1	23.6	1.57	1.57	1.57	980	92
	3 038 S13264-00 Liver-Std-2 2 1	211000	175000	52600	82.9	24.9	1.57	1.57	1.57	1520	284
	3 039 S13264-00 Liver-Std-3 2 1	294000	242000	74000	82.3	25.2	1.58	1.57	1.57	2100	448
	3 040 S13264-00 Liver-Std-4 2 1	465000	378000	122000	81.3	26.2	1.54	1.54	1.54	3560	856
	3 041 S13264-00 Liver-Std-5 2 1	630000	515000	172000	81.7	27.3	1.57	1.57	1.57	4460	771
	3 042 S13264-00 Liver-Std-6 2 1	724000	590000	193000	81.5	26.7	1.58	1.58	1.57	4590	1050
	3 043 S13264-00 Liver-Std-7 2 1	864000	723000	235000	83.7	27.2	1.59	1.58	1.58	6480	978
	3 044 S13264-00 Liver-Std-8 2 1	1020000	840000	274000	82.4	26.9	1.58	1.57	1.57	7770	1060
				Average:	**82.6**	26.3	**1.58**	**1.57**	**1.57**	NA	
4	4 009 S13264-00 Liver-Std-1 1 1	97400	84800	26600	87.1	27.3	1.58	1.57	1.57	897	143
	4 010 S13264-00 Liver-Std-2 1 1	178000	151000	46200	84.8	26.0	1.58	1.58	1.58	1810	342
	4 011 S13264-00 Liver-Std-3 1 1	260000	218000	69600	83.8	26.8	1.60	1.60	1.60	1610	454
	4 012 S13264-00 Liver-Std-4 1 1	418000	351000	109000	84.0	26.1	1.57	1.57	1.57	2300	722
	4 013 S13264-00 Liver-Std-5 1 1	570000	478000	151000	83.9	26.5	1.58	1.58	1.57	2550	828
	4 014 S13264-00 Liver-Std-6 1 1	642000	548000	175000	85.4	27.3	1.58	1.58	1.57	4330	799
	4 015 S13264-00 Liver-Std-7 1 1	768000	663000	216000	86.3	28.1	1.58	1.58	1.58	3370	1210
	4 016 S13264-00 Liver-Std-8 1 1	885000	755000	251000	85.3	28.4	1.59	1.58	1.58	4180	1980
	4 037 S13264-00 Liver-Std-1 2 1	97100	84500	25200	87.0	26.0	1.59	1.58	1.58	1010	125
	4 038 S13264-00 Liver-Std-2 2 1	176000	147000	43500	83.5	24.7	1.59	1.59	1.59	2340	257
	4 039 S13264-00 Liver-Std-3 2 1	249000	210000	63500	84.3	25.5	1.59	1.58	1.58	1450	336
	4 040 S13264-00 Liver-Std-4 2 1	404000	348000	111000	86.1	27.5	1.58	1.57	1.57	2940	630
	4 041 S13264-00 Liver-Std-5 2 1	550000	479000	149000	87.1	27.1	1.59	1.58	1.58	4430	698
	4 042 S13264-00 Liver-Std-6 2 1	629000	534000	170000	84.9	27.0	1.58	1.57	1.57	3430	1150
	4 043 S13264-00 Liver-Std-7 2 1	779000	630000	206000	80.9	26.4	1.59	1.59	1.58	5930	1170
	4 044 S13264-00 Liver-Std-8 2 1	895000	728000	249000	81.3	27.8	1.58	1.58	1.58	3740	1520
				Average:	**84.7**	26.8	**1.58**	**1.57**	**1.58**	NA	

*RAPAR: Relative Abundance Peak Area Ratio to m/z 300

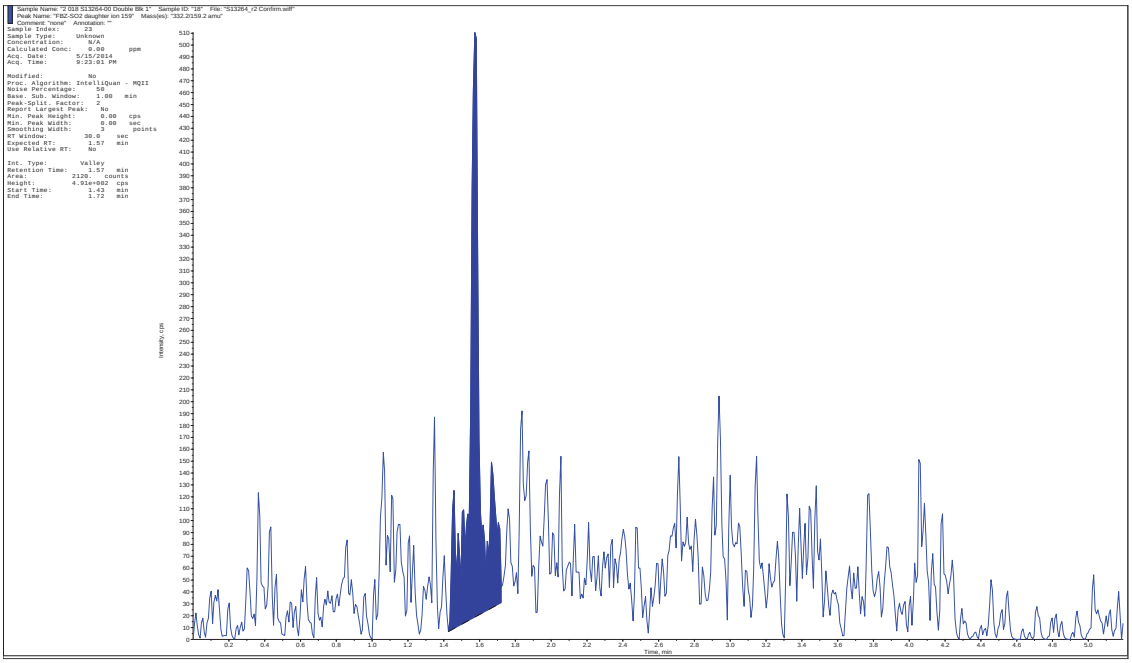
18.4 Summary Confirmatory Results for QCs and Treated Tissue Samples

Summary Confirmatory Results for QCs and Treated Tissue Samples														
Run ID	Sample ID	Relative Abundance Peak Area Ratio to m/z 300				Retention Time (min)						S/N Ratio (> 50)	
		m/z 159		m/z 104		m/z 300		m/z 159		m/z 104		m/z 159	m/z 104
		Individual	Acc. Range	Individual	Acc. Range	Individual	Acc. Range	Individual	Acc. Range	Individual	Acc. Range	Individual	Individual
2	S13264-00 QC1 1	86.2	75.8-95.8	26.4	17.8-37.8	1.57	1.49-1.65	1.57	1.49-1.65	1.57	1.49-1.65	3230	330
	S13264-00 QC1 2	87.3	75.8-95.8	26.8	17.8-37.8	1.58	1.49-1.65	1.57	1.49-1.65	1.57	1.49-1.65	3150	345
3	S13264-00 QC1 1	80.7	72.6-92.6	26.1	16.3-36.3	1.58	1.50-1.66	1.58	1.49-1.65	1.57	1.49-1.65	1070	203
	S13264-00 QC1 2	82.4	72.6-92.6	26.6	16.3-36.3	1.59	1.50-1.66	1.58	1.49-1.65	1.58	1.49-1.65	1310	210
4	S13264-00 QC1 1	80.9	74.7-94.7	24.8	16.8-36.8	1.58	1.50-1.66	1.58	1.49-1.65	1.57	1.50-1.66	1300	159
	S13264-00 QC1 2	87.5	74.7-94.7	26.9	16.8-36.8	1.58	1.50-1.66	1.57	1.49-1.65	1.57	1.50-1.66	1000	176
2	S13264-00 QC2 1	85.2	75.8-95.8	27.3	17.8-37.8	1.57	1.49-1.65	1.57	1.49-1.65	1.57	1.49-1.65	5570	597
	S13264-00 QC2 2	84.9	75.8-95.8	27.2	17.8-37.8	1.57	1.49-1.65	1.57	1.49-1.65	1.57	1.49-1.65	3590	494
3	S13264-00 QC2 1	81.4	72.6-92.6	25.7	16.3-36.3	1.58	1.50-1.66	1.58	1.49-1.65	1.58	1.49-1.65	1980	469
	S13264-00 QC2 2	80.2	72.6-92.6	25.3	16.3-36.3	1.58	1.50-1.66	1.58	1.49-1.65	1.58	1.49-1.65	2310	401
4	S13264-00 QC2 1	84.0	74.7-94.7	26.1	16.8-36.8	1.59	1.50-1.66	1.59	1.49-1.65	1.58	1.50-1.66	2040	478
	S13264-00 QC2 2	82.9	74.7-94.7	26.0	16.8-36.8	1.58	1.50-1.66	1.58	1.49-1.65	1.57	1.50-1.66	1490	475
2	S13264-00 QC3 1	86.5	75.8-95.8	28.5	17.8-37.8	1.58	1.49-1.65	1.57	1.49-1.65	1.57	1.49-1.65	7570	1110
	S13264-00 QC3 2	85.5	75.8-95.8	28.4	17.8-37.8	1.57	1.49-1.65	1.57	1.49-1.65	1.57	1.49-1.65	11100	1460
3	S13264-00 QC3 1	83.8	72.6-92.6	26.6	16.3-36.3	1.58	1.50-1.66	1.58	1.49-1.65	1.58	1.49-1.65	5210	944
	S13264-00 QC3 2	82.1	72.6-92.6	26.6	16.3-36.3	1.58	1.50-1.66	1.57	1.49-1.65	1.57	1.49-1.65	3990	799
4	S13264-00 QC3 1	84.8	74.7-94.7	28.4	16.8-36.8	1.59	1.50-1.66	1.58	1.49-1.65	1.58	1.50-1.66	2800	998
	S13264-00 QC3 2	87.6	74.7-94.7	27.3	16.8-36.8	1.58	1.50-1.66	1.58	1.49-1.65	1.57	1.50-1.66	2690	847
2	S13264-00 QC4 1	88.0	75.8-95.8	29.1	17.8-37.8	1.57	1.49-1.65	1.57	1.49-1.65	1.57	1.49-1.65	13800	1590
	S13264-00 QC4 2	84.5	75.8-95.8	27.8	17.8-37.8	1.57	1.49-1.65	1.57	1.49-1.65	1.57	1.49-1.65	15200	2040
3	S13264-00 QC4 1	82.0	72.6-92.6	27.1	16.3-36.3	1.58	1.50-1.66	1.58	1.49-1.65	1.57	1.49-1.65	7510	1370
	S13264-00 QC4 2	83.4	72.6-92.6	27.7	16.3-36.3	1.58	1.50-1.66	1.57	1.49-1.65	1.57	1.49-1.65	6110	1360
4	S13264-00 QC4 1	84.7	74.7-94.7	27.5	16.8-36.8	1.58	1.50-1.66	1.58	1.49-1.65	1.57	1.50-1.66	3220	1360
	S13264-00 QC4 2	85.3	74.7-94.7	27.5	16.8-36.8	1.60	1.50-1.66	1.59	1.49-1.65	1.59	1.50-1.66	4980	1290
2	S13264-00 T2-M-1/4	86.4	75.8-95.8	27.0	17.8-37.8	1.57	1.49-1.65	1.57	1.49-1.65	1.57	1.49-1.65	7210	757
	S13264-00 T2-M-1/4	82.9	75.8-95.8	27.6	17.8-37.8	1.58	1.49-1.65	1.57	1.49-1.65	1.57	1.49-1.65	5410	769
3	S13264-00 T2-M-1/4	80.4	72.6-92.6	25.8	16.3-36.3	1.58	1.50-1.66	1.58	1.49-1.65	1.58	1.49-1.65	2550	489
4	S13264-00 T2-M-1/4	86.9	74.7-94.7	27.2	16.8-36.8	1.58	1.50-1.66	1.57	1.49-1.65	1.57	1.50-1.66	1840	537
	S13264-00 T2-M-1/4	89.2	74.7-94.7	26.6	16.8-36.8	1.59	1.50-1.66	1.58	1.49-1.65	1.58	1.50-1.66	1890	472
2	S13264-00 T3-M-2/4	83.3	75.8-95.8	28.6	17.8-37.8	1.57	1.49-1.65	1.57	1.49-1.65	1.57	1.49-1.65	13600	2010
3	S13264-00 T3-M-2/4	83.4	72.6-92.6	27.1	16.3-36.3	1.57	1.50-1.66	1.57	1.49-1.65	1.57	1.49-1.65	5070	1070
	S13264-00 T3-M-2/4	81.5	72.6-92.6	26.7	16.3-36.3	1.58	1.50-1.66	1.58	1.49-1.65	1.57	1.49-1.65	6510	1190
4	S13264-00 T3-M-2/4	85.0	74.7-94.7	27.9	16.8-36.8	1.59	1.50-1.66	1.59	1.49-1.65	1.59	1.50-1.66	2850	1010
	S13264-00 T3-M-2/4	84.2	74.7-94.7	26.9	16.8-36.8	1.59	1.50-1.66	1.58	1.49-1.65	1.58	1.50-1.66	2950	942

