2.0 MATERIALS AND METHODS

2.1 Protocol

Procedures used in this study followed those described in the Smithers Viscient protocol entitled "Validation of the Analytical Method for the Determination of BAS315I and Metabolites in Soil" (Appendix 1). The study was conducted under Good Laboratory Practices (GLP) regulations and principles as described in 40 CFR 160 (U.S. EPA, 1989) and the OECD principles on GLP (OECD, 1998), and followed the guidance documents SANCO/825/00rev 8.1 (EC, 2010) and OCSPP 850.6100 (U.S. EPA, 2012).

2.2 Test Substances

The test substance, BAS 315 I (Compound A), was received on 20 December 2016 from BASF Corporation, Durham, North Carolina. The following information was provided:

Name:	BAS 315 I (Compound A)
Synonyms:	BAS 315 I; Hydramethylnon; Reg. No. 4111109
Batch No.:	L83-26
CAS No.:	67485-29-4
Purity:	99.5% (Certificate of Analysis, Appendix 2)
Expiration Date:	1 October 2020

Upon receipt at Smithers Viscient, the test substance (SMV No. 8662) was stored refrigerated in the original container. Concentrations were adjusted for the purity of the test substance.

The test substance, BASF Reg. No. 255418 (M12), was received on 20 December 2016 from BASF Corporation, Durham, North Carolina. The following information was provided:

Name:	BASF Reg. No. 255418 (M12)
Batch No.:	AC9745-96A
CAS No.:	42160-07-6
Purity:	99.6% (Certificate of Analysis, Appendix 2)
Expiration Date:	1 November 2018

Upon receipt at Smithers Viscient, the test substance (SMV No. 8664) was stored refrigerated in the original container. Concentrations were adjusted for the purity of the test substance.

The test substance, M11, was received on 3 February 2017 from Ricerca Biosciences, Concord, Ohio. The following information was provided:

Name:	M11
Synonyms:	Hydramethylnon Metabolite P; Compound P
Lot No.:	55658-25-30
CAS No.:	Not available (newly synthesized molecule)
Purity:	94.49% (Certificate of Analysis, Appendix 2)
Retest Date:	20 December 2019

Upon receipt at Smithers Viscient, the test substance (SMV No. 8744) was stored in a freezer in the original container. Concentrations were adjusted for the purity of the test substance.

The test substance, M6, was received on 3 February 2017 from Ricerca Biosciences, Concord, Ohio. The following information was provided:

Name:	M6
Synonyms:	Hydramethylnon Metabolite M6; Compound M6
Lot No.:	55715-06-04
CAS No.:	Not available (newly synthesized molecule)
Purity:	95.94% (Certificate of Analysis, Appendix 2)
Retest Date:	20 December 2019

Upon receipt at Smithers Viscient, the test substance (SMV No. 8741) was stored in a freezer in the original container. Concentrations were adjusted for the purity of the test substance.

The test substance, M1a, was received on 3 February 2017 from Ricerca Biosciences, Concord, Ohio. The following information was provided:

Name:	M1a
Synonyms:	Hydramethylnon Metabolite M1a; Compound M1a
Lot No.:	55878-16-05
CAS No.:	Not available (newly synthesized molecule)
Purity:	99.42% (Certificate of Analysis, Appendix 2)
Retest Date:	10 January 2020

Upon receipt at Smithers Viscient, the test substance (SMV No. 8742) was stored in a freezer in the original container. Concentrations were adjusted for the purity of the test substance.

The test substance, M1b, was received on 3 February 2017 from Ricerca Biosciences, Concord, Ohio. The following information was provided:

Name:	M1b
Synonyms:	Hydramethylnon Metabolite M1b; Compound M1b
Lot No.:	55898-8-34
CAS No.:	Not available (newly synthesized molecule)
Purity:	94.5% (Certificate of Analysis, Appendix 2)
Retest Date:	20 December 2019

Upon receipt at Smithers Viscient, the test substance (SMV No. 8743) was stored refrigerated in the original container. Concentrations were adjusted for the purity of the test substance.

Determination of stability and characterization, verification of the test substance identity, maintenance of records on the test substances, and archival of a sample of the test substances are the responsibility of the Study Sponsor.

