Final Report

Study Title

Chlormequat Chloride Formulation – Independent
Laboratory Validation of Analytical Method
14105.6104 for the Determination of Chlormequat
Chloride in Soil

Study Guideline(s)

EPA 850.6100 (2012)
SANCO/3029/99 rev. 4 (2000)

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INTRODUCTION

The objective of this study was to independently validate the analytical method validated in Smithers Viscient, Wareham Study No. 14105.6104, for measuring residues of Chlormequat Chloride in two soils of differing USDA Textural Classification, in accordance with EPA 850.6100 (2012) and SANCO/3029/99 rev.4 (2000) guidelines.

The validation report for study 14105.6104 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 3201883-01D, including the instrumentation available at Smithers Viscient (ESG) Ltd., Harrogate. This was followed for method validation, and re-issued as SMV 3201883-01V when validation was complete.

Control samples of KS and CA soil were fortified with Chlormequat Chloride at 0.05 and 0.5 mg/kg in quintuplicate and analysed. Samples were extracted with methanol: 1M (pH 7) potassium carbonate (50:50 v:v) followed by dilution into the calibration range with acetonitrile: water: trifluoroacetic acid (80:20:0.1 v/v/v).

To assess matrix effects, calibration standards were prepared in control soil final extract and in acetonitrile: water: trifluoroacetic acid (80:20:0.1 v/v/v).

Samples were analysed using high performance liquid chromatography with triple quadrupole mass spectrometry detection (LC-MS/MS).

Matrix effects, linearity and specificity of the method were determined. Precision and accuracy was calculated at each validation level in each soil for Chlormequat Chloride.

One primary and one confirmatory LC-MS/MS transition were analysed for Chlormequat Chloride.
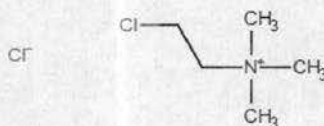
MATERIALS AND METHODS

The study was conducted in accordance with the protocol and one amendment, with no deviations.

Test Substances

Test Substance Name:	**Chlormequat Chloride**
IUPAC Name:	2-chloroethyl(trimethyl)azanium;chloride
CAS Number:	999-81-5
EC Number:	213-666-4
Structure:	

Molecular Formula:	$C_5H_{13}Cl_2N$
Molecular Mass:	158.09 g/mol
Sponsor Lot Number:	7162100
Appearance:	Colourless crystalline solid
Purity:	100%
Storage Conditions:	Room Temperature (15.0 to 30.0°C)
Recertification Date:	30 January 2021

A Certificate of Analysis for the test substance is presented in Appendix 1.

Test System

Control samples of soil with differing USDA Textural Classification were supplied by Smithers Viscient, Wareham. The soils used were TFD-KS-1 (clay loam) and TFD-CA-1 (sandy loam). The soils were given the unique identifications CS 72/17 and CS 73/17 for the KS soil and CA soil respectively and stored refrigerated (2 to 8°C).

Soil characterisation data are listed in the table below:

Soil Name	Textural class[1]	% Sand, Silt, Clay[2]	CEC (meq/100 g)	% Organic Carbon	pH in H_2O	pH in 0.01M $CaCl_2$
TFD-KS-1	clay loam	29, 44, 27	27.8	1.4	7.3	6.6
TFD-CA-1	sandy loam	60, 29, 11	13.0	0.6	8.3	7.6

[1,2] USDA classification.

The certificates of analysis for each soil are presented in Appendix 2.

The moisture contents of the soils were determined to be 11.9% and 10.0% of the dry soil weight for the KS and CA soil respectively.

Materials

Acetonitrile	HPLC grade, Honeywell
Acetonitrile	LC-MS grade, Honeywell
Methanol	HPLC grade, Honeywell
Water	Milli-Q (with LCPAK polisher), In house
Potassium carbonate	BioXtra, Sigma
Hydrochloric acid (HCl)	ACS reagent, Sigma
Formic acid	ACS reagent, Sigma
Trifluoroacetic acid (TFA)	Reagent grade, Sigma
Ammonium formate	Reagent grade, Sigma

Equivalent materials may be used.