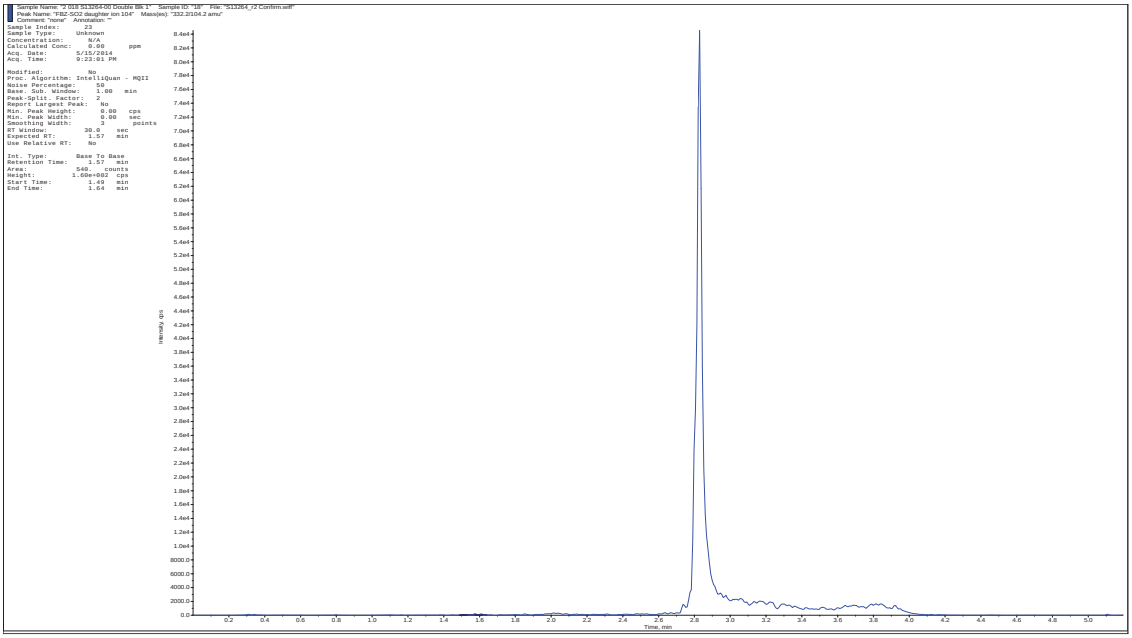
18.5 Example Chromatograms from Confirmatory Analysis

