# 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 Groundwater and Surface Water" (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 OCSPP 850.6100 (U.S. EPA, 2012) and SANCO/825/00 rev 8.1 (EC, 2010).

## 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, Reg. No. 4435553 (Compound E), was received on 20 December 2016 from BASF Corporation, Durham, North Carolina. The following information was provided:

| | |
|---|---|
| Name: | Reg. No. 4435553 (Compound E) |
| Batch No.: | L83-278 |
| CAS No.: | Not Listed |
| Purity: | 99.4% (Certificate of Analysis, Appendix 2) |
| Expiration Date: | 1 February 2019 |

Upon receipt at Smithers Viscient, the test substance (SMV No. 8663) was stored refrigerated in the original container. Concentrations were adjusted for the purity of the test substance.

The test substance, Compound UK, was received on 8 November 2017 from Ricerca Biosciences, Concord, Ohio. The following information was provided:

| | |
|---|---|
| Name: | Compound UK |
| Batch No.: | Not Available |
| CAS No.: | Not Available |
| Purity: | 93.0% (Determined in house, Appendix 3) |
| Expiration Date: | Not Available |

Upon receipt at Smithers Viscient, the test substance (SMV No. 9147) was stored in a freezer in the original container. Concentrations were adjusted for the purity of the test substance.

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

| | |
|---|---|
| Name: | Compound R |
| Synonym: | Hydramethylnon Metabolite R |
| Lot No.: | 55658-28-35 |
| CAS No.: | Not Listed |
| Purity: | 95.98% (Certificate of Analysis, Appendix 2) |
| Retest Date: | 20 December 2019 |

Upon receipt at Smithers Viscient, the test substance (SMV No. 8745) was stored in a freezer in the original container. Concentrations were adjusted for the purity of the test substance.

The test substance, Compound C, was received on 23 February 2016 from Sigma-Aldrich, Allentown, Pennsylvania. The following information was provided:

| | |
|---|---|
| Name: | Compound C |
| Synonyms: | Hydramethylnon Metabolite C; 4-(Trifluoromethyl)benzoic acid; 4-Carboxybenzotrifluoride |

| | |
|---|---|
| Lot No.: | MKBW7296V |
| CAS No.: | 455-24-3 |
| Purity: | 97.68% (Certificate of Analysis, Appendix 2) |
| Retest Date: | 20 December 2019 |

Upon receipt at Smithers Viscient, the test substance (SMV No. 8114) was stored at room temperature in the original container in a dark, ventilated cabinet. Concentrations were adjusted for the purity of the test substance.

The test substance, Compound F, was received on 19 April 2016 from Sigma-Aldrich Corp., Milwaukee, Wisconsin. The following information was provided:

| | |
|---|---|
| Name: | Compound F |
| Synonyms: | Hydramethylnon Metabolite F; trans-4-(Trifluoromethyl)cinnamic acid |
| Lot No.: | 1402309V |
| CAS No.: | 16642-92-5 |
| Purity: | 100% (Certificate of Analysis, Appendix 2) |
| Retest Date: | 20 December 2019 |

Upon receipt at Smithers Viscient, the test substance (SMV No. 8205) was stored at room temperature in the original container in a dark, ventilated cabinet. Concentrations were not 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. | Ammonium Hydroxide: | J.T. Baker, reagent grade |
| 6. | Formic Acid: | BDH, reagent grade |
| 7. | Dimethylformamide: | EMD, reagent grade |
| 8. | 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<br>AB MDS Sciex 4000 mass spectrometer equipped with an AB MDS Sciex ESI Turbo V source<br>Shimadzu LC-20AD solvent delivery pumps<br>Shimadzu DGU-20A3 vacuum degasser<br>Shimadzu DGU-20A5R vacuum degasser<br>Shimadzu SIL-20ACHT autoinjector<br>Shimadzu CTO-20A column compartment<br>Shimadzu CTO-20AC column oven<br>Shimadzu CBM-20A communications bus<br>Analyst version 1.6.3 software for data acquisition |
| 2. | Balance: | Mettler Toledo XSE205DU |
| 3. | Laboratory equipment: | Positive displacement pipets, volumetric flasks, disposable glass and plastic pipets, graduated cylinders, stir bar and stir plate, vortexer, amber vials with crimp caps, disposable glass vials, 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 Matrices

The matrices used during this method validation were groundwater and surface water.

Groundwater information:
Groundwater used in the study was unfiltered well water. The water was determined to have a pH of 6.31 measured using a Yellow Springs Instruments (YSI) pH100 pH meter and a dissolved oxygen concentration of 5.62 mg/L measured using a YSI Pro 20 dissolved oxygen meter. All documentation relating to the preparation, storage, and handling is maintained by Smithers Viscient.

Surface water information:
The surface water used for this method validation analysis was collected from the Taunton River (SMV Lot No.12 Jul 17 Wat-B, collected on 12 July 2017 for the main report and SMV Lot No.19 Mar 18 Wat-A, collected on 19 March 2018 for Appendix 5) in Taunton, Massachusetts. The water was collected from an area of the river with approximately 30 to 60 cm of overlying water and was determined to have a pH of 6.2 measured using a Yellow Springs Instruments (YSI) pH100 pH meter and a dissolved oxygen concentration of 6.2 mg/L measured using a YSI Pro 20 dissolved oxygen meter. All documentation relating to the preparation, storage, and handling is maintained by Smithers Viscient.

Representative samples of groundwater and surface water were characterized in house. The results of these characterizations are presented in Appendix 4.

## 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 methanol/purified reagent water (v/v) liquid reagent solution was typically prepared by combining 50.0 mL of acetonitrile and 50.0 mL of purified reagent water. The solution was mixed well using a stir bar and stir plate for five minutes.

