## INTRODUCTION

The objective of this study was to independently validate the analytical method used in Smithers Viscient, Wareham Study No. 14113.6131 (Validation of an Environmental Chemistry Method for the Determination of Cycloate, Cycloate Sulfoxide, and N-ethylcyclohexylamine in Soil by LC-MS/MS). The analytical method was validated with regards to accuracy and precision, linearity, specificity and limit of quantification.

This study was conducted to support the registration of the test substances.

The method validation described in this report is designed to conform to SANCO 3029/99 rev 4 (2000) Guidance for generating and reporting methods of analysis in support of pre-registration data requirements for Annex II (part A, Section 4) and Annex III (part A, Section 5) of Directive 91/414, OCSPP 850.6100: Environmental Chemistry Methods and Associated Independent Laboratory Validation.

Analytical method 14113.6131 was supplied by Smithers Viscient, Wareham on behalf of the sponsor. The method was re-written in Smithers Viscient, Harrogate format as draft method SMV 3202259-01D, including the instrumentation available at Smithers Viscient, Harrogate. This was followed for method validation, and re-issued as SMV 3202259-01V when validation was complete.

The supplied method was followed with only minor changes (equipment and reagents were substituted for suitable equivalents, shaker speed was limited to 200 rpm rather than 300/250 rpm, Cycloate extracts were evaporated to 25-100 µL rather than 25 µL and Cycloate extracts were ultrasonicated to re-constitute in acetonitrile rather than vortex-mixed for 15 seconds).

Control samples of soil were separately fortified with Cycloate, Cycloate sulfoxide and ECHA at 10 µg/kg in septuplicate and at 100 µg/kg in quintuplicate and analysed. Cycloate samples were extracted with water and toluene, concentrated under nitrogen and reconstituted with acetonitrile. The extract was diluted into calibration range with acetonitrile: water (50:50 v/v).

Cycloate sulfoxide samples were extracted with saturated sodium chloride in methanol: water (50:50 v/v) and toluene. The extract was diluted into calibration range with acetonitrile: water (50:50 v/v).

ECHA samples were extracted with methanol, 3M sodium hydroxide in water and toluene. The extract was diluted into the calibration range with acetonitrile followed by water to a final ratio of acetonitrile: water (50:50 v/v).

To assess matrix effects, calibration standards were prepared in control soil final extract and in acetonitrile: water (50:50 v/v).

Samples were analysed using liquid chromatography with tandem mass spectrometry detection (LC-MS/MS).

Matrix effects, linearity and specificity of the method were determined. Precision and accuracy were calculated at each validation level for each compound.

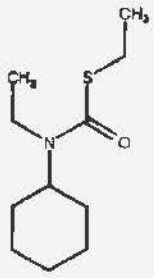
One primary and one confirmatory LC-MS/MS transition were analysed for Cycloate, Cycloate sulfoxide and ECHA.

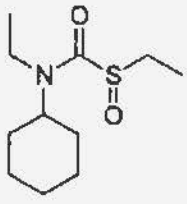
The study was initiated on 22 August 2018 (date the protocol was signed by the Study Director) and completed on the date the final report was signed by the Study Director. The practical phase of the study was conducted by Smithers Viscient (ESG) and was started on 22 August 2018 (stock preparation) and completed on 17 September 2018 (LC-MS/MS analysis).

## MATERIALS AND METHODS

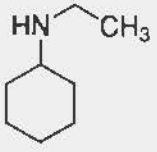
The study was conducted in accordance with the protocol and one amendment. All deviations are given in Appendix 7.

### Test Substance

| | |
|---|---|
| **Test Substance Name:** | **Cycloate** |
| CAS Number: | 1134-23-2 |
| Molecular Formula: | $C_{11}H_{21}NOS$ |
| Molecular Weight: | 215.39 g/mol |
| Sponsor Lot Number: | 5608300 |
| Structure: |  |
| Purity: | 98.1% |
| Storage Conditions: | Room temperature (15-30°C) |
| Recertification Date: | 18 January 2019 |

| | |
|---|---|
| **Test Substance Name:** | **Cycloate sulfoxide** |
| CAS Number: | N/A |
| Molecular Formula: | $C_{11}H_{21}NO_2S$ |
| Molecular Weight: | 231.35 g/mol |
| Sponsor Lot Number: | ET18361-12 |
| Structure: |  |
| Purity: | 98.88% |
| Storage Conditions: | Room temperature (15-30°C) |
| Recertification Date: | 28 November 2018[1] |

[1] The recertification date is 1 year from manufacture (28 November 2017) as confirmed by the sponsor.

| | |
|---|---|
| **Test Substance Name:** | **N-Ethylcyclohexylamine (ECHA)** |
| CAS Number: | 5459-93-8 |
| Sponsor Lot Number: | 14128CO |
| Molecular Formula: | $C_6H_{11}NHC_2H_5$ |
| Molecular Weight: | 127.23 g/mol |
| Structure: |  |
| Purity: | 98.7% |
| Storage Conditions: | Room temperature (15-30°C) |
| Recertification Date: | 28 August 2018[1] |

[1] The ECHA validation was performed on 24 August 2018, which was before the recertification date had passed.

The Certificates of Analysis for the test substances are presented in Appendix 1.

**Test System**

A control sample of soil was provided by Smithers Viscient, Wareham (DU-L-PF soil ID# 10JAN18-SoilB). The soil was given the unique identification 18/0000000/17 and stored refrigerated.