18.5.1 Double Blank

Product Ion 159

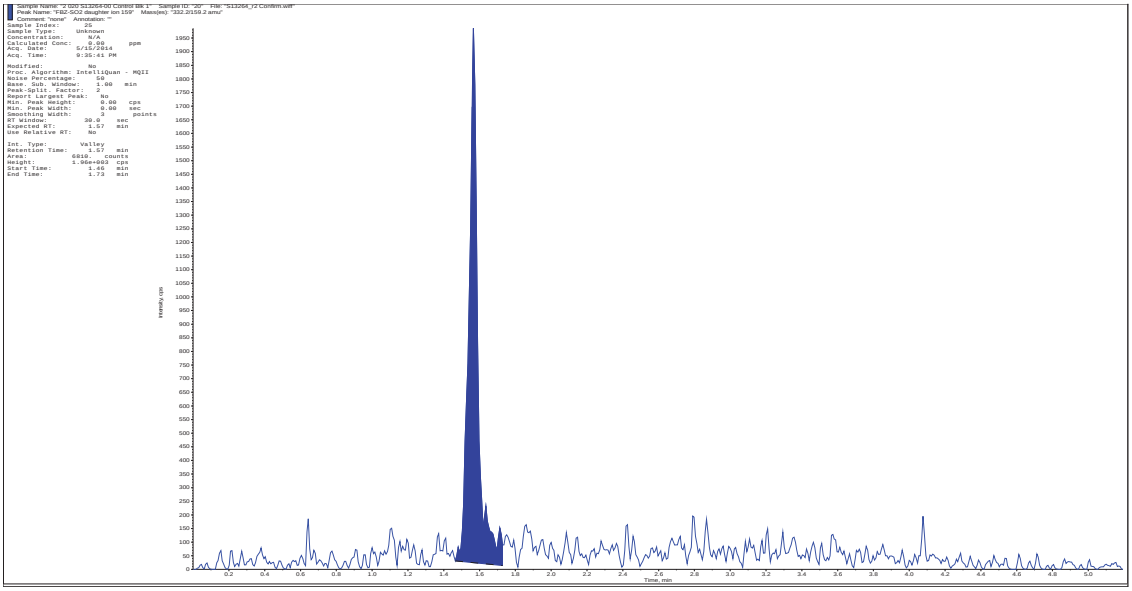


Product Ion 104

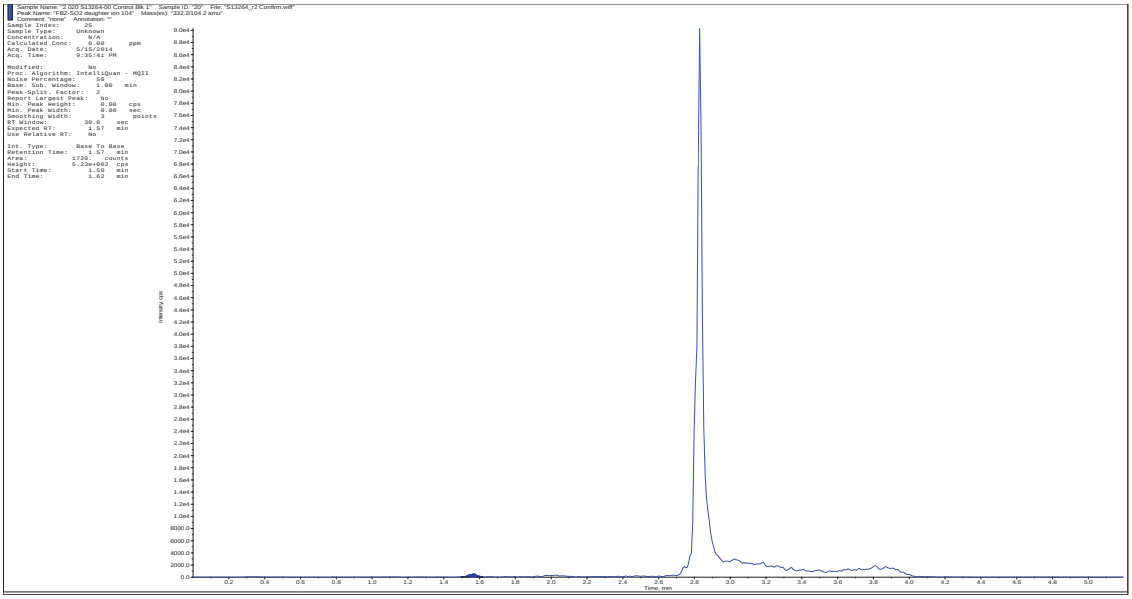


18.5.2 Control Blank

Product Ion 159

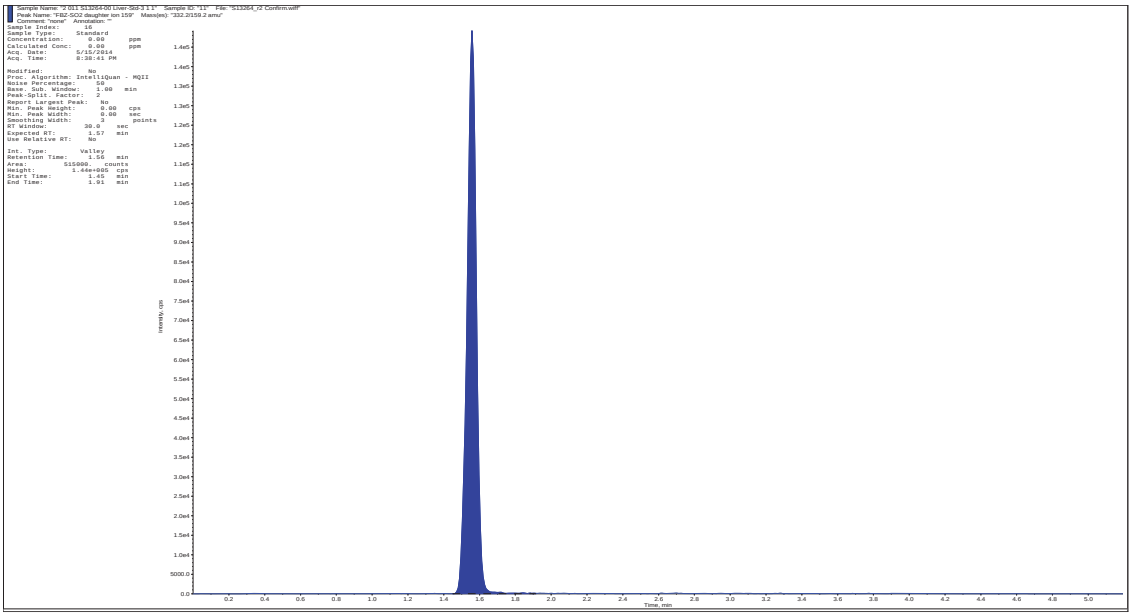


Product Ion 104

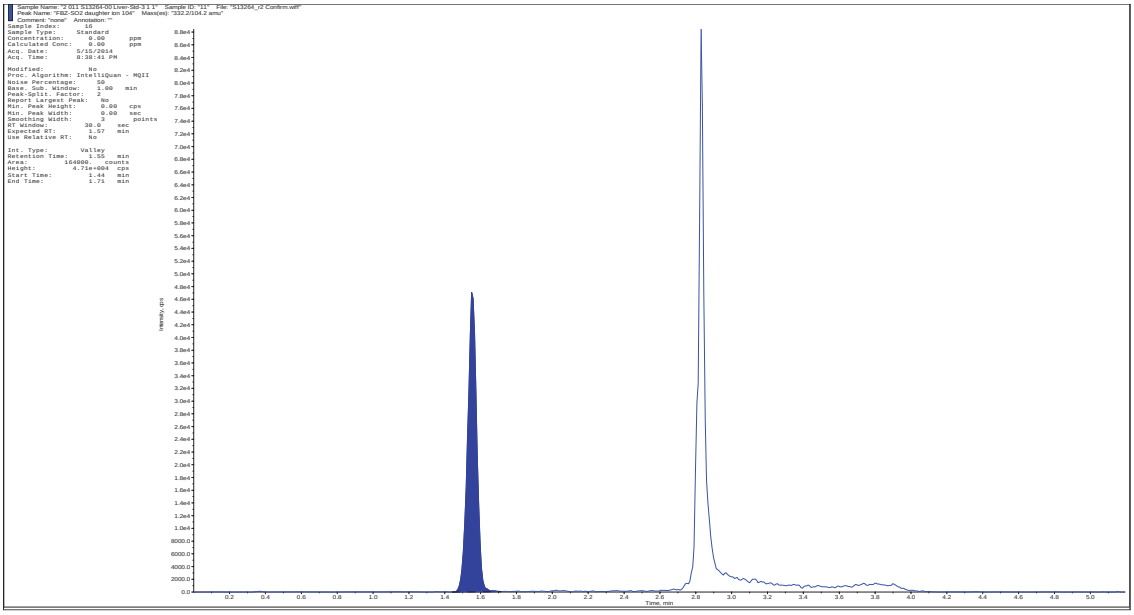


18.5.3 Standard Equivalent to 3 ppm

Product Ion 159

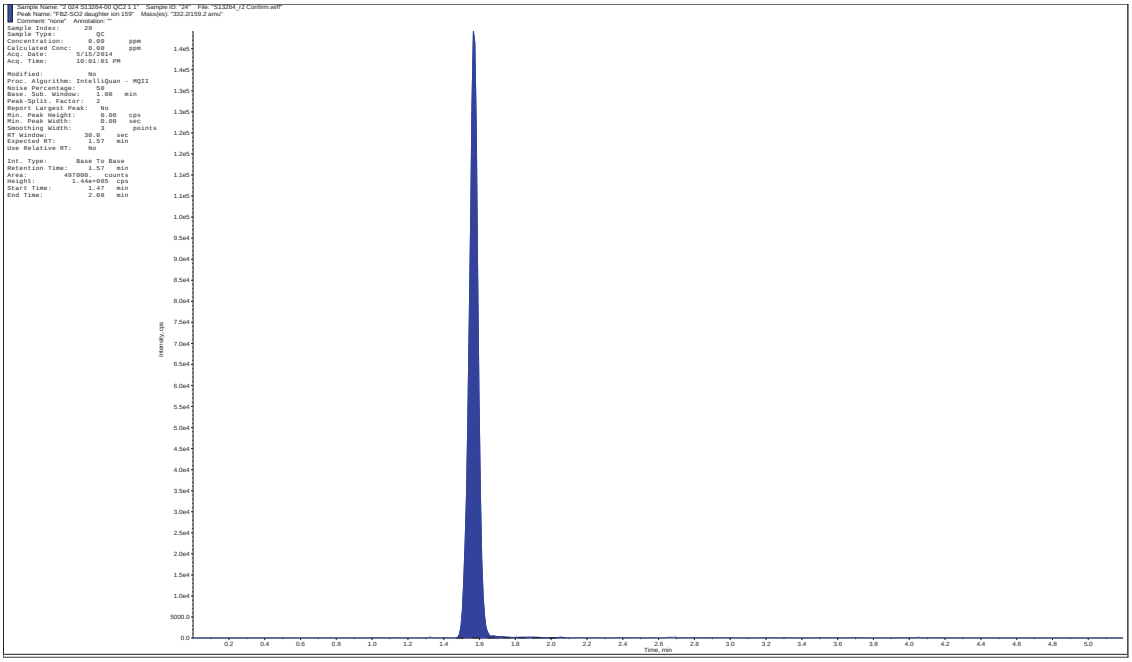


Product Ion 104

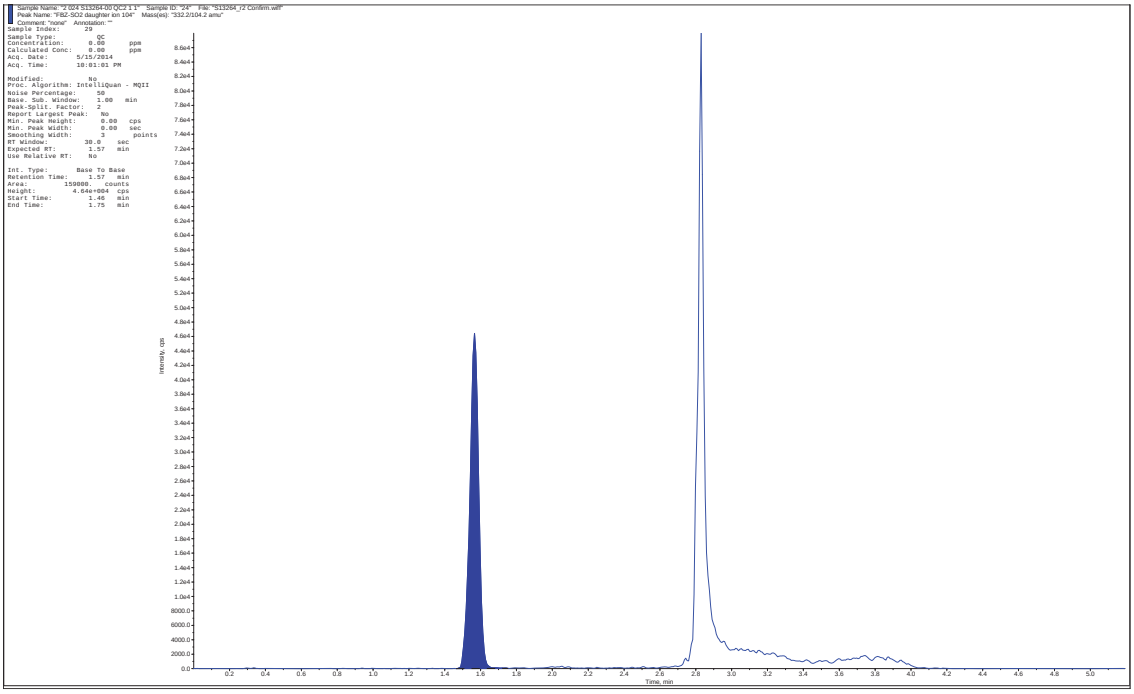


18.5.4 Fortified Sampe at 3.2 ppm

Product Ion 159

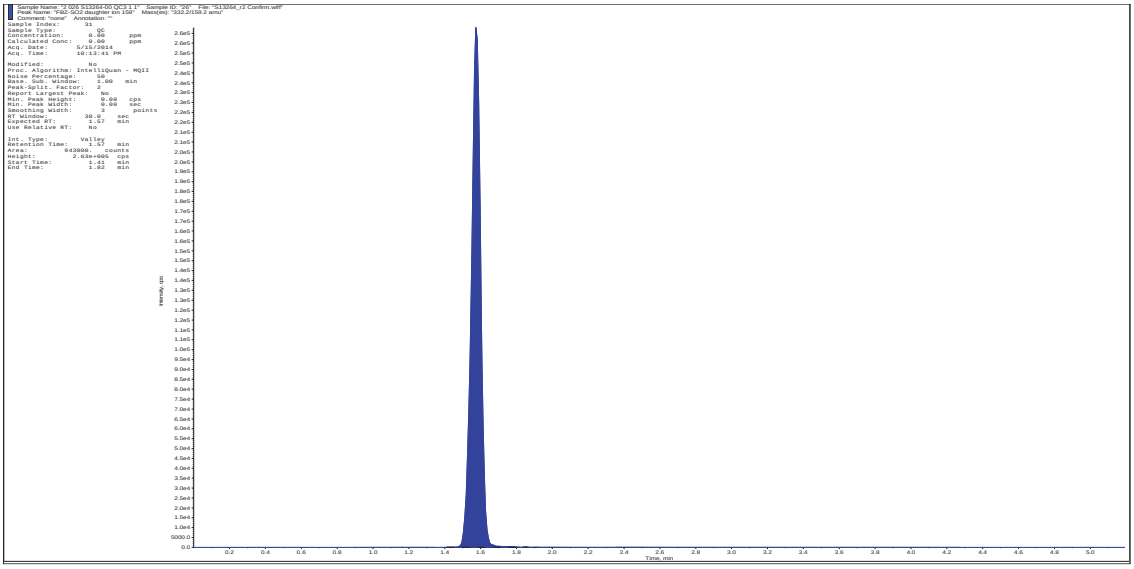


Product Ion 104

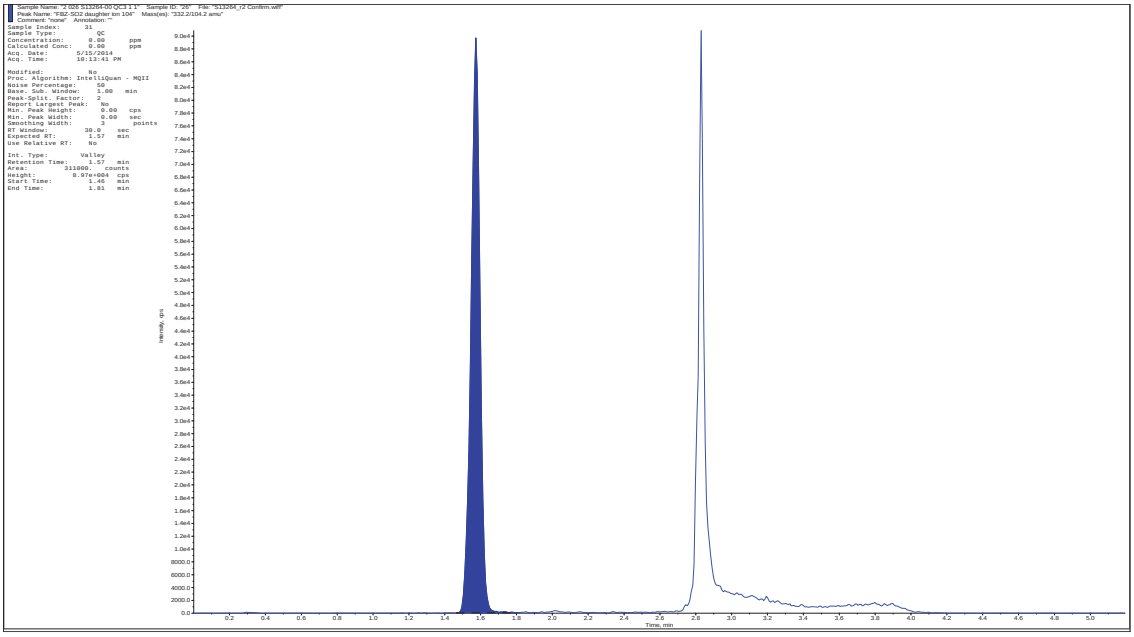


18.5.5 **Fortified Sampe at 6.4 ppm**

Product Ion 159

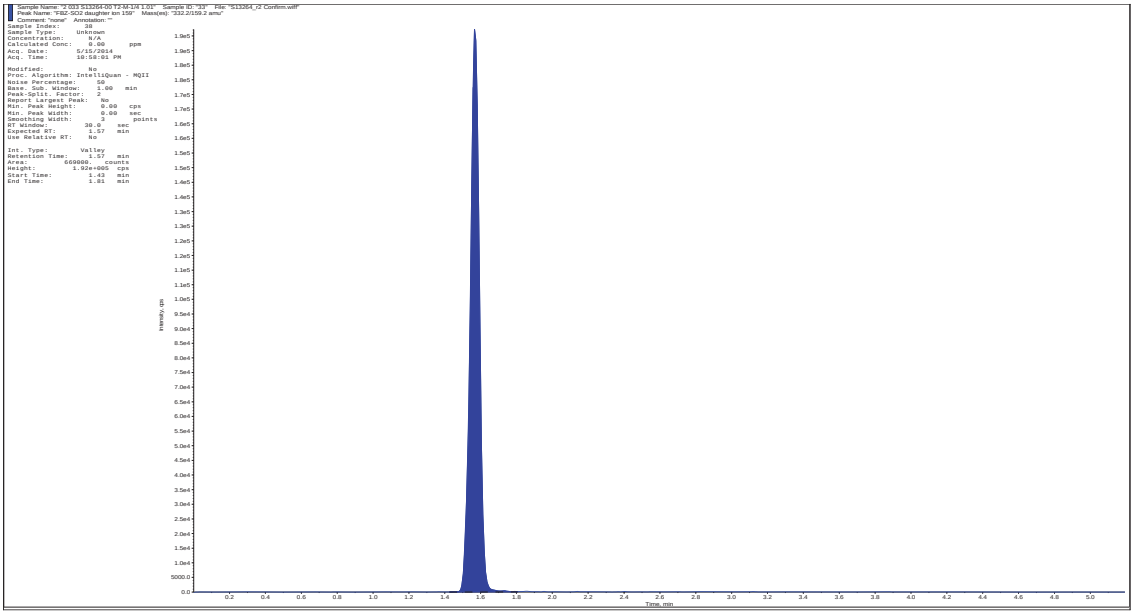


Product Ion 104

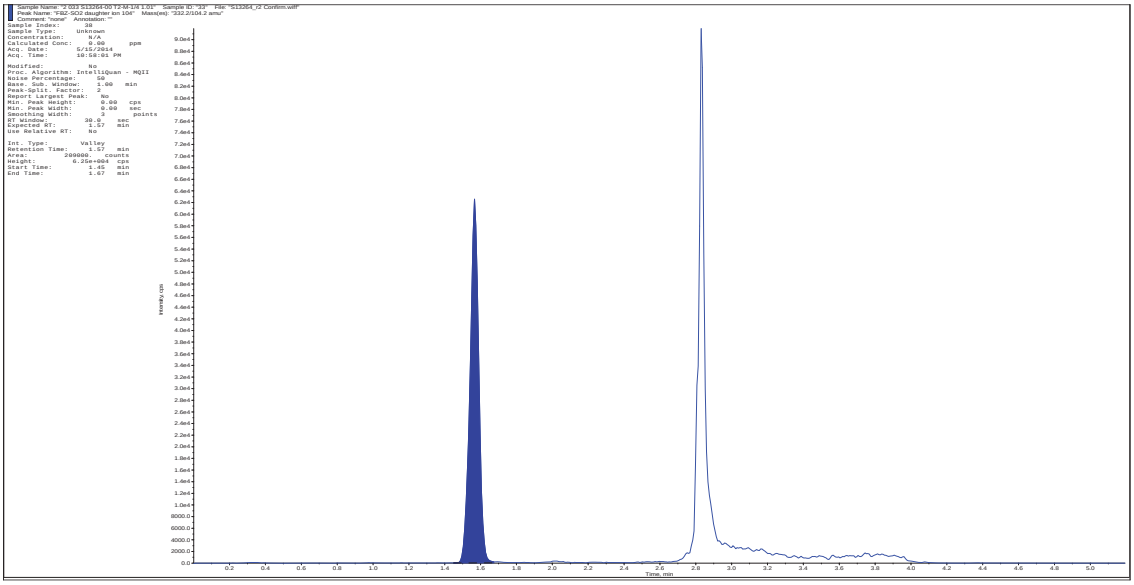


18.5.6 Incurred Treatment Group 2

Product Ion 159

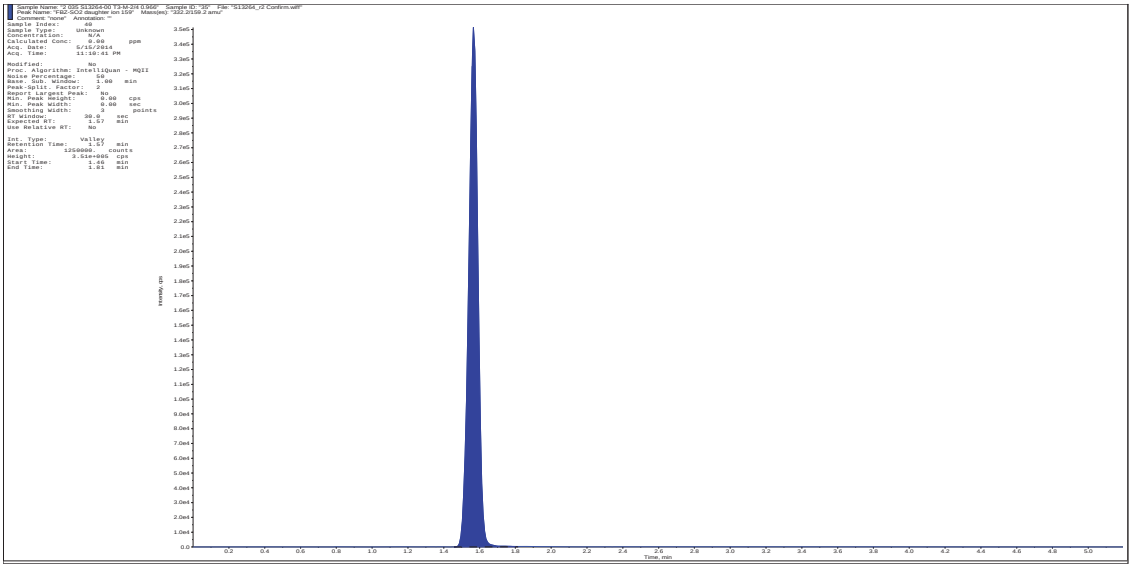


Product Ion 104

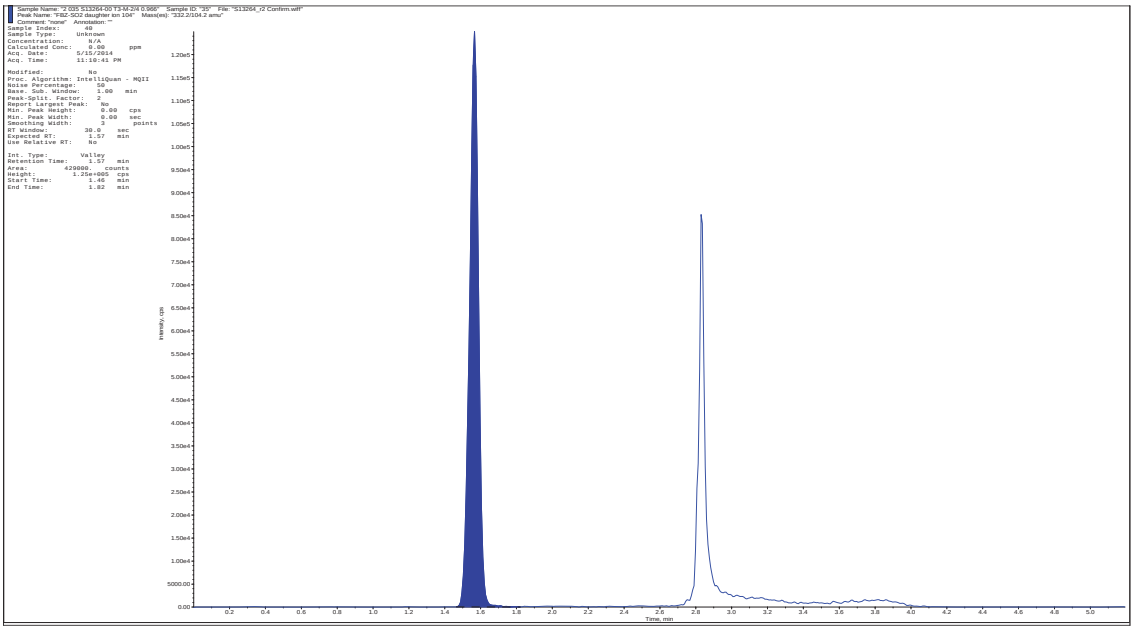


18.5.7 Incurred Treatment Group 3

Product Ion 159



Product Ion 104



19 DETERMINATIVE ANALYSIS RESULTS AND CHROMATOGRAMS DETERMINATIVE ANALYSIS

The data contained in Sections 19.1 and 19.2 were obtained during an inter-laboratory method transfer of the method.

19.1 Summary Determinative Results for Controls and QCs Analyzed in the Reference Laboratory

Summary Determinative Results for Controls and QCs						
Run ID	Sample ID	Calc Conc (ppm)	%Accuracy			%CV
			Nominal (ppm)	Individual	Average	
2	Double Blank-1	NA	NA	NA	NA	NA
	Double Blank-2	NA		NA		
3	Double Blank-3	NA		NA		
	Double Blank-4	NA		NA		
4	Double Blank-5	NA		NA		
	Double Blank-6	NA		NA		
2	Control Blank-1	-0.345*	NA	NA	NA	NA
	Control Blank-2	-0.348*		NA		
3	Control Blank-3	0.0323*		NA		