A 50/50 methanol/test matrix (v/v) liquid reagent solution was typically prepared by combining 100 mL of methanol and 100 mL of test matrix. The solution was mixed well using a stir bar and stir plate for five minutes.

A 20/80 methanol/test matrix (v/v) liquid reagent solution was typically prepared by combining 40.0 mL of methanol and 160 mL of test matrix. The solution was mixed well using a stir bar and stir plate for five minutes.

A 95/5 methanol/ammonium hydroxide (v/v) liquid reagent solution was prepared by combining 475 mL of methanol and 25.0 mL of ammonium hydroxide. The solution was mixed using a stir bar and stir plate for five minutes.

A 20/80 caustic methanol/purified reagent water (v/v) liquid reagent solution was typically prepared by combining 100 mL of 95/5 methanol/ammonium hydroxide (v/v) and 400 mL of purified reagent water. The solution was mixed well using a stir bar and stir plate for five minutes.

A 20/80 methanol/purified reagent water (v/v) liquid reagent solution was typically prepared by combining 20.0 mL of methanol and 80.0 mL of purified reagent water. The solution was mixed well using a stir bar and stir plate for five minutes.

A 90/10 acetonitrile/purified reagent water (v/v) liquid reagent solution was typically prepared by combining 225 mL of acetonitrile and 25.0 mL of purified reagent water. The solution was mixed well following preparation.

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

## 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 typically 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 | Secondary stock solution |
| 8663A | 0.0505 | 0.0502 | Dimethylformamide | 50.0 | 1000 | Secondary stock solution |
| 9147A | 0.00570 | 0.00530 | Acetonitrile | 5.00 | 1060 | Secondary stock solution |
| 8745A | 0.0523 | 0.0502 | Methanol | 50.0 | 1000 | Secondary stock solution |
| 8114D | 0.0514 | 0.0502 | 90/10 acetonitrile/ purified reagent water (v/v) | 50.0 | 1000 | Secondary stock solution |
| 8205D | 0.0500 | 0.0500 | 90/10 acetonitrile/ purified reagent water (v/v) | 50.0 | 1000 | Secondary stock solution |

Secondary stock solutions were typically 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 |
|---|---|---|---|---|---|---|---|
| 8662D | 1000 | 0.500 | 50.0 | Acetonitrile | 8662D-2 | 10.0 | Sub-stock solution |
| 8663A | 1000 | 0.500 | 50.0 | Acetonitrile | 8663A-2 | 10.0 | Sub-stock solution |
| 9147A | 1060 | 0.472 | 50.0 | Acetonitrile | 9147A-1 | 10.0 | Sub-stock solution |
| 8745A | 1000 | 0.500 | 50.0 | Methanol | 8745A-2 | 10.0 | Sub-stock solution |
| 8114D | 1000 | 0.500 | 50.0 | Acetonitrile | 8114D-2 | 10.0 | Sub-stock solution |
| 8205D | 1000 | 0.500 | 50.0 | Acetonitrile | 8205D-2 | 10.0 | Sub-stock solution |

Sub-stock solutions were typically 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 |
|---|---|---|---|---|---|---|---|
| 8662D-2 | 10.0 | 0.0100 | 10.0 | Methanol | Mix Stk 1 | 0.0100 | Matrix-matched calibration standards and recovery samples |
| 8663A-2 | 10.0 | 0.0100 | | | | | |
| 9147A-1 | 10.0 | 0.0100 | | | | | |
| 8745A-2 | 10.0 | 0.100 | 10.0 | Methanol | Stk 1 | 0.100 | Calibration Standards and recovery samples |
| 8114D-2 | 10.0 | 0.0100 | 10.0 | Methanol | Mix Stk 1 | 0.0100 | Matrix-matched calibration standards and recovery samples |
| 8205D-2 | 10.0 | 0.0100 | | | | | |

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 and Compounds E and UK

Calibration standards were prepared in 50/50 methanol/test matrix (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 |
|---|---|---|---|---|---|
| Mix Stk 1 | 0.0100 | 0.0300 | 10.0 | 0.0300 | Std 1 |
| | | 0.0500 | 10.0 | 0.0500 | Std 2 |
| | | 0.0700 | 10.0 | 0.0700 | Std 3 |
| | | 0.100 | 10.0 | 0.100 | Std 4 |
| | | 0.150 | 10.0 | 0.150 | Std 5 |
| | | 0.300 | 10.0 | 0.300 | Std 6 |

### 2.8.2 Calibration Standards - Compound R

Calibration standards were prepared in 20/80 caustic methanol/purified reagent water (v/v) by fortifying with the 0.100 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 1 | 0.100 | 0.0250 | 10.0 | 0.250 | Std 1 |
| | | 0.0500 | 10.0 | 0.500 | Std 2 |
| | | 0.0700 | 10.0 | 0.700 | Std 3 |
| | | 0.100 | 10.0 | 1.00 | Std 4 |
| | | 0.150 | 10.0 | 1.50 | Std 5 |
| | | 0.200 | 10.0 | 2.00 | Std 6 |

### 2.8.3 Calibration Standards - Compounds C and F

Calibration standards were prepared in 20/80 methanol/test matrix (v/v) 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 |
|---|---|---|---|---|---|
| Mix Stk 1 | 0.0100 | 0.0500 | 10.0 | 0.0500 | Std 1 |
| | | 0.100 | 10.0 | 0.100 | Std 2 |
| | | 0.200 | 10.0 | 0.200 | Std 3 |
| | | 0.300 | 10.0 | 0.300 | Std 4 |
| | | 0.400 | 10.0 | 0.400 | Std 5 |
| | | 0.500 | 10.0 | 0.500 | Std 6 |

