

Bayer Method MM-002-W15-02

An Analytical Method for the Determination of Residues of Mesosulfuron-methyl (AE F130060) and its metabolites AE F160459, AE F160460, AE F140584, AE F147447 and AE F092944 in Water Using LC/MS/MS

1.0 SUMMARY

An analytical method was developed to determine the residues of mesosulfuron-methyl (AE F130060) and its metabolites AE F160459, AE F160460, AE F140584, AE F147447 and AE F092944 in water.

Residues of mesosulfuron-methyl (AE F130060) and its metabolites AE F140584 and AE F092944 are amended with an isotopic internal standard and analyzed by direct injection.

Residues of AE F147447, AE F160459 and AE F160460 are concentrated on a Strata-XL SPE cartridge and then analyzed. All compounds were analyzed by LC/MS/MS with quantification based on a comparison of peak areas with those of known standards.

The method limit of quantitation (LOQ) in water for all compounds is 0.25 ng/mL.

2.0 BACKGROUND

The analytical method presented in this report is designed to measure residues of mesosulfuron-methyl (AE F130060) and its metabolites AE F160459, AE F160460, AE F140584, AE F147447 and AE F092944 using isotopically labeled internal standards and LC/MS/MS detection.

3.0 APPARATUS

(Functional equivalents may be substituted)

- Various general laboratory glassware and utensils.
- MicroMan pipettors and tips
- TurboVap
- Thermo Scientific Aquasil C18, 100 mm X 3.0 mm, 3 µm particle size, (Part No: 77503-103030)
- Imtakt Scherzo SM-C18, 100 mm X 4.6 mm, 3 µm particle size, (Part No: SM004)
- ABSciex API 6500 chromatograph/mass spectrometer (LC/MS/MS) equipped with electrospray ionization (ESI) interface, Shimadzu HPLC pumps and a CTC PAL autosampler, and Analyst 1.6.2 data collection software (ABSciex)

4.0 REAGENTS AND CONSUMABLES

(Functional equivalents may be substituted)

- Water (HPLC grade)
- Acetonitrile (ACN, HPLC grade)
- Methanol (MeOH, HPLC grade)
- Ammonium formate (Fisher Part No. A639-500)
- Formic acid (LC/MS grade, Fisher Part No. A117-50)
- 2% formic acid in water; prepare by adding 10 mL of formic acid to 490 mL of water and mixing well.
- 9:1 Water/MeOH; prepare by adding 100 mL of MeOH to 900 mL of water and mixing well.
- 1:1 ACN/water; prepare by adding 500 mL of ACN to 500 mL of water and mixing well.
- 9:1 ACN/water; prepare by adding 900 mL of ACN to 100 mL of water and mixing well.
- Water/methanol (9:1, v:v) containing 10 mM Ammonium formate and 120 µL/L formic acid; prepare by adding 900 mL water, 100 mL MeOH, 0.63 g ammonium formate and 0.120 mL of formic acid to a 1 L graduated cylinder and mixing well.
- Water/methanol (1:9, v:v) containing 10 mM Ammonium formate and 120 µL/L formic acid; prepare by adding 100 mL water, 900 mL MeOH, 0.63 g ammonium formate and 0.120 mL of formic acid to a 1 L graduated cylinder and mixing well.
- 5 mM ammonium formate; prepare by adding 0.315 g ammonium formate to 1 L of water and mixing well.
- 0.2 M ammonium formate in water/methanol (1:1, v/v); prepare by adding 6.3 g of ammonium formate to 500 mL water and 500 mL methanol. Mix well.
- Vial (20 mL glass, Fisher Part No. 03-337-4)
- Strata-XL C18 SPE cartridges (200 mg, 6 mL; Phenomenex Part No. 8B-S043-FCH)
- Culture tubes (20 x 150 mm, Fisher Part No. 14-961-33)
- HPLC vials and caps (2-mL, National Scientific, Part Nos. C4011-5W and C4011-55)

5.0 PREPARATION OF STANDARD SOLUTIONS

Analytical standards of mesosulfuron-methyl (AE F130060) and its metabolites AE F160459, AE F160460, AE F140584, AE F147447 and AE F092944 and the isotopic internal standards AE F092944-d₆, 2-Carbomethoxybenzenesulfonamide-d₄, mesosulfuron-methyl-d₆ and AE F147447-¹³C₅ are needed. These standards may be obtained from Bayer CropScience, 2 T.W. Alexander Drive, Research Triangle Park, NC 27709. Additional details about these chemicals are given in [Appendix 1](#).