	Control Blank-4	-0.113*		NA		
4	Control Blank-5	-0.191*		NA		
	Control Blank-6	-0.208*		NA		
2	QC1-1	1.37	1.6	85.6	89.1	6.09
	QC1-2	1.32		82.5		
3	QC1-3	1.46		91.2		
	QC1-4	1.57		98.1		
4	QC1-5	1.44		90.0		
	QC1-6	1.39		86.9		
2	QC2-1	2.96	3.2	92.5	93.3	2.91
	QC2-2	3.04		95.0		
3	QC2-3	3.08		96.2		
	QC2-4	3.01		94.1		
4	QC2-5	3.00		93.7		
	QC2-6	2.83		88.4		
2	QC3-1	6.27	6.4	98.0	102 (97.2***)	12.4 (1.4***)
	QC3-2	8.16**		128**		
3	QC3-3	6.16		96.2		
	QC3-4	6.31		98.6		
4	QC3-5	6.30		97.7		
	QC3-6	6.09		95.3		
2	QC4-1	9.86	10.6	93.0	92.5	1.69
	QC4-2	9.72		91.7		
3	QC4-3	9.96		94.0		
	QC4-4	9.73		91.8		
4	QC4-5	9.98		94.2		
	QC4-6	9.55		90.1		

*Value is BLOQ (< 1 ppm); NA: Not Applicable
**The average percent accuracy and precision includes all 6 values
***The average percent accuracy and precision includes 5 values only; QC3-2 was excluded

19.2 Summary Determinative Results for Untreated and Incurred Samples

19.2.1 Summary of Determinative Method Concentration Data for Untreated and Incurred Samples Analyzed in the Reference Laboratory

Precision & Accuracy							
	QC Level	Conc. (ppm)	%CV	Mean %Accuracy	Incur Animal#	Conc (ppm)	%CV
Inter-Batch (n = 6)	QC1	1.60	6.09	89.1	Mean Assay Conc. (n=5)		
	QC2	3.20	2.91	93.3	FDA Sample1	BLOQ	N/A
	QC3	6.40	12.4*(1.4**)	102*(97.2**)	FDA Sample2	3.77	3.13
	QC4	10.6	1.69	92.5	FDA Sample3	8.12	3.37

*The average percent accuracy and precision included all 6 values
**The average percent accuracy and precision includes 5 values only; QC3-2 was excluded

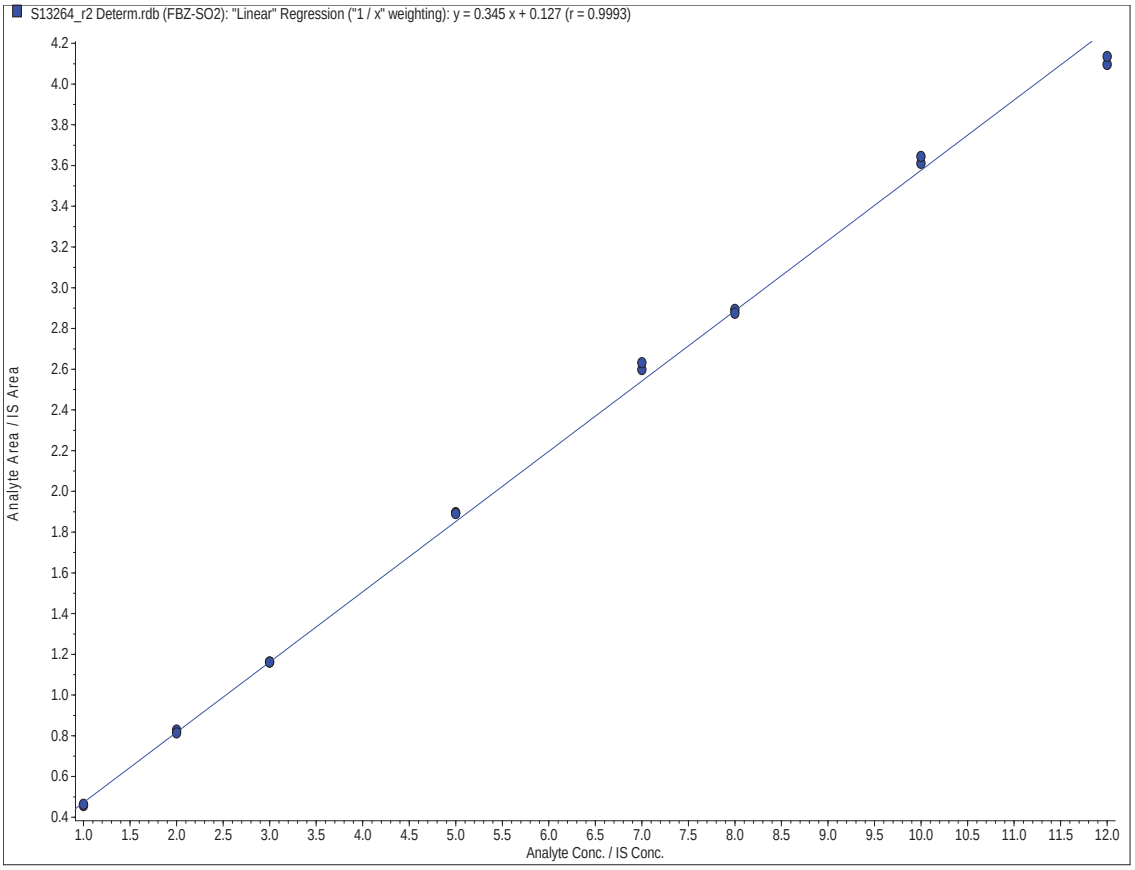
19.2.2 Summary of Determinative Method Concentration Data for Untreated and Incurred Samples Analyzed in Testing Laboratory A