Equipment

Shimadzu Nexera series HPLC system with ABSciex Triple TOF 5600+ MS/MS detector.

Analytical Method

The report for study 14105.6104 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 3201883-01D, including the instrumentation available at Smithers Viscient, Harrogate. This was followed for method validation, and re-issued as SMV 3201883-01V when validation was complete. The method used LC-MS/MS analysis. The complete analytical method is presented in Appendix 6.

Preparation of Reagents

Acetonitrile: Water (50:50 v/v)
50 mL HPLC grade acetonitrile was mixed with 50 mL Milli-Q water.

Acetonitrile: Water: TFA (80:20:0.1 v/v)
400 mL HPLC grade acetonitrile was mixed with 100 mL Milli-Q water and 0.5 mL TFA.

1M HCl
165 mL HCl was diluted to 2000 mL with Milli-Q water.

1M Potassium Carbonate
138 g potassium carbonate was dissolved in 1000 mL Milli-Q water.

1M Potassium Carbonate (pH 7)
806 mL 1M potassium carbonate was <u>carefully</u> mixed with 1194 mL 1M HCl (<u>a large volume of gas may be quickly evolved</u>).

Methanol: 1M (pH 7) Potassium Carbonate (50:50 v/v)
1000 mL HPLC grade methanol was mixed with 1000 mL 1M potassium carbonate (pH 7).

2M Ammonium Formate
12.6 g ammonium formate was dissolved in 100 mL Milli-Q water.

50mM (pH 3) Ammonium Formate
25 mL ammonium formate was diluted to 1000 mL with Milli-Q water and adjusted to pH 3.0 with formic acid.

Reagents may be scaled as appropriate.

Preparation of Primary Stocks
Primary stock solutions of Chlormequat Chloride were prepared in volumetric flasks as described in the following table:

Stock ID	Amount Weighed (mg)	Purity (%)	Solvent	Final Volume (mL)	Concentration (μg/mL)[1]
Stock 1	10.19	100	Milli-Q water	10	1000
Stock 2	10.40			10	1000

[1] Corrected for Purity, to three significant figures.
Duplicate stocks were prepared for correlation purposes.

Primary stocks were transferred into amber glass bottles, stored refrigerated and given a nominal expiry date of three months.

Preparation Secondary Stocks
Secondary stock solutions of Chlormequat Chloride were prepared in volumetric flasks as described in the following table:

Stock Concentration (μg/mL)	Volume Taken (mL)	Solvent	Final Volume (mL)	Concentration (μg/mL)
1000	1	Acetonitrile: water (50:50 v/v)	10	100
100	1		10	10
10	1		10	1

Secondary stocks were transferred into amber glass bottles, stored refrigerated and given a nominal expiry date of 1 month.

Preparation of Sub-Stocks
Sub-stock solutions of Chlormequat Chloride were prepared in volumetric flasks as described in the following table:

Stock Concentration (μg/mL)	Volume Taken (mL)	Solvent	Final Volume (mL)	Concentration (μg/mL)
1	0.1	Acetonitrile: water: TFA (80:20:0.1 v/v/v)	10	0.01[1]

[1] Equivalent to 10 μg/L.

Sub-stock solutions were prepared on the day of use, transferred into disposable glass vials and stored refrigerated until the analysis was complete.

Preparation of Matrix Matched Standards for Matrix Assessment

Matrix matched standards of Chlormequat Chloride were prepared in disposable glass vials as described in the following tables:

KS soil

Stock Concentration (µg/L)	Volume Taken (mL)	Solvent	Final Volume (mL)	Concentration (µg/L)
10	1	Control KS soil final extract	10	1
10	1		10	1
10	1		10	1

CA soil

Stock Concentration (µg/L)	Volume Taken (mL)	Solvent	Final Volume (mL)	Concentration (µg/L)
10	1	Control CA soil final extract	10	1
10	1		10	1
10	1		10	1

Preparation of Non-Matrix Matched Standards for Matrix Assessment

Non-matrix matched standards of Chlormequat Chloride were prepared in disposable glass vials as described in the following table:

Stock Concentration (µg/L)	Volume Taken (mL)	Solvent	Final Volume (mL)	Concentration (µg/L)
10	1	Acetonitrile: water: TFA (80:20:0.1 v/v/v)	10	1
10	1		10	1
10	1		10	1

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

Preparation of Calibration Standards

Calibration standards of Chlormequat Chloride were prepared in volumetric flasks (10 mL) or HPLC vials (1 mL) as described in the following table:

Stock Concentration (µg/L)	Volume Taken (mL)	Solvent	Final Volume (mL)	Concentration (µg/L)
10	2.5		10	2.5
2.5	0.6		1	1.5
2.5	0.4	Acetonitrile: water: TFA (80:20:0.1 v/v/v)	1	1
2.5	0.2		1	0.5
2.5	0.04		1	0.1
2.5	0.02		1	0.05
2.5	0.01		1	0.025

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, and analysed twice during the batch, in random order interspersed with the samples.

Sample Preparation and Fortification

10.0 g dry weight of soil was weighed into a 50 mL Nalgene centrifuge tube. Samples were fortified with Chlormequat Chloride standard in acetonitrile: water (50:50 v/v) as described in the following tables:

KS soil

Sample ID	Sample Weight (g)	Stock Concentration (µg/mL)	Volume Added (mL)	Fortified Concentration (mg/kg)
Reagent Blank A	N/A	N/A	N/A	N/A
Control A & C-D	10	N/A	N/A	N/A
F0.05 A-E	10	10	0.05	0.05
F0.5 A-E	10	100	0.05	0.5

N/A = Not Applicable.
Control A was used to prepared matrix matched standards for matrix assessment.

CA soil

Sample ID	Sample Weight (g)	Stock Concentration (µg/mL)	Volume Added (mL)	Fortified Concentration (mg/kg)
Reagent Blank B	N/A	N/A	N/A	N/A
Control B & E-F	10	N/A	N/A	N/A
F0.05 F-J	10	10	0.05	0.05
F0.5 F-J	10	100	0.05	0.5

N/A = Not Applicable.
Control B was used to prepared matrix matched standards for matrix assessment.

Soil Extraction

10.0 g soil was weighed into a 50 mL Nalgene centrifuge tube. The samples were extracted four times with 30 mL methanol: 1M (pH 7) potassium carbonate (50:50 v/v) by placing in an ultrasonic bath for 15 minutes, shaking at 200 rpm for 30 minutes and centrifuging at 1500 rpm for 10 minutes. The extracts were combined in a plastic pot and made to 120 mL with methanol: 1M (pH 7) potassium carbonate (50:50 v/v). The sample extract was diluted into calibration range with acetonitrile: water: TFA (80:20:0.1 v/v) and transferred into an HPLC vial. The extraction and dilution procedures are detailed in the following tables:

KS soil

Sample ID	Fortified Concentration (mg/kg)	Sample Weight (g)	Extract Volume (mL)	Dilution (mL-mL)	Dilution Factor
Reagent Blank A	N/A	N/A	120	0.1-10	1200
Control A & C-D	N/A	10.0	120	0.1-10[1]	1200
F0.05 A-E	0.05	10.0	120	0.1-10	1200
F0.5 A-E	0.5	10.0	120	0.1-10	1200

N/A = Not Applicable.
[1] Three aliquots of control A extract were diluted to prepare matrix matched standards for matrix assessment.

CA soil

Sample ID	Fortified Concentration (mg/kg)	Sample Weight (g)	Extract Volume (mL)	Dilution (mL-mL)	Dilution Factor
Reagent Blank B	N/A	N/A	120	0.1-10	1200
Control B & E-F	N/A	10.0	120	0.1-10[1]	1200
F0.05 F-J	0.05	10.0	120	0.1-10	1200
F0.5 F-J	0.5	10.0	120	0.1-10	1200

N/A = Not Applicable.
[1] Three aliquots of control B extract were diluted to prepare matrix matched standards for matrix assessment.

