1.0 INTRODUCTION

The purpose of this study was to validate an analytical method used to determine the content of cycloate and its metabolites, cycloate sulfoxide and N-ethylcyclohexylamine (ECHA), in DU soil. The method was validated (20 February to 15 May 2018) to quantify the concentrations of cycloate, cycloate sulfoxide, and ECHA in DU soil. The analytical method was validated with regards to specificity, linearity, accuracy, precision, limit of quantitation (LOQ), limit of detection (LOD), method detection limit (MDL), and confirmation of analyte identification.

The method was validated in DU soil by fortification with cycloate and its metabolites, cycloate sulfoxide and ECHA, at concentrations of 10.0 (LOQ) and 100 (10X LOQ) µg/kg. The cycloate recovery samples were extracted with purified reagent water and toluene, concentrated under nitrogen, reconstituted with acetonitrile, followed by dilution with 50/50 acetonitrile/purified reagent water (v/v). The 10X LOQ recovery samples were further diluted into the calibration standard range with 50/50 acetonitrile/purified reagent water (v/v). The cycloate sulfoxide recovery samples were extracted with a saturated solution of sodium chloride in 50/50 methanol/purified reagent water (v/v) and toluene, followed by dilution into the calibration standard range with 50/50 acetonitrile/purified reagent water. The ECHA recovery samples were extracted with methanol, 3.0 M sodium hydroxide in purified reagent water, and toluene. Samples were then diluted into the calibration standard range with acetonitrile followed by purified reagent water to a final ratio of 50/50 acetonitrile/purified reagent water. All samples were analyzed using liquid chromatography with tandem mass spectrometry detection (LC-MS/MS).

The study was initiated on 9 February 2018, the day the Study Director signed the protocol, and was completed on the day the Study Director signed the final report. The experimental portion of the validation was conducted from 20 February to 15 May 2018 at Smithers Viscient (SMV), located in Wareham, Massachusetts. All original raw data, the protocol, and the final report produced during this study are stored in Smithers Viscient's archives at the above location.

2.0 MATERIALS AND METHODS

2.1 Protocol

Procedures used in this study followed those described in the Smithers Viscient protocol entitled "Validation of an Environmental Chemistry Method for the Determination of Cycloate, Cycloate Sulfoxide, and N-ethylcyclohexylamine in Soil by LC-MS/MS" (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/3029/99 rev. 4 (EC, 2000) and OCSPP 850.6100 (U.S. EPA, 2012).

2.2 Test Substances

The test substance, cycloate, was received on 30 November 2016 from Chem Service Inc., West Chester, Pennsylvania. The following information was provided:

Name:	Cycloate
Lot No.:	5608300
CAS No.:	1134-23-2
Purity:	98.1%
Recertification Date:	18 January 2019

Upon receipt at Smithers Viscient, the test substance (SMV No. 8624) was stored at room temperature in a dark, ventilated cabinet in the original container. Concentrations were not adjusted for the purity of the test substance.

The test substance, cycloate sulfoxide, was received on 28 November 2017 from Golden Pacific Laboratories LLC, Fresno, California. The following information was provided:

Name:	Cycloate sulfoxide
Synonym:	TM 1
Batch No.:	ET18361-12
CAS No.:	Not Listed
Purity:	98.88%
Expiration Date:	Not Listed

Upon receipt at Smithers Viscient, the test substance (SMV No. 9183) was stored at room temperature in a dark, ventilated cabinet in the original container. Concentrations were adjusted for the purity of the test substance.

The test substance, N-Ethylcyclohexylamine, was received on 28 August 2017 from Sigma Aldrich, Inc., Milwaukee, Wisconsin. The following information was provided:

Name:	N-Ethylcyclohexylamine
Synonym:	N-Cyclohexylethylamine
Lot No.:	14128CO
CAS No.:	5459-93-8
Purity:	98.7%
Expiration Date:	28 August 2018

Upon receipt at Smithers Viscient, the test substance (SMV No. 9074) was stored at room temperature in a dark, ventilated cabinet in the original container. Concentrations were adjusted for the purity of the test substance.