The toxicities of these chemicals have not been precisely determined. Thus, each chemical must be treated as a potential health hazard. Exposure to these chemicals should be reduced to the lowest reasonable level.

NOTE: The following procedure is an example description of how these standard solutions may be prepared. Alternate or additional standards of appropriate weight and volume may be prepared as needed.

Class "A" volumetric glassware or calibrated pipets should be used in the preparation of all analytical standards. All standard solutions should be stored in a refrigerator in glass containers when not in use. Solutions should be allowed to warm to room temperature prior to use. Corrections for standard purities should be applied when expressing standard concentrations.

5.1 Primary Stock Standard Solution

Prepare individual ~100 µg/mL stock solutions of mesosulfuron-methyl (AE F130060), AE F160459, AE F160460, AE F140584, AE F147447 and AE F092944. Standards used to prepare initial stock solutions should be weighed on an analytical balance capable of accurately weighing samples to ± 0.01 mg. Standards are typically provided in ~5.0 mg aliquots. The standards are quantitatively transferred to a 50 mL volumetric flask using acetonitrile for mesosulfuron-methyl, AE F140584, and AE F 92944 and 1:1 ACN/water for AE F160459, AE F160460, and AE F147447. Dilute to volume with the corresponding solvent and mix well. All stock solutions should be stored in a freezer.

Prepare a mixed stock solution containing 10 µg/mL of mesosulfuron-methyl (AE F130060), AE F140584 and AE F092944 by taking an appropriate volume (~5 mL) of each of the primary stock solutions and diluting to 50 mL with acetonitrile.

Prepare a mixed stock solution containing 10 µg/mL of AE F160459, AE F160460, and AE F147447 by taking an appropriate volume (~5 mL) of each of the primary stock solutions and diluting to 50 mL with acetonitrile.

NOTE: Corrections for standard purities should be applied when expressing standard concentrations.

5.2 Fortification Standard Solutions

1 µg/mL mixed solution

Transfer 5 mL of the 10 µg/mL mixed stock standard solution into a 50 mL volumetric flask. Dilute to volume with acetonitrile. Mix well.

100 ng/mL mixed solution

Transfer 5 mL of the 1 µg/mL mixed stock standard solution into a 50 mL volumetric flask. Dilute to volume with acetonitrile. Mix well.

5.3 Isotopic Internal Standard Solutions

Prepare individual ~100 µg/mL stock solutions of AE F092944-d₆, 2-Carbomethoxybenzenesulfonamide-d₄, mesosulfuron-methyl-d₆ and AE F147447-¹³C₅. Standards used to prepare initial stock solutions should be weighed on an analytical balance capable of accurately weighing samples to ± 0.01 mg. Standards are typically provided in ~2.5 mg aliquots. The standards are quantitatively transferred to a 25 mL volumetric flask using acetonitrile, and diluted to volume with acetonitrile. All stock solutions should be stored in a freezer.

Prepare a mixed 1 µg/mL internal standard solution containing a mixture of AE F092944-d₆, 2-Carbomethoxybenzenesulfonamide-d₄ and mesosulfuron-methyl-d₆ by taking an appropriate volume (~0.5 mL) of each of the stock internal standard solutions and diluting to 50 mL with acetonitrile.

Prepare a 1 µg/mL internal standard solution containing AE F147447-¹³C₅ by taking an appropriate volume (~0.5 mL) of the stock internal standard solution and diluting to 50 mL with acetonitrile.