2.3 Reagents

1.	0.1% Formic acid in water:	Fisher, reagent grade
2.	0.1% Formic acid in acetonitrile:	Fisher, reagent grade
3.	Methanol:	EMD, reagent grade
4.	Acetonitrile:	EMD, reagent grade
5.	Purified reagent water:	Prepared from a Millipore MilliQ Direct 8 water purification system (meets ASTM Type II requirements)

2.4 Instrumentation and Laboratory Equipment

1.	Instruments:	MDS Sciex API 5000 mass spectrometer equipped with an MDS Sciex ESI Turbo V source AB MDS Sciex 4000 mass spectrometer equipped with an AB MDS Sciex ESI Turbo V source Shimadzu LC-20AD binary pumps Shimadzu DGU-20A3 vacuum degasser Shimadzu DGU-20A5R vacuum degasser Shimadzu SIL-20ACHT autosampler Shimadzu CTO-20AC column oven Shimadzu CTO-20A column oven Shimadzu CBM-20A communications bus Analyst version 1.6.3 software for data acquisition
2.	Balances:	Mettler Toledo XSE205DU; Mettler Toledo PG-2002-S
3.	Centrifuge:	Thermo Scientific Sorvall Legend XFR Centrifuge
4.	Moisture balance:	Sartorius Moisture Analyzer MA-45;
5.	Shaker table:	VWR Standard Analog Shaker Table 3500STD;
6.	Laboratory equipment:	Positive displacement pipets, volumetric flasks, disposable glass and plastic pipets, 50 mL Nalgene centrifuge tubes, graduated cylinders, stir bars, stir plates, vortex mixer, amber vials with crimp caps, and amber glass bottles with Teflon-lined caps

Other equipment or instrumentation may be used in future testing but may require optimization to achieve the desired separation and sensitivity.

2.5 Test Matrix

The test system evaluated during this study was soil representative of the type of matrix this method was intended to analyze. The soil used for the main method validation was Rochester loamy sand soil (SMV Lot No. 012616A) and for the re-validation validation (Appendix 4) was sandy loam soil (SMV Lot No. RMN-SL-PF 0-6" 5-29-18) from Rochester, Massachusetts. Prior to testing, Rochester loamy sand soil (SMV Lot No. 012616A) and sandy loam soil (SMV Lot No. RMN-SL-PF 0-6" 5-29-18) moisture content was determined using a Sartorius and Mettler Toledo HB43-S moisture analyzer. Characterization of the soils were performed by Agvise Laboratories, Northwood, North Dakota. Soil characterization data is listed in the table below. The results of this characterization is presented in Appendix 3.

Soil Type	% Sand, Silt, Clay	Bulk Density (gm/cc)	Cation Exchange Capacity (meq/100 g)	% Organic Matter (Walkley Black)	pH in 1/1 soil/water Ratio	Moisture Content (%)
Loamy sand soil	78, 18, 4	1.06	9.7	4.9	6.8	23.39 - 25.65
Sandy loam soil	75, 18, 7	1.08	14.1	3.4	6.6	16.01

2.6 Preparation of Liquid Reagents

The volumes listed in this section were those used during the validation. For future testing, the actual volumes used may be scaled up or down as necessary.

A 50/50 acetonitrile/purified reagent water (v/v) liquid reagent solution was typically prepared by combining 500 mL of acetonitrile and 500 mL of purified reagent water. The solution was mixed well using a stir bar and stir plate for five minutes.

A 95/5 acetonitrile/purified reagent water (v/v) liquid reagent solution was prepared by combining 950 mL of acetonitrile and 50.0 mL of purified reagent water. The solution was mixed using a stir bar and stir plate for five minutes.

A 30/30/40 acetonitrile/methanol/purified reagent water (v/v/v) autosampler needle wash solution was prepared by combining 1500 mL of acetonitrile, 1500 mL of methanol, and 2000 mL of purified reagent water. The solution was mixed well before use.

2.7 Preparation of Stock Solutions

The volumes and masses listed in this section were those used during each separate validation. For future testing, the actual volumes and masses used may be scaled up or down as necessary.

Primary stock solutions were prepared as described in the table below:

Primary Stock ID	Amount of Substance Weighed (g), Net Weight	Amount of Substance Weighed (g), as Active Ingredient	Stock Solvent	Final Volume (mL)	Primary Stock Concentration (mg/L)	Primary Stock Use
8662D	0.0504	0.0501	Acetonitrile	50.0	1000	Sub-stock solution
8664A	0.0502	0.0500	Acetonitrile	50.0	1000	Sub-stock solution
8744A	0.0530	0.0501	50/50 acetonitrile/ purified reagent water (v/v)	50.0	1000	Sub-stock solution
8741A	0.0522	0.0501	Acetonitrile	50.0	1000	Sub-stock solution
8742A	0.0503	0.0500	Acetonitrile	50.0	1000	Secondary stock solution and sub-stock solution
8743A	0.0529	0.0500	Acetonitrile	50.0	1000	Sub-stock solution

A secondary stock solution was prepared as per the table below:

Fortifying Stock ID	Fortifying Stock Concentration (mg/L)	Volume of Fortification (mL)	Final Volume (mL)	Stock Solvent	Stock ID	Stock Concentration (mg/L)	Stock Use
8742A	1000	0.500	50.0	Acetonitrile	8742A-2	10.0	Sub-stock solution

Sub-stock solutions were prepared as per the tables below:

Fortifying Stock ID	Fortifying Stock Concentration (mg/L)	Volume of Fortification (mL)	Final Volume (mL)	Stock Solvent	Stock ID	Stock Concentration (mg/L)	Stock Use
8662D	1000	0.100	10.0	Acetonitrile	Mixed Stk 1	10.0	10× LOQ level recovery samples and sub-stock solution
8664A	1000	0.100					
8744A	1000	0.100					
8741A	1000	0.100					
8743A	1000	0.100					
Mixed Stk 1	10.0	1.00	10.0	Acetonitrile	Mixed Stk 2	1.00	LOQ level recovery samples and sub-stock solution
Mixed Stk 2	1.00	0.100	10.0	Acetonitrile	Mixed Stk 3	0.0100	Calibration standards and Matrix-effects standards
8742A-2	10.0	1.00	10.0	Acetonitrile	Stk 1	1.00	Recovery samples and sub-stock solution
Stk 1	1.00	0.100	10.0	Acetonitrile	Stk 2	0.0100	Calibration and matrix-effects standards

All primary and secondary stock solutions were stored refrigerated (2 to 8 °C) in amber glass bottles fitted with Teflon-lined caps. Sub-stock solutions were prepared fresh on the day of use and discarded after use.

2.8 Preparation of Calibration Standards

2.8.1 Calibration Standards – BAS 315 I, M12, M11, M6, and M1b

Calibration standards were prepared in 50/50 acetonitrile/purified reagent water (v/v) by fortifying with the 0.0100 mg/L mixed sub-stock solution to yield test substance concentrations listed in the table below.

Test Substance Stock ID	Stock Concentration (mg/L)	Volume of Fortification (mL)	Final Volume (mL)	Standard Concentration (µg/L)	Sample ID
Mixed Stk 3	0.0100	0.100	10.0	0.100	Std 1
		0.200	10.0	0.200	Std 2
		0.300	10.0	0.300	Std 3
		0.500	10.0	0.500	Std 4
		0.700	10.0	0.700	Std 5
		1.00	10.0	1.00	Std 6

2.8.2 Calibration Standards – M1a

Calibration standards were prepared in matrix blank by fortifying with the 0.0100 mg/L sub-stock solution to yield test substance concentrations listed in the table below.

Test Substance Stock ID	Stock Concentration (mg/L)	Volume of Fortification (mL)	Final Volume (mL)	Standard Concentration (µg/L)	Sample ID
Stk 2	0.0100	0.100	10.0	0.100	Std 1
		0.200	10.0	0.200	Std 2
		0.300	10.0	0.300	Std 3
		0.500	10.0	0.500	Std 4
		0.700	10.0	0.700	Std 5
		1.00	10.0	1.00	Std 6

2.8.3 Matrix Effect Investigation – BAS 315 I, M12, M11, M6, and M1b

In an effort to observe any potential matrix effects, an aliquot of control sample final fraction was fortified in triplicate and analyzed at each transition. These matrix-matched standards were compared to non-matrix matched standards fortified at the same concentration. Calibration standards used to assess possible matrix effects were prepared as described in the following tables.

2.8.3.1 Matrix-Matched Standards

Test Substance Stock ID	Stock Concentration (mg/L)	Volume of Fortification (mL)	Final Volume[a] (mL)	Standard Concentration (µg/L)	Sample ID
Mixed Stk 3	0.0100	0.200	10.0	0.200	MM-Std 1
		0.200	10.0	0.200	MM-Std 2
		0.200	10.0	0.200	MM-Std 3

[a] Diluted with the final dilution of the matrix-matched control sample 986-6267-02.

2.8.3.2 Non-Matrix-Matched Standards

Test Substance Stock ID	Stock Concentration (mg/L)	Volume of Fortification (mL)	Final Volume (mL)[a]	Standard Concentration (µg/L)	Sample ID
Mixed Stk 3	0.0100	0.200	10.0	0.200	Std A
		0.200	10.0	0.200	Std B
		0.200	10.0	0.200	Std C

[a] Diluted with 50/50 acetonitrile/purified reagent water (v/v).

2.8.4 Matrix Effect Investigation – M1a

In an effort to observe any potential matrix effects, an aliquot of control sample final fraction was fortified in triplicate and analyzed at each transition. These matrix-matched standards were compared to non-matrix matched standards fortified at the same concentration. Calibration standards used to assess possible matrix effects were prepared as described in the following tables.