Characterisation of the soil was performed by Agvise Laboratories, Northwood, North Dakota (not determined under this study). Characterisation data was taken from Smithers Viscient, Wareham study 14113.6131 and is presented in the following table:

| | |
|---|---|
| Collection location: | Grand Forks, ND |
| % Organic carbon: | 7.1% |
| USDA textural class: | Loam |
| Particle size distribution: | 31% sand, 44% silt, 25% clay |
| pH (soil: water ratio 1:1): | 6.7 |
| % Water holding capacity (at 1/3 bar): | 45.1% |

The moisture content of the soil was determined at Smithers Viscient, Harrogate and was determined to be 29.4% of the dry soil weight.

**Reagents**

| | |
|---|---|
| Acetonitrile | HPLC grade, Honeywell |
| Methanol | HPLC grade, Honeywell |
| Toluene | HPLC grade, Honeywell |
| Water | Milli-Q with LCPAK polisher, In House |
| 0.1% Formic acid in water | MS grade, Honeywell |
| 0.1% Formic acid in acetonitrile | MS grade, Honeywell |
| Sodium chloride (NaCl) | Reagent grade, Fisher |
| Sodium hydroxide (NaOH) | Reagent grade, Fisher |

Equivalent or better reagents may have been used.

**Equipment**
Shimadzu Nexera series HPLC system with AB Sciex API 5000 MS/MS detector
Allegra X-15R centrifuge

**Analytical Method**
The report for study 14113.6131 was supplied by Smithers Viscient, Wareham on behalf of the sponsor. The method was re-written in Smithers Viscient, Harrogate format as draft method SMV 3202259-01D, including the instrumentation available at Smithers Viscient, Harrogate. This was followed for method validation, and re-issued as SMV 3202259-01V when validation was complete. The method used LC-MS/MS analysis. The complete analytical procedure is presented in Appendix 5. One person can complete the extraction of 15 samples (for either Cycloate, Cycloate sulfoxide or ECHA) in 1 day (8 hour working period). Automated LC-MS/MS analysis of 15 sample extracts can be completed in a further 6 hours from submission. Schematic diagrams of the analytical method are presented in Appendix 6.

***Preparation of Reagents***
*Acetonitrile: water (50:50 v/v)*
500 mL HPLC grade acetonitrile was mixed with 500 mL Milli-Q water.

*Saturated NaCl in methanol: water (50:50 v/v)*
> 90 g NaCl was added to 250 mL Milli-Q water until no more dissolved, and mixed with 250 mL methanol.

*3M NaOH in water*
60 g NaOH was dissolved in 500 mL Milli-Q water.

***Preparation of Primary Stocks***
Primary stock solutions of Cycloate, Cycloate sulfoxide and ECHA were prepared as described in the following table:

| Stock ID | Test Substance | Amount Weighed (mg) | Purity (%) | Solvent | Final Volume (mL) | Concentration (µg/mL)[1] |
|---|---|---|---|---|---|---|
| Stock 1 | Cycloate | 10.60 | 98.1 | Acetonitrile | 10.398 | 1000 |
| Stock 2 | | 10.94 | 98.1 | | 10.732 | 1000 |
| Stock 3 | Cycloate sulfoxide | 11.03 | 98.88 | | 10.907 | 1000 |
| Stock 4 | | 11.54 | 98.88 | | 11.411 | 1000 |
| Stock 5 | ECHA | 10.22 | 98.7 | | 10.087 | 1000 |
| Stock 6 | | 10.89 | 98.7 | | 10.748 | 1000 |

[1] Corrected for Purity.
Duplicate stocks were prepared for correlation purposes.

Primary stocks were stored refrigerated in amber glass bottles and given a nominal expiry of three months.

***Preparation of Secondary Stocks***
Secondary stock solutions were separately prepared as described in the following table:

| Test Substance | Stock Concentration (µg/mL) | Volume Taken (mL) | Solvent | Final Volume (mL) | Concentration (µg/mL) |
|---|---|---|---|---|---|
| Cycloate | 1000 | 0.1 | Acetonitrile | 10 | 10 |
| Cycloate sulfoxide | 1000 | 0.1 | | 10 | 10 |
| ECHA | 1000 | 0.1 | | 10 | 10 |

Secondary stock solutions were stored refrigerated in amber glass bottles and given a nominal expiry of one month.

***Preparation of Sub-Stocks***
Sub-stock solutions were prepared as described in the following table:

| Test Substance | Stock Concentration (µg/mL) | Volume Taken (mL) | Solvent | Final Volume (mL) | Concentration (µg/mL) |
|---|---|---|---|---|---|
| Cycloate | 10 | 1 | Acetonitrile | 10 | 1 |
| | 1 | 1 | | 10 | 0.1 |
| | 0.1 | 1 | | 10 | 0.01[1] |
| Cycloate sulfoxide | 10 | 1 | | 10 | 1 |
| | 1 | 1 | | 10 | 0.1 |
| | 0.1 | 1 | | 10 | 0.01[1] |
| ECHA | 10 | 1 | | 10 | 1 |
| | 1 | 1 | | 10 | 0.1 |
| | 0.1 | 1 | | 10 | 0.01[1] |

[1] Equivalent to 10 µg/L.

Sub-stock solutions were prepared on the day of use and stored refrigerated until the corresponding analysis was complete.

