CONDUCT OF THE STUDY

The study was conducted at the Ricerca Biosciences, LLC AgChem Product Development Department Laboratories according to the Ricerca Biosciences, LLC protocol, Document Number 034249-0, "Independent Laboratory Validation (ILV) Study of 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" (Appendix E).

Personnel involved with the study were:

E. Klosi	Scientist
C. Reed	Associate Scientist I

INTRODUCTION

This protocol describes the experiments for an Independent Laboratory Validation (ILV) of Bayer Method MM-002-W15-01, 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].

OBJECTIVE/PURPOSE

The United States Environmental Protection Agency (USEPA) published a data reporting guideline in the Federal Register on April 19, 1995 for Environmental Chemistry Methods (ECM). It included a requirement for registrants to validate methods at an independent laboratory prior to sending them to the Office of Pesticide Programs. The purpose of this study is to perform an independent laboratory validation (ILV) of "Independent Laboratory Validation (ILV) Study of 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", Bayer method: MM-002-W15-01.

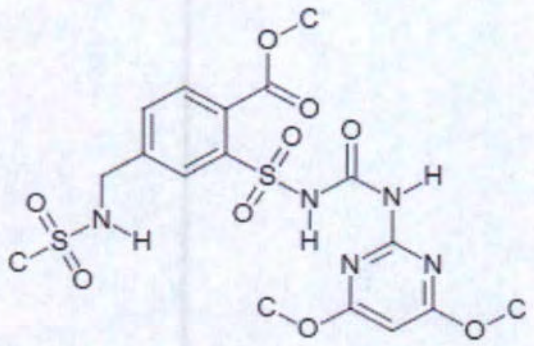
METHODS AND MATERIALS

TEST SUBSTANCE

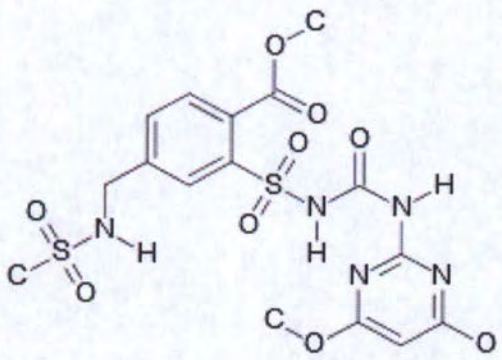
The sponsor supplied the test substances, Mesosulfuron-methyl (AE F130060) and its metabolites AE F160459, AE F160460, AE F140584, AE F147447 and AE F092944.

The test substances were stored frozen. Information concerning the test substance including purity is provided as follows:

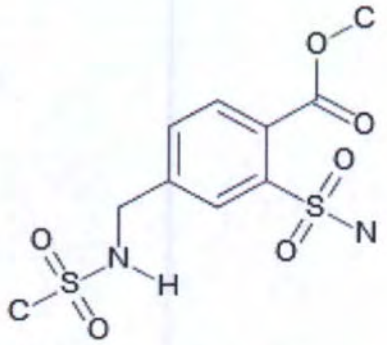
- **Mesosulfuron-methyl**



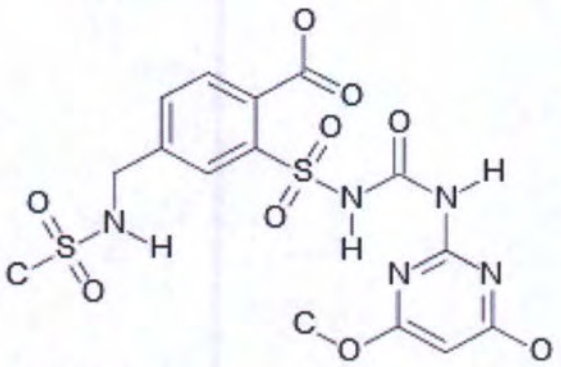
Compound	AE F130060
Common Name:	Mesosulfuron-methyl
Chemical Name:	Methyl 2-[[[[(4,6-dimethyoxy-2-pyrimidinyl)amino]carbonyl]amino]sulfonyl]-4-[[(methylsulfonyl)amino]methyl]benzoate
CAS No:	208465-21-8
Molecular Formula:	$C_{17}H_{21}N_5O_9S_2$
Purity:	98.5%
Lot Number:	0311200801
Expiration Date:	3/21/16