Determination of stability and characterization, verification of the test substance identities, 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.	Toluene:	EMD, reagent grade
2.	Acetonitrile:	EMD, reagent grade
3.	Methanol:	EMD, reagent grade
4.	Sodium chloride:	Fisher, reagent grade

5.	Sodium hydroxide:	Fisher, reagent grade
6.	0.1% Formic acid in water:	Fisher, reagent grade
7.	0.1% Formic acid in acetonitrile:	Fisher, 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:	**Cycloate validation:** AB MDS Sciex API 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 CBM-20A communications bus Analyst version 1.6.3 software for data acquisition **Cycloate sulfoxide validation:** MDS Sciex API 6500+ QTRAP mass spectrometer equipped with an ESI Turbo V source Shimadzu SIL-20ACXR autoinjector Shimadzu DGU-20A5R vacuum degasser Shimadzu LC-20ADXR solvent delivery pumps Shimadzu CTO-20AC column oven Shimadzu CBM-20A communications bus Analyst 1.6.3 software for data acquisition **ECHA validation:** MDS Sciex API 5000 mass spectrometer equipped with an ESI Turbo V ion source Shimadzu SIL-20ACXR autoinjector Shimadzu DGU-20A5R vacuum degasser Shimadzu DGU-20A5R vacuum degasser Shimadzu LC-20ADXR solvent delivery pumps Shimadzu CTO-20AC column oven Shimadzu CBM-20A communications bus Analyst 1.6 software for data acquisition
2.	Balances:	Mettler Toledo XSE205DU, Mettler Toledo PG-2002-S
3.	Moisture Balances:	Mettler Toledo HB43-S, Sartorius MA-45
4.	Shaker Table:	VWR 3500
5.	Centrifuge:	Thermo Scientific Sorvall Legend XFR

6.	pH Meter:	YSI Ecosense pH100A
7.	Laboratory equipment:	Positive displacement pipets, volumetric flasks, disposable glass vials, disposable glass pipets, Teflon centrifuge tubes, graduated cylinders, Pasteur pipets, autosampler vials, and amber glass bottles with Teflon-lined cap

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 matrix used during this method validation was abbreviated as DU soil based on the suppliers soil identification number (DU-L-PF). Characterization of soil was performed by Agvise Laboratories, Northwood, North Dakota. A second batch of soil from the same location and supplier was obtained since the stock supply was depleted. Soil characterization data for the second batch is provided below.

Soil utilized for cycloate validation:

Parameter	Soil
Smithers Viscient Batch No.:	DU-L-PF 10JAN18 Soil-B
Collection location:	Grand Forks, ND
Percent organic carbon:	7.1%
USDA textural class:	Loam
Particle size distribution:	31% sand 44% silt 25% clay
pH (1/1 soil/water ratio):	6.7
Percent water holding capacity (at 1/3 bar):	45.1%
Percent Moisture:	24.52%

Soil utilized for cycloate sulfoxide and ECHA validation:

Parameter	Soil
Smithers Viscient Batch No.:	DU-L 14DEC16 Soil-B
Collection location:	Grand Forks, ND
Percent organic carbon:	3.2%
USDA textural class:	Clay Loam
Particle size distribution:	40% sand 28% silt 32% clay
pH (1/1 soil/water ratio):	5.4
Percent water holding capacity (at 1/3 bar):	31.5%
Percent Moisture (cycloate sulfoxide validation):	18.97%
Percent Moisture (ECHA validation):	24.29%

2.6 Preparation of Liquid Reagent Solutions

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 saturated solution of NaCl in 50/50 methanol/purified reagent water (v/v) liquid reagent solution was typically prepared by combining 125.37 g of NaCl with 250 mL of purified reagent water and was mixed thoroughly. A 250 mL of methanol was then transferred to the saturated aqueous solution. The solution was mixed well using a stir bar and stir plate for five minutes.

A 3.0M NaOH in purified reagent water liquid reagent solution was typically prepared by combining 61.0154 g of NaOH with 500 mL of purified reagent water. The solution was mixed well before use.

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 before use.

2.7 Preparation of Stock Solutions

The volumes and masses listed in this section are representative of the stocks prepared during testing, but may not reflect the exact quantities for each separate validation. Volumes and masses may be changed; however, the proportions must remain the same.