***Preparation of Matrix Matched Standards for Matrix Assessment***
Matrix matched standards of Cycloate, Cycloate sulfoxide and ECHA were prepared in disposable glass vials as described in the following tables:

Cycloate

| Stock Concentration (µg/L) | Volume Taken (mL) | Solvent | Final Volume (mL) | Concentration (µg/L) |
|---|---|---|---|---|
| 10 | 0.2 | Control soil final extract | 10 | 0.2 |
| 10 | 0.2 | | 10 | 0.2 |
| 10 | 0.2 | | 10 | 0.2 |

Cycloate sulfoxide

| Stock Concentration (µg/L) | Volume Taken (mL) | Solvent | Final Volume (mL) | Concentration (µg/L) |
|---|---|---|---|---|
| 10 | 0.02 | Control soil final extract | 10 | 0.02 |
| 10 | 0.02 | | 10 | 0.02 |
| 10 | 0.02 | | 10 | 0.02 |

ECHA

| Stock Concentration (µg/L) | Volume Taken (mL) | Solvent | Final Volume (mL) | Concentration (µg/L) |
|---|---|---|---|---|
| 10 | 0.04 | Control soil final extract | 10 | 0.04 |
| 10 | 0.04 | | 10 | 0.04 |
| 10 | 0.04 | | 10 | 0.04 |

***Preparation of Non-Matrix Matched Standards for Matrix Assessment***
Non-matrix standards of Cycloate, Cycloate sulfoxide and ECHA were prepared in disposable glass vials as described in the following tables:

Cycloate

| Stock Concentration (µg/L) | Volume Taken (mL) | Solvent | Final Volume (mL) | Concentration (µg/L) |
|---|---|---|---|---|
| 10 | 0.2 | Acetonitrile: water (50:50 v/v) | 10 | 0.2 |
| 10 | 0.2 | | 10 | 0.2 |
| 10 | 0.2 | | 10 | 0.2 |

Cycloate sulfoxide

| Stock Concentration (µg/L) | Volume Taken (mL) | Solvent | Final Volume (mL) | Concentration (µg/L) |
|---|---|---|---|---|
| 10 | 0.02 | Acetonitrile: water (50:50 v/v) | 10 | 0.02 |
| 10 | 0.02 | | 10 | 0.02 |
| 10 | 0.02 | | 10 | 0.02 |

ECHA

| Stock Concentration (µg/L) | Volume Taken (mL) | Solvent | Final Volume (mL) | Concentration (µg/L) |
|---|---|---|---|---|
| 10 | 0.04 | Acetonitrile: water (50:50 v/v) | 10 | 0.04 |
| 10 | 0.04 | | 10 | 0.04 |
| 10 | 0.04 | | 10 | 0.04 |

The matrix matched standards were analysed alternately with the non-matrix standards and the mean peak areas compared.

***Preparation of Calibration Standards***

Non-Matrix matched calibration standards of Cycloate, Cycloate sulfoxide and ECHA were prepared as described in the following tables:

Cycloate

| Stock Concentration (µg/L) | Volume Taken (mL) | Solvent | Final Volume (mL) | Concentration (µg/L) |
|---|---|---|---|---|
| 10 | 0.5 | Acetonitrile: water (50:50 v/v) | 10 | 0.5 |
| 0.5 | 0.8 | | 1 | 0.4 |
| 0.5 | 0.6 | | 1 | 0.3 |
| 0.5 | 0.4 | | 1 | 0.2 |
| 0.5 | 0.2 | | 1 | 0.1 |
| 0.5 | 0.1 | | 1 | 0.05 |

Cycloate sulfoxide

| Stock Concentration (µg/L) | Volume Taken (mL) | Solvent | Final Volume (mL) | Concentration (µg/L) |
|---|---|---|---|---|
| 10 | 0.05 | Acetonitrile: water (50:50 v/v) | 10 | 0.05 |
| 0.05 | 0.8 | | 1 | 0.04 |
| 0.05 | 0.6 | | 1 | 0.03 |
| 0.05 | 0.4 | | 1 | 0.02 |
| 0.05 | 0.2 | | 1 | 0.01 |
| 0.05 | 0.1 | | 1 | 0.005 |

ECHA

| Stock Concentration (µg/L) | Volume Taken (mL) | Solvent | Final Volume (mL) | Concentration (µg/L) |
|---|---|---|---|---|
| 10 | 0.1 | Acetonitrile: water (50:50 v/v) | 10 | 0.1 |
| 0.1 | 0.8 | | 1 | 0.08 |
| 0.1 | 0.6 | | 1 | 0.06 |
| 0.1 | 0.4 | | 1 | 0.04 |
| 0.1 | 0.2 | | 1 | 0.02 |
| 0.1 | 0.1 | | 1 | 0.01 |

Calibration standards were prepared on the day of use, transferred into disposable glass vials and stored refrigerated until the analysis was complete.

A single set of calibration standards was prepared for each validation batch, which was analysed twice during the batch, in random order interspersed with the samples.

***Sample Fortification***

5 g dry weight of soil was weighed into a Nalgene centrifuge tube. Soil samples were fortified with Cycloate, Cycloate sulfoxide or ECHA standard in acetonitrile at 10 µg/kg in septuplicate and at 100 µg/kg in quintuplicate. Duplicate control water samples and a reagent blank were also prepared, as described in the following tables:

Cycloate

| Sample ID | Stock Concentration (µg/mL) | Volume Added (mL) | Sample Weight (g) | Fortified Concentration (µg/kg) |
|---|---|---|---|---|
| Reagent blank A & D | N/A | N/A | N/A | N/A |
| Control A, D-E & J-K | N/A | N/A | 5 | N/A |
| F10 A-G & V-AB | 0.1 | 0.5 | 5 | 10 |
| F100 A-E | 1 | 0.5 | 5 | 100 |

N/A = Not Applicable.
Con A was used for matrix assessment.

Cycloate sulfoxide

| Sample ID | Stock Concentration (µg/mL) | Volume Added (mL) | Sample Weight (g) | Fortified Concentration (µg/kg) |
|---|---|---|---|---|
| Reagent blank B | N/A | N/A | N/A | N/A |
| Control B & F-G | N/A | N/A | 5 | N/A |
| F10 H-N | 0.1 | 0.5 | 5 | 10 |
| F100 F-J | 1 | 0.5 | 5 | 100 |

N/A = Not Applicable.
Con B was used for matrix assessment.