Precision & Accuracy (Liver)							
	QC Level	Conc. (ppm)	%CV	Mean %Accuracy	Blinded Samples	Conc (ppm)	%CV
Inter-Batch (n = 6)	QC1	1.60	0.802	96.7	Mean Assay Conc. (n=5)		
	QC2	3.20	1.36	99.4	FDA Sample1	BLOQ	N/A
	QC3	6.40	1.49	100	FDA Sample2	4.22	0.723
	QC4	10.6	0.918	100	FDA Sample3	9.18	1.08

19.2.3 Summary of Determinative Method Concentration Data for Untreated and Incurred Samples Analyzed in Testing Laboratory B

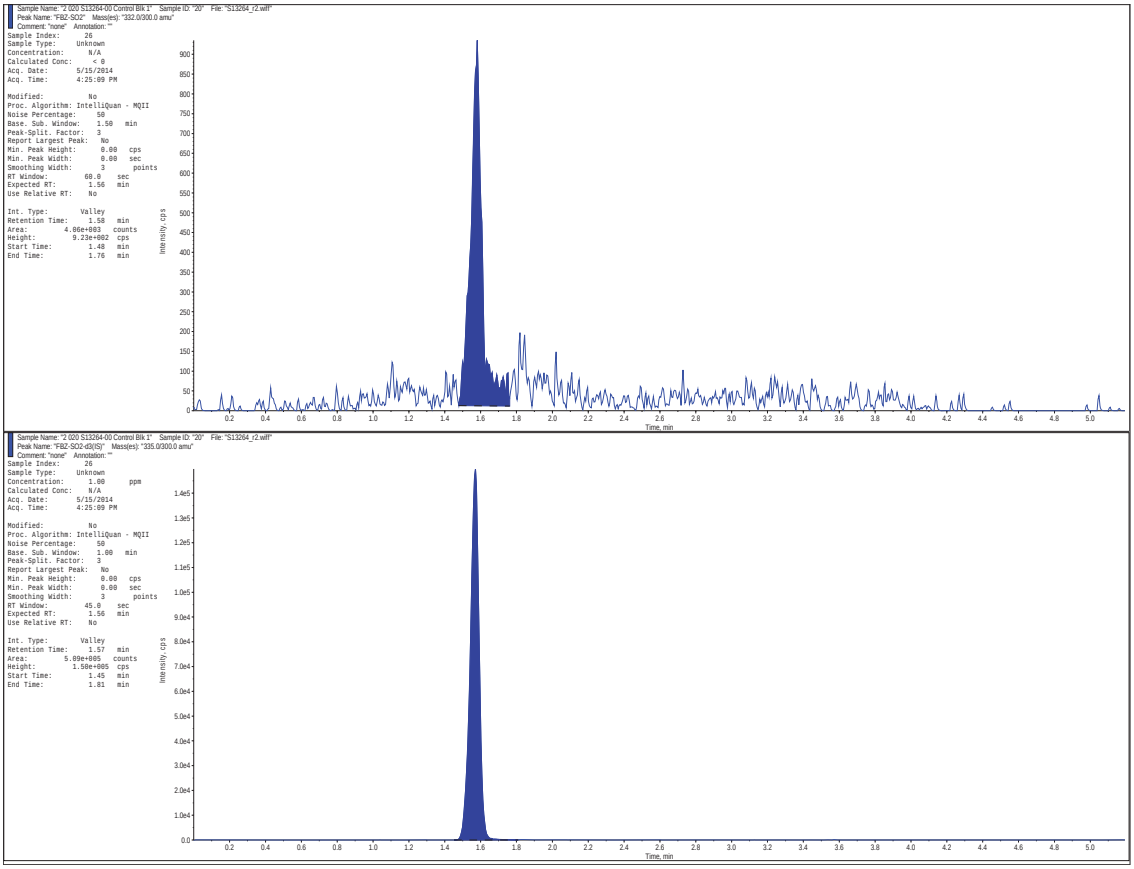
Precision & Accuracy (Liver)							
	QC Level	Conc. (ppm)	%CV	Mean %Accuracy	Blinded Samples	Conc (ppm)	%CV
Inter-Batch (n = 6)	QC1	1.60	2.97	91.2	Mean Assay Conc. (n=5)		
	QC2	3.20	3.02	96.6	FDA Sample1	BLOQ	N/A
	QC3	6.40	1.79	97.5	FDA Sample2	4.13	1.96
	QC4	10.6	1.81	97.7	FDA Sample3	9.16	0.51

19.3 Calibration Curve



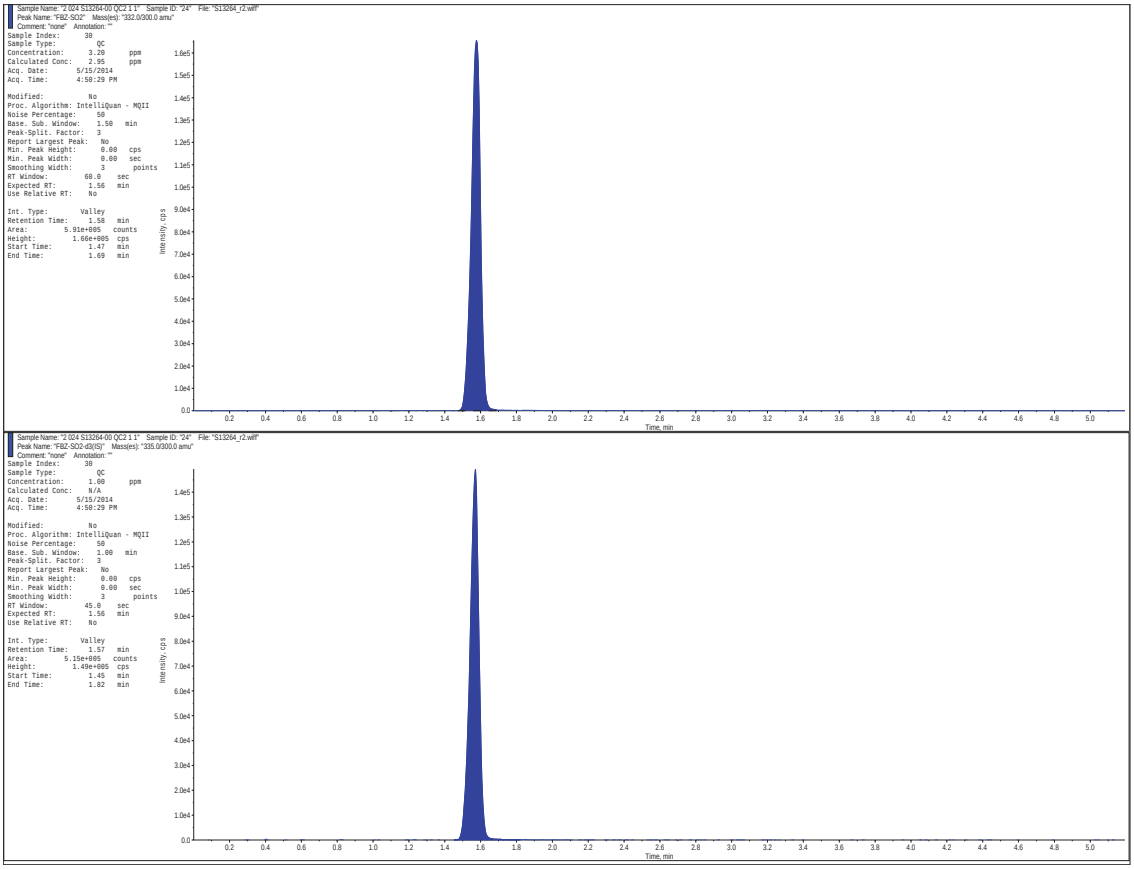
19.4 Control Blank (with IS at 3 ppm)