5.4 Calibration Standard Solutions

5.4.1 Mesosulfuron-methyl, AE F092944 and AE F140584

Prepare working calibration solutions consisting of 0.1, 0.5, 1, 5, 10, 50, and 100 ppb of mesosulfuron-methyl (AE F130060), AE F140584, and AE F092944 by diluting to 25 mL with 9:1 Water/MeOH. Before bringing the calibration solutions to volume, add by pipet 0.25 mL of the 1 µg/mL mixed internal standard solution to each of the calibration solutions. (see Section 5.3 Isotopic Internal Standard Solutions)

Concentration of Standard Solution used for dilution (µg/mL)	Concentration of Internal Standard Solution used for dilution (µg/mL)	Aliquot Native mix Taken (mL)	Aliquot Internal Standard Taken (mL)	Concentration of Calibration Solution (ppb)	Concentration of Internal Standard (ppb)
10	1	0.250	0.25	100	10
10	1	0.125	0.25	50	10
1	1	0.250	0.25	10	10
1	1	0.125	0.25	5	10
0.1	1	0.250	0.25	1	10
0.1	1	0.125	0.25	0.5	10
0.1	1	0.025	0.25	0.1	10

Further calibration solutions may be prepared as needed, depending on the analytical range for the samples. At least six calibration standards are needed.

5.4.2 AE F147447, AE F160459 and AE F160460

Prepare working calibration solutions consisting of 1, 5, 10, 25, 50 and 100 ppb of AE F160459, AE F160460 and AE F147447 by diluting to 25 mL with 9:1 Water/MeOH. Before bringing the calibration solutions to volume, add by pipet 2.5 mL of the 1 µg/mL AE F147447 internal standard solution to each of the calibration solutions. (see Section 5.3 Isotopic Internal Standard Solutions).

Concentration of Standard Solution used for dilution (µg/mL)	Concentration of Internal Standard Solution used for dilution (µg /mL)	Aliquot Native mix Taken (mL)	Aliquot Internal Standard Taken (mL)	Dilution Volume (mL)	Concentration of Calibration Solution (ng/mL)	Concentration of Internal Standard (ng/mL)
10	1	0.250	2.5	25	100	100
10	1	0.125	2.5	25	50	100
1	1	0.625	2.5	25	25	100
1	1	0.250	2.5	25	10	100
1	1	0.125	2.5	25	5	100
0.1	1	0.250	2.5	25	1	100

Further calibration solutions may be prepared as needed, depending on the analytical range for the samples. At least six calibration standards are needed. Fresh calibration solutions should be prepared weekly.

6.0 PROCEDURES**6.1 Water extraction for Mesosulfuron-methyl, AE F092944 and AE F140584**

Appendix 2A shows the analytical scheme for the extraction of mesosulfuron-methyl (AE F130060), AE F092944 and AE F140584 in water. The detailed stepwise procedure is as follows:

1. Place 10 mL of water into a 20 mL glass vial or other suitable container.
2. Fortify the recovery samples at the desired fortification level with the appropriate mixed standard solution (see Section 5.2 Fortification Standard Solutions).
3. Add 0.1 mL of the 1 µg/mL mixed internal standard solution to each sample. Mix well.
4. Place an aliquot into an hplc vial for analysis by LC/MS/MS using the conditions described in Section 7.2.1.

6.2 Water extraction for AE F147447, AE F160459 and AE F160460

Appendix 2B shows the analytical scheme for the extraction of AE F147447, AE F160459, and AE F160460 in water. The detailed stepwise procedure is as follows:

1. Place 10 mL of water into a 20 mL glass vial or other suitable container.
2. Fortify the recovery samples at the desired fortification level with the appropriate mixed standard solution (see Section 5.2 Fortification Standard Solutions).
3. Add 0.05 mL of the 1 µg/mL internal standard solution to each sample.
4. Add 0.2 mL of formic acid to each sample. Mix well.
5. Apply to a preconditioned Strata-XL SPE cartridge (pre-condition with one cartridge volume of methanol followed by one cartridge volume of 2% formic acid in water). Do not use vacuum to pull sample through cartridge.
6. Rinse vial with 1 mL of 2% formic acid in water and add to cartridge. Do not use vacuum to pull solvent through cartridge.
7. Elute samples into a clean culture tube with 2 mL of 9:1 ACN/water. Do not use vacuum to pull solvent through cartridge. Vacuum may be used to pull remaining solvent from cartridge.
8. Evaporate sample to dryness using a TurboVap at ~50 °C.
9. Add 0.5 mL of 9:1 Water/MeOH to each sample and vortex to dissolve.
10. Place an aliquot into an hplc vial for analysis by LC/MS/MS using the conditions described in Section 7.2.2.