### 2.8.4 Matrix Effect Investigation - BAS 315 I and Compounds E and UK

In an effort to observe any potential matrix effects, 50/50 methanol/test matrix (v/v) 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[a] (mL) | Standard Concentration (µg/L) | Sample ID |
|---|---|---|---|---|---|
| Mix Stk 1 | 0.0100 | 0.0500 | 10.0 | 0.0500 | MM-Std A |
| | | 0.0500 | 10.0 | 0.0500 | MM-Std B |
| | | 0.0500 | 10.0 | 0.0500 | MM-Std C |

[a] Diluted with 50/50 methanol/test matrix (v/v)

### 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 |
|---|---|---|---|---|---|
| Mix Stk 1 | 0.0100 | 0.0500 | 10.0 | 0.0500 | Std A |
| | | 0.0500 | 10.0 | 0.0500 | Std B |
| | | 0.0500 | 10.0 | 0.0500 | Std C |

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

### 2.8.5 Matrix Effect Investigation - Compound R

In an effort to observe any potential matrix effects, prepared matrix blanks were 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.

### 2.8.5.1 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 1 | 0.100 | 0.0250 | 5.00 | 0.500 | MM-Std 1 |
| | | 0.0250 | 5.00 | 0.500 | MM-Std 2 |
| | | 0.0250 | 5.00 | 0.500 | MM-Std 3 |

[a] Diluted with the final dilution of the matrix blanks

### 2.8.5.2 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 1 | 0.100 | 0.0250 | 5.00 | 0.500 | Std A |
| | | 0.0250 | 5.00 | 0.500 | Std B |
| | | 0.0250 | 5.00 | 0.500 | Std C |

[a] Diluted with 20/80 caustic methanol/purified reagent water (v/v)

### 2.8.6 Matrix Effect Investigation - Compounds C and F

In an effort to observe any potential matrix effects, 20/80 methanol/test matrix (v/v) were 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.

### 2.8.6.1 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 |
|---|---|---|---|---|---|
| Mix Stk 1 | 0.0100 | 0.100 | 10.0 | 0.100 | MM-Std A |
| | | 0.100 | 10.0 | 0.100 | MM-Std B |
| | | 0.100 | 10.0 | 0.100 | MM-Std C |

[a] Diluted with 20/80 methanol/test matrix (v/v)

### 2.8.6.2 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 |
|---|---|---|---|---|---|
| Mix Stk 1 | 0.0100 | 0.100 | 10.0 | 0.100 | Std A |
| | | 0.100 | 10.0 | 0.100 | Std B |
| | | 0.100 | 10.0 | 0.100 | Std C |

[a] Diluted with 20/80 methanol/purified reagent water (v/v)

## 2.9 Sample Fortification and Preparation

### 2.9.1 BAS 315 I and Compounds E, and UK

The recovery samples were prepared in each matrix (groundwater and surface water) with BAS 315 I and Compounds E, and UK at concentrations of 0.100 (LOQ) and 1.00 (10XLOQ) µg/L. Recovery samples for the two matrices were prepared separately ("de novo") at these concentrations. Seven replicates were produced for the LOQ concentration and five replicates were produced for the High concentration. Two samples were left unfortified to serve as controls and were diluted in the same fashion as the LOQ concentration recovery samples. In addition, one reagent blank was prepared and processed in the same manner as the control samples. The preparation procedure for each separate matrix is outlined in the tables below. BAS315 I and Compound UK 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 5.

**Groundwater recovery samples:**

| Sample ID 986-6265- | Sample Type | Sub-Stock Concentration (µg/L) | Volume of Fortification (mL) | Final Volume (mL) | Fortified Concentration (µg/L) |
|---|---|---|---|---|---|
| 75 | Reagent Blk | NA[a] | NA | 5.00[b] | 0.00 |
| 76 & 77 | Control | NA | NA | 5.00[c] | 0.00 |
| 78, 79, 80, 81, 82, 83, & 84 | LOQ | 0.0100 | 0.0500 | 5.00[c] | 0.100 |
| 82, 86, 87, 88, & 89 | 10XLOQ | 0.0100 | 0.500 | 5.00[c] | 1.00 |

[a] NA = Not Applicable
[b] Reagent: Methanol
[c] Matrix: Groundwater

**Surface water recovery samples:**

| Sample ID 986-6265- | Sample Type | Sub-Stock Concentration (mg/L) | Volume of Fortification (mL) | Final Volume (mL) | Fortified Concentration (µg/L) |
|---|---|---|---|---|---|
| 60 | Reagent Blk | NA[a] | NA | 5.00[b] | 0.00 |
| 61 & 62 | Control | NA | NA | 5.00[c] | 0.00 |
| 63, 64, 65, 66, 67, 68, & 69 | LOQ | 0.0100 | 0.0500 | 5.00[c] | 0.100 |
| 70, 71, 72, 73, & 74 | 10XLOQ | 0.0100 | 0.500 | 5.00[c] | 1.00 |

[a] NA = Not Applicable
[b] Reagent: Methanol
[c] Matrix: Surface water

### 2.9.2 Compound R

The recovery samples were prepared in each matrix (groundwater and surface water) with Compound R at concentrations of 0.100 (LOQ) and 1.00 (10XLOQ) µg/L. Recovery samples for each matrix were prepared separately ("de novo") at these concentrations. Seven replicates were produced for the LOQ samples and five replicates were produced for the 10XLOQ. Two samples were left unfortified to serve as controls and were processed in the same fashion as the LOQ concentration recovery samples. Three samples were left unfortified to serve as matrix

blanks and were processed in the same manner as the control samples in order to assess matrix effects. In addition, one reagent blank was prepared using the elution solvent and processed in the same manner as the control samples. The preparation procedure for each separate matrix is outlined in the tables below. If test samples of unknown concentration are analyzed, then it is recommended that the processing scheme used for the LOQ samples be employed, and then diluted as necessary. If test samples are suspected of containing significant concentrations of Compound R, then the processing scheme used for the 10XLOQ samples can be employed using less sample volume.