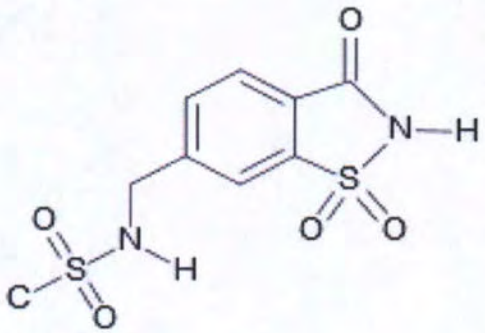
Compound	AE F160459
Common Name:	Des-O-Methyl Mesosulfuron-methyl
Chemical Name:	Methyl 2-[[[[(4-hydroxy-6-methoxy-2-pyrimidinyl)amino]carbonyl]amino]sulfonyl]-4-[[(methylsulfonyl)amino]methyl]benzoate
CAS No:	Not provided
Molecular formula:	C_{16} H_{19} N_5 O_9 S_2
Purity:	95.4%
Lot Number:	K-2162
Expiration Date	11/6/18



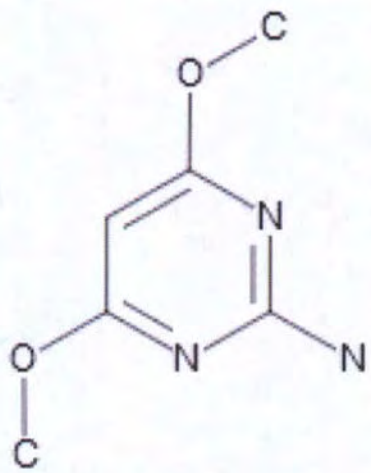
Compound	AE F140584
Common Name:	Mesosulfuron Sulfonamide
Chemical Name:	Methyl 2-(aminosulfonyl)-4-[[(methylsulfonyl)amino]methyl]benzoate
CAS No:	393509-80-3
Molecular formula:	C_{10} H_{14} N_2 O_6 S_2
Purity:	95.5%
Lot Number:	K-2163
Expiration Date	5/23/17



Compound	AE F160460
Common Name:	Des-O-Methyl DesMethyl Ester Mesosulfuron
Chemical Name:	2-[[[[(4-Hydroxy-6-methoxy-2-pyrimidinyl)amino]carbonyl]amino]sulfonyl]-4-[[(methylsulfonyl)amino]methyl]benzoic acid
CAS No:	Not provided
Molecular formula:	C_{15} H_{17} N_5 O_9 S_2
Purity:	95.6%
Lot Number:	K-2173
Expiration Date	2/19/18



Compound	AE F147447
Common Name:	Saccharin methanesulfonamide
Chemical Name:	*N*-[(2,3-Dihydro-1,1-dioxido-3-oxo-1,2-benzisothiazol-6-yl)methyl]methanesulfonamide
CAS No:	Not provided
Molecular formula:	$C_9 H_{10} N_2 O_5 S_2$
Purity:	98.1%
Lot Number:	K-2174
Expiration Date	10/7/18



Compound	AE F092944
Common Name:	ADMP
Chemical Name:	4,6-Dimethoxy-2-pyrimidinamine
Cas No:	36315-01-2
Molecular formula:	$C_6 H_9 N_3 O_2$
Purity:	99.6
Lot Number:	1001200318
Expiration Date	3/18/20

TEST SYSTEM

The test system, pond water, was supplied Bayer CropScience. The characterization report of the pond water is included in Appendix B. The pond water was stored refrigerated before use.

SOLVENTS AND REAGENTS

- Formic acid, Fisher Optima LC/MS Grade
- Acetonitrile (ACN), Sigma Chromasolv HPLC Grade
- Water, Sigma Chromasolv HPLC Grade
- Methanol, Sigma Chromasolv HPLC Grade
- 9:1 ACN/water; prepare by adding 900 mL of ACN to 100 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.
- 2% formic acid in water; prepare by adding 10 mL of formic acid to 490 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: 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.