7.0 ANALYSIS BY LC/MS/MS

7.1 Analytical Procedure

- Step 1. Using the recommended procedures listed below, analyze an aliquot of each of the calibration standard solutions (if necessary, additional standard solutions).
- Step 2. Analyze an aliquot of each of the analytical samples.
Note: Up to 20 sample analyses can be made after the analysis of the standard solutions. In the case of over 20 samples, extra standard solutions could be added between sample analyses.
- Step 3. Again, analyze an aliquot of each of the calibration standard solutions (and, if necessary, additional standard solutions).
- Step 4. When necessary, analyze additional samples and standard solutions. Always finish the procedure with the analysis of a set of standard solutions.

7.2 HPLC Conditions

Note: The analyst must optimize chromatographic conditions to obtain satisfactory chromatography.

7.2.1 HPLC Conditions for Mesosulfuron-methyl, AE F092944 and AE F140584

The following recommended conditions were used on an AB Sciex 6500 instrument equipped with Shimadzu HPLC pumps.

Mobile Phase A: Water/methanol (9:1, v:v) containing 10 mM Ammonium formate and 120 μ L/L formic acid
Mobile Phase B: Water/methanol (1:9, v:v) containing 10 mM Ammonium formate and 120 μ L/L formic acid

HPLC column: Thermo Scientific Aquasil C18, 100 mm X 3 mm, 3 μ m
Column Temp: 50 $^{\circ}$ C
Injection volume: 50 μ L (Adjust for LC/MS/MS system being used)

Time (min)	Mobile Phase A	Mobile Phase B	Flow rate μ L/min
0.0	95	5	800
0.5	95	5	800
4.0	0	100	800
5.0	0	100	800
5.1	95	5	800

Approximate retention times are listed below:

AE F140584 2.3 min
AE F092944 3.1 min
Mesosulfuron-methyl 3.8 min

7.2.2 HPLC Conditions for AE F147447, AE F160459 and AE F160460

The following recommended conditions were used on an AB Sciex 6500 instrument equipped with Shimadzu HPLC pumps.

Mobile Phase A: 5 mM ammonium formate in water
 Mobile Phase B: 0.2 M ammonium formate in water/Methanol (1:1, v/v)

HPLC column: Intakt Scherzo SM-C18, 100 mm X 4.6 mm, 3 µm
 Column Temp: 50 °C
 Injection volume: 50 µL (Adjust for LC/MS/MS system being used)

Time (min)	Mobile Phase A	Mobile Phase B	Flow rate µL/min
0.0	50	50	1000
0.5	50	50	1000
4.5	0	100	1000
5.5	0	100	1000
5.6	50	50	1000

Approximate retention times are listed below:

AE F147447 2.0 min
 AE F160459 4.0 min
 AE F160460 4.2 min

7.3 Mass Spectrometer Conditions

Note: The analyst must optimize the mass spectrometer conditions to obtain satisfactory system response. The following conditions were used on an AB Sciex 6500 instrument.