**Groundwater recovery samples:**

| Sample ID 986-6265- | Sample Type | Sub-Stock Concentration (mg/L) | Volume of Fortification (mL) | Final Volume (mL) | Formic Acid Volume (mL) | Fortified Concentration (µg/L) |
|---|---|---|---|---|---|---|
| 01 | Reagent Blk | NA[a] | NA | NA | NA | 0.00 |
| MM-Std 1, 2, & 3 | Matrix Blank | NA | NA | 25.0 | 0.100 | 0.00 |
| 02 & 03 | Control | NA | NA | 25.0 | 0.100 | 0.00 |
| 04, 05, 06, 07, 08, 09, & 10 | LOQ | 0.100 | 0.0250 | 25.0 | 0.100 | 0.100 |
| 11, 12, 13, 14, & 15 | 10XLOQ | 0.100 | 0.0500 | 5.00 | 0.0200 | 1.00 |

[a] NA = Not Applicable

**Surface water recovery samples:**

| Sample ID 986-6265- | Sample Type | Sub-Stock Concentration (mg/L) | Volume of Fortification (mL) | Final Volume (mL) | Formic Acid Volume (mL) | Fortified Concentration (µg/L) |
|---|---|---|---|---|---|---|
| 16 | Reagent Blk | NA[a] | NA | NA | NA | 0.00 |
| MM-Std 1, 2, & 3 | Matrix Blank | NA | NA | 25.0 | 0.100 | 0.00 |
| 17 & 18 | Control | NA | NA | 25.0 | 0.100 | 0.00 |
| 19, 20, 21, 22, 23, 24, & 25 | LOQ | 0.100 | 0.0250 | 25.0 | 0.100 | 0.100 |
| 26, 27, 28, 29, & 30 | 10XLOQ | 0.100 | 0.0500 | 5.00 | 0.0200 | 1.00 |

[a] NA = Not Applicable

### 2.9.3 Compounds C and F

The recovery samples were prepared in each matrix (groundwater and surface water) with Compounds C and F at concentrations of 0.100 (LOQ) and 1.00 (10XLOQ) µg/L. Recovery samples for the two matrices were prepared separately ("de novo") at these concentrations. Seven replicates were produced for the LOQ concentration and five replicates were produced for the High concentration. Two samples were left unfortified to serve as controls and were diluted in the same fashion as the LOQ concentration recovery samples. In addition, one reagent blank was prepared and processed in the same manner as the control samples. The preparation procedure for each separate matrix is outlined in the tables below.

**Groundwater recovery samples:**

| Sample ID 986-6265- | Sample Type | Sub-Stock Concentration (µg/L) | Volume of Fortification (mL) | Final Volume (mL) | Fortified Concentration (µg/L) |
|---|---|---|---|---|---|
| 31 | Reagent Blk | NA[a] | NA | 8.00[b] | 0.00 |
| 32 & 33 | Control | NA | NA | 8.00[c] | 0.00 |
| 34, 35, 36, 37, 38, 39, & 40 | LOQ | 0.0100 | 0.0800 | 8.00[c] | 0.100 |
| 41, 42, 43, 44, & 45 | 10XLOQ | 0.0100 | 0.800 | 8.00[c] | 1.00 |

[a] NA = Not Applicable
[b] Reagent: Methanol
[c] Matrix: Groundwater

**Surface water recovery samples:**

| Sample ID 986-6265- | Sample Type | Sub-Stock Concentration (mg/L) | Volume of Fortification (mL) | Final Volume (mL) | Fortified Concentration (µg/L) |
|---|---|---|---|---|---|
| 46 | Reagent Blk | NA[a] | NA | 8.00[b] | 0.00 |
| 47 & 48 | Control | NA | NA | 8.00[c] | 0.00 |
| 49, 50, 51, 52, 53, 54, & 55 | LOQ | 0.0100 | 0.0800 | 8.00[c] | 0.100 |
| 56, 57, 58, 59, & 60 | 10XLOQ | 0.0100 | 0.800 | 8.00[c] | 1.00 |

[a] NA = Not Applicable
[b] Reagent: Methanol
[c] Matrix: Surface water

## 2.10 Sample Processing

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

### 2.10.1 BAS 315 I and Compounds E, and UK

To minimize the potential for losses of the test substance during processing, the aqueous recovery samples were not sub-sampled prior to dilution. The first dilution with methanol was performed by the addition of methanol to the entire volume of the aqueous sample in the container in which it was fortified to a final composition of 50/50 methanol/test matrix (v/v). The 10XLOQ recovery samples were subsequently diluted into the calibration standard range with 50/50 methanol/test matrix (v/v). The dilution procedure is outlined in the tables below. BAS315 I and Compound UK 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 5.