Top: Analyte
Bottom: IS

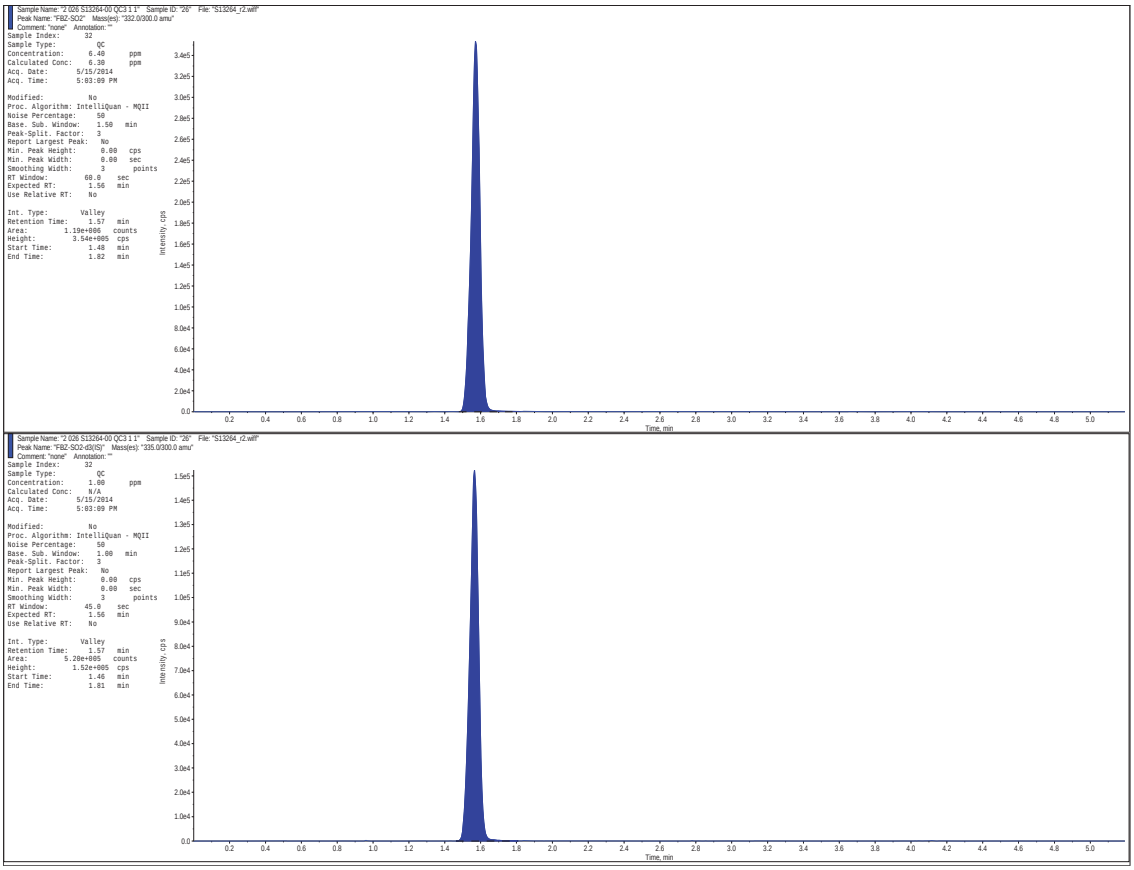


19.5 Fortified Sample (3.2 ppm)

Top: Analyte
Bottom: IS

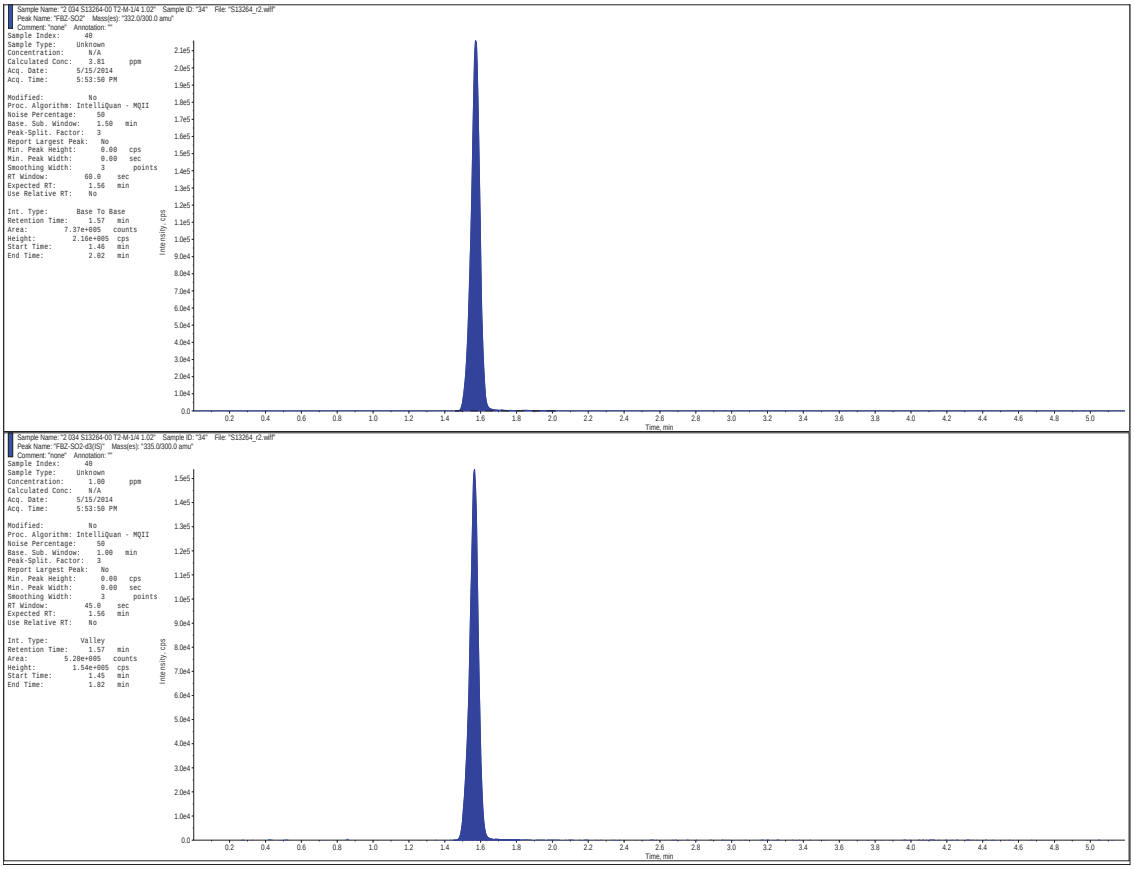


19.6 Fortified Sample (6.4 ppm)

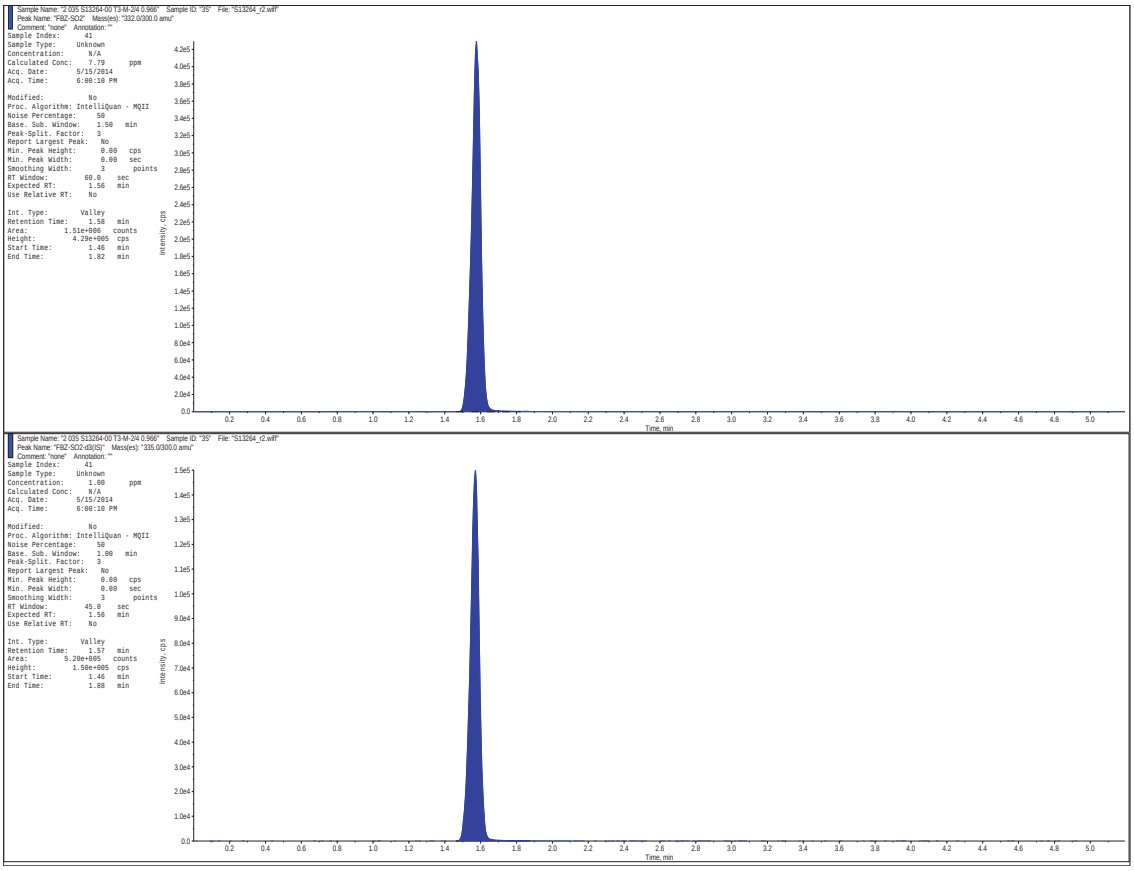


19.7 Incurred Sample Group 2

Top: Analyte
Bottom: IS



19.8 Incurred Sample Group 3



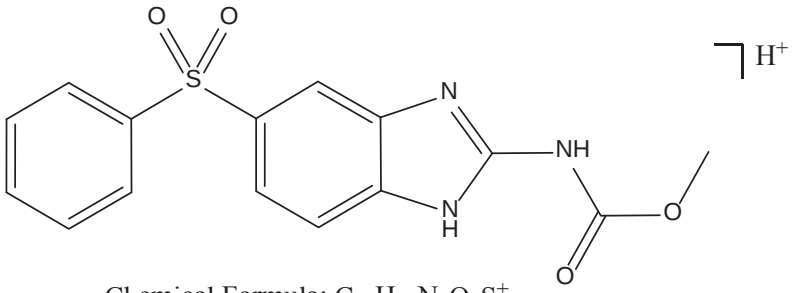
20 FRAGMENTATION REPORT FROM FT-ICR

A Bruker solariX™ 9.4-T Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometer with a quadrupole front end was used to conduct the experiments. The sample was introduced *via* Advion Triversa NanoMate™ robot and used as provided. A CAD (Collision-Activated Dissociation) experiment was performed in the collision cell portion of the Bruker FT-ICR instrument with argon as the collision gas, while the isolation occurred in the first quadrupole.

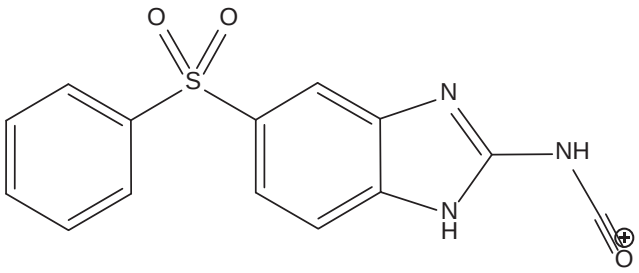
20.1 Proposed Fragmentation Pathways for Fenbendazole Sulfone at m/z 332

Proposed analyte product ions are 300, 159, and 104. The proposed product ion for determinative analysis is 300. The proposed product ions for confirmatory analysis are 159 and 104. The collision energy used was 40 eV.

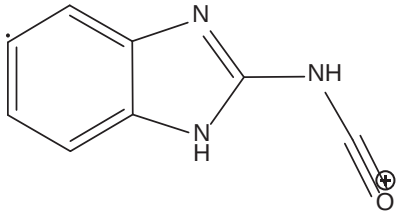
See following figure.



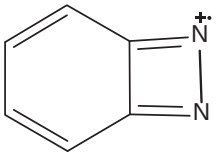
Chemical Formula: $C_{15}H_{14}N_3O_4S^+$
Exact Mass: 332.0700



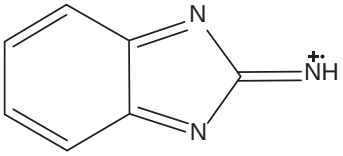
Chemical Formula: $C_{14}H_{10}N_3O_3S^+$
Exact Mass: 300.0437
error: 0.3 ppm



Chemical Formula: $C_8H_5N_3O^{\bullet+}$
Exact Mass: 159.0427
error: 0.1 ppm



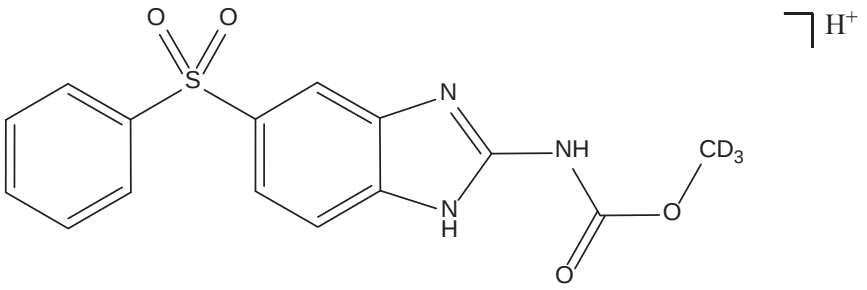
Chemical Formula: $C_6H_4N_2^{\bullet+}$
Exact Mass: 104.0369
error: 0.1 ppm



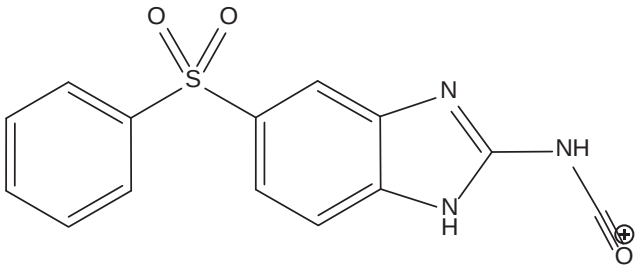
Chemical Formula: $C_7H_5N_3^{\bullet+}$
Exact Mass: 131.0478
error: 0.2 ppm

20.2 Proposed Fragmentation Pathways for Fenbendazole Sulfone-D_3 at m/z 335

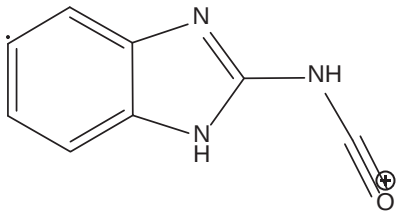
Proposed IS product ion is 300, for determinative analysis only. Internal standard is not used for confirmatory analysis. The collision energy used was 30 eV.



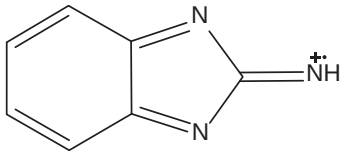
Chemical Formula: $C_{15}H_{11}D_3N_3O_4S^+$
Exact Mass: 335.0888



Chemical Formula: $C_{14}H_{10}N_3O_3S^+$
Exact Mass: 300.0437
error: 0.6 ppm



Chemical Formula: $C_8H_5N_3O^{\bullet+}$
Exact Mass: 159.0427
error: 0.3ppm



Chemical Formula: $C_7H_5N_3^{\bullet+}$
Exact Mass: 131.0478
error: 0.2 ppm

21 MATERIAL SAFETY DATA SHEETS (MSDS) FOR FENBENDAZOLE SULFONE AND FENBENDAZOLE SULFONE-D_3

21.1 MSDS for Fenbendazole Sulfone

SIGMA-ALDRICH sigma-aldrich.com

Material Safety Data Sheet

Version 5.0
Revision Date 03/22/2013
Print Date 11/26/2013

1. PRODUCT AND COMPANY IDENTIFICATION

Product name	:	Fenbendazole sulfone
Product Number	:	32544
Brand	:	Fluka
Supplier	:	Sigma-Aldrich 3050 Spruce Street SAINT LOUIS MO 63103 USA
Telephone	:	+1 800-325-5832
Fax	:	+1 800-325-5052
Emergency Phone # (For both supplier and manufacturer)	:	(314) 776-6555
Preparation Information	:	Sigma-Aldrich Corporation Product Safety - Americas Region 1-800-521-8956

2. HAZARDS IDENTIFICATION

Emergency Overview

OSHA Hazards
Harmful by ingestion., Skin sensitiser, Irritant

GHS Classification
Acute toxicity, Oral (Category 4)
Skin irritation (Category 2)
Skin sensitisation (Category 1)

GHS Label elements, including precautionary statements

Pictogram

Signal word: Warning

Hazard statement(s)

H302	Harmful if swallowed.
H315	Causes skin irritation.
H317	May cause an allergic skin reaction.