2.8.4.1 Matrix-Matched Standards

Test Substance Stock ID	Stock Concentration (mg/L)	Volume of Fortification (mL)	Final Volume (mL)[a]	Standard Concentration (µg/L)	Sample ID
Stk 2	0.0100	0.200	10.0	0.200	MM-Std 1
		0.200	10.0	0.200	MM-Std 2
		0.200	10.0	0.200	MM-Std 3

[a] Diluted with the final dilution of the matrix-matched controls sample 986-6267-17.

2.8.4.2 Non-Matrix-Matched Standards

Test Substance Stock ID	Stock Concentration (mg/L)	Volume of Fortification (mL)	Final Volume (mL)[a]	Standard Concentration (µg/L)	Sample ID
Stk 2	0.0100	0.200	10.0	0.200	Std A
		0.200	10.0	0.200	Std B
		0.200	10.0	0.200	Std C

[a] Diluted with 50/50 acetonitrile/purified reagent water (v/v).

2.9 Sample Fortification and Preparation

2.9.1 BAS 315 I, M12, M11, M6, and M1b

The recovery samples were prepared in loamy sand soil with BAS 315 I, M12, M11, M6, and M1b at concentrations of 50.0 and 500 µg/kg. A total of 14 recovery samples (5.00 g dry weight) were weighed into individual 50-mL Nalgene centrifuge tubes and were fortified with the appropriate test substance mixed sub-stock solution at concentrations of 50.0 and 500 µg/kg. Seven replicates were prepared for the 50.0 µg/kg (LOQ) concentration level and five replicates were prepared for the 500 µg/kg concentration level. In addition, two samples were left unfortified to serve as controls and were extracted in the same fashion as the LOQ recovery samples. One reagent blank was also prepared (no test substance or matrix) in order to assess interference from extraction solvents. The dosing procedure is detailed in the following table. BAS315 I and metabolite M6 were re-validated using the more abundant carbon isotope. This was done in order to provide a more straightforward and efficient method. The new method was demonstrated to be more reproducible and robust. This re-validation can be found in Appendix 4.

Sample ID 986-6267-	Sample Type	Sub-Stock Concentration (mg/L)	Volume of Fortification (mL)	Dry Weight (g)	Fortified Concentration (µg/kg)
01	Reagent Blk	NA[a]	NA	NA	0.00
02 & 03	Control	NA	NA	5.00	0.00
04, 05, 06, 07, 08, 09, & 10	LOQ	1.00	0.250	5.00	50.0
11, 12, 13, 14, & 15	10× LOQ	10.0	0.250	5.00	500

[a] NA = Not Applicable

2.9.2 M1a

The recovery samples were prepared in loamy sand soil with M1a test substance at concentrations of 50.0 and 500 µg/kg. A total of 14 recovery samples (5.00 g dry weight) were weighed into individual 50-mL centrifuge tubes and were fortified with the appropriate test substance sub-stock solution at concentrations of 50.0 and 500 µg/kg. Seven replicates were prepared for the 50.0 µg/kg (LOQ) concentration level and five replicates were prepared for the 500 µg/kg concentration level. In addition, two samples were left unfortified to serve as controls and were extracted in the same fashion as the LOQ recovery samples. One reagent blank was also prepared (no test substance or matrix) in order to assess interference from extraction solvents. The dosing procedure is detailed in the following table.

Sample ID 986-6267-	Sample Type	Sub-Stock Concentration (mg/L)	Volume of Fortification (mL)	Dry Weight (g)	Fortified Concentration (µg/kg)
16	Reagent Blk	NA[a]	NA	NA	0.00
17 & 18	Control	NA	NA	5.00	0.00
19, 20, 21, 22, 23, 24, & 25	LOQ	1.00	0.250	5.00	50.0
26, 27, 28, 29, & 30	10× LOQ	1.00	2.50	5.00	500

[a] NA = Not Applicable

2.10 Soil Extraction and Dilution

Samples were processed according to the flow scheme shown in Figure 1.