**Groundwater recovery samples:**

| Sample ID 986-6265- | Sample Type | Nominal Concentration (µg/L) | Sample Volume (mL) | Final Volume (mL)[a] | Sample Volume (mL) | Final Volume (mL)[b] | Dilution Factor |
|---|---|---|---|---|---|---|---|
| 75 | Reagent Blk | 0.00 | 5.00 | 10.0 | NA[c] | NA | 2.00 |
| 76 & 77 | Control | 0.00 | 5.00 | 10.0 | NA | NA | 2.00 |
| 78, 79, 80, 81, 82, 83, & 84 | LOQ | 0.100 | 5.00 | 10.0 | NA | NA | 2.00 |
| 82, 86, 87, 88, & 89 | 10XLOQ | 1.00 | 5.00 | 10.0 | 2.00 | 10.0 | 10.0 |

[a] Diluted with methanol
[b] Diluted with 50/50 methanol/groundwater (v/v)
[c] NA = Not Applicable

**Surface water recovery samples:**

| Sample ID 986-6265- | Sample Type | Nominal Concentration (µg/L) | Sample Volume (mL) | Final Volume (mL)[a] | Sample Volume (mL) | Final Volume (mL)[b] | Dilution Factor |
|---|---|---|---|---|---|---|---|
| 60 | Reagent Blk | 0.00 | 5.00 | 10.0 | NA[c] | NA | 2.00 |
| 61 & 62 | Control | 0.00 | 5.00 | 10.0 | NA | NA | 2.00 |
| 63, 64, 65, 66, 67, 68, & 69 | LOQ | 0.100 | 5.00 | 10.0 | NA | NA | 2.00 |
| 70, 71, 72, 73, & 74 | 10XLOQ | 1.00 | 5.00 | 10.0 | 2.00 | 10.0 | 10.0 |

[a] Diluted with methanol
[b] Diluted with 50/50 methanol/surface water (v/v)
[c] NA = Not Applicable

### 2.10.2 Compound R

Waters Oasis MCX SPE columns (150 mg, 6 cc) were conditioned by rinsing with two column volumes of methanol followed by two column volumes of purified reagent water. The columns were not allowed to go dry until before elution. An aliquot of formic acid was added to each aqueous sample (i.e., a 0.100 mL to a 25.0 mL sample and a 0.0200 mL to a 5.00 mL sample). The samples were loaded onto the columns, and allowed to flow through under vacuum at no greater than 1 drop/sec. The columns were slowly brought to dryness without additional drying or rinsing as this would result in analyte losses. The test substance was eluted from the SPE columns with 2.50 mL of 95/5 methanol/ammonium hydroxide (v/v) under vacuum at no greater than 1 drop/sec and then collected into glass conical tubes. When eluting, the sorbent was saturated with elution solvent and allowed to sit for thirty seconds before applying vacuum. The samples were taken to volume (0.200 mL) under a gentle stream of nitrogen at 50.0 °C. The samples were reconstituted in 20/80 caustic methanol/purified reagent water (v/v), which was added to each sample with vortexing (15 seconds) to aid in reconstitution. The dilution procedure is outlined in the tables below.

**Groundwater recovery samples:**

| Sample ID 986-6265- | Sample Type | Nominal Concentration (µg/L) | Sample Volume (mL) | Reconstitution Volume (mL)[a] | Dilution Factor |
|---|---|---|---|---|---|
| 01 | Reagent Blk | 0.00 | NA[b] | 5.00 | 0.200 |
| MM-Std 1, 2, & 3 | Matrix Blank | 0.00 | 25.0 | 5.00 | 0.200 |
| 02 & 03 | Control | 0.00 | 25.0 | 5.00 | 0.200 |
| 04, 05, 06, 07, 08, 09, & 10 | LOQ | 0.100 | 25.0 | 5.00 | 0.200 |
| 11, 12, 13, 14, & 15 | 10XLOQ | 1.00 | 5.00 | 5.00 | 1.00 |

[a] Diluted with 20/80 caustic methanol/purified reagent water (v/v)
[b] NA = Not Applicable

**Surface water recovery samples:**

| Sample ID 986-6265- | Sample Type | Nominal Concentration (µg/L) | Sample Volume (mL) | Reconstitution Volume (mL)[a] | Dilution Factor |
|---|---|---|---|---|---|
| 16 | Reagent Blk | 0.00 | NA[b] | 5.00 | 0.200 |
| MM-Std 1, 2, & 3 | Matrix Blank | 0.00 | 25.0 | 5.00 | 0.200 |
| 17 & 18 | Control | 0.00 | 25.0 | 5.00 | 0.200 |
| 19, 20, 21, 22, 23, 24, & 25 | LOQ | 0.100 | 25.0 | 5.00 | 0.200 |
| 26, 27, 28, 29, & 30 | 10XLOQ | 1.00 | 5.00 | 5.00 | 1.00 |

[a] Diluted with 20/80 caustic methanol/purified reagent water (v/v)
[b] NA = Not Applicable

### 2.10.3 Compounds C and F

To minimize the potential for losses of the test substance during processing, the aqueous recovery samples were not sub-sampled prior to dilution. The first dilution with methanol was performed by the addition of methanol to the entire volume of the aqueous sample in the container in which it was fortified to a final composition of 20/80 methanol/test matrix (v/v). The 10XLOQ recovery samples were subsequently diluted into the calibration standard range with 20/80 methanol/test matrix (v/v). The dilution procedure is outlined in the tables below.