STOCK, FORTIFICATION, AND CALIBRATION SOLUTIONS

STOCK SOLUTIONS

Stock solutions of Mesosulfuron-methyl, AE F092944 and AE F140584 were prepared at ~100 µg/mL in acetonitrile. The stock solutions were stored frozen when not in use.

Stock solutions of AE F147447, AE F160459 and AE F160460 were prepared at ~100 µg/mL in 1:1 ACN/water. The stock solutions were stored frozen when not in use.

Internal standard solutions of Mesosulfuron-methyl-dimethoxy-d_6, 2-Carbomethoxybenzenesulfonamide-3,4,5,6-d_4 and AE F092944-d_6 were prepared at ~100 µg/mL in 1:1 Acetonitrile/water. The stock solutions were stored frozen when not in use.

FORTIFICATION SOLUTIONS

The fortification solutions were stored refrigerated when not in use.

Mixed stock solution of Mesosulfuron-methyl, AE F092944 and AE F140584				
Stock	**Purity Corrected concentration (µg/mL)**	**Aliquot taken (mL)**	**Final total volume mL**	**Final total Concentration**
AE F130060	100.47	4.98	50	10 µg/mL
AE F140584	97.41	5.13		10 µg/mL
AE F092944	101.0	4.95		10 µg/mL

Mixed stock solution of AE F160459, AE F160460, and AE F147447				
Stock	**Purity Corrected concentration (µg/mL)**	**Aliquot taken (mL)**	**Final total volume mL**	**Final total Concentration**
AE F160459	97.50	5.13	50	10 µg/mL
AE F160460	95.98	5.21		10 µg/mL
AE F147447	98.1	5.10		10 µg/mL

FORTIFICATION STANDARD SOLUTIONS FOR EACH MIXED STOCK 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

Internal Standard solution				
Stock	**Purity Corrected concentration (µg/mL)**	**Aliquot taken (mL)**	**Final total volume mL**	**Final total Concentration**
Mesosulfuron-methyl-dimethoxy-d_6	98.4	0.508	50	1 µg/mL
2-Carbomethoxybenzenesulfonamide-3,4,5,6-d_4	101.7	0.492		1 µg/mL
AE F092944-d_6	96.08	0.520		1 µg/mL

The intermediate solutions were stored refrigerated when not in use.

INTERMEDIATE/CALIBRATION SOLUTIONS

Calibration Standard Solutions 1

Mesosulfuron-methyl, AE F092944 and AE F140584 with internal standards

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.

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

The calibration solutions were stored refrigerated when not in use.

Calibration Standard Solutions 2 Attempt 1

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 F147447by diluting to 25 mL with 9:1 Water/MeOH.

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

Calibration Standard Solutions 2 Attempt 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 F147447by 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.

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 Internal Standard (ppb)	Concentration of Calibration Solution (ppb)
10	1	0.250	0.25	10	100
10	1	0.125	0.25	10	50
1	1	0.625	0.25	10	25
1	1	0.250	0.25	10	10
1	1	0.125	0.25	10	5
0.1	1	0.250	0.25	10	1

Calibration Standard Solutions 2 Attempt 3
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 mixed internal standard solution to each of the calibration 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 Internal Standard (ppb)	Concentration of Calibration Solution (ppb)
10	1	0.250	2.5	100	100
10	1	0.125	2.5	100	50
1	1	0.625	2.5	100	25
1	1	0.250	2.5	100	10
1	1	0.125	2.5	100	5
0.1	1	0.250	2.5	100	1

ANALYTICAL METHODOLOGY

FORTIFICATION OF POND WATER- PROCEDURE 1

Mesosulfuron-methyl, AE F092944 and AE F140584

1. Place 10 mL of pond 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 per table below.
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.