2.10.1 BAS 315 I, M12, M11, M6, and M1b

A 20.0-mL aliquot of 95/5 acetonitrile/purified reagent water (v/v) was added to each loamy sand soil recovery sample (5.00 g dry weight) and samples were placed on a shaker table for 20 minutes at 250 rpm. The samples were then centrifuged at 3000 rpm for 10 minutes and the extracts were transferred to 50.0-mL volumetric flasks. The extraction and centrifugation

procedures were repeated with an additional 20.0-mL aliquot of 95/5 acetonitrile/purified reagent water (v/v). The extracts were combined, taken to volume (50.0 mL) with 95/5 acetonitrile/purified reagent water (v/v) and mixed well. The loamy sand soil recovery sample extracts were further diluted into the calibration standard range with 50/50 acetonitrile/purified reagent water (v/v). All recovery samples were transferred to autosampler vials for analysis. Secondary dilution volumes can be scaled up or down as necessary. The extraction and dilution procedures are detailed below. BAS315 I and metabolite M6 were re-validated using the more abundant carbon isotope. This was done in order to provide a more straightforward and efficient method. The new method was demonstrated to be more reproducible and robust. This re-validation can be found in Appendix 4.

Sample ID 986-6267-	Nominal Concentration (µg/kg)	Dry Weight (g)	Final Volume[a] (mL)	Sample Volume (mL)	Final Volume[b] (mL)	Dilution Factor
01	0.00	NA[c]	50.0	0.400	10.0	25.0
02 & 03	0.00	5.00	50.0	0.400	10.0	250
MM-Std 1[d], MM-Std 2[d], & MM-Std 3[d]	0.00	NA	NA	0.400	10.0	250
04, 05, 06, 07, 08, 09, & 10	50.0	5.00	50.0	0.400	10.0	250
11, 12, 13, 14, & 15	500	5.00	50.0	0.100	10.0	1000

[a] Extracted with 95/5 acetonitrile/purified reagent water (v/v).
[b] Diluted with 50/50 acetonitrile/purified reagent water (v/v).
[c] NA = Not Applicable
[d] Taken from control sample 986-6267-02.

2.10.2 M1a

A 20.0-mL aliquot of 100% acetonitrile was added to each loamy sand soil recovery sample (5.00 g dry weight) and samples were placed on a shaker table for 20 minutes at 250 rpm. The samples were then centrifuged at 3000 rpm for 10 minutes and the extracts were transferred to 50.0-mL volumetric flasks. The extraction and centrifugation procedures were repeated with an

additional 20.0-mL aliquot of 100% acetonitrile. The extracts were combined, taken to volume (50.0 mL) with 100% acetonitrile, and mixed well. The control and LOQ recovery sample extracts were further diluted into the calibration standard range with 50/50 acetonitrile/purified reagent water (v/v). 10× LOQ samples were diluted into matrix blank diluent. All recovery samples were transferred to autosampler vials for analysis. Secondary dilution volumes can be scaled up or down as necessary. The extraction and dilution procedures are detailed below.

Sample ID 986-6267-	Nominal Concentration (µg/kg)	Dry Weight (g)	Final Volume[a] (mL)	Sample Volume (mL)	Final Volume (mL)	Dilution Factor
Matrix Blank	0.00	NA[b]	NA	8.00[c]	200[d]	250
16	0.00	NA	50.0	0.400	10.0[d]	25.0
17 & 18	0.00	5.00	50.0	0.400	10.0[d]	250
19, 20, 21, 22, 23, 24, & 25	50.0	5.00	50.0	0.400	10.0[d]	250
26, 27, 28, 29, & 30	500	5.00	50.0	0.100	10.0[e]	1000

[a] Extracted with 100% acetonitrile.
[b] NA = Not Applicable
[c] Taken from control sample 986-6267-17.
[d] Diluted with 50/50 acetonitrile/purified reagent water (v/v).
[e] Diluted with Matrix Blank.

2.11 Analysis

2.11.1 Instrumental Conditions BAS 315 I, M12, M6, and M1b

The LC-MS/MS analysis was conducted utilizing the following instrumental conditions:

LC parameters:

Column:	Agilent Poroshell EC-C8, 2.7 µm, 3.0 × 50 mm
Mobile Phase A:	0.1% formic acid in water
Mobile Phase B:	0.1% formic acid in acetonitrile

Gradient:

Time (min.)	Flow rate (mL/min.)	Solvent A (%)	Solvent B (%)
0.50	0.800	95.0	5.00
0.60	0.800	50.0	50.0
3.00	0.800	0.00	100
4.00	0.800	0.00	100
4.10	0.800	95.0	5.00
5.00	0.800	95.0	5.00

Run Time: 5.0 minutes
Injector Wash Solvent: 30/30/40 acetonitrile/methanol/purified reagent water (v/v/v)
Column Temperature: 40 °C
Sample Temperature: 10 °C
Injection Volume: 50.0 µL
Retention Times: see table below

Analyte	Analysis	Retention Time
BAS 315 I	Primary	2.03
	Confirmatory	2.03
M12	Primary	2.70
	Confirmatory	2.70
M6	Primary	2.55
	Confirmatory	2.55
M1b	Primary	1.88
	Confirmatory	1.87