**Groundwater recovery samples:**

| Sample ID 986-6265- | Sample Type | Nominal Concentration (µg/L) | Sample Volume (mL) | Final Volume (mL)[a] | Sample Volume (mL) | Final Volume (mL)[b] | Dilution Factor |
|---|---|---|---|---|---|---|---|
| 31 | Reagent Blk | 0.00 | 8.00 | 10.0 | NA[c] | NA | 1.25 |
| 32 & 33 | Control | 0.00 | 8.00 | 10.0 | NA | NA | 1.25 |
| 34, 35, 36, 37, 38, 39, & 40 | LOQ | 0.100 | 8.00 | 10.0 | NA | NA | 1.25 |
| 41, 42, 43, 44, & 45 | 10XLOQ | 1.00 | 8.00 | 10.0 | 4.00 | 10.0 | 3.13 |

[a] Diluted with methanol
[b] Diluted with 20/80 methanol/groundwater (v/v)
[c] NA = Not Applicable

**Surface water recovery samples:**

| Sample ID 986-6265- | Sample Type | Nominal Concentration (µg/L) | Sample Volume (mL) | Final Volume (mL)[a] | Sample Volume (mL) | Final Volume (mL)[b] | Dilution Factor |
|---|---|---|---|---|---|---|---|
| 46 | Reagent Blk | 0.00 | 8.00 | 10.0 | NA[c] | NA | 1.25 |
| 47 & 48 | Control | 0.00 | 8.00 | 10.0 | NA | NA | 1.25 |
| 49, 50, 51, 52, 53, 54, & 55 | LOQ | 0.100 | 8.00 | 10.0 | NA | NA | 1.25 |
| 56, 57, 58, 59, & 60 | 10XLOQ | 1.00 | 8.00 | 10.0 | 4.00 | 10.0 | 3.13 |

[a] Diluted with methanol
[b] Diluted with 20/80 methanol/surface water (v/v)
[c] NA = Not Applicable

## 2.11 Analysis

### 2.11.1 Instrumental Conditions - BAS 315 I and Compounds E and UK

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

**LC parameters:**

| | |
|---|---|
| Column: | Phenomenex Synergi Fusion RP 80Å, 4.0 µm, 5 × 2.0 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 (%) |
|---|---|---|---|
| 0.01 | 0.500 | 85.0 | 15.0 |
| 0.50 | 0.500 | 85.0 | 15.0 |
| 3.00 | 0.500 | 0.00 | 100 |
| 4.00 | 0.500 | 0.00 | 100 |
| 4.10 | 0.500 | 85.0 | 15.0 |
| 5.00 | 0.500 | 85.0 | 15.0 |

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: 100 µL
Retention Times: see table below

| Analyte | Analysis | Approximate Retention Time (minutes) | |
|---|---|---|---|
| | | Groundwater | Surface Water |
| BAS 315 I | Primary | 2.7 | 2.7 |
| | Confirmatory | 2.7 | 2.7 |
| Compound E | Primary | 2.5 | 2.4 |
| | Confirmatory | 2.5 | 2.6 |
| Compound UK | Primary | 2.5 | 2.5 |
| | Confirmatory | 2.5 | 2.5 |

**MS parameters:**

Instrument: MDS Sciex API 5000 mass spectrometer
Ionization Mode: Positive (+) ESI
Ion Spray Voltage: 5500 V
Scan Type: MRM
Dwell Time: 75.0 milliseconds
Source Temperature: 650 °C
Curtain Gas: 15.0
Ion Source – Gas 1 / Gas 2: 60.0/60.0
Collision Gas: 8.00
Declustering Potential: 100
Resolution Q1/Q3: Unit/Unit

**BAS 315 I[a]:**

| | Primary Transition | Confirmatory Transition |
|---|---|---|
| Q1/Q3 Masses (amu): | 496.20/324.10 | 496.20/369.10 |
| Collision Cell Entrance Potential: | 12.5 | 11.5 |
| Collision Energy: | 43.0 | 46.0 |
| Collision Cell Exit Potential: | 10.0 | 20.0 |

**Compound E:**

| | Primary Transition | Confirmatory Transition |
|---|---|---|
| Q1/Q3 Masses (amu): | 351.44/331.08 | 351.44/275.05 |
| Collision Cell Entrance Potential: | 10.0 | 10.0 |
| Collision Energy: | 31.0 | 41.0 |
| Collision Cell Exit Potential: | 7.00 | 13.0 |

**Compound UK[a]:**

| | Primary Transition | Confirmatory Transition |
|---|---|---|
| Q1/Q3 Masses (amu): | 512.16/324.21 | 512.16/315.29 |
| Collision Cell Entrance Potential: | 10.0 | 10.0 |
| Collision Energy: | 48.0 | 49.0 |
| Collision Cell Exit Potential: | 9.00 | 15.0 |

[a] The $C^{13}$ transition was monitored in this instance, however both the $C^{12}$ and $C^{13}$ transitions can be used as necessary. See re-validation located in Appendix 5.

### 2.11.2 Instrumental Conditions - Compound R

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

**LC parameters:**

Column: Waters Atlantis T3, 3.0 µm, 4.6 × 100 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 (%) |
|---|---|---|---|
| 0.01 | 1.200 | 100 | 0.00 |
| 0.50 | 1.200 | 100 | 0.00 |
| 6.00 | 1.200 | 40.0 | 60.0 |
| 6.50 | 1.200 | 40.0 | 60.0 |
| 6.60 | 1.200 | 100 | 0.00 |
| 8.00 | 1.200 | 100 | 0.00 |

Run Time: 8.0 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: 100 µL
Retention Times: see table below

| Analyte | Analysis | Approximate Retention Time (minutes) | |
|---|---|---|---|
| | | Groundwater | Surface Water |
| Compound R | Primary | 3.4 | 3.4 |
| | Confirmatory | 3.4 | 3.4 |

**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: | 5.00 |
| Collision Cell Entrance Potential: | 10.0 |
| Collision Cell Exit Potential: | 10.0 |
| Declustering Potential: | 50.0 |
| Resolution Q1/Q3: | Unit/Unit |

| | Primary Transition | Confirmatory Transition |
|---|---|---|
| Q1/Q3 Masses (amu): | 142.13/70.02 | 142.13/72.17 |
| Collision Energy: | 20.0 | 23.0 |