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

Primary Stock ID	Amount Weighed (g), Net Weight	Amount Weighed (g), as Active Ingredient	Stock Solvent	Final Volume (mL)	Primary Stock Concentration (mg/L)	Primary Stock Use
8624-4A	0.0512	0.0502	Acetonitrile	50.0	1000	Secondary stock solution
9183C	0.0506	0.0500	Acetonitrile	50.0	1000	Secondary stock solution
9074D	0.0507	0.0500	Acetonitrile	50.0	1000	Secondary stock solution

Secondary stock solutions were typically prepared as described in 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
8624-4A	1000	0.500	50.0	Acetonitrile	8624-4A-1	10.0	Sub-stock solutions
9183C	1000	0.500	50.0	Acetonitrile	9183C-1	10.0	Sub-stock solutions
9074D	1000	0.500	50.0	Acetonitrile	9074D-1	10.0	Sub-stock solutions

Sub-stock solutions were typically prepared as described in 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
8624-4A-1	10.0	0.100	10.0	Acetonitrile	Tech Stk 1	0.100	LOQ-level recovery samples during the cycloate validation
8624-4A-1	10.0	1.00	10.0	Acetonitrile	Tech Stk 2	1.00	10X LOQ-level recovery samples during the cycloate validation
8624-4A-1	10.0	0.0100	10.0	Acetonitrile	Ana Stk 1	0.0100	Calibration standards during the cycloate validation
9183C-1	10.0	0.100	10.0	Acetonitrile	Tech Stk 1	0.100	LOQ-level recovery samples during the cycloate sulfoxide validation
9183C-1	10.0	1.00	10.0	Acetonitrile	Tech Stk 2	1.00	10X LOQ-level recovery samples during the cycloate sulfoxide validation
9183C-1	10.0	0.0100	10.0	Acetonitrile	Ana Stk 1	0.0100	Calibration standards during the cycloate sulfoxide validation
9074D-1	10.0	0.100	10.0	Acetonitrile	Tech Stk 1	0.100	LOQ-level recovery samples during the ECHA validation
9074D-1	10.0	1.00	10.0	Acetonitrile	Tech Stk 2	1.00	10X LOQ-level recovery samples during the ECHA validation
9074D-1	10.0	0.0100	10.0	Acetonitrile	Ana Stk 1	0.0100	Calibration standards during the ECHA validation

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 - Cycloate

Calibration standards were prepared in 50/50 acetonitrile/purified reagent water (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
Ana 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

2.8.2 Calibration Standards - Cycloate Sulfoxide

Calibration standards were prepared in 50/50 acetonitrile/purified reagent water (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
Ana Stk 1	0.0100	0.00500	10.0	0.00500	Std 1
		0.0100	10.0	0.0100	Std 2
		0.0200	10.0	0.0200	Std 3
		0.0300	10.0	0.0300	Std 4
		0.0400	10.0	0.0400	Std 5
		0.0500	10.0	0.0500	Std 6

2.8.3 Calibration Standards - ECHA

Calibration standards were prepared in 50/50 acetonitrile/purified reagent water (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
Ana Stk 1	0.0100	0.0100	10.0	0.0100	Std 1
		0.0200	10.0	0.0200	Std 2
		0.0400	10.0	0.0400	Std 3
		0.0600	10.0	0.0600	Std 4
		0.0800	10.0	0.0800	Std 5
		0.100	10.0	0.100	Std 6

2.8.4 Matrix Effect Investigation - Cycloate

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 - Cycloate

Test Substance Stock ID	Stock Concentration (mg/L)	Volume of Fortification (mL)	Final Volume[a] (mL)	Standard Concentration (µg/L)	Sample ID
Ana Stk 1	0.0100	0.100	10.0	0.100	MM-Std 1
		0.100	10.0	0.100	MM-Std 2
		0.100	10.0	0.100	MM-Std 3

[a] Diluted with the final dilution of the matrix-matched control sample 14113-6131-02.

2.8.4.2 Non-Matrix-Matched Standards - Cycloate

Test Substance Stock ID	Stock Concentration (mg/L)	Volume of Fortification (mL)	Final Volume[a] (mL)	Standard Concentration (µg/L)	Sample ID
Ana 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 50/50 acetonitrile/purified reagent water (v/v).

2.8.5 Matrix Effect Investigation - Cycloate Sulfoxide

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.

2.8.5.1 Matrix-Matched Standards - Cycloate Sulfoxide

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

[a] Diluted with the final dilution of the matrix-matched control sample 14113-6131-32.