MS parameters:

Instrument: MDS Sciex API 5000 mass spectrometer
Ionization Mode: Positive (+) ESI
Ion Spray Voltage: 5500 V
Scan Type: MRM
Dwell Time: 100 milliseconds
Source Temperature: 600 °C
Curtain Gas: 15.0
Ion Source – Gas 1 / Gas 2: 60.0/60.0
Collision Gas: 7.00
Resolution Q1/Q3: Unit/Unit

BAS 315 I:

	Primary Transition	Confirmatory Transition
Q1/Q3 Masses (amu):	496.20/324.10	496.20/396.10
Declustering Potential:	110	110
Collision Cell Entrance Potential:	12.5	11.5
Collision Energy:	43.0	46.0
Collision Cell Exit Potential:	10.0	20.0

M12:

	Primary Transition	Confirmatory Transition
Q1/Q3 Masses (amu):	371.21/159.09	371.32/199.04
Declustering Potential:	80.0	80.0
Collision Cell Entrance Potential:	7.00	7.00
Collision Energy:	35.0	41.0
Collision Cell Exit Potential:	10.0	10.0

M6:

	Primary Transition	Confirmatory Transition
Q1/Q3 Masses (amu):	384.20/364.20	384.20/151.10
Declustering Potential:	100	100
Collision Cell Entrance Potential:	6.50	9.00
Collision Energy:	37.6	68.0
Collision Cell Exit Potential:	9.00	25.0

M1b:

	Primary Transition	Confirmatory Transition
Q1/Q3 Masses (amu):	511.10/491.10	511.10/364.10
Declustering Potential:	100	100
Collision Cell Entrance Potential:	10.0	15.0
Collision Energy:	40.0	53.1
Collision Cell Exit Potential:	7.00	12.0

2.11.2 Instrumental Conditions M11

The LC-MS/MS analysis was conducted utilizing the following instrumental conditions:

LC parameters:

Column:	Waters T3, 3.0 µm, 4.6 × 100 mm
Mobile Phase A:	0.1% formic acid in water
Mobile Phase B:	100% acetonitrile

Gradient:

Time (min.)	Flow rate (mL/min.)	Solvent A (%)	Solvent B (%)
1.00	1.200	100	0.00
1.10	1.200	60.0	40.0
4.00	1.200	0.00	100
4.50	1.200	0.00	100
4.60	1.200	100	0.00
6.00	1.200	100	0.00

Run Time: 6.0 minutes
Injector Wash Solvent: 30/30/40 acetonitrile/methanol/purified reagent water (v/v/v)
Column Temperature: 40 °C
Sample Temperature: 10 °C
Injection Volume: 25.0 µL
Retention Time: see table below

Analyte	Analysis	Retention Time (minutes)
M11	Primary	2.67
	Confirmatory	2.67

MS parameters:

Instrument: MDS Sciex API 5000 mass spectrometer
Ionization Mode: Positive (+) ESI
Ion Spray Voltage: 5500 V
Scan Type: MRM
Dwell Time: 200 milliseconds
Source Temperature: 600 °C
Curtain Gas: 15.0
Ion Source – Gas 1 / Gas 2: 60.0/60.0
Collision Gas: 7.00
Resolution Q1/Q3: Unit/Unit
Declustering Potential: 80.0
Collision Cell Entrance Potential: 5.00

	Primary Transition	Confirmatory Transition
Q1/Q3 Masses (amu):	129.17/69.08	129.17/70.03
Collision Energy:	26.0	23.0
Collision Cell Exit Potential:	10.0	10.0

2.11.3 Instrumental Conditions M1a

The LC-MS/MS analysis was conducted utilizing the following instrumental conditions:

LC parameters:

Column: Agilent Poroshell EC-C8, 2.7 µm, 3.0 × 50 mm
Mobile Phase A: 0.1% formic acid in water
Mobile Phase B: 0.1% formic acid in acetonitrile
Gradient:

Time (min.)	Flow rate (mL/min.)	Solvent A (%)	Solvent B (%)
0.50	0.800	95.0	5.00
3.00	0.800	00.0	100
4.00	0.800	00.0	100
4.10	0.800	95.0	5.00
5.00	0.800	95.0	5.00

Run Time: 5.0 minutes
Injector Wash Solvent: 30/30/40 acetonitrile/methanol/purified reagent water (v/v/v)
Column Temperature: 40 °C
Sample Temperature: 5 °C
Injection Volume: 25.0 µL
Retention Time: see table below