ECHA

| Sample ID | Stock Concentration (µg/mL) | Volume Added (mL) | Sample Weight (g) | Fortified Concentration (µg/kg) |
|---|---|---|---|---|
| Reagent blank C | N/A | N/A | N/A | N/A |
| Control C & H-I | N/A | N/A | 5 | N/A |
| F10 O-U | 0.1 | 0.5 | 5 | 10 |
| F100 K-O | 1 | 0.5 | 5 | 100 |

N/A = Not Applicable.
Con C was used for matrix assessment.

***Sample Extraction***

*Cycloate*

10 mL water was added to each sample directly after fortification and vortex mixed for approximately 5 seconds. 15 mL toluene was added and vortex mixed for approximately 15 seconds, then placed on a shaker set to 200 rpm for 1 hour and centrifuged at 3000 rpm for 10 minutes. Approximately 10 mL of toluene extract was removed from the top layer of the sample and transferred into a glass jar. The sample extraction was repeated with 10 mL toluene, and the extracts combined. The toluene extract was made to 50 mL volume in a measuring cylinder. 1 mL extract was transferred to a borosilicate glass tube and evaporated to 25-100 µL under a gentle stream of nitrogen (no heating). It was critical that the extract was not reduced to dryness (resulting in low recovery), or that too much toluene remained (resulting in partition of the extract). The sample was reconstituted with 1 mL acetonitrile and ultrasonicated for 5 minutes. The sample was diluted into calibration range with acetonitrile: water (50:50 v/v) and transferred into an HPLC vial for analysis.

The dilution procedure is given in the following table:

| Sample ID | Fortified Concentration (µg/kg) | Sample Weight (g) | Extract Volume (mL) | Dilution (mL-mL) | Dilution Factor |
|---|---|---|---|---|---|
| Reagent blank A & D | N/A | N/A | 50 | 1-10 | 100 |
| Control A, D-E & J-K | N/A | 5 | 50 | 1-10[1] | 100 |
| F10 A-G & V-AB | 10 | 5 | 50 | 1-10 | 100 |
| F100 A-E | 100 | 5 | 50 | 1-10 then 0.3-1 | 333 |

N/A = Not Applicable.
[1] 3 aliquots of Control A extract were evaporated and diluted for matrix assessment.

*Cycloate sulfoxide*
10 mL saturated NaCl in methanol: water (50:50 v/v) was added to each sample directly after fortification and vortex mixed for approximately 5 seconds. 15 mL toluene was added and vortex mixed for approximately 15 seconds, then placed on a shaker set to 200 rpm for 30 minutes and centrifuged at 3000 rpm for 5 minutes. Exactly 10 mL toluene was removed from the top layer of the sample and transferred into a glass jar. The sample extraction was repeated with 10 mL toluene three more times, and the extracts combined to give a total of 40 mL toluene extract. An aliquot of the toluene extract was diluted into calibration range with acetonitrile: water (50:50 v/v) and transferred into an HPLC vial for analysis.

The dilution procedure is given in the following table:

| Sample ID | Fortified Concentration (µg/kg) | Sample Weight (g) | Extract Volume (mL) | Dilution (mL-mL) | Dilution Factor |
|---|---|---|---|---|---|
| Reagent blank B | N/A | N/A | 40 | 0.08-10 | 1000 |
| Control B & F-G | N/A | 5 | 40 | 0.08-10[1] | 1000 |
| F10 H-N | 10 | 5 | 40 | 0.08-10 | 1000 |
| F100 F-J | 100 | 5 | 40 | 0.025-10 | 3200 |

N/A = Not Applicable.
[1] 3 aliquots of Control B extract were diluted for matrix assessment.

*ECHA*
5 mL methanol was added to each sample directly after fortification and vortex mixed for approximately 5 seconds. 5 mL 3M NaOH was added and vortex mixed for approximately 5 seconds. 10 mL toluene was added and vortex mixed for approximately 15 seconds, then placed on a shaker set to 200 rpm for 10 minutes and centrifuged at 3000 rpm for 5 minutes. Exactly 5 mL toluene was removed from the top layer of the sample and transferred into a 20 mL glass vial. The sample extraction was repeated with 5 mL toluene three more times, and the extracts combined to give a total of 20 mL toluene extract. A portion of extract was diluted with 5 mL acetonitrile and vortex mixed for approximately 30 seconds. The sample was left for at least 10 minutes to allow the analyte to partition out of the toluene. 5 mL water was added to the acetonitrile and mixed, to give a ratio of acetonitrile: water (50:50 v/v). A portion of the final extract was transferred to an HPLC vial for analysis.

The dilution procedure is given in the following table:

| Sample ID | Fortified Concentration (µg/kg) | Sample Weight (g) | Extract Volume (mL) | Dilution (mL-mL) | Dilution Factor |
|---|---|---|---|---|---|
| Reagent blank C | N/A | N/A | 20 | 0.06-10 | 667 |
| Control C & H-I | N/A | 5 | 20 | 0.06-10[1] | 667 |
| F10 O-U | 10 | 5 | 20 | 0.06-10 | 667 |
| F100 K-O | 100 | 5 | 20 | 0.025-10 | 1600 |

N/A = Not Applicable.
[1] 3 aliquots of Control C extract were diluted for matrix assessment.