Instrument Conditions

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

HPLC Parameters:

Instrument	Shimadzu Nexera series HPLC system
Column#	Waters BEH Amide, 2.5 μm, 2.1 × 100 mm
Mobile Phase A#	50mM (pH 3) ammonium formate
Mobile Phase B#	Acetonitrile (LC-MS grade)

	Time (min)	Flow Rate (mL/min)	Mobile Phase A (%)	Mobile Phase B (%)
Gradient	0.00	1	3	97
	0.50	1	3	97
	2.50	1	60	40
	2.51	1	60	40
	3.00	0.5	60	40
	3.10	0.5	3	97
	5.00	0.5	3	97

Run Time	5 minutes
Column Temperature	40°C
Autosampler Temperature	5°C
Injection Volume	10 μL
Retention Time	Approx. 1.3 minutes (Chlormequat Chloride)

Valco Valve Diverter	Time (min)	Position
	0	A (to waste)
	1	B (to MS)
	4	A (to waste)

MS/MS Parameters:

Instrument	AB Sciex 5000 Triple Quadrupole Mass Spectrometer
Ionisation Type#	Electrospray (ESI)
Polarity#	Positive
Scan Type#	MRM
Ion Spray Voltage	5500 V
Curtain Gas (CUR)	25
Gas Flow 1 (GS1)	40
Gas Flow 2 (GS2)	40
Vaporiser Temperature (TEM)	500°C
Interface Heater (ihe)	On
Collision Gas (CAD)	5
Entrance Potential (EP)	10
Declustering Potential (DP)	100
Collision Exit Potential (CXP)	13

Compound Name	MRM Transition Ions Monitored	Collision Energy (CE)	Dwell Time (ms)
Chlormequat Chloride (Primary)	122.0/58.1	40	150
Chlormequat Chloride (Confirmatory)	122.0/62.9	32	150

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 was collected using Analyst 1.6.2.

Calculation of Results

Results were calculated using Analyst 1.6.2. When the calibration fit is linear, 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)

Procedural recovery from fortified samples is calculated as follows:

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

The sample concentration in mg/kg = concentration in μg/kg/1000

The standard concentration in μg/mL = concentration in μg/L/1000

The Limit of Detection (LOD) based upon the sample concentration equivalent to three times the baseline noise of a control sample was calculated as follows:

LOD = 3 × height of control baseline noise × control dilution factor × calibration standard concentration (μg/mL) / height of calibration standard peak

The Method Detection Limit (MDL) 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:

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

Specificity was acceptable if the amounts found in the control samples were ≤ 30% of the LOQ.

Linearity

Linearity was acceptable if the lowest calibration standard concentration was $\leq 80\%$ of the equivalent LOQ final extract concentration and the highest calibration standard concentration was $\geq 120\%$ of the $10 \times$ LOQ final extract concentration (after dilution if applicable). The correlation coefficient (r) was acceptable if it was ≥ 0.995. If matrix effects were determined to be significant, matrix matched calibration standards would be used.

Limit of Detection (LOD) Assessment

An estimate of the LOD was made at $3 \times$ baseline noise for the primary and confirmatory transitions.

Method Detection Limit (MDL) Assessment

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 for triplicate standards prepared in control soil final extract and in blank solvent. This applied to the primary and confirmatory transitions and for both soils.

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

A difference of $\geq 20\%$ was considered significant.

RESULTS

Recovery
Mean recoveries were within the acceptable range of 70 to 110% at each concentration (primary and confirmatory transitions) for each soil.

Precision
Precision was acceptable (RSD ≤ 20%) at each concentration (primary and confirmatory transitions) for each soil.

Specificity
Chlormequat Chloride was not present in KS or CA soil at > 30% of the LOQ.

Linearity
The response of the LC-MS/MS was linear using a 1/x weighting over the range of 0.025 to 2.5 µg/L for Chlormequat Chloride, which is equivalent to soil concentrations of 0.03 to 3 mg/kg (using a dilution factor of 1200). The correlation coefficients (r) were ≥ 0.995. The lowest calibration point was ≤ 80% of the LOQ

final extract concentration, and the highest calibration point was ≥ 120% × LOQ final extract concentration.

Limit of Quantification (LOQ)
The limit of quantification based upon the lowest level validated confirmed the LOQ to be 0.05 mg/kg for Chlormequat Chloride in soil.