Precautionary statement(s)

P261	Avoid breathing dust/ fume/ gas/ mist/ vapours/ spray.
P264	Wash skin thoroughly after handling.
P270	Do not eat, drink or smoke when using this product.
P272	Contaminated work clothing should not be allowed out of the workplace.
P280	Wear protective gloves.
P301 + P312	IF SWALLOWED: Call a POISON CENTER or doctor/ physician if you feel unwell.
P302 + P352	IF ON SKIN: Wash with plenty of soap and water.
P321	Specific treatment (see supplemental first aid instructions on this label).
P330	Rinse mouth.
P333 + P313	If skin irritation or rash occurs: Get medical advice/ attention.
P362	Take off contaminated clothing and wash before reuse.
P501	Dispose of contents/ container to an approved waste disposal plant.

HMIS Classification

Health hazard:	2
Flammability:	0
Physical hazards:	0

NFPA Rating

Health hazard:	2
Fire:	0
Reactivity Hazard:	0

Potential Health Effects

Inhalation	May be harmful if inhaled. May cause respiratory tract irritation.
Skin	Harmful if absorbed through skin. May cause skin irritation.
Eyes	May cause eye irritation.
Ingestion	Harmful if swallowed.

3. COMPOSITION/INFORMATION ON INGREDIENTS

Synonyms : (5-Benzenesulfonyl-1H-benzoimidazol-2-yl)-carbamic acid methyl ester

Formula : $C_{15}H_{13}N_3O_4S$

Molecular Weight : 331.35 g/mol

Component		Concentration
Fenbendazole sulfone		
CAS-No.	54029-20-8	-

4. FIRST AID MEASURES

General advice
Consult a physician. Show this safety data sheet to the doctor in attendance.Move out of dangerous area.

If inhaled
If breathed in, move person into fresh air. If not breathing, give artificial respiration. Consult a physician.

In case of skin contact
Wash off with soap and plenty of water. Consult a physician.

In case of eye contact
Flush eyes with water as a precaution.

If swallowed
Never give anything by mouth to an unconscious person. Rinse mouth with water. Consult a physician.

5. FIREFIGHTING MEASURES

Suitable extinguishing media
Use water spray, alcohol-resistant foam, dry chemical or carbon dioxide.

Special protective equipment for firefighters
Wear self contained breathing apparatus for fire fighting if necessary.

Hazardous combustion products
Hazardous decomposition products formed under fire conditions. - Carbon oxides, nitrogen oxides (NOx), Sulphur oxides

6. ACCIDENTAL RELEASE MEASURES

Personal precautions
Use personal protective equipment. Avoid dust formation. Avoid breathing vapours, mist or gas. Ensure adequate ventilation. Avoid breathing dust.

Environmental precautions
Do not let product enter drains.

Methods and materials for containment and cleaning up
Pick up and arrange disposal without creating dust. Sweep up and shovel. Keep in suitable, closed containers for disposal.

7. HANDLING AND STORAGE

Precautions for safe handling
Avoid contact with skin and eyes. Avoid formation of dust and aerosols.
Provide appropriate exhaust ventilation at places where dust is formed. Normal measures for preventive fire protection.

Conditions for safe storage
Keep container tightly closed in a dry and well-ventilated place.

8. EXPOSURE CONTROLS/PERSONAL PROTECTION

Contains no substances with occupational exposure limit values.

Personal protective equipment

Respiratory protection
For nuisance exposures use type P95 (US) or type P1 (EU EN 143) particle respirator.For higher level protection use type OV/AG/P99 (US) or type ABEK-P2 (EU EN 143) respirator cartridges. Use respirators and components tested and approved under appropriate government standards such as NIOSH (US) or CEN (EU).

Hand protection
Handle with gloves. Gloves must be inspected prior to use. Use proper glove removal technique (without touching glove's outer surface) to avoid skin contact with this product. Dispose of contaminated gloves after use in accordance with applicable laws and good laboratory practices. Wash and dry hands.

Eye protection
Face shield and safety glasses Use equipment for eye protection tested and approved under appropriate government standards such as NIOSH (US) or EN 166(EU).

Skin and body protection
Complete suit protecting against chemicals, The type of protective equipment must be selected according to the concentration and amount of the dangerous substance at the specific workplace.

Hygiene measures
Handle in accordance with good industrial hygiene and safety practice. Wash hands before breaks and at the end of workday.

9. PHYSICAL AND CHEMICAL PROPERTIES

Appearance

Form	solid
Colour	colourless

Safety data

pH	no data available
Melting point/freezing point	> 320 °C (> 608 °F)
Boiling point	no data available
Flash point	no data available
Ignition temperature	no data available
Auto-ignition temperature	no data available
Lower explosion limit	no data available
Upper explosion limit	no data available
Vapour pressure	no data available

Density	no data available
Water solubility	no data available
Partition coefficient: n-octanol/water	log Pow: 2.0
Relative vapour density	no data available
Odour	odourless
Odour Threshold	no data available
Evapouration rate	no data available

10. STABILITY AND REACTIVITY

Chemical stability
Stable under recommended storage conditions.

Possibility of hazardous reactions
no data available

Conditions to avoid
no data available

Materials to avoid
Strong acids and strong bases, Strong oxidizing agents

Hazardous decomposition products
Hazardous decomposition products formed under fire conditions. - Carbon oxides, nitrogen oxides (NOx), Sulphur oxides
Other decomposition products - no data available

11. TOXICOLOGICAL INFORMATION

Acute toxicity

Oral LD50
no data available

Inhalation LC50
no data available

Dermal LD50
no data available

Other information on acute toxicity
no data available

Skin corrosion/irritation
no data available

Serious eye damage/eye irritation
no data available

Respiratory or skin sensitisation
no data available

Germ cell mutagenicity
no data available

Carcinogenicity

IARC:	No component of this product present at levels greater than or equal to 0.1% is identified as probable, possible or confirmed human carcinogen by IARC.
ACGIH:	No component of this product present at levels greater than or equal to 0.1% is identified as a carcinogen or potential carcinogen by ACGIH.
NTP:	No component of this product present at levels greater than or equal to 0.1% is identified as a

known or anticipated carcinogen by NTP.

OSHA: No component of this product present at levels greater than or equal to 0.1% is identified as a carcinogen or potential carcinogen by OSHA.

Reproductive toxicity

no data available

Teratogenicity

no data available

Specific target organ toxicity - single exposure (Globally Harmonized System)
no data available

Specific target organ toxicity - repeated exposure (Globally Harmonized System)
no data available

Aspiration hazard
no data available

Potential health effects

Inhalation	May be harmful if inhaled. May cause respiratory tract irritation.
Ingestion	Harmful if swallowed.
Skin	Harmful if absorbed through skin. May cause skin irritation.
Eyes	May cause eye irritation.

Signs and Symptoms of Exposure
To the best of our knowledge, the chemical, physical, and toxicological properties have not been thoroughly investigated.

Synergistic effects
no data available

Additional Information
RTECS: Not available

12. ECOLOGICAL INFORMATION

Toxicity

no data available

Persistence and degradability
no data available

Bioaccumulative potential
no data available

Mobility in soil
no data available

PBT and vPvB assessment
no data available

Other adverse effects

no data available

13. DISPOSAL CONSIDERATIONS

Product
Offer surplus and non-recyclable solutions to a licensed disposal company.

Contaminated packaging
Dispose of as unused product.

14. TRANSPORT INFORMATION

DOT (US)
Not dangerous goods

IMDG
Not dangerous goods

IATA
Not dangerous goods

15. REGULATORY INFORMATION

OSHA Hazards
Harmful by ingestion., Skin sensitiser, Irritant

SARA 302 Components
SARA 302: No chemicals in this material are subject to the reporting requirements of SARA Title III, Section 302.

SARA 313 Components
SARA 313: This material does not contain any chemical components with known CAS numbers that exceed the threshold (De Minimis) reporting levels established by SARA Title III, Section 313.

SARA 311/312 Hazards
Acute Health Hazard

Massachusetts Right To Know Components
No components are subject to the Massachusetts Right to Know Act.

Pennsylvania Right To Know Components

	CAS-No.	Revision Date
Fenbendazole sulfone	54029-20-8	

New Jersey Right To Know Components

	CAS-No.	Revision Date
Fenbendazole sulfone	54029-20-8	

California Prop. 65 Components
This product does not contain any chemicals known to State of California to cause cancer, birth defects, or any other reproductive harm.

16. OTHER INFORMATION

Further information

The above information is believed to be correct but does not purport to be all inclusive and shall be used only as a guide. The information in this document is based on the present state of our knowledge and is applicable to the product with regard to appropriate safety precautions. It does not represent any guarantee of the properties of the product. Sigma-Aldrich Corporation and its Affiliates shall not be held liable for any damage resulting from handling or from contact with the above product. See www.sigma-aldrich.com and/or the reverse side of invoice or packing slip for additional terms and conditions of sale.

21.2 MSDS for Fenbendazole Sulfone-D_3

SIGMA-ALDRICH sigma-aldrich.com

Material Safety Data Sheet

Version 5.0
Revision Date 01/26/2011
Print Date 11/22/2013

1. PRODUCT AND COMPANY IDENTIFICATION

Product name	:	Fenbendazole sulfone-d_3		
Product Number	:	32545		
Brand	:	Fluka		
Product Use	:	For laboratory research purposes.		
Supplier	:	Sigma-Aldrich 3050 Spruce Street SAINT LOUIS MO 63103 USA	Manufacturer :	Sigma-Aldrich Corporation 3050 Spruce St. St. Louis, Missouri 63103 USA
Telephone	:	+1 800-325-5832		
Fax	:	+1 800-325-5052		
Emergency Phone # (For both supplier and manufacturer)	:	(314) 776-6555		
Preparation Information	:	Sigma-Aldrich Corporation Product Safety - Americas Region 1-800-521-8956		

2. HAZARDS IDENTIFICATION

Emergency Overview

OSHA Hazards
Toxic by ingestion, Skin sensitiser, Irritant

GHS Classification
Acute toxicity, Oral (Category 4)
Skin irritation (Category 2)
Skin sensitization (Category 1)

GHS Label elements, including precautionary statements

Pictogram

Signal word Warning

Hazard statement(s)

H302	Harmful if swallowed.
H315	Causes skin irritation.
H317	May cause an allergic skin reaction.

Precautionary statement(s)

P280	Wear protective gloves.

HMIS Classification

Health hazard:	2
Flammability:	0
Physical hazards:	0

NFPA Rating

Health hazard:	2
Fire:	0
Reactivity Hazard:	0

Potential Health Effects

Inhalation	May be harmful if inhaled. Causes respiratory tract irritation.
Skin	May be harmful if absorbed through skin. Causes skin irritation.
Eyes	Causes eye irritation.
Ingestion	Toxic if swallowed.

3. COMPOSITION/INFORMATION ON INGREDIENTS

Synonyms : (5-Benzenesulfonyl-1H-benzoimidazol-2-yl)-carbamic acid methyl-D3 ester

Formula : $C_{15}D_3H_{10}N_3O_4S$ $C_{15}D_3H_{10}N_3O_4S$
Molecular Weight : 334.36 g/mol

CAS-No.	EC-No.	Index-No.	Concentration
Fenbendazole sulfone-d3 VETRANAL®			
1228182-49-7	-	-	-

4. FIRST AID MEASURES

General advice
Consult a physician. Show this safety data sheet to the doctor in attendance.Move out of dangerous area.

If inhaled
If breathed in, move person into fresh air. If not breathing, give artificial respiration. Consult a physician.

In case of skin contact
Wash off with soap and plenty of water. Consult a physician.

In case of eye contact
Flush eyes with water as a precaution.

If swallowed
Never give anything by mouth to an unconscious person. Rinse mouth with water. Consult a physician.

5. FIRE-FIGHTING MEASURES

Conditions of flammability
Not flammable or combustible.

Suitable extinguishing media
Use water spray, alcohol-resistant foam, dry chemical or carbon dioxide.

Special protective equipment for fire-fighters
Wear self contained breathing apparatus for fire fighting if necessary.

Hazardous combustion products
Hazardous decomposition products formed under fire conditions. - Carbon oxides, nitrogen oxides (NOx), Sulphur oxides

6. ACCIDENTAL RELEASE MEASURES

Personal precautions
Use personal protective equipment. Avoid dust formation. Avoid breathing vapors, mist or gas. Ensure adequate ventilation. Avoid breathing dust.

Environmental precautions
Do not let product enter drains.