7.3.1 Mesosulfuron-methyl, AE F092944 and AE F140584**Period 1 (0-2.8 min):****Period 1 Experiment 1****Negative ion mode for AE F140584**

CUR: Curtain Gas 50
 CAD: Collision Gas 6
 GS1: Ion Source Gas 1 50
 GS2: Ion Source Gas 2 50
 TEM: Source Temp. 400°C
 IS: Ion Transfer Voltage -4500

Period 1 Experiment 2**Positive ion mode for 2-Carbomethoxybenzenesulfonamide IS**

CUR: Curtain Gas	50
CAD: Collision Gas	6
GS1: Ion Source Gas 1	50
GS2: Ion Source Gas 2	50
TEM: Source Temp.	400°C
IS: Ion Transfer Voltage	4500

Period 2 (2.8-6.0 min): Positive ion mode for AE F092944 and Mesosulfuron-methyl

CUR: Curtain Gas	50
CAD: Collision Gas	6
GS1: Ion Source Gas 1	50
GS2: Ion Source Gas 2	50
TEM: Source Temp.	400°C
IS: Ion Transfer Voltage	5500

7.3.2 AE F147447, AE F160459 and AE F160460**Period 1 (0-3.0 min):****Negative ion mode for AE F147447**

CUR: Curtain Gas	50
CAD: Collision Gas	6
GS1: Ion Source Gas 1	50
GS2: Ion Source Gas 2	50
TEM: Source Temp.	300°C
IS: Ion Transfer Voltage	-4500

Period 2 (3.0-6.0 min): Positive ion mode for AE F160460 and AE F160459

CUR: Curtain Gas	50
CAD: Collision Gas	6
GS1: Ion Source Gas 1	50
GS2: Ion Source Gas 2	50
TEM: Source Temp.	300°C
IS: Ion Transfer Voltage	5500

7.4 Mass Spectrometer Data Collection

For tuning the instrument, use a 1 ug/mL standard solution prepared in mobile phase B.

7.4.1 Mesosulfuron-methyl, AE F092944 and AE F140584

The daughter ions used in this method were chosen due to their optimum sensitivity on the instrument used for this study. **The analyst must optimize the mass spectrometer data collection to obtain satisfactory system response.** The following recommended ion transitions and conditions were example conditions used on an AB Sciex 6500 instrument:

Analyte Name	Polarity	Q1 Mass (amu)	Q3 Mass (amu)	Dwell (msec)	DP	EP	CE	CXP
AE F140584	-	320.9	289.0	50	60	10	28	10
AE F140584 Confirmatory	-	320.9	209.0	50	60	10	44	10
2-Carbomethoxy benzenesulfonamide IS	+	219.9	203.0	50	1	10	11	10
AE F092944	+	156.2	100.0	50	60	10	23	6
AE F092944 IS	+	162.2	103.0	50	60	10	23	6
AE F092944 Confirmatory	+	156.2	124.0	50	60	10	25	6
Mesosulfuron-methyl	+	504.2	181.9	50	60	10	31	6
Mesosulfuron-methyl IS	+	510.2	187.9	50	60	10	31	6
Mesosulfuron-methyl confirmatory	+	504.2	83.0	50	60	10	83	6

As there is no internal standard available for AE F140584, the 2-Carbomethoxy benzenesulfonamide internal standard is used for quantifying this compound.

7.4.2 AE F147447, AE F160459 and AE F160460

The daughter ions used in this method were chosen due to their optimum sensitivity on the instrument used for this study. **The analyst must optimize the mass spectrometer data collection to obtain satisfactory system response.** The following recommended ion transitions and conditions were example conditions used on an AB Sciex 6500 instrument:

Analyte Name	Polarity	Q1 Mass (amu)	Q3 Mass (amu)	Dwell (msec)	DP	EP	CE	CXP
AE F147447	-	288.8	209.0	50	60	10	40	16
AE F147447 Confirmatory	-	288.8	181.0	50	60	10	54	16
AE F147447 IS	-	294.8	210.0	50	60	10	40	16
AE F160460	+	476.0	168.0	50	60	10	29	6
AE F160460 Confirmatory	+	476.0	142.1	50	60	10	31	6
AE F160459	+	490.0	168.0	50	60	10	29	6
AE F160459 Confirmatory	+	490.0	100.0	50	60	10	73	6

8.0 CALCULATION OF RESULTS

The example calculation displayed below was used by the laboratory developing this method. Alternate calculation procedures appropriate to the reporting requirements may be substituted.

Residue concentrations were determined using calibration curves which were generated after each analysis using Analyst software (Version 1.6.2) using linear regression with 1/x weighting.