2.8.5.2 Non-Matrix-Matched Standards - Cycloate Sulfoxide

Test Substance Stock ID	Stock Concentration (mg/L)	Volume of Fortification (mL)	Final Volume[a] (mL)	Standard Concentration (µg/L)	Sample ID
Ana Stk 1	0.0100	0.0100	10.0	0.0100	Std A
		0.0100	10.0	0.0100	Std B
		0.0100	10.0	0.0100	Std C

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

2.8.6 Matrix Effect Investigation - ECHA

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.

2.8.6.1 Matrix-Matched Standards - ECHA

Test Substance Stock ID	Stock Concentration (mg/L)	Volume of Fortification (mL)	Final Volume[a] (mL)	Standard Concentration (µg/L)	Sample ID
Ana Stk 1	0.0100	0.0150	10.0	0.0150	MM-Std 1
		0.0150	10.0	0.0150	MM-Std 2
		0.0150	10.0	0.0150	MM-Std 3

[a] Diluted with the final dilution of the matrix-matched control sample 14113-6131-47.

2.8.6.2 Non-Matrix-Matched Standards - ECHA

Test Substance Stock ID	Stock Concentration (mg/L)	Volume of Fortification (mL)	Final Volume (mL)[a]	Standard Concentration (µg/L)	Sample ID
Ana Stk 1	0.0100	0.0150	10.0	0.0150	Std A
		0.0150	10.0	0.0150	Std B
		0.0150	10.0	0.0150	Std C

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

2.9 Sample Fortification and Preparation

For each test substance (cycloate, cycloate sulfoxide, and ECHA), 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 sub-stock solution at concentrations of 10.0 (LOQ) and 100 (10X LOQ) µg/kg (dry weight). Seven replicates were prepared for the 10.0 µg/kg (LOQ) concentration level and five replicates were prepared for the 100 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 tables.

Cycloate:

Sample ID 14113-6131-	Sample Type	Stock Concentration (mg/L)	Fortification Volume (mL)	Dry Weight (g)	Fortified Concentration (µg/kg)
01	Reagent Blank	NA[a]	NA	NA	0.00
02 & 03	Control	NA	NA	5.00	0.00
04, 05, 06, 07, 08, 09, & 10	LOQ	0.100	0.500	5.00	10.0
11, 12, 13, 14, & 15	10X LOQ	1.00	0.500	5.00	100

[a] NA = Not Applicable

Cycloate sulfoxide:

Sample ID 14113-6131-	Sample Type	Stock Concentration (mg/L)	Fortification Volume (mL)	Dry Weight (g)	Fortified Concentration (µg/kg)
31	Reagent Blank	NA[a]	NA	NA	0.00
32 & 33	Control	NA	NA	5.00	0.00
34, 35, 36, 37, 38, 39, & 40	LOQ	0.100	0.500	5.00	10.0
41, 42, 43, 44, & 45	10X LOQ	1.00	0.500	5.00	100

[a] NA = Not Applicable

ECHA:

Sample ID 14113-6131-	Sample Type	Stock Concentration (mg/L)	Fortification Volume (mL)	Dry Weight (g)	Fortified Concentration (µg/kg)
46	Reagent Blank	NA[a]	NA	NA	0.00
47 & 48	Control	NA	NA	5.00	0.00
49, 50, 51, 52, 53, 54, & 55	LOQ	0.100	0.500	5.00	10.0
56, 57, 58, 59, & 60	10X LOQ	1.00	0.500	5.00	100

[a] NA = Not Applicable

2.10 Soil Extraction and Dilution

2.10.1 Soil Extraction and Dilution - Cycloate

A 10.0-mL aliquot of purified reagent water was added to each reagent blank and sample directly after preparation and samples were vortex mixed for five seconds. A 15.0-mL aliquot of toluene was then added to each sample and they were vortex mixed for 15 seconds, then placed on a shaker table at 300 rpm for one hour. Following shaking, the samples were then centrifuged at 3000 rpm for 10 minutes. Each sample consisted of three layers: the bottom layer was the soil, the middle layer was the purified reagent water, and the top layer was the toluene (which contained the test substance). Approximately 10.0 mL was removed from the toluene layer of each sample and transferred to labelled 50.0-mL volumetric flasks. The extraction and mixing procedures were repeated with an additional 10.0-mL aliquot of toluene. The extracts were combined, taken to volume (50.0 mL) with toluene, and mixed well. An aliquot (1.00 mL) of each sample was removed and transferred to separate conical vials. Three additional aliquots