***Instrument Conditions***

LC-MS/MS analysis was performed using the following instrument conditions:

*Cycloate*

HPLC Parameters:

| | | | |
|---|---|---|---|
| Instrument | Shimadzu Nexera series HPLC system | | |
| Column# | Waters Atlantis T3, 3 µm, 4.6 × 100 mm | | |
| Mobile Phase A# | 0.1% Formic acid in water | | |
| Mobile Phase B# | 0.1% Formic acid in acetonitrile | | |
| Flow Rate | 0.8 mL/min | | |
| Gradient | Time (min) | Mobile Phase A (%) | Mobile Phase B (%) |
| | 0.0 | 98 | 2 |
| | 0.5 | 98 | 2 |
| | 0.6 | 50 | 50 |
| | 6.0 | 0 | 100 |
| | 7.0 | 0 | 100 |
| | 7.1 | 98 | 2 |
| | 8.5 | 98 | 2 |
| Run Time | 8.5 minutes | | |
| Column Temperature | 40°C | | |
| Autosampler Temperature | 10°C | | |
| Injection Volume | 50 µL | | |
| Retention Time | Approx. 6.2 minutes (Cycloate) | | |
| Valco Valve Diverter | Time (min) | Position | |
| | 0 | A (to waste) | |
| | 1 | B (to MS) | |
| | 7.5 | A (to waste) | |

MS/MS Parameters:

| | | | | |
|---|---|---|---|---|
| Instrument | AB Sciex API 5000 Triple Quadrupole Mass Spectrometer | | | |
| Ionisation Type# | Electrospray (ESI) | | | |
| Polarity# | Positive | | | |
| Scan Type# | Multiple reaction monitoring (MRM) | | | |
| Ion Spray Voltage | 5500 V | | | |
| Collision Gas (CAD) | 5 | | | |
| Curtain Gas (CUR) | 25 | | | |
| Gas Flow 1 (GS1) | 40 | | | |
| Gas Flow 2 (GS2) | 40 | | | |
| Vaporiser Temperature (TEM) | 550°C | | | |
| Interface Heater (ihe) | On | | | |
| Entrance Potential (EP) | 10 | | | |
| Collision Exit Potential (CXP) | 13 | | | |
| Compound Name | MRM Transition Ions Monitored | Declustering Potential (DP) | Collision Energy (CE) | Dwell Time (ms) |
| Cycloate (Primary) | 215.9/83.0 | 120 | 22.26 | 100 |
| Cycloate (Confirmatory) | 215.9/154.1 | 120 | 18.06 | 100 |

Parameters marked # may not be modified. Minor adjustments to the remaining parameters may be required in order to fully optimise the system.

*Cycloate Sulfoxide*

HPLC Parameters:

| | | | |
|---|---|---|---|
| Instrument | Shimadzu Nexera series HPLC system | | |
| Column# | Waters Atlantis T3, 3 µm, 4.6 × 100 mm | | |
| Mobile Phase A# | 0.1% Formic acid in water | | |
| Mobile Phase B# | 0.1% Formic acid in acetonitrile | | |
| Flow Rate | 1.2 mL/min | | |
| Gradient | Time (min) | Mobile Phase A (%) | Mobile Phase B (%) |
| | 0.0 | 95 | 5 |
| | 1.0 | 95 | 5 |
| | 4.0 | 0 | 100 |
| | 5.0 | 0 | 100 |
| | 5.1 | 95 | 5 |
| | 6.0 | 95 | 5 |
| Run Time | 6 minutes | | |
| Column Temperature | 35°C | | |
| Autosampler Temperature | 10°C | | |
| Injection Volume | 25 µL | | |
| Retention Time | Approx. 3.8 minutes (Cycloate sulfoxide) | | |
| Valco Valve Diverter | Time (min) | Position | |
| | 0 | A (to waste) | |
| | 1 | B (to MS) | |
| | 5 | A (to waste) | |

MS/MS Parameters:

| | | | | |
|---|---|---|---|---|
| Instrument | AB Sciex API 5000 Triple Quadrupole Mass Spectrometer | | | |
| Ionisation Type# | Electrospray (ESI) | | | |
| Polarity# | Positive | | | |
| Scan Type# | Multiple reaction monitoring (MRM) | | | |
| Ion Spray Voltage | 5500 V | | | |
| Collision Gas (CAD) | 5 | | | |
| Curtain Gas (CUR) | 25 | | | |
| Gas Flow 1 (GS1) | 40 | | | |
| Gas Flow 2 (GS2) | 40 | | | |
| Vaporiser Temperature (TEM) | 650°C | | | |
| Interface Heater (ihe) | On | | | |
| Entrance Potential (EP) | 10 | | | |
| Collision Exit Potential (CXP) | 13 | | | |
| Compound Name | MRM Transition Ions Monitored | Declustering Potential (DP) | Collision Energy (CE) | Dwell Time (ms) |
| Cycloate sulfoxide (Primary) | 253.9/226.1 | 135 | 18.8 | 200 |
| Cycloate sulfoxide (Confirmatory) | 253.9/177.1 | 135 | 18.0 | 200 |

Parameters marked # may not be modified. Minor adjustments to the remaining parameters may be required in order to fully optimise the system.