Analyte	Analysis	Retention Time (minutes)
M1a	Primary	2.60
	Confirmatory	2.59

MS parameters:

Instrument: AB MDS Sciex 4000 mass spectrometer
Ionization Mode: Positive (+) ESI
Ion Spray Voltage: 5500 V
Scan Type: MRM
Dwell Time: 200 milliseconds
Source Temperature: 600 °C
Curtain Gas: 15.0
Ion Source – Gas 1 / Gas 2: 60.0/60.0
Collision Gas: 7.00
Declustering Potential: 50.0
Resolution Q1/Q3: Unit/Unit

	Primary Transition	Confirmatory Transition
Q1/Q3 Masses (amu):	511.10/369.10	511.10/142.20
Collision Cell Entrance Potential:	10.0	15.0
Collision Energy:	38.0	45.0
Collision Cell Exit Potential:	10.0	12.0

Other instrumentation may be used but may require optimization to achieve the desired separation and sensitivity. It is important to note that the parameters above have been established for this particular instrumentation and may not be applicable for other similar equipment that may be used.

2.11.4 Preparation of Calibration Standard Curve

Two sets of calibration standards were analyzed with each recovery sample set; one set prior to analysis of the recovery samples, and the second set immediately following the analysis of the recovery samples. M11 calibration standards were interspersed among analysis of the recovery samples, every two to six injections. Injection of samples and calibration standards onto the LC-MS/MS system was performed by programmed automated injection.

2.12 Evaluation of Precision, Accuracy, Specificity, and Linearity

The accuracy was reported in terms of percent recovery of the fortified recovery samples. Recoveries of 70.0 to 120% (for the individual mean concentrations) are acceptable. The precision was reported in terms of the relative standard deviation (RSD) for the recovery samples. RSD values ≤20% were considered acceptable for the recovery samples. Specificity of the method was determined by examination of the control samples for peaks at the same retention times as BAS 315 I, M12, M11, M6, M1a, and M1b which might interfere with the quantitation of the analytes. Linearity of the method was determined by the coefficient of determination (r^2), y-intercept, and slope of the regression line. Representative product ion spectrum chromatograms are presented in Figure 2 though Figure 7.

2.13 Limit of Quantitation (LOQ)

The method was validated at the Limit of Quantitation (LOQ). This was defined as the lowest fortification level. Blank values (reagent blanks and untreated control samples) did not exceed 30% of the LOQ.

2.14 Limit of Detection (LOD) and Method Detection Limit (MDL)

The LOD was calculated using the standard deviation of the average recovery in units of concentration of the seven samples fortified at the LOQ, multiplied by a one-tailed t-statistic at the 99% confidence level for n-1 replicates. Representative calculations for the LOD can be found in Section 3.0.

The Method Detection Limit (MDL) was defined as the lowest concentration in test samples which can be detected based on the concentration of the low calibration standard and the dilution factor of the control solutions. Representative calculations for the MDL can be found in Section 3.0.

2.15 Sample Stability

The sample extracts were determined to be stable from the time they were processed until the instrumental analysis was complete. They were proven stable for 12 hours, and this is confirmed by their recoveries.

3.0 CALCULATIONS

For BAS 315 I, M12, M11, M6, and M1b, a calibration curve was constructed by plotting the analyte concentration (µg/L) of the calibration standards against the peak area of the analyte in the calibration standards. The equation of the line (equation 1) was algebraically manipulated to give equation 2. The concentration of test substance in each recovery sample was calculated

using the slope and intercept from the linear regression analysis, the detector response, and the dilution factor of the recovery sample. Equations 2 and 3 were then used to calculate measured concentrations and analytical results.

(1) $y = mx + b$

(2) $DC(x) = \frac{(y - b)}{m}$

(3) $A = DC \times DF$

where:

x	=	analyte concentration
y	=	detector response (peak area) from the chromatogram
b	=	y-intercept from the regression analysis
m	=	slope from the regression analysis
DC (x)	=	detected concentration (µg/L) in the sample
DF	=	dilution factor (final volume of the sample divided by the original sample mass, mL/g)
A	=	analytical result (µg/kg), concentration in the original sample

Example Calculation from sample 986-6267-37 for the primary transition of BAS 315 I in loamy sand soil (Figure 4E and Figure 4M) which is part of the re-validation located in Appendix 4.