(1.00 mL each) from one of the control samples (Sample ID: 14113-6131-02) were removed in the same manner and concentrated with the rest of the samples for the matrix effects investigation. All samples were taken to a volume of no less than 25 µL and no more than 100 µL under a gentle stream of nitrogen at room temperature. An aliquot (see table below) of acetonitrile was added to each sample and they were vortex mixed for 15 seconds and ultrasonicated to aid in reconstitution. It is suspected that the evaporation of the toluene extracts is a critical step in the method. Evaporating to dryness could result in low sample recovery. Leaving greater than 100 µL toluene in the final extract could also reduce the sample recovery by causing the organic phase to partition out of the final extract. The samples were further diluted into the calibration standard range with 50/50 acetonitrile/purified reagent water (v/v) as needed. All recovery samples were transferred to autosampler vials for analysis. The extraction and dilution procedures are detailed below.

Sample ID 14113-6131-	Sample Type	Nominal Concentration (µg/kg)	Dry Weight (g)	Final Volume[a] (mL)	Sample Volume (mL)	Reconstituted Volume[b] (mL)	Final Volume[c] (mL)	Sample Volume (mL)	Final Volume[c] (mL)	Dilution Factor
01	Reagent Blank	0.00	NA[d]	50.0	1.00	1.00	10.0	NA	NA	100
02 & 03	Control	0.00	5.00	50.0	1.00	1.00	10.0	NA	NA	100
MM-Std 1[e], MM-Std 2[e], & MM-Std 3[e]	Matrix-matched standard	0.00	NA	NA	1.00	1.00	10.0	NA	NA	100
04, 05, 06, 07, 08, 09, & 10	LOQ	10.0	5.00	50.0	1.00	1.00	10.0	NA	NA	100
11, 12, 13, 14, & 15	10X LOQ	100	5.00	50.0	1.00	1.00	10.0	3.00	10.0	333

[a] Dilution solvent: toluene
[b] Dilution solvent: acetonitrile
[c] Dilution solvent: 50/50 acetonitrile/purified reagent water (v/v)
[d] NA = Not Applicable
[e] Taken from control sample (Sample ID: 14113-6131-02)

2.10.2 Soil Extraction and Dilution - Cycloate Sulfoxide

A 10.0-mL aliquot of saturated solution of sodium chloride in 50/50 methanol/purified reagent water (v/v) was added to each reagent blank and sample directly after preparation and the samples were vortex mixed for five seconds. A 15.0-mL aliquot of toluene was then added to each sample and they were vortex mixed for 15 seconds, then placed on a shaker table at 250 rpm for 30 minutes. Following shaking, the samples were then centrifuged at 3000 rpm for

five minutes. The samples consisted of three layers: the bottom layer was the soil, the middle layer was the saturated solution of sodium chloride in 50/50 methanol/purified reagent water (v/v), and the top layer was the toluene (which contained the test substance). A 10.0-mL aliquot was removed from the toluene layer and transferred to disposable vials with PTFE-lined caps. The extraction and centrifugation procedures were repeated three more times with additional 10.0-mL aliquots of toluene, for a total of four extractions. After each extraction, exactly 10.0 mL was removed from the toluene layer and combined with the other extracts for a total volume of 40.0 mL for each sample. The samples were diluted into the calibration standard range with 50/50 acetonitrile/purified reagent water (v/v). Three additional aliquots (0.0800 mL each) were removed from one of the control sample extracts (Sample ID: 14113-6131-32) for the matrix effects investigation and diluted in the same manner as the controls. All recovery samples were transferred to autosampler vials for analysis. The extraction and dilution procedures are detailed below.

Sample ID 14113-6131-	Sample Type	Nominal Concentration (µg/kg)	Dry Weight (g)	Final Volume[a] (mL)	Sample Volume (mL)	Final Volume[b] (mL)	Dilution Factor
31	Reagent Blank	0.00	0.00	40.0	0.0800	10.0	1000
32 & 33	Control	0.00	5.00	40.0	0.0800	10.0	1000
MM-Std 1[c], MM-Std 2[c], & MM-Std 3[c]	Matrix-matched standard	0.00	NA[d]	NA	0.0800	10.0	1000
34, 35, 36, 37, 38, 39, & 40	LOQ	10.0	5.00	40.0	0.0800	10.0	1000
41, 42, 43, 44, & 45	10X LOQ	100	5.00	40.0	0.0250	10.0	3200