The standards were fit to the linear equation:

$$Y = MX + B \text{ with } 1/x \text{ weighting.}$$

where: X is the concentration of the reference standard in ng/mL

M is the calibration line slope

B is the calibration line intercept

Y is the native peak area or native peak area : isotopic peak area ratio

After regression coefficients were calculated, the residue in ng/g was determined using the following equation,

$$\text{Residue found (ng/mL)} = \frac{(Y-B) \times D}{M}$$

For Mesosulfuron-methyl, AE F092944 and AE F140584: Dilution Factor (D) = 1

For AE F147447, AE F160459 and AE F160460: Dilution Factor (D) = 0.05

After regression coefficients were calculated, the residue in ng/mL was determined. The ng/mL of residue in the sample was calculated using the following equation,

$$\text{Residue found (ng/mL)} = (Y-B) / M$$

Analyst software was used to calculate the amount of mesosulfuron-methyl (AE F130060), AE F160459, AE F160460, AE F140584, AE F147447 and AE F092944 in ng/mL for each sample and the percent recovery for the spiked samples.

8.1 Fortification Experiments

Note: Fortification experiments may be performed as needed to monitor method efficiency and reproducibility, but are not required when analysis of samples is performed for tolerance enforcement. Fortification experiments are intended to be used for data collection methods or establishing and validating method efficiency.

With each sample set, analyze an untreated control sample and one or more fortified control samples. Calculate recoveries using the following equation:

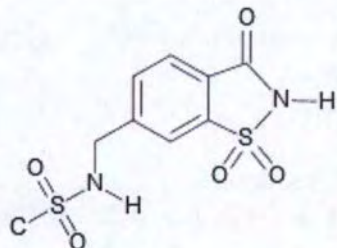
$$\text{Recovery (\%)} = \frac{(R - S)}{T} \times 100$$

Where: R = ppb of target analyte found in fortified sample
S = ppb of target analyte found in control sample, real or apparent
T = theoretical ppb in fortified sample

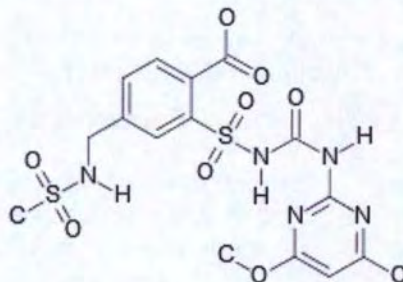
Recoveries are determined by analyzing fortified control samples alone or in conjunction with a sample set. Samples may be fortified prior to extraction at the LOQ or other appropriate level with fortification solutions. Calculate the final residue for the control (S) and fortified control (R) samples.

Appendix 1 Test and Reference Substances

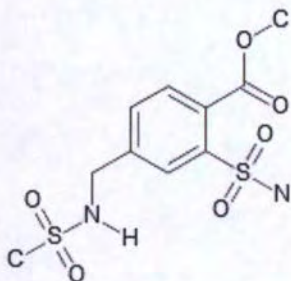
The toxicities of these chemicals have not been precisely determined. Thus, each chemical must be treated as a potential health hazard. Exposure to these chemicals should be reduced to the lowest reasonable level.

**AE F147447**

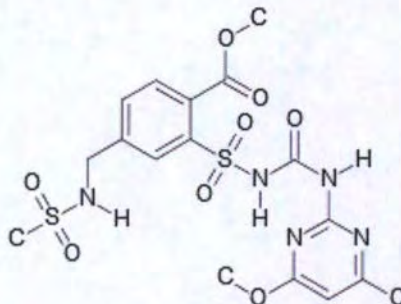
N-[(2,3-Dihydro-1,1-dioxido-3-oxo-1,2-benzisothiazol-6-yl)methyl]methanesulfonamide

**AE F160460**

2-[[[(4-Hydroxy-6-methoxy-2-pyrimidinyl)amino]carbonyl]amino]sulfonyl]-4-[[methylsulfonyl]amino]methyl]benzoic acid