(1) $469460 = 2527014x - 12559.0116$

(2) $0.19075\ \mu g/L = \frac{2527014 + 12559.0116}{2527014}$

(3) $47.687\ \mu g/kg = 0.19075\ \mu g/L \times 250$

For M1a, a calibration curve was constructed by plotting the natural logarithm (ln) of the analyte concentration (µg/L) of the calibration standards against the natural logarithm (ln) of the peak

(4) $\ln y = m(\ln x) + b$

(5) $\ln x = (\ln y - b) / m$

(6) $DC(x) = \text{inverse}(\ln x)$

(7) $A = DC \times DF$

area ratio of the analyte to the internal standard in the calibration standards. The equation of the line (equation 4) was algebraically manipulated to give equation 5. The concentration of test substance in each recovery sample was calculated using the slope and intercept of the regression analysis, and the natural logarithm of the peak area and the dilution factor of the recovery sample. Equations 5, 6, and 7 were then used to calculate measured concentrations and analytical results.

where:

lnx	=	natural logarithm of sample concentration
lny	=	natural logarithm of detector response ratio
m	=	slope from regression analysis
b	=	y-intercept from regression analysis
DC (x)	=	detected concentration (µg/L) in the sample
DF	=	dilution factor (final volume of the sample divided by the original sample mass, mL/g)
A	=	analytical result (µg/kg)

The LOD was calculated using the following equation (U.S. EPA, 2016, 1994):

$$(8) \qquad LOD = t_{0.99} \times SD$$

where:

$t_{0.99}$	=	One-tailed t-statistic at the 99% confidence level for n-1 replicates (i.e., 3.143 for seven replicates)
SD	=	Standard deviation of the detected concentrations of n samples spiked at the estimated LOQ
LOD	=	Limit of detection for the analysis

The MDL was calculated using the following equation:

$$(9) \qquad MDL = MDL_{LCAL} \times DF_{CNTL}$$

where:

MDL_{LCAL}	=	The lowest concentration calibration standard (i.e., 0.100 µg/L)
DF_{CNTL}	=	Dilution factor of the control samples (final volume of the sample divided by the original sample mass, mL/g)
MDL	=	Method detection limit reported (0.100 µg/L × 250 = 25.0 µg/kg).

Figure 1. Flow scheme for the processing of samples.

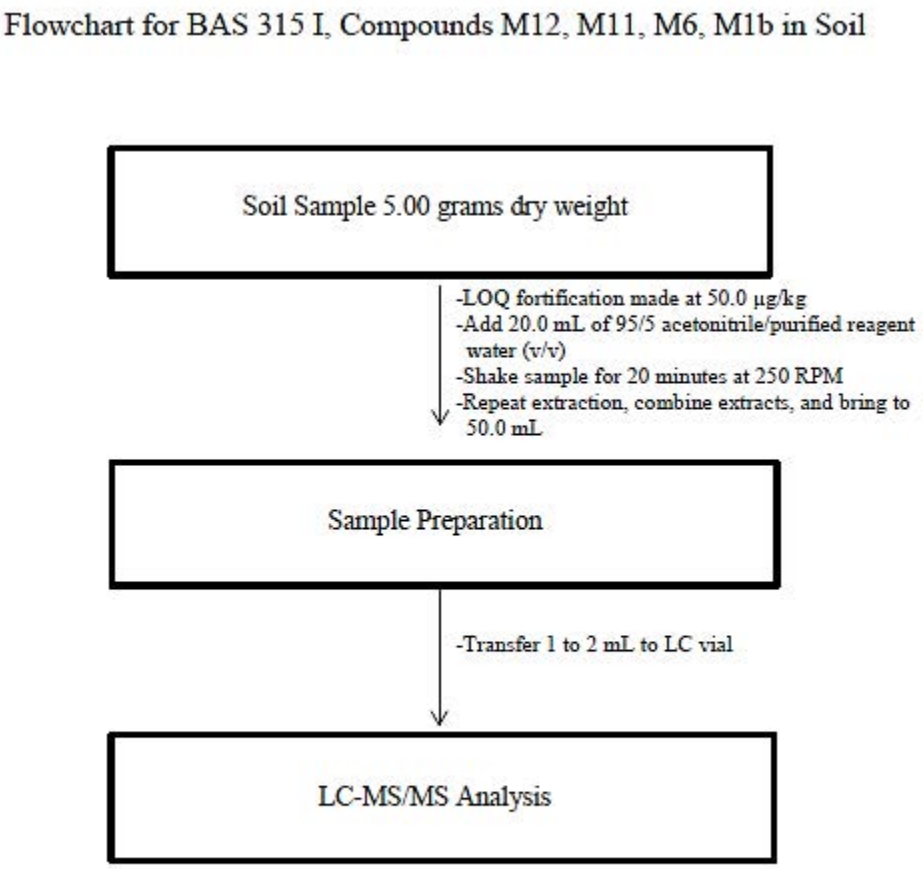

Figure 1. Continued. Flow scheme for the processing of samples.