Limit of Detection
The limit of detection based upon the sample concentration equivalent to 3 × baseline noise was calculated in KS and CA soil for Chlormequat Chloride (primary and confirmatory). The results are presented in the summary table at the beginning of the results section.

Method Detection Limit (MDL)
The MDL was calculated to be 0.03 mg/kg for Chlormequat Chloride (based upon a lowest standard concentration of 0.025 µg/L and a dilution factor of 1200).

Matrix Effects
An assessment of matrix effects was made by comparison of peak areas from triplicate standards prepared in control soil final extract and in blank solvent. The difference from the mean non-matrix standard peak areas was calculated. Matrix assessment results are presented in Table 5 and Table 6.

Matrix effects were insignificant (< 20% difference from non-matrix standards) for the primary transition, but were significant for the confirmatory transition for Chlormequat Chloride in KS and CA soil, therefore matrix matched calibration standards were used to cover both transitions.

Appendix 6
Analytical Procedure

Analytical Procedure

Procedure Title	Determination of Chlormequat Chloride in Soil by LC-MS/MS
Procedure Code	SMV 3201883-01V
Page Number	1 of 14

The methodology described in this procedure has been validated in KS and CA soils at 0.05 and 0.5 mg/kg.

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REVISION HISTORY

SMV 3201883-01V   New document produced following independent laboratory
validation of Smithers Viscient, Wareham study 14105.6104

SAFETY PRECAUTIONS

Operators should take the normal precaution of wearing gloves, laboratory coats and
safety glasses when handling compound and matrix samples.

Safety assessments (Control of Substances Hazardous to Health, COSHH) have been
made of those procedural steps involving preparation of solutions, reagents and
analysis of matrix samples. Appropriate safety codes have been included in the text
and are defined in the section titled General Handling Control Categories.

The hazards and risks of the substances hazardous to health used in this method have
been considered. Provided the method is accurately followed and the control measures
specified in the method are correctly used, there should be no foreseeable hazards to
health.

INTRODUCTION

This method describes the procedure for determining concentrations of Chlormequat
chloride in two soils by LC-MS/MS.

Samples are extracted four times with methanol: 1M (pH 7) potassium carbonate
(50:50 v/v). The extract is diluted into calibration range with acetonitrile: water:
trifluoroacetic acid (80:20:0.1 v/v/v). Sample extracts are quantified by LC-MS/MS.

Matrix effects for Chlormequat chloride in soil were determined by comparing peak
areas of calibration standards prepared in control soil final extract and in acetonitrile:
water: trifluoroacetic acid (80:20:0.1 v/v/v).

Matrix effects are considered significant if the matrix matched standard area is ≥ 20%
different to the non-matrix standard area. If matrix effects are significant, matrix
matched calibration standards will be used for method validation.

- 2 -

APPARATUS, MATERIALS, REAGENTS AND SOLUTIONS

Apparatus and Glassware
- Shimadzu Nexera series HPLC system with ABSciex API 5000 MS/MS detector.
- HPLC column: Waters XBridge BEH Amide, 130Å, 2.5 µm, 2.1 × 100 mm
- Analytical balance
- pH meter
- Shaker
- Centrifuge
- 50 mL Nalgene centrifuge tubes
- Positive displacement pipettes
- Volumetric flasks
- Amber glass vials
- Disposable glass vials
- HPLC vials

Equivalent equipment may be used if required

Materials
Acetonitrile	HPLC grade, Honeywell
Acetonitrile	LC-MS grade, Honeywell
Methanol	HPLC grade, Honeywell
Water	Milli-Q (with LCPAK polisher), In house
Potassium carbonate	BioXtra, Sigma
Hydrochloric acid (HCl)	ACS reagent, Sigma
Formic acid	ACS reagent, Sigma
Trifluoroacetic acid (TFA)	Reagent grade, Sigma
Ammonium formate	Reagent grade, Sigma

Equivalent materials may be used if required

Reagents
Acetonitrile: water (50:50 v/v)
Mix 50 mL HPLC grade acetonitrile with 50 mL Milli-Q water.

Acetonitrile: water: TFA (80:20:0.1 v/v/v)
Mix 400 mL HPLC grade acetonitrile with 100 mL Milli-Q water and 0.5 mL TFA.