*ECHA*

HPLC Parameters:

| | | | |
|---|---|---|---|
| Instrument | Shimadzu Nexera series HPLC system | | |
| Column# | Waters Atlantis T3, 3 µm, 4.6 × 100 mm | | |
| Mobile Phase A# | 0.1% Formic acid in water | | |
| Mobile Phase B# | 0.1% Formic acid in acetonitrile | | |
| Flow Rate | 1.2 mL/min | | |
| Gradient | Time (min) | Mobile Phase A (%) | Mobile Phase B (%) |
| | 0.0 | 98 | 2 |
| | 0.5 | 98 | 2 |
| | 3.0 | 0 | 100 |
| | 4.0 | 0 | 100 |
| | 4.1 | 98 | 2 |
| | 5.0 | 98 | 2 |
| Run Time | 5 minutes | | |
| Column Temperature | 40°C | | |
| Autosampler Temperature | 15°C | | |
| Injection Volume | 50 µL | | |
| Retention Time | Approx. 1.2 minutes (ECHA) | | |
| Valco Valve Diverter | Time (min) | Position | |
| | 0 | A (to waste) | |
| | 1 | B (to MS) | |
| | 4 | A (to waste) | |

MS/MS Parameters:

| | | | | |
|---|---|---|---|---|
| Instrument | AB Sciex API 5000 Triple Quadrupole Mass Spectrometer | | | |
| Ionisation Type# | Electrospray (ESI) | | | |
| Polarity# | Positive | | | |
| Scan Type# | Multiple reaction monitoring (MRM) | | | |
| Ion Spray Voltage | 5500 V | | | |
| Collision Gas (CAD) | 5 | | | |
| Curtain Gas (CUR) | 25 | | | |
| Gas Flow 1 (GS1) | 40 | | | |
| Gas Flow 2 (GS2) | 40 | | | |
| Vaporiser Temperature (TEM) | 650°C | | | |
| Interface Heater (ihe) | On | | | |
| Entrance Potential (EP) | 10 | | | |
| Collision Exit Potential (CXP) | 13 | | | |
| Compound Name | MRM Transition Ions Monitored | Declustering Potential (DP) | Collision Energy (CE) | Dwell Time (ms) |
| ECHA (Primary) | 128.2/83.0 | 124 | 20.6 | 200 |
| ECHA (Confirmatory) | 128.2/55.1 | 124 | 27.1 | 200 |

Parameters marked # may not be modified. Minor adjustments to the remaining parameters may be required in order to fully optimise the system.

LC-MS/MS data were collected using Analyst 1.6.2.

***Calculation of Results***
LC-MS/MS data were calculated using Analyst 1.6.2. When the calibration fit is linear as in this study, Analyst uses the following formula to calculate the concentration of test substance present in the sample extract:

$$x = \frac{(y - c)}{m} \times DF$$

Where:

$x$ = concentration of test substance in sample extract (µg/kg)
$y$ = peak area due to test substance
$c$ = $y$ intercept on calibration graph
$m$ = gradient of the calibration graph
DF = sample dilution factor

The sample dilution factor is calculated as follows:

DF = Final extract volume (mL) / Amount of soil in final extract (g)

Where:

Final extract volume = volume of the final diluted sample
Amount of soil in final extract = equivalent amount of soil in the extract after final dilution

Procedural recovery from fortified samples is calculated as follows:

Recovery (%) = Sample concentration / Fortified concentration × 100

The Limit of Detection (LOD) in µg/kg was calculated according to the following equation (U.S. EPA, 2016, 1994, Definition and Procedure for the Determination of the Method Detection Limit, Revision 1.11 and Revision 2):

LOD = (t 0.99 × SD) + mean apparent residue in the control samples

Where SD = standard deviation for 7 replicate samples at the LOQ
t 0.99 = one-tailed statistic at the 99% confidence interval for 7 replicates (3.143)

The Method Detection Limit (MDL) in µg/kg was calculated by multiplying the lowest calibration standard (in µg/L) by the dilution factor for the control.

***Validation Pass Criteria***
The validation was deemed acceptable if the following criteria were met for the primary and confirmatory transitions monitored for each compound.

*Mean Recovery and Precision*
Recovery and precision were acceptable if each fortification level had a mean recovery between 70 and 110% and a %RSD (relative standard deviation) ≤ 20%.

*Specificity/Selectivity*
Specificity was acceptable if the amounts found in blank samples were ≤ 30% of the LOQ.

*Linearity*
Linearity was acceptable if the lowest calibration standard concentration was ≤ 80% of the equivalent LOQ final extract concentration. The highest calibration standard concentration was ≥ 120% of the 10 × LOQ extract concentration (after dilution if applicable). The correlation coefficient (r) was acceptable if it was ≥ 0.995.

***LOD (Limit of Detection) Assessment***
The LOD was calculated according to the U.S. EPA, 2016, 1994, Definition and Procedure for the Determination of the Method Detection Limit, Revision 1.11 and Revision 2.

***MDL (Method Detection Limit)***
The MDL was calculated as the sample concentration equivalent to the lowest calibration standard.

***Matrix Assessment***
An assessment of matrix effects was made by comparison of the peak areas from triplicate standards prepared in blank solvent and in each control matrix final extract. This was assessed for the primary and confirmatory transitions.