[a] Dilution solvent: toluene
[b] Dilution solvent: 50/50 acetonitrile/purified reagent water (v/v)
[c] Taken from control sample (Sample ID: 14113-6131-32)
[d] NA = Not Applicable

2.10.3 Soil Extraction and Dilution - ECHA

A 5.00-mL aliquot of methanol was added to each reagent blank and sample directly after preparation and the samples were vortex mixed for five seconds. Then, a 5.00-mL aliquot of 3.0 M sodium hydroxide in purified reagent water was added to each reagent blank and sample and the samples were vortex mixed for five seconds. A 10.0-mL aliquot of toluene was then added to each sample and they were vortex mixed for 15 seconds, then placed on a shaker table

at 250 rpm for 10 minutes. Following shaking, the samples were then centrifuged at 3000 rpm for five minutes. The samples consisted of three layers: the bottom layer was the soil, the middle layer was the 50/50 methanol/3.0 M sodium hydroxide in purified reagent water (v/v), and the top layer was the toluene (which contained the test substance). A 5.00-mL aliquot was removed from the toluene layer and transferred to disposable vials with PTFE-lined caps. The extraction and centrifugation procedures were repeated three more times with additional 5.00-mL aliquots of toluene, for a total of four extractions. After each extraction, exactly 5.00 mL was removed from the toluene layer and combined with the other extracts for a total volume of 20.0 mL for each sample. The samples were further diluted into the calibration standard range with 50/50 acetonitrile/purified reagent water (v/v), which was done by adding each constituent separately. The samples were first diluted with 5.00 mL of acetonitrile and vortex mixed for 30 seconds. The samples were allowed to sit for at least ten minutes allowing the analyte to partition out of the toluene. The samples were then brought to volume with purified reagent water. Three additional aliquots (0.0600 mL each) were removed from one of the control sample extracts (Sample ID: 14113-6131-47) for the matrix effects investigation and diluted in the same manner as the controls. All recovery samples were transferred to autosampler vials for analysis. The extraction and dilution procedures are detailed below.

Sample ID 14113-6131-	Sample Type	Nominal Concentration (µg/kg)	Dry Weight (g)	Final Volume[a] (mL)	Sample Volume (mL)	Final Volume[b] (mL)	Dilution Factor
46	Reagent Blank	0.00	0.00	20.0	0.0600	10.0	667
47 & 48	Control	0.00	5.00	20.0	0.0600	10.0	667
MM-Std 1[c], MM-Std 2[c], & MM-Std 3[c]	Matrix-matched standard	0.00	NA[d]	NA	0.0600	10.0	667
49, 50, 51, 52, 53, 54, & 55	LOQ	10.0	5.00	20.0	0.0600	10.0	667
56, 57, 58, 59, & 60	10X LOQ	100	5.00	20.0	0.0250	10.0	1600

[a] Dilution solvent: toluene
[b] Dilution solvent: 5.00 mL of acetonitrile, then brought to volume with purified reagent water
[c] Taken from control sample (Sample ID: 14113-6131-47)
[d] NA = Not Applicable

2.11 Analysis

2.11.1 Instrumental Conditions

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

Cycloate in DU soil

LC parameters:

Column: Waters T3 Atlantis 3 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	0.800	98.0	2.00
0.50	0.800	98.0	2.00
0.60	0.800	50.0	50.0
6.00	0.800	0.00	100
7.00	0.800	0.00	100
7.10	0.800	98.0	2.00
8.50	0.800	98.0	2.00

Run Time: 8.50 minutes
Injector Rinse 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 Time: approximately 6.5 minutes

MS parameters:

Instrument: AB MDS Sciex API 4000 mass spectrometer
Ionization Mode: Positive (+) ESI
Ion Spray Voltage: 5500 V
Scan Type: MRM
Source Temperature: 550 °C
Curtain Gas: 15.0
Ion Source – Gas 1 / Gas 2: 50.0 / 50.0
Collision Gas: 4.00
Collision Cell Exit Potential: 15.0
Resolution (Q1/Q3): Unit/Unit

	Primary Transition	Confirmatory Transition
Q1/Q3 Masses (Da):	215.99/83.05	215.99/154.18
Dwell Time (msec):	100	100
Declustering Potential:	40.0	40.0
Collision Cell Entrance Potential:	10.0	10.0
Collision Energy:	24.0	17.0