1M HCl
Dilute 165 mL HCl to 2000 mL with Milli-Q water.

1M potassium carbonate in water
Dissolve 138 g potassium carbonate in 1000 mL Milli-Q water.

1M (pH 7) potassium carbonate
Very carefully mix 806 mL 1M potassium carbonate with 1194 mL 1M HCl (**a lot of gas will be quickly evolved**).

- 3 -

Methanol: 1M (pH 7) potassium carbonate (50:50 v/v)
Mix 1000 mL HPLC grade methanol with 1000 mL 1M (pH 7) potassium carbonate

2M ammonium formate in water
Dissolve 12.6 g ammonium formate in 100 mL Milli-Q water.

50mM (pH 3) ammonium formate
Dilute 25 mL 2M ammonium formate to 1000 mL with Milli-Q water. Adjust to pH
3.0 using formic acid.

Reagents may be scaled as appropriate.

Standard Solution Preparation [1b, 4a]
Primary Standard Stock
Prepare duplicate stock solutions of Chlormequat chloride at 1000 µg/mL in Milli-Q
water: Accurately weigh ≥ 10 mg test substance, corrected for purity and transfer into
a 10 mL volumetric flask. Adjust the volume to give exactly 1000 µg/mL. Transfer
into amber glass bottles. The primary stocks should be stored refrigerated and given a
nominal expiry date of 3 months.

Standard Correlation
Dilute the duplicate primary stocks to the mid-point of the calibration line. Correlate
the standard solutions by injecting each of the two calibration standards 5 times into
the LC-MS/MS. Ensure that the two solutions are injected alternately in the run
sequence. The results for the correlation should be ± 5% of the overall mean
calculated by peak areas.

Review of Results
Review the data and document the correlation calculations. If the correlation is out of
specification, either repeat the injections, re-dilute, or prepare two new stock
standards and repeat the procedures in sections *<<Initial Weighing of Stock
Solutions>>* to *<<Review of Results>>*.

If the acceptance criteria from section *<<Standard Correlation>>* have been met,
then the calibration solutions are acceptable for use. If required, fortification solutions
for method validation will be made from the same stock standard, or its dilutions,
from which the calibration line has been prepared.

Secondary Standard Stocks
Prepare secondary stock solutions of Chlormequat chloride as described in the
following table:

Stock Concentration (µg/mL)	Volume Taken (mL)	Solvent	Final Volume (mL)	Concentration (µg/mL)
1000	1	Acetonitrile:	10	100
100	1	water	10	10
10	1	(50:50 v/v)	10	1

Transfer into amber glass bottles. The secondary stocks should be stored refrigerated
and given a nominal expiry date of 1 month.

Sub-Stocks
Prepare sub-stock solutions of Chlormequat chloride as described in the following table:

Stock Concentration (μg/mL)	Volume Taken (mL)	Solvent	Final Volume (mL)	Concentration (μg/mL)
1	0.1	Acetonitrile: water: TFA (80:20:0.1 v/v/v)[1]	10	0.01[2]

[1] If matrix matched calibration standards are required, the solvent should be substituted for control soil final extract.
2 Equivalent to 10 μg/L.

Transfer into disposable glass vials. The sub-stock solutions should be prepared on the day of use.

Preparation of Matrix Matched Standards for Matrix Assessment
Prepare matrix matched standards of Chlormequat chloride in disposable glass vials as described in the following tables:

KS soil

Stock Concentration (μg/L)	Volume Taken (mL)	Solvent	Final Volume (mL)	Concentration (μg/L)
10	1	Control KS soil final extract	10	1
10	1		10	1
10	1		10	1

CA soil

Stock Concentration (μg/L)	Volume Taken (mL)	Solvent	Final Volume (mL)	Concentration (μg/L)
10	1	Control CA soil final extract	10	1
10	1		10	1
10	1		10	1

Matrix matched standards for matrix assessment should be prepared on the day of use.