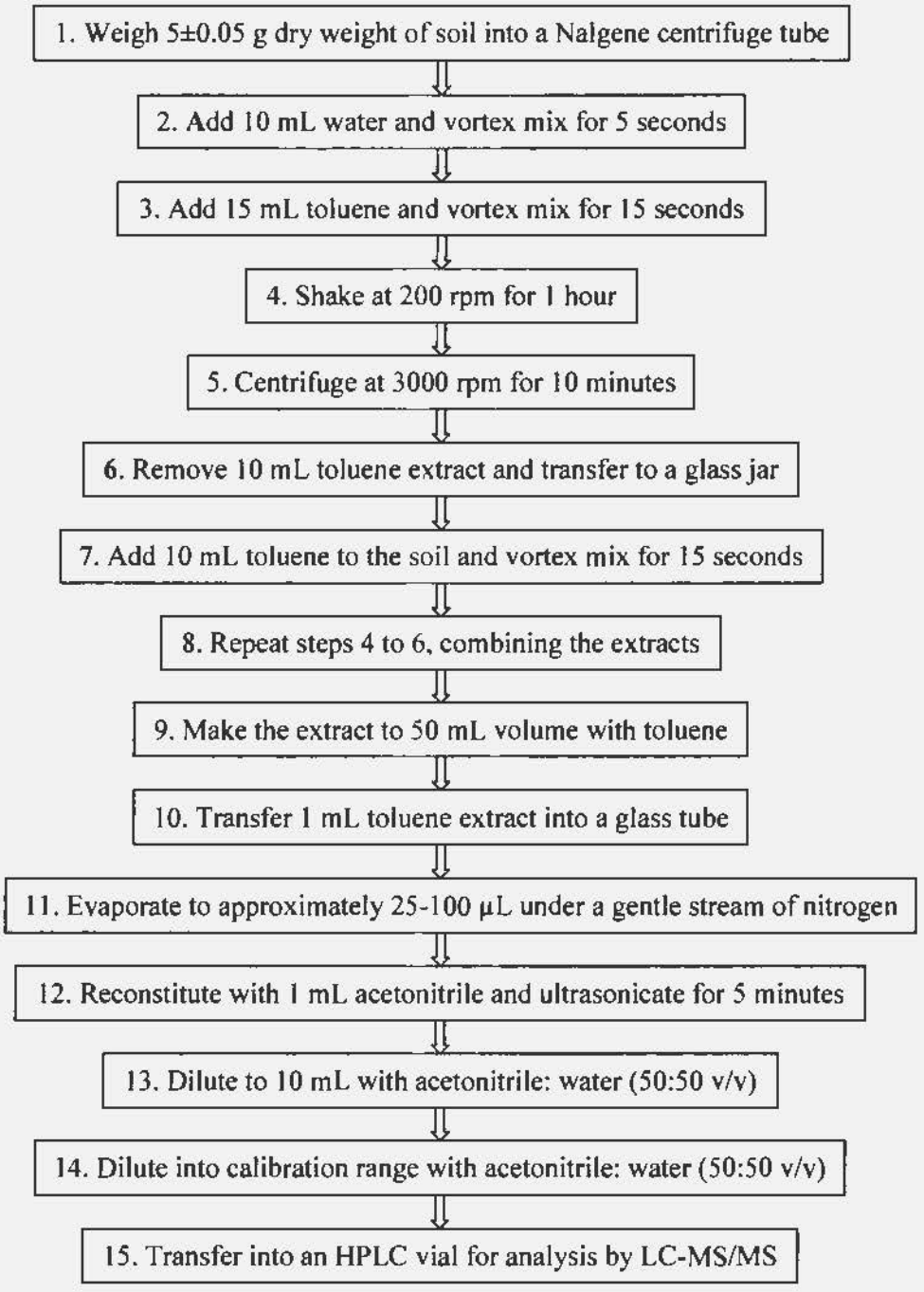
Results were presented as a % difference from the mean non-matrix standard value.

A difference of ≥ 20% was considered significant.

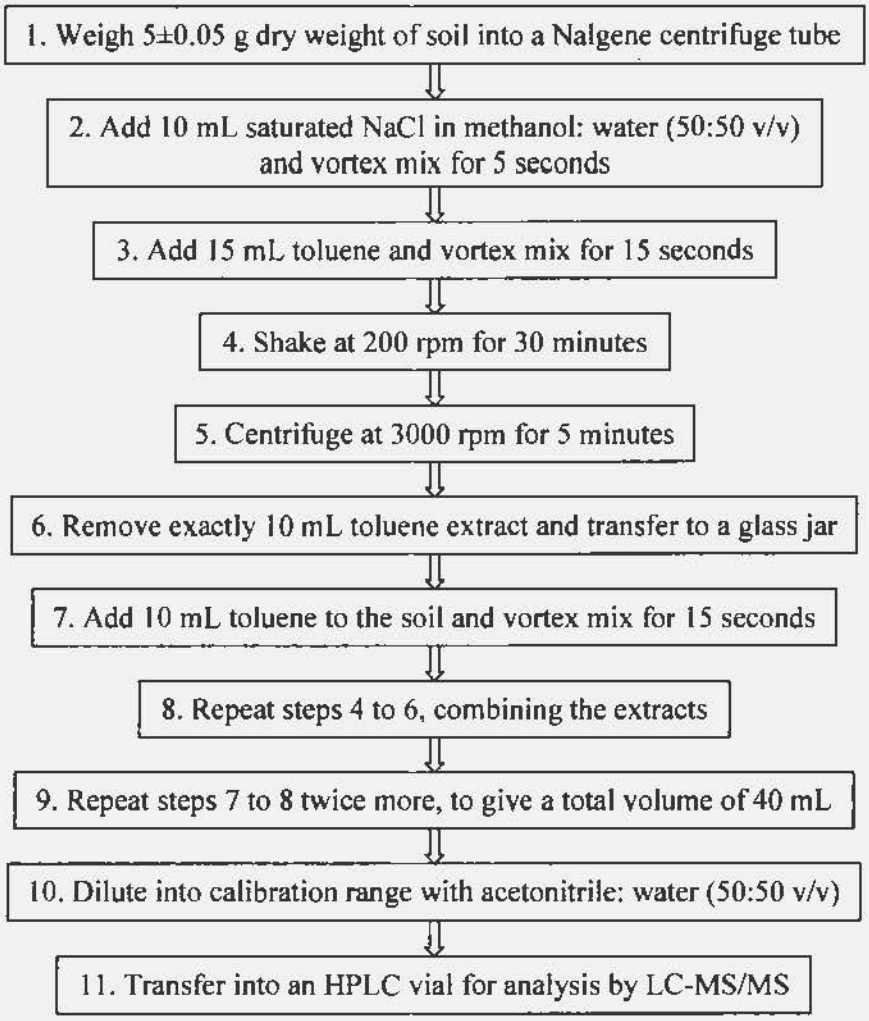
Cycloate, Cycloate sulfoxide and ECHA were analysed using non-matrix matched calibration standards because matrix effects were insignificant.