### 2.11.3 Instrumental Conditions - Compounds C and F

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

**LC parameters:**

Column: Waters Xbridge BEH C18, 2.5 µm, 2.1 × 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 (%) |
|---|---|---|---|
| 0.01 | 0.350 | 95.0 | 5.00 |
| 0.50 | 0.350 | 95.0 | 5.00 |
| 3.50 | 0.350 | 00.0 | 100 |
| 4.00 | 0.350 | 00.0 | 100 |
| 4.10 | 0.350 | 95.0 | 5.00 |
| 5.00 | 0.350 | 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: 100 µL
Retention Times: see table below

| Analyte | Analysis | Approximate Retention Time (minutes) | |
|---|---|---|---|
| | | Groundwater | Surface Water |
| Compound C | Primary | 3.2 | 3.2 |
| | Confirmatory | 3.2 | 3.2 |
| Compound F | Primary | 3.3 | 3.3 |
| | Confirmatory | 3.3 | 3.3 |

**MS parameters:**

Instrument: AB MDS Sciex 4000 mass spectrometer
Ionization Mode: Negative (−) ESI
Ion Spray Voltage: -4200 V
Scan Type: MRM
Dwell Time: 75.0 milliseconds
Source Temperature: 650 °C
Curtain Gas: 15.0
Ion Source – Gas 1 / Gas 2: 60.0/20.0
Collision Gas: 4.00
Collision Cell Exit Potential: -10.0
Resolution Q1/Q3: Unit/Unit

**Compound C:**

| | Primary Transition | Confirmatory Transition |
|---|---|---|
| Q1/Q3 Masses (amu): | 189.01/144.86 | 235.08/188.82 |
| Declustering Potential: | -40.0 | -28.0 |
| Collision Cell Entrance Potential: | -5.00 | -6.00 |
| Collision Energy: | -22.0 | -10.0 |

**Compound F:**

| | Primary Transition | Confirmatory Transition |
|---|---|---|
| Q1/Q3 Masses (amu): | 215.04/170.98 | 261.09/214.88 |
| Declustering Potential: | -50.0 | -20.0 |
| Collision Cell Entrance Potential: | -8.00 | -8.00 |
| Collision Energy: | -19.0 | -10.0 |

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. Calibration standards were interspersed among analysis of the recovery samples, every two to six injections. For Compounds C and F, one set was analyzed prior to analysis of the recovery samples and the second set immediately following the analysis of the recovery samples. Injection of recovery samples and calibration standards onto the LC-MS/MS system was performed by programmed automated injection.

## 2.12 Evaluation of Accuracy, Precision, Linearity, and Specificity

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. Linearity of the method was determined by the coefficient of determination ($r^2$), y-intercept, and slope of the regression line. Specificity of the method was determined by examination of the control samples for peaks at the same retention times as BAS 315 I and Compounds E, UK, R, C, and F which might interfere with the quantitation of the analytes. Representative product ion spectrum chromatograms are presented in Figure 2 through 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 Time Required for Analysis

This validation study included the validation of two water matrices (surface water and groundwater). Each water matrix validation included three sets of samples used for LC-MS/MS analysis. Each set of samples consisted of 12 fortified, two unfortified samples, one reagent blank, and six calibration standards (21 samples total). A single analyst completed a set of 21 samples in one working day (eight hours) with LC-MS/MS analysis performed overnight into the next day (approximately 8 hours).

### 2.16 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 and Compounds E, UK, and R, 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 volume) |
| A | = | analytical result (µg/L), concentration in the original sample |

Example Calculation from sample 986-6265-81 for the primary transition of Compound E in groundwater (Figure 15 and Figure 70)

(4) $92509 = 1771301x + 698.7425$

(5) $0.051832\ \mu g/L = \frac{92509 - 698.7425}{1771301}$

(6) $0.10366\ \mu g/L = 0.051832\ \mu g/L \times 2.00$

For Compounds C and F, 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 area of the analyte 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 4, 5, and 6 were then used to calculate measured concentrations and analytical results.

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

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

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

(10) $A = DC \times DF$

where:

| | | |
|---|---|---|
| lnx | = | natural logarithm of sample concentration |
| lny | = | natural logarithm of detector response |
| 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 volume) |
| A | = | analytical result (µg/L) |

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

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

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 method detection limit (MDL) is defined as the lowest concentration that can be detected by this method in test solution samples. The MDL is calculated (equation 12) based on the concentration of the low calibration standard and the dilution factor of the control samples.

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

where:

| | | |
|---|---|---|
| $MDL_{LCAL}$ | = | lowest concentration calibration standard (e.g., 0.0300 µg/L) |
| $DF_{CNTL}$ | = | dilution factor of the control samples (smallest dilution factor used; e.g., 2.00) |
| MDL | = | method detection limit reported for the analysis (e.g., 0.0300 µg/L × 2.00 = 0.0600 µg/L) |

# PROTOCOL DEVIATION

The protocol states that determination of matrix effects should be assessed as outlined in the analytical methods for both primary and confirmatory LC-MS/MS methods. Matrix effects should be evaluated at the LOQ level for each test substance. Calibration with standards in solvent may be used if experiments clearly demonstrate that matrix effects are not significant (i.e., matrix effects <20%), In the event that there are no matrix effects, matrix-matched standards may also be used if deemed appropriate. For compounds C and F, the matrix assessment was inadvertently not performed at the concentration the LOQ samples were diluted to in the standard curve. The assessment was done at 0.100 µg/L as opposed to 0.0800 µg/L. Since no significant matrix effect was observed at 0.100 µg/L and it is unlikely that the effect would become significant if the matrix blanks were fortified at 0.0800 µg/L. Additionally, the intent of the matrix assessment is to determine if there is a bias in measured concentrations when comparing samples with matrix to solvent based standards without matrix. Since the calibration standards were matrix matched along with all of the samples any effect would be cancelled out; therefore, this deviation did not have a negative impact on the overall outcome of the results or interpretation of the study.