Preparation of Non-Matrix Matched Standards for Matrix Assessment
Prepare non-matrix matched standards of Chlormequat chloride in disposable glass vials as described in the following table:

Stock Concentration (μg/L)	Volume Taken (mL)	Solvent	Final Volume (mL)	Concentration (μg/L)
10	1	Acetonitrile: water: TFA (80:20:0.1 v/v/v)	10	1
10	1		10	1
10	1		10	1

Non-matrix matched standards for matrix assessment should be prepared on the day of use.

- 5 -

Calibration Standards

Prepare calibration standards of Chlormequat chloride in volumetric flasks (10 mL) or
HPLC vials (1 mL) as described in the following table:

Stock Concentration (µg/L)	Volume Taken (mL)	Solvent	Final Volume (mL)	Concentration (µg/L)
10	2.5		10	2.5
2.5	0.6		1	1.5
2.5	0.4	Acetonitrile:	1	1
2.5	0.2	water: TFA	1	0.5
2.5	0.04	(80:20:0.1 v/v/v)[1]	1	0.1
2.5	0.02		1	0.05
2.5	0.01		1	0.025

[1] If matrix matched calibration standards are required, the solvent should be substituted for control soil
final extract.

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

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

- 6 -

PROCEDURES

All procedures will be carried out in compliance with departmental SOPs, following departmental safety procedures in conjunction with COSHH assessments.

All work should be carried out under the minimum control categories listed under the safety precautions section. Additional controls are listed with the individual steps of the procedure.

Fortification of Control Samples for Method Validation [1b, 4a]

Weigh 10.0 g dry weight of soil into a 50 mL Nalgene centrifuge tube.
Fortify samples with Chlormequat chloride standard in acetonitrile: water (50:50 v/v) as described in the following tables:

KS soil

Number of Replicates	Sample Type	Stock Concentration (µg/mL)	Volume Added (mL)	Sample Weight (g)	Fortified Concentration (mg/kg)
1	Reagent blank	N/A	N/A	N/A	N/A
2	Control	N/A	N/A	10.0	N/A
5	LOQ	10	0.05	10.0	0.05
5	10 × LOQ	100	0.05	10.0	0.5

N/A = Not Applicable.

CA soil

Number of Replicates	Sample Type	Stock Concentration (µg/mL)	Volume Added (mL)	Sample Weight (g)	Fortified Concentration (mg/kg)
1	Reagent blank	N/A	N/A	N/A	N/A
2	Control	N/A	N/A	10.0	N/A
5	LOQ	10	0.05	10.0	0.05
5	10 × LOQ	100	0.05	10.0	0.5

N/A = Not Applicable.

Sample Extraction [1b, 4a]

1. Weight 10.0 g soil into a 50 mL Nalgene centrifuge tube.
2. Add 30 mL methanol: 1M (pH 7) potassium carbonate (50:50 v/v).
3. Place in an ultrasonic bath for 15 minutes.
4. Place on a shaker set to 200 rpm for 30 minutes.
5. Centrifuge at 1500 rpm for 10 minutes.
6. Transfer the supernatant into a plastic pot.
7. Repeat steps 2 to 6 three more times, for a total of four extractions, combining the extracts.
8. Dilute the combined extracts to 120 mL with methanol: 1M (pH 7) potassium carbonate (50:50 v/v).
9. Dilute into calibration range with acetonitrile: water: TFA (80:20:0.1 v/v).
10. Transfer into an HPLC vial for analysis.

Recommended dilution procedure is given in the following table:

Analytical Procedure SMV 3201883-01 V

Sample Type	Fortified Concentration (mg/kg)	Sample Weight (g)	Extract Volume (mL)	Dilution (mL-mL)	Dilution Factor
Reagent blank	N/A	N/A	120	0.1-10	1200
Control	N/A	10.0	120	0.1-10[1]	1200
LOQ	0.05	10.0	120	0.1-10	1200
10 × LOQ	0.5	10.0	120	0.1-10	1200

N/A = Not Applicable.
[1] Dilute an additional 3 aliquots of control extract for matrix assessment.