## Appendix 6
## Schematic Diagram of the Analytical Method

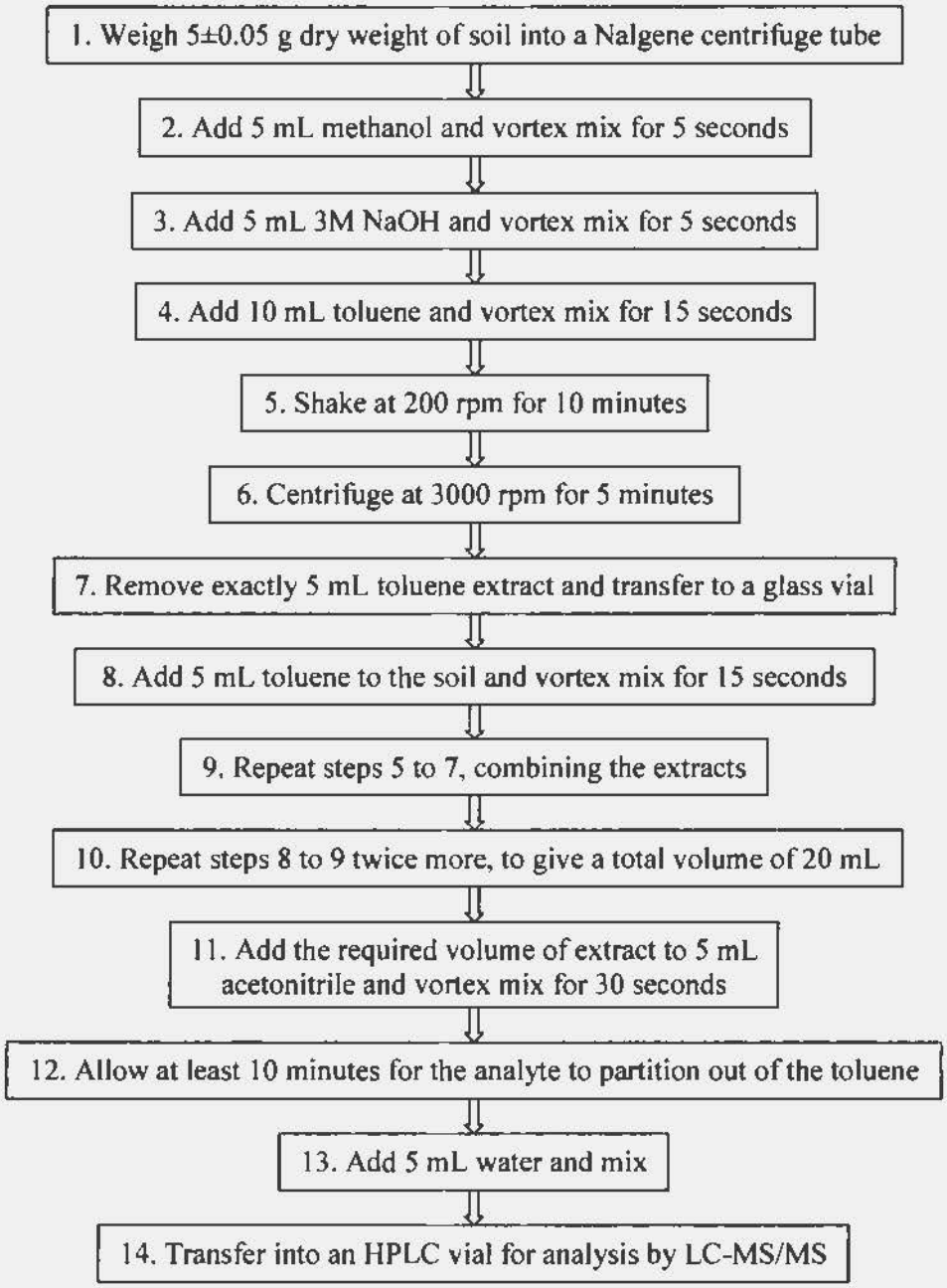
Cycloate:



Cycloate sulfoxide:



ECHA


1. Weigh 5±0.05 g dry weight of soil into a Nalgene centrifuge tube
2. Add 5 mL methanol and vortex mix for 5 seconds
3. Add 5 mL 3M NaOH and vortex mix for 5 seconds
4. Add 10 mL toluene and vortex mix for 15 seconds
5. Shake at 200 rpm for 10 minutes
6. Centrifuge at 3000 rpm for 5 minutes
7. Remove exactly 5 mL toluene extract and transfer to a glass vial
8. Add 5 mL toluene to the soil and vortex mix for 15 seconds
9. Repeat steps 5 to 7, combining the extracts
10. Repeat steps 8 to 9 twice more, to give a total volume of 20 mL
11. Add the required volume of extract to 5 mL acetonitrile and vortex mix for 30 seconds
12. Allow at least 10 minutes for the analyte to partition out of the toluene
13. Add 5 mL water and mix
14. Transfer into an HPLC vial for analysis by LC-MS/MS

## Appendix 7
## Deviations

The protocol specified the MRM transitions to two decimal places, as given in the primary analytical method. It was not the intention to use these exact masses, as the actual mass measured is dependent on the tuning and optimisation of the mass spectrometer, however no such statement was included in the protocol. The MRM transitions used in this study were only given to one decimal place, as this is the level of precision displayed when tuning. This protocol deviation did not affect the integrity of the study.