Sample ID	Fortification volume	Internal Standard$_4$ volume (mL) 1 µg/mL	Sample Volume (mL)
Reagent Blank(90:10)MeOH:H_2O	25 µL$_3$	0.1	10
Control 1	25 µL$_3$	0.1	10
Control 2	25 µL$_3$	0.1	10
LOQ-A	25 µL$_1$	0.1	10
LOQ-B	25 µL$_1$	0.1	10
LOQ-C	25 µL$_1$	0.1	10
LOQ-D	25 µL$_1$	0.1	10
LOQ-E	25 µL$_1$	0.1	10
10X LOQ-A	25 µL$_2$	0.1	10
10X LOQ-B	25 µL$_2$	0.1	10
10X LOQ-C	25 µL$_2$	0.1	10
10X LOQ-D	25 µL$_2$	0.1	10
10X LOQ-E	25 µL$_2$	0.1	10

$_1$ 100 ng/mL Mesosulfuron-methyl, AE F092944 and AE F140584
$_2$ 1 µg/mL Mesosulfuron-methyl, AE F092944 and AE F140584
$_3$ 90:10 MeOH: H_2O
$_4$1 µg/mL Mesosulfuron-methyl-dimethoxy-d_6, 2-Carbomethoxybenzenesulfonamide-3,4,5,6-d_4 and AE F092944-d_6

FORTIFICATION OF POND WATER- PROCEDURE 2 ATTEMPT 1

Water extraction for AE F147447, AE F160459 and AE F160460

SPE CARTRIDGE CLEAN-UP

1. Place 10 mL of pond 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.
3. Add 0.2 mL of formic acid to each sample. Mix well.
4. 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.
5. Rinse vial with 1 mL of 2% formic acid in water and add to cartridge. Do not use vacuum to pull solvent through cartridge.
6. 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.
7. Evaporate sample to dryness using a TurboVap at ~50 °C.
8. Add 0.5 mL of 9:1 Water/MeOH to each sample and vortex to dissolve.
9. Place an aliquot into an hplc vial for analysis by LC/MS/MS;

Sample ID	Fortification volume	Sample Volume (mL)
Reagent Blank(90:10)MeOH:H_2O	25 μL_3	10
Control 1	25 μL_3	10
Control 2	25 μL_3	10
LOQ-A	25 μL_1	10
LOQ-B	25 μL_1	10
LOQ-C	25 μL_1	10
LOQ-D	25 μL_1	10
LOQ-E	25 μL_1	10
10X LOQ-A	25 μL_2	10
10X LOQ-B	25 μL_2	10
10X LOQ-C	25 μL_2	10
10X LOQ-D	25 μL_2	10
10X LOQ-E	25 μL_2	10

$_1$ 100 ng/mL AE F147447, AE F160459 and AE F160460
$_2$ 1 µg/mL AE F147447, AE F160459 and AE F160460
$_3$ 90:10 MeOH: H_2O

FORTIFICATION OF POND WATER- PROCEDURE 2 ATTEMPT 2

Water extraction for AE F147447, AE F160459 and AE F160460

SPE CARTRIDGE CLEAN-UP

1. Place 10 mL of pond 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 solutions and internal standard.
3. Add 0.2 mL of formic acid to each sample. Mix well.
4. 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.
5. Rinse vial with 1 mL of 2% formic acid in water and add to cartridge. Do not use vacuum to pull solvent through cartridge.
6. 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.
7. Evaporate sample to dryness using a TurboVap at ~50 °C.
8. Add 0.5 mL of 9:1 Water/MeOH to each sample and vortex to dissolve.
9. Place an aliquot into an hplc vial for analysis by LC/MS/MS.

Sample ID	Internal Standard[4] volume (mL) 1 µg/mL	Fortification volume	Sample Volume (mL)
Reagent Blank(90:10)MeOH:H_2O	n/a	25 µL[3]	10
Control 1	n/a	25 µL[3]	10
Control 2	n/a	25 µL[3]	10
LOQ-A	0.05	25 µL[1]	10
LOQ-B	0.05	25 µL[1]	10
LOQ-C	0.05	25 µL[1]	10
LOQ-D	0.05	25 µL[1]	10
LOQ-E	0.05	25 µL[1]	10
10X LOQ-A	0.05	25 µL[2]	10
10X LOQ-B	0.05	25 µL[2]	10
10X LOQ-C	0.05	25 µL[2]	10
10X LOQ-D	0.05	25 µL[2]	10
10X LOQ-E	0.05	25 µL[2]	10

[1] 100 ng/mL AE F147447, AE F160459 and AE F160460
[2] 1 µg/mL AE F147447, AE F160459 and AE F160460
[3] 90:10 MeOH: H_2O
[4] 1 µg/mL AE F147447 IS

FORTIFICATION OF POND WATER- PROCEDURE 2 ATTEMPT 3

Water extraction for AE F147447, AE F160459 and AE F160460

SPE CARTRIDGE CLEAN-UP

---Pre-rinse all glass vials and culture tubes with 2% formic acid and allow to dry.
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 and internal standard solutionper table below.
3. Add 0.2 mL of formic acid to each sample. Mix well.