LC-MS/MS CONDITIONS

HPLC Parameters:

Instrument	Shimadzu Nexera series HPLC system
Column#	Waters BEH Amide, 2.5 µm, 2.1 × 100 mm
Mobile Phase A#	50mM (pH 3) ammonium formate
Mobile Phase B#	Acetonitrile (LC-MS grade)

Gradient

Time (min)	Flow Rate (mL/min)	Mobile Phase A (%)	Mobile Phase B (%)
0.0	1	3	97
0.5	1	3	97
2.5	1	60	40
2.51	1	60	40
3.0	0.5	60	40
3.1	0.5	3	97
5.0	0.5	3	97

Run Time	5 minutes
Column Temperature	40°C
Autosampler Temperature	5°C
Injection Volume	10 µL
Retention Time	Approx. 1.3 minutes (Chlormequat chloride)

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#	MRM
Ion Spray Voltage	5500 V
Curtain Gas (CUR)	25
Gas Flow 1 (GS1)	40
Gas Flow 2 (GS2)	40
Vaporiser Temperature (TEM)	500°C
Interface Heater (ihe)	On
Collision Gas (CAD)	5
Entrance Potential (EP)	10
Declustering Potential (DP)	100
Collision Cell Exit Potential (CXP)	13

Compound Name	MRM Transition Ions Monitored	Collision Energy (CE)	Dwell Time (ms)
Chlormequat chloride (Primary)	122.0/58.1	40	150
Chlormequat chloride (Confirmatory)	122.0/62.9	32	150

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

CALCULATION OF RESULTS

All peak measurements and calculations are performed on Analyst 1.6.2. From the measured peak area, where 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 (µg/kg)
y = area of peak due to test substance
m = gradient
c = Y intercept on calibration graph
DF = sample dilution factor

The dilution factor is calculated from the following calculation:

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

Procedural recovery data from fortified samples are calculated via the following equation:

$$\text{Recovery}(\%) = \frac{A}{S} \times 100$$

Where:-

A = concentration found in fortified sample (µg/kg)
S = concentration added to fortified sample (µg/kg)

The sample concentration in mg/kg = concentration in µg/kg/1000

- 10 -

METHOD CRITERIA

For the analysis by LC-MS/MS to be considered successful the following criteria should be met.

- At least 5 calibration standards will be used in the determination of the calibration line.
- The correlation coefficient (r) for the calibration line will be ≥ 0.995 with a 1/x weighting.
- All sample extracts will be within the appropriate range of calibration standards.
- Mean recovery from fortified samples will be considered acceptable within the range of 70 to 110%.
- The control sample should not contain interference > 30% of the LOQ.

- 11 -

GENERAL HANDLING CONTROL CATEGORIES

CATEGORY Main Division		CONTROL Name and Specification
1		GLOVES
	a	Disposable latex
	b	Disposable nitrile
	c	Rubber gloves
	d	Specific type for the job (see assessment giving details)
2		PROTECTIVE CLOTHING
	a	Laboratory coat or equivalent
	b	Disposable overalls
	c	Oversleeves
	d	Overshoes
	e	Plastic apron
3		EYE/FACE PROTECTION
	a	Safety glasses to BS 2092/2 C or better
	b	Face shield to BS 2092/2 C or better
	c	Safety goggles to BS 2092/2 C or better
4		ENGINEERING CONTROLS
	a	Open bench in ventilated area
	b	Fume cupboard to BS 7258
	c	Laminar flow cabinet to BS 5295 Class 1
	d	Re-circulating fume chamber
	e	Radioisotope lab
	f	Biohazard lab
	g	Glove box
5		RESPIRATORY PROTECTIVE EQUIPMENT
	a	Disposable filtering facemask (HSE approved), i - organic vapour ii - dust iii – combination organic vapour/dust MUST SPECIFY TYPE
	b	Powered respirators/helmets with safety visor to BS 2092/2 C or better (HSE approved)
	c	Respirator with specified canister (HSE approved)
6		SPECIFIC IMMUNISATION REQUIRED (GIVE DETAILS)
7		ALLERGIC PERSONS PROHIBITED (SPECIFY ALLERGY)
8		REFER TO MATERIAL SAFETY DATA SHEET
9		KNOWN OR SUSPECTED REPRODUCTIVE HAZARD TO EITHER SEX (must specify details)
10		POISON – ensure antidote is available and is within its expiry date (must specify details)

- 13 -