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

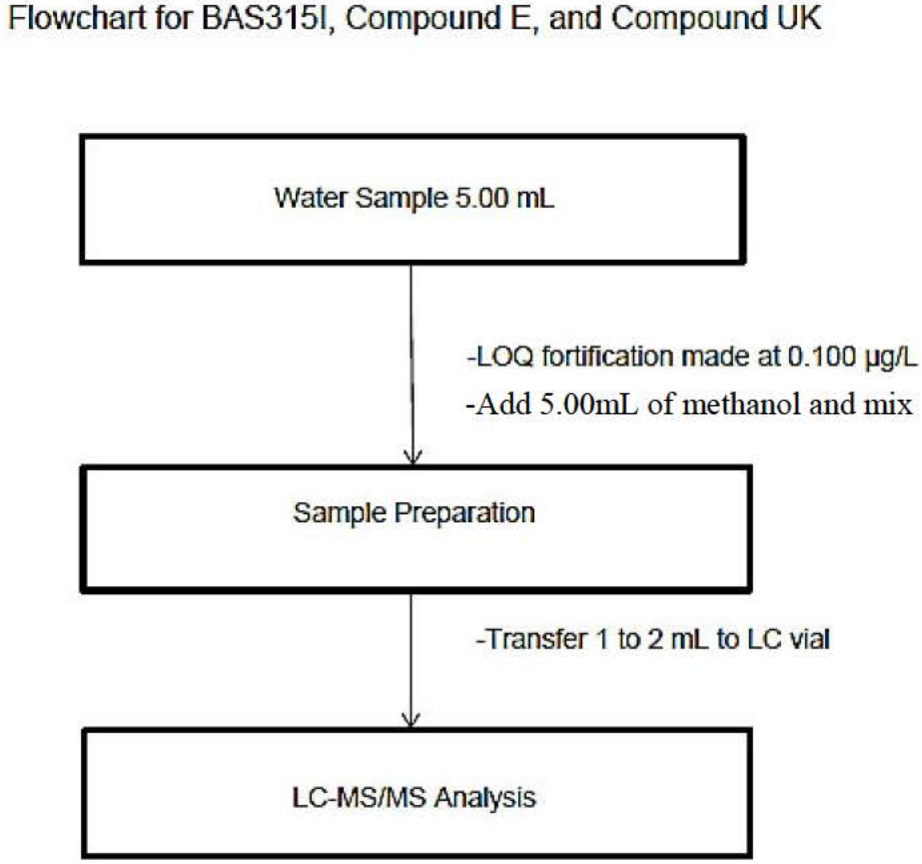

**Figure 1. Continued. Flow scheme for the processing of samples.**

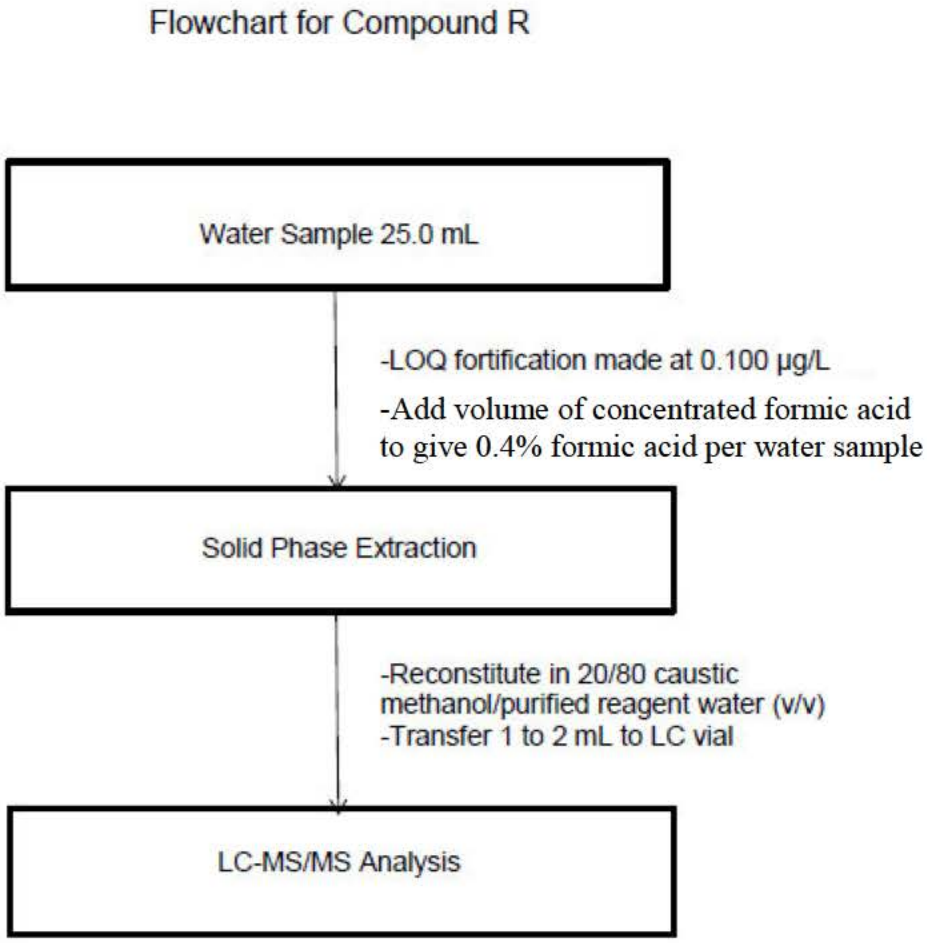

**Figure 1. Continued. Flow scheme for the processing of samples.**