Cycloate sulfoxide in DU soil

LC parameters:

Column: Waters T3 Atlantis 3 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	1.20	95.0	5.00
1.00	1.20	95.0	5.00
4.00	1.20	0.00	100
5.00	1.20	0.00	100
5.10	1.20	95.0	5.00
6.00	1.20	95.0	5.00

Run Time: 6.00 minutes
Injector Rinse Solvent: 30/30/40 acetonitrile/methanol/purified reagent water (v/v/v)
Column Temperature: 35 °C
Sample Temperature: 10 °C
Injection Volume: 25.0 µL
Retention Time: approximately 4.0 minutes

MS parameters:

Instrument: Sciex API 6500+ QTrap mass spectrometer
Ionization Mode: Positive (+) ESI
Ion Spray Voltage: 5500 V
Scan Type: MRM
Source Temperature: 650 °C
Curtain Gas: 20.0
Ion Source – Gas 1 / Gas 2: 50.0 / 50.0
Collision Gas: Medium
Collision Cell Exit Potential: 15.0
Declustering Potential: 56.0
Resolution (Q1/Q3): Unit/Unit

	Primary Transition	Confirmatory Transition
Q1/Q3 Masses (Da):	254.16/226.12	254.16/177.16
Dwell Time (msec):	200	200
Collision Cell Entrance Potential:	9.00	9.00
Collision Energy:	18.0	21.0

ECHA in DU soil

LC parameters:

Column: Waters T3 Atlantis 3 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	1.20	98.0	2.00
0.50	1.20	98.0	2.00
3.00	1.20	0.00	100
4.00	1.20	0.00	100
4.10	1.20	98.0	2.00
5.00	1.20	98.0	2.00

Run Time: 5.00 minutes
Injector Rinse Solvent: 30/30/40 acetonitrile/methanol/purified reagent water (v/v/v)
Column Temperature: 40 °C
Sample Temperature: 15 °C
Injection Volume: 50.0 µL
Retention Time: approximately 1.3 minutes

MS parameters:

Instrument: AB Sciex API 5000 mass spectrometer
Ionization Mode: Positive (+) ESI
Ion Spray Voltage: 5500 V
Scan Type: MRM
Source Temperature: 650 °C
Curtain Gas: 30.0
Ion Source – Gas 1 / Gas 2: 50.0 / 50.0
Collision Gas: 8.00
Collision Cell Exit Potential: 15.0
Declustering Potential: 50.0
Resolution (Q1/Q3): Unit/Unit

	Primary Transition	Confirmatory Transition
Q1/Q3 Masses (Da):	128.23/82.74	128.23/55.01
Dwell Time (msec):	200	200
Collision Cell Entrance Potential:	10.0	8.00
Collision Energy:	25.0	30.0

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.

2.11.2 Preparation of Calibration Standard Curve

Two sets of calibration standards were analyzed with each sample set. Calibration standards were interspersed among analysis of the recovery samples, every two to six injections. Injection of recovery samples and calibration standards onto the chromatographic 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 110% (for the mean recovery at each fortification level) are acceptable. The precision was reported in terms of the relative standard deviation (RSD) for the recovery samples. RSD values less than 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 cycloate, cycloate sulfoxide, and ECHA, 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.

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 one-tailed t-statistic at the 99% confidence level for n-1 replicates plus the average residue in the untreated controls in µg/kg. 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.

3.0 CALCULATIONS

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.

$$(1) \quad y = mx + b$$

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

$$(3) \quad A = DC \times DF$$

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/kg), concentration in the original sample

The LOD was calculated using the following equation:

$$(4) \quad LOD = t_{0.99} \times S + \text{Average Residue in Untreated Controls}$$

where:

t	=	one-tailed t-statistic at the 99% confidence level for n-1 replicates (i.e., 3.143; U.S. EPA, 1994)
S	=	standard deviation 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 5) based on the concentration of the low calibration standard and the dilution factor of the control samples.

$$(5) \quad MDL = MDL_{LCAL} \times DF_{CNTL}$$

where:

MDL_{LCAL}	=	lowest concentration calibration standard (e.g., 0.0500 µg/L)
DF_{CNTL}	=	dilution factor of the control samples (smallest dilution factor used, e.g., 100)
MDL	=	method detection limit reported for the analysis (e.g., 0.0500 µg/L × 100 = 5.00 µg/kg)