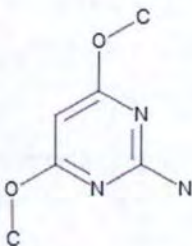
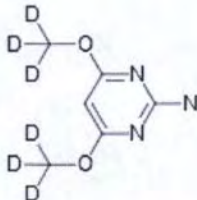
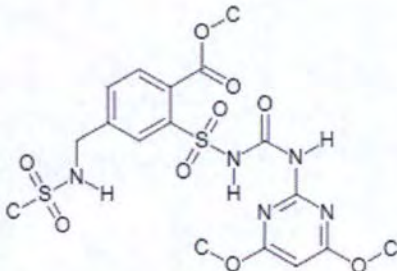
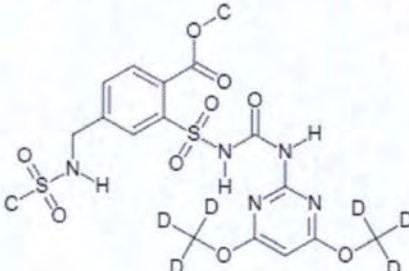
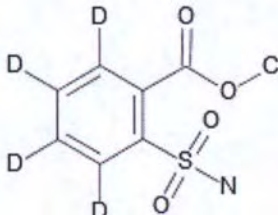
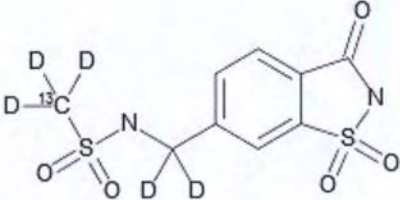
**AE F140584**

Methyl 2-(aminosulfonyl)-4-[[[(methylsulfonyl)amino]methyl]benzoate

**AE F160459**

Methyl 2-[[[(4-hydroxy-6-methoxy-2-pyrimidinyl)amino]carbonyl]amino]sulfonyl]-4-[[methylsulfonyl]amino]methyl]benzoate

Appendix 1 Test and Reference Substances (continued)

 <p>AE F092944 4,6-Dimethoxy-2-pyrimidinamine</p>	 <p>AE F092944-d₆ 4,6-Di(methoxy-d₃)-2-pyrimidinamine</p>
 <p>Mesosulfuron-methyl (AE F130060) Methyl 2-[[[(4,6-dimethoxy-2-pyrimidinyl)amino]carbonyl]amino]sulfonyl]-4-[[[(methylsulfonyl)amino]methyl]benzoate</p>	 <p>Mesosulfuron-methyl -d₆ Methyl 2-[[[(4,6-dimethoxy-d₆-2-pyrimidinyl)amino]carbonyl]amino]sulfonyl]-4-[[[(methylsulfonyl)amino]methyl]benzoate</p>
 <p>2-Carbomethoxybenzenesulfonamide-d₄ Methyl 6-(aminosulfonyl)benzoate-d₄</p>	 <p>AE F147447-¹³C,d₅ 1,1,1-trideuterio-N-[dideuterio-(1,1,3-trioxo-1,2-benzothiazol-6-yl)methyl]methanesulfonamide</p>

Appendix 2A. Extraction Scheme for Water Samples (Mesosulfuron-methyl, AE F092944 and AE F140584)

Place 10 mL of water into a suitable glass vial



Add 0.1 mL of the 1 µg/mL internal standard solution. Mix well.



Transfer to vial for LC/MS/MS analysis

Appendix 2B.

Extraction Scheme for Water Samples (AE F147447, AE F160459 and AE F160460)

Place 10 mL of water into a suitable glass vial



Add 0.05 mL of internal standard solution.



Add 0.2 mL of formic acid. Mix well.



Apply to pre-conditioned Strata-XL cartridge



Rinse vial and cartridge with 1 mL of 2% formic acid in water



Elute cartridge with 2 mL of 9:1 ACN/water



Evaporate sample to dryness



Dissolve in 0.5 mL of 9:1 water/methanol



Transfer to vial for LC/MS/MS analysis

Appendix 6 Revision History

Method Number	Revision	Description
MM-002-W15-01	01	Completed on development of method
MM-002-W15-02	02	Added AE F147447 internal standard to method