Methods and materials for containment and cleaning up
Pick up and arrange disposal without creating dust. Sweep up and shovel. Keep in suitable, closed containers for disposal.

7. HANDLING AND STORAGE

Precautions for safe handling
Avoid contact with skin and eyes. Avoid formation of dust and aerosols.
Provide appropriate exhaust ventilation at places where dust is formed.

Conditions for safe storage
Keep container tightly closed in a dry and well-ventilated place.

8. EXPOSURE CONTROLS/PERSONAL PROTECTION

Contains no substances with occupational exposure limit values.

Personal protective equipment

Respiratory protection
For nuisance exposures use type P95 (US) or type P1 (EU EN 143) particle respirator.For higher level protection use type OV/AG/P99 (US) or type ABEK-P2 (EU EN 143) respirator cartridges. Use respirators and components tested and approved under appropriate government standards such as NIOSH (US) or CEN (EU).

Hand protection
Handle with gloves. Gloves must be inspected prior to use. Use proper glove removal technique (without touching glove's outer surface) to avoid skin contact with this product. Dispose of contaminated gloves after use in accordance with applicable laws and good laboratory practices. Wash and dry hands.

Eye protection
Face shield and safety glasses Use equipment for eye protection tested and approved under appropriate government standards such as NIOSH (US) or EN 166(EU).

Skin and body protection
Complete suit protecting against chemicals, The type of protective equipment must be selected according to the concentration and amount of the dangerous substance at the specific workplace.

Hygiene measures
Handle in accordance with good industrial hygiene and safety practice. Wash hands before breaks and at the end of workday.

9. PHYSICAL AND CHEMICAL PROPERTIES

Appearance

Form	solid
Colour	colourless

Safety data

pH	no data available
Melting/freezing point	> 310 °C (> 590 °F)
Boiling point	no data available
Flash point	no data available
Ignition temperature	no data available
Autoignition temperature	no data available
Lower explosion limit	no data available
Upper explosion limit	no data available
Vapour pressure	no data available
Density	no data available
Water solubility	no data available
Partition coefficient: n-octanol/water	no data available
Relative vapour density	no data available
Odour	odourless

Odour Threshold	no data available
Evaporation rate	no data available

10. STABILITY AND REACTIVITY

Chemical stability
Stable under recommended storage conditions.

Possibility of hazardous reactions
no data available

Conditions to avoid
no data available

Materials to avoid
Strong acids and strong bases, Strong oxidizing agents

Hazardous decomposition products
Hazardous decomposition products formed under fire conditions. - Carbon oxides, nitrogen oxides (NOx), Sulphur oxides
Other decomposition products - no data available

11. TOXICOLOGICAL INFORMATION

Acute toxicity

Oral LD50
no data available

Inhalation LC50
no data available

Dermal LD50
no data available

Other information on acute toxicity
no data available

Skin corrosion/irritation
no data available

Serious eye damage/eye irritation
no data available

Respiratory or skin sensitization
no data available

Germ cell mutagenicity
no data available

Carcinogenicity

IARC:	No component of this product present at levels greater than or equal to 0.1% is identified as probable, possible or confirmed human carcinogen by IARC.
ACGIH:	No component of this product present at levels greater than or equal to 0.1% is identified as a carcinogen or potential carcinogen by ACGIH.
NTP:	No component of this product present at levels greater than or equal to 0.1% is identified as a known or anticipated carcinogen by NTP.
OSHA:	No component of this product present at levels greater than or equal to 0.1% is identified as a carcinogen or potential carcinogen by OSHA.

Reproductive toxicity

no data available

Teratogenicity

no data available

Specific target organ toxicity - single exposure (Globally Harmonized System)
no data available

Specific target organ toxicity - repeated exposure (Globally Harmonized System)
no data available

Aspiration hazard
no data available

Potential health effects

Inhalation	May be harmful if inhaled. Causes respiratory tract irritation.
Ingestion	Toxic if swallowed.
Skin	May be harmful if absorbed through skin. Causes skin irritation.
Eyes	Causes eye irritation.

Signs and Symptoms of Exposure
To the best of our knowledge, the chemical, physical, and toxicological properties have not been thoroughly investigated.

Synergistic effects
no data available

Additional Information
RTECS: Not available

12. ECOLOGICAL INFORMATION

Toxicity

no data available

Persistence and degradability
no data available

Bioaccumulative potential
no data available

Mobility in soil
no data available

PBT and vPvB assessment
no data available

Other adverse effects

no data available

13. DISPOSAL CONSIDERATIONS

Product
Offer surplus and non-recyclable solutions to a licensed disposal company. Contact a licensed professional waste disposal service to dispose of this material. Dissolve or mix the material with a combustible solvent and burn in a chemical incinerator equipped with an afterburner and scrubber.

Contaminated packaging
Dispose of as unused product.

14. TRANSPORT INFORMATION

DOT (US)
Not dangerous goods

IMDG
Not dangerous goods

IATA
Not dangerous goods

15. REGULATORY INFORMATION

OSHA Hazards
Toxic by ingestion, Skin sensitiser, Irritant

DSL Status
This product contains the following components that are not on the Canadian DSL nor NDSL lists.

	CAS-No.
Fenbendazole sulfone-d3 VETRANAL®	1228182-49-7

SARA 302 Components
SARA 302: No chemicals in this material are subject to the reporting requirements of SARA Title III, Section 302.

SARA 313 Components
SARA 313: This material does not contain any chemical components with known CAS numbers that exceed the threshold (De Minimis) reporting levels established by SARA Title III, Section 313.

SARA 311/312 Hazards
Acute Health Hazard

Massachusetts Right To Know Components
No components are subject to the Massachusetts Right to Know Act.

Pennsylvania Right To Know Components

	CAS-No.	Revision Date
Fenbendazole sulfone-d3 VETRANAL®	1228182-49-7	

New Jersey Right To Know Components

	CAS-No.	Revision Date
Fenbendazole sulfone-d3 VETRANAL®	1228182-49-7	

California Prop. 65 Components
This product does not contain any chemicals known to State of California to cause cancer, birth defects, or any other reproductive harm.

16. OTHER INFORMATION

Further information

The above information is believed to be correct but does not purport to be all inclusive and shall be used only as a guide. The information in this document is based on the present state of our knowledge and is applicable to the product with regard to appropriate safety precautions. It does not represent any guarantee of the properties of the product. Sigma-Aldrich Co., shall not be held liable for any damage resulting from handling or from contact with the above product. See reverse side of invoice or packing slip for additional terms and conditions of sale.

22 OTHER VALIDATION DATA

22.1 Matrix Effect Data

At 1.0 ppm level, the mean matrix effects of the three replicates for the 6 individual lots of control chicken liver were from -21.8% to -11.7% for the analyte. At 9.0 ppm, the mean matrix effects of the three replicates for the 6 individual lots of control chicken liver were from -13.4% to -0.773% for the analyte. Refer to reference section 24.1.

22.2 Validation Experiments Conducted

The following experiments were conducted during method validation:

- Exhaustive extraction test
- System suitability test
- Limit of detection (LOD)
- Linearity (calibration curve) and range
- Precision and Accuracy (Core Runs)
- Lower limit of quantitation (LOQ) in Spiked Matrix
- Specificity / Selectivity
- Matrix effect & Interference compounds
- Extraction recovery
- Ruggedness/Robustness Testing
  - Using alternate analytical column (Thermo Acclaim 120, C-18, 3 µm, 2.1 x 50 mm)
  - Using alternate mobile phase by increasing mobile phase A (water with 0.1% formic acid) and decreasing Mobile Phase B (acetonitrile with 0.1% formic acid) by relatively 10% (gradient starting percentage changed to 75:25 from 70:30)
  - Using alternate LC-MS/MS platform (Thermo Vantage LC-MS/MS system equipped with an API source, Thermo Accela pump, and Open Access autosampler)
- Stability Studies
- Confirmatory analysis

Selectivity experiments in fortified matrix were evaluated in MAH Study Number N09-031-01: Report of the validation of a determinative and confirmatory procedure for the detection of fenbendazole sulfone in muscle and liver tissue of chicken. Refer to reference section 24.2.

23 CHANGES FROM PREVIOUS VERSIONS

Method Version	Effective Date	Changes to Previous Version	
		Changes	**Reason(s)**
V6.0	13-May-16	In title page, added new addresses for testing facility and sponsor	Clarification
		In section 11.2.2, added optimization note for confirmatory procedure and deleted the last sentence to avoid confusion	Per CVM recommendation
		In section 18.2, removed the conditional phase "depending on the instrument sensitivity"	Per CVM recommendation
		In section 19.2, added the concentration data from the respective testing laboratories	Per CVM recommendation
		In section 23, added method changes from V3.0 to V4.0, from V4.0 to V5.0, and from V5.0 to V6.0	To reflect the updates
		In section 24, added Intervet study SN S13264-00	Omitted
V5.0	20-Oct-14	In section 19, added section 19.1 for summary determinative results for controls and QCs. Added section 19.2 for summary determinative results for untreated and incurred samples	Omitted
V4.0	01-Oct-14	In section 17, added section 17.2 for IS monitoring and LC-MS/MS system cleanness	Clarification
		In section 18.5, added sections 18.5.5, 18.5.5, and 18.5.7 for more example chromatograms from confirmatory analysis	Clarification
		In section 19, added more example chromatograms from determinative analysis	Clarification
V3.0	21Apr14	In Table 5-2, deleted the alternative way to prepare mobile phases	To reflect the actual use during validation
		In Table 6-3, added the LC Quan version	For clarification
		In section 7.1.1, added a critical note for reference standard material weighing	For clarification
		In section 7.1.3, modified the preferred dilution for stock comparison and added a dilution scheme	For clarification
		In sections 7.2 and 7.5, added two standard levels (10.0 ppm and 12.0 ppm)	To increase the calibration range
		In sections 7.3 and 7.6, added one QC level (10.6 ppm)	To cover a wider matrix QC range
		In section 7.5, added equation and explanation for the conversion factor between solvent concentrations (in ng/mL) and tissue equivalent concentrations (in ppm)	Per CVM request and for clarification
		In section 9.2f, added autosampler vials as alternate containers for sample extracts	Per CVM request and for wider application
		In section 9.2f, modified the statement for the conversion factor	For clarification

		In section 12.3, modified integration process	Per CVM request
		In section 19.2, replaced the wrong chromatogram	For correction
		In section 22.1, added matrix effect data	Per CVM request
		In section 22.2, listed experiments conducted during method validation and details for ruggedness tests	For clarification
		In section 24, listed reference studies	For clarification
V2	02Dec13	Changed standard curve range from 1.0 – 10.0 ppm, to 1.0 – 8.0 ppm	To fit the new tolerance
		Changed fortification levels from 1.0, 3.0, 6.0, and 9.0 ppm, to 1.6, 3.2, and 6.4 ppm	To fit the new tolerance
		Changed working solution preparation in Table 7-2-1, 7-3-1, and 7-6-1 accordingly	Due to the change of standard curve and fortification levels
		In section 18, added confirmatory method	For clarification
		In section 16.5, added long term freezer storage stability data	For clarification
		In section 16.4, added freeze-thaw cycle stability data	For clarification
		In section 9.2, added a statement for the relationship between the tissue equivalent level and the liquid concentration	For clarification
		In section 5.3.2, added CAS number for FBZ-SO_2-D_3	For clarification
V1	30Sep13	NA	NA

24 REFERENCES

24.1 Intervet study SN S13010-00, Validation of LC-MS/MS Determinative and Confirmatory Procedures for the Detection of Fenbendazole Sulfone as a Marker for Fenbendazole in Broiler Chicken Liver. (b) (4), submitted to the CVM on 03-Oct-2013

24.2 Intervet study SN N09-070-01, Generation of Incurred Residue Samples of Fenbendazole Sulfone in Chicken Liver and Muscle Tissues Samples from Fenbendazole Administered Chickens and Determination of Long-Term Frozen Storage Stability of Incurred Fenbendazole Sulfone Residues and Fortified Fenbendazole Sulfone in Chicken Liver and Muscle Tissue. And Intervet study SN N09-031-01, Report of the validation of a determinative and confirmatory procedure for the detection of fenbendazole sulfone in muscle and liver tissue of chicken. (b) (4), submitted to the CVM on 31-Oct-2013

24.3 Intervet study SN S13264-00, Inter-Laboratory Method Trial for the Determination of the Marker Residue, Fenbendazole Sulfone in Chicken Liver. (b) (4), submitted to the CVM on 14-Nov-2014