4. 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.
5. Rinse vial with 1 mL of 2% formic acid in water and add to cartridge. Do not use vacuum to pull solvent through cartridge.
6. 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.
7. Evaporate sample to dryness using a TurboVap at ~50 °C.
8. Add 0.5 mL of 9:1 Water/MeOH+10% ACN to each sample and vortex to dissolve.
9. Place an aliquot into an hplc vial for analysis by LC/MS/MS

Sample ID	Internal Standard[4] volume (mL) 1 µg/mL	Fortification volume	Sample Volume (mL)
Reagent Blank(90:10)MeOH:H_2O	n/a	25 µL[3]	10
Control 1	n/a	25 µL[3]	10
Control 2	n/a	25 µL[3]	10
LOQ-A	0.05	25 µL[1]	10
LOQ-B	0.05	25 µL[1]	10
LOQ-C	0.05	25 µL[1]	10
LOQ-D	0.05	25 µL[1]	10
LOQ-E	0.05	25 µL[1]	10
10X LOQ-A	0.05	25 µL[2]	10
10X LOQ-B	0.05	25 µL[2]	10
10X LOQ-C	0.05	25 µL[2]	10
10X LOQ-D	0.05	25 µL[2]	10
10X LOQ-E	0.05	25 µL[2]	10

[1] 100 ng/mL AE F147447, AE F160459 and AE F160460
[2] 1 µg/mL AE F147447, AE F160459 and AE F160460
[3] 90:10 MeOH: H_2O
[4] 1 µg/mL AE F147447 IS

LC-MS/MS ANALYSIS

Separation of the analyte from the matrix was achieved by high performance liquid chromatography (HPLC). Quantitative LC-MS/MS analysis of the analytes in the samples utilized a highly specific and sensitive MRM (Multiple Reaction Monitoring) method. The analytes were identified by the coincidence of the retention time with that of the calibration standards, and quantified by integration of the peak area relative to the calibration curves.

The following are the LC-MS/MS system (controlled by the operating software, Analyst™ version 1.6.2, a validated system) and the parameters used.

HPLC: Shimadzu (Nexera X2)
Shimadzu Pump LC-30AD
Shimadzu System Controller

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

Column: Thermo Scientific Aquasil C18, 100 mm X 3 mm, 3 µm

Injection Volume: 50 µL

Solvent System:

Solvent A = Water/methanol (9:1, v:v) containing 10 mM Ammonium formate and 120 µL/L formic acid
Solvent B = Water/methanol (1:9, v:v) containing 10 mM Ammonium formate and 120 µL/L formic acid
Wash solvent = ACN-water (50:50 v:v)

Solvent Program:

Time (minutes)	Flow Rate (mL/min)	%A	%B
0.0	0.8	95	5
0.5	0.8	95	5
4.0	0.8	0	100
5.0	0.8	0	100
5.1	0.8	95	5

Retention Time:

AE F140584 2.6 min
AE F092944 3.41 min
Mesosulfuron-methyl 4.10 min

HPLC Conditions for AE F147447, AE F160459 and AE F160460

Column: Imtakt Scherzo SM-C18, 100 mm X 4.6 mm, 3 µm

Injection Volume: 50 µL

Solvent System:

Solvent A = 5 mM ammonium formate in water
Solvent B = 0.2 M ammonium formate in water/Methanol (1:1, v/v)
Wash solvent = ACN-water (50:50 v:v)

Solvent Program:

Time (minutes)	Flow Rate (mL/min)	%A	%B
0.0	1	50	50
0.5	1	50	50
4.5	1	0	100
5.5	1	0	100
5.6	1	50	50

Retention Time:

AE F147447 2.51 min
AE F160459 4.76 min
AE F160460 5.05 min

Mass Spectrometer: SCIEX API 6500

Mass Spectrometer settings: **Mesosulfuron-methyl, AE F092944 and AE F140584**

Period 1 (0-3.2min):
Period 1 Experiment 1
Negative ion mode for AE F140584
CUR: Curtain Gas 30
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 (3.2-6.2 min): Positive ion mode for AE F092944 and Mesosulfuronmethyl
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

Mass Spectrometer settings: **AE F147447, AE F160459 and AE F160460**

Period 1 (0-3.2 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 (3.2-6.7 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

MRM:

Analyte ID	Polarity	Q1 Mass (amu)	Q3 Mass (amu)	Dwell Time (msec)	DP	EP	CE	CXP
AE F140584	-	320.9	289.0	40	-70	-10	-18	-15
AE F140584 Confirmatory	-	320.9	209.0	40	-70	-10	-46	-13
2-Carbomethoxy benzenesulfonamide IS	+	220.154	203.090	40	-106	10	11	12
AE F092944	+	156.08	100.093	40	36	10	25	8
AE F092944 IS	+	162.2	103.0	40	60	10	23	6
AE F092944 Confirmatory	+	156.08	124.062	40	36	10	27	14
Mesosulfuron-methyl	+	504.035	182.062	40	91	10	31	14
Mesosulfuron-methyl IS	+	510.2	187.9	40	60	10	83	6
Mesosulfuron-methyl confirmatory	+	504.080	83.070	40	91	10	79	8

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

Analyte ID	Polarity	Q1 Mass (amu)	Q3 Mass (amu)	Dwell Time (msec)	DP	EP	CE	CXP
AE F147447	-	288.957	208.961	40	-60	-10	-42	-1
AE F147447 Confirmatory	-	288.957	181.049	40	-60	-10	-44	-9
AE F147447 IS	-	294.8	210.0	40	-130	-10	-38	-16
AE F160460	+	476.0	168.0	40	60	10	29	6
AE F160460 Confirmatory	+	476.0	142.1	40	60	10	31	6
AE F160459	+	490.0	168.0	40	60	10	29	6
AE F160459 Confirmatory	+	490.0	100.0	40	60	10	73	6

METHODS OF CALCULATION

Recoveries

CALCULATION OF RESULTS

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. The standards were fit to the linear equation:

An example calculation for the recovery of Mesosulfuron Methyl (at LOQ) from water is shown below:

The general linear equation for a calibration curve is

$y = mx + b$, (x= calclulated concentration, m=slope, b=intercept)

thus $x = \frac{y'-b}{m}$ where y' is $\frac{\text{Area of analyte}}{\text{Area of Internal standard}}$

In our example

The calibration curve equation for the primary transition of Mesosulfuron methyl was $y = 6.86x + 0.128$, ($r = 0.9999$)

In the case of 10 x LOQ A,

$$y' = \frac{3350000}{181000} = 18.508$$

$$x = \frac{y'-b}{m} = \frac{18.508 - 0.128}{6.86} = 2.68$$

The calculated concentration is calculated from the curve by way of the equation

The percent recovery of Mesosulfuron methyl for 10 x LOQ A primary transition was calculated as follows:

$$\text{Percent recovery} = \frac{\text{Calculated concentration}}{\text{Analyte Concentration}} \times 100 = \frac{2.68}{2.5} = 107\%$$

APPENDIX D:

Communications with the Sponsor

EK – E. Klosi, CR – C. Reed, AM – A. Miller, DN – D. Netzband

December 10, 2015	E.K. sent protocol draft to AM.
December 30, 2015	E.K. informs AM about the progress of procedure 1 with compounds AE F140584, AEF092944, and Mesosulfuron methyl, and asks for clarifications in order to quantify compound AE F140584 as a function of its IS within Analyst 1.6.2.
December 30, 2015	DN answers the question regarding the internal standard.
December 30, 2015	EK informs DN that the part of the method regarding procedure 1 seems to pass the acceptance criteria, but suggests evaluating the standard curve for AE F140584 with a quadratic ($1/x^2$) fit. Also the data regarding the ILV are sent to DN.
December 30, 2015	DN recommends excluding the 50 and 100 ng/mL standards for AE F140584, and indicates his preference for a linear fit with 1/x weighting.
December 30, 2015	EK responds by accepting to drop the standards suggested and use a linear fit.
January 4, 2016	EK informs AM and DN about the first attempt with the second procedure of the ILV which regards compounds AE F147477, AE F160459 and AE F160459. It is observed that there is some peak shift noticeable for the samples, however the retention times of the standards are reproducible. It is suggested that a tighter control on the pH of the sample might result in a better outcome for the retention time of the samples. EK also welcomes any feedback as to how to proceed.
January 5, 2016	AM responds with a few suggestions and remarks on the unusual differences in peak area for LOQ and 10X LOQ, in the case of AE F160460, and suggests that it might be due to spiking error. AM asks for the standard preparation sheets, and reiterates the original method's spiking levels. It is also suggested to analyze a spiked control extract and spiked solvent at the LOQ level for all compounds in order to determine if there are issues with the sample preparation procedure or the instrumentation. The existence of an issue with the pH is considered unlikely as the SPE eluents are evaporated to dryness and any formic acid remaining in the eluent should also be evaporated. In addition there was a question as to how the dilution factor was

	accounted for in the experiment files that were sent to Bayer.
January 5, 2016	EK welcomes the suggestion and affirms that they will be incorporated in the rerun of the ILV. It is also pointed out that there was an error regarding the calculated concentrations provided to Analyst. As a result the recoveries are actually half the ones calculated. EK asks for a conference call to discuss the different suggestions and any further clarifications
January 5, 2016	AM writes that prior to starting the second trial, a control extract from trial 1 and a solvent blank should be spiked at 0.25 ppb and analyzed to see if there is a good response on the instrument. Also, the SPE column should be profiled to see if some compound is being lost either in the eluent or if it is sticking to the cartridge.
January 6, 2016	EK sends the data for the experiments suggested by AM the day before, after which there was a conference call between EK, CR, AM and DN. In the call AM and DN asked how old the fortification and calibration solutions were, and suggested that they should not be older than a week. Their suggestion was accepted. AM and DN added that they would send overnight an internal standard for compound (AE F147447). This should help with understanding better if recovery losses are due to masking. At the suggestion of EK and CR, it was agreed that formic acid would be added after the reconstitution step in order to see the effect of pH on retention time.
January 7, 2016	AM sends a method amendment document which adds the AE F147447 internal standard information. It is advised to store this internal standard in a freezer (-20 C).
January 8, 2016	AM sends a CoA for the AE F147447 internal standard as well as a clarification regarding its lot number.
January 12, 2016	EK sends AM the result files for the second ILV. Additional experiments are also performed in order to elucidate the loss of recovery. Explanations of these experiments are provided and their data sent to AM. These later experiments looked at the adsorption of AE F147447 on glass vials (which are used in the experiment) compared to polypropylene vials. EK suggests that given the information at hand, it would probably be best to either wash the glass vials with formic acid before starting, or add 1% formic acid in the solution at the very first step of the method.
January 13, 2016	AM points out that it seems from the files sent to her from the second ILV that 1 µg/mL internal standard was added to the samples instead of the 0.1 µg/mL. When the IS concentration is corrected in Analyst for using a 1 µg/mL IS, then the recoveries for AE F147447 are good.
January 14, 2016	EK requests a conference call, which took part later in the day. Participants: AM, DN, EK, CR. During the call it was agreed (at the suggestion of EK and CR) to carry out the third attempt of the ILV the next day.

	It was ascertained that there was in fact a misunderstanding from EK and CR as to the actual concentration of the IS for AE F147477 in the second ILV. It was agreed to change the resolution on the instrument for Q3 to "Low" for the first period containing AE F147447. The concentration of the IS AE F147447 being judged too low in light of the second ILV's data, it was agreed to change it to 10 times higher (the actual concentration we used in the second ILV) and AM would send a revised protocol to reflect this change.
January 18, 2016	EK informs AM about the results of the third ILV and sends the result files. EK asks for AM's opinion on how to proceed. It is agreed to discuss the results in a phone call. In a reply e-mail, AM asks whether internal standards were added to the control samples, to which EK replies that no internal standards were added.
January 18, 2016	In a short phone call between AM, DN, EK, and CR it was agreed to proceed with the write up of the final report.