50747516

**Study Title**

Validation of the Analytical Method for the Determination of Chlormequat Chloride in Soil Matrix by LC-MS/MS

**Test Guidelines**

OCSPP 850.6100
SANCO/3029/99 rev. 4 (2000)

# 1.0 INTRODUCTION

The purpose of this study was to validate an analytical method used to determine the content of chlormequat chloride in two different soil types. The analytical method was validated with regards to specificity, linearity, accuracy, precision, limit of quantitation (LOQ), limit of detection (LOD), and method detection limit (MDL).

The method was validated by fortification of soil with chlormequat chloride at concentrations of 0.050 (LOQ) and 0.50 (10X LOQ) mg/kg. All recovery samples were extracted four times with 50/50 methanol/1 M (pH 7) potassium carbonate and diluted into calibration range with 80/20/0.1 acetonitrile/purified reagent water/trifluoroacetic acid. All samples were analyzed using liquid chromatography with tandem mass spectrometry detection (LC-MS/MS).

# 2.0 MATERIALS AND METHODS

## 2.1 Protocol

Procedures used in this study followed those described in the Smithers Viscient protocol entitled "Validation of the Analytical Method for the Determination of Chlormequat Chloride in Soil Matrix by LC-MS/MS" (Appendix 1). The study was conducted under Good Laboratory Practice (GLP) Standards 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 Substance

The test substance, chlormequat chloride, was received on 5 January 2017 from Chem Service, Inc., West Chester, Pennsylvania. The following information was provided:

| | |
|---|---|
| Name: | chlormequat chloride |
| Lot No.: | 5731400 |
| CAS No.: | 999-81-5 |
| Purity: | 98.02% (Certificate of Analysis, Appendix 2) |
| Recertification Date: | 31 January 2019 |

Upon receipt at Smithers Viscient, the test substance (SMV No. 8683) 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 identity, maintenance of records on the test substance, and archival of a sample of the test substance are the responsibility of the Study Sponsor.

## 2.3 Reagents

| | | |
|---|---|---|
| 1. | Acetonitrile: | EMD, reagent grade |
| 2. | Methanol: | EMD, reagent grade |
| 3. | Potassium carbonate: | Fisher Chemical |
| 4. | Hydrochloric acid: | EMD, reagent grade |
| 5. | Formic acid: | Sigma, reagent grade |
| 6. | Trifluoroacetic acid: | EMD, reagent grade |
| 7. | Ammonium formate: | Sigma, reagent grade |
| 8. | Purified reagent water: | Prepared from a Millipore Milli-Q Direct 8 or Barnstead water purification system (meets ASTM Type II requirements) |

Reagents of similar grade and comparable purity may be substituted for the specific reagents above in future testing with this method as long as acceptable performance is demonstrated.

## 2.4 Instrumentation and Laboratory Equipment

| | | |
|---|---|---|
| 1. | Instrument: | Sciex TripleTOF 5600 mass spectrometer equipped with a Sciex DuoSpray (ESI and APCI) ion source<br>Shimadzu SIL-20ACXR autoinjector<br>Shimadzu DGU-20A5R vacuum degasser<br>Shimadzu LC-20ADXR solvent delivery pumps<br>Shimadzu CTO-20AC column compartment<br>Shimadzu CBM-20A system controller<br>Analyst TF 1.6 software for data acquisition |
| 2. | Balances: | Mettler Toledo XSE205DU; Ohaus EX224, EP4102, and 4202 |
| 3. | Centrifuges: | Thermo Scientific Sorvall ST40, Eppendorf 5418 |
| 4. | Shaker table: | Thermo Scientific SHKA2000 |
| 5. | Moisture balance: | Sartorius Moisture Analyzer MA-150 |
| 6. | 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 caps |

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

## 2.5 Test Soils

The soils used for the method validation were KS Soil (SMV Lot No. R05-10-17-137) from Larned, Kansas, and CA Soil (SMV Lot No. R05-03-17-130) from Porterville, California. Prior to testing, soil moisture content was determined to be 18.08% for the KS Soil and 12.34% for the CA Soil using a Sartorius MA-150 moisture analyzer. Soil characterization data are listed in the table below.

| Soil | Soil Type | % Sand, Silt, Clay | Bulk Density (gm/cc) | Cation Exchange Capacity (meq/100 g) | % Organic Matter (Walkley Black) | pH in 1/1 Soil/Water Ratio |
|---|---|---|---|---|---|---|
| KS Soil | Loam | 46, 36, 18 | 1.01 | NA | 4.0 | 6.0 |
| CA Soil | Loamy Sand | 75, 20, 5 | 1.16 | 8.5 | 1.07 | 7.8 |

Soil Characterized by Agvise Laboratories, Northwood, North Dakota.
NA = Not Applicable; parameter not characterized.

## 2.6 Preparation of Liquid Reagent and Mobile Phase 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 liquid reagent solution was prepared by combining 2000 mL of acetonitrile and 2000 mL of purified reagent water.

A 1 M hydrochloric acid solution was prepared by diluting 165 mL of hydrochloric acid with 2000 mL with purified reagent water.

A 1 M potassium carbonate solution was prepared by diluting 276.42 g of potassium carbonate to 2000 mL with purified reagent water.

A 1 M (pH 7) potassium carbonate solution was prepared by combining 500 mL of 1 M potassium carbonate solution with 740 mL of 1 M hydrochloric acid. The prepared solution had a pH of 7 as measured with pH strips (EMD).

A 50/50 methanol/1 M (pH 7) potassium carbonate solution was prepared by combining 1240 mL of 1 M (pH 7) potassium carbonate solution with 1240 mL of methanol.

An 80/20/0.1 acetonitrile/purified reagent water/trifluoroacetic acid liquid reagent solution was prepared by combining 1600 mL of acetonitrile, 400 mL of purified reagent water, and 2 mL of trifluoroacetic acid.

A 2000 mM ammonium formate liquid reagent solution was prepared by diluting 31.5293 g of ammonium formate to 250 mL with purified reagent water.

A 50 mM (pH 3) ammonium formate mobile phase solution was prepared by diluting 50 mL of 2000 mM ammonium formate to 2 L with purified reagent water and adding formic acid until the

prepared solution had a pH of 2.98 as measured with a Yellow Springs Instrument (YSI) pH100 pH meter.

## 2.7 Preparation of Stock Solutions

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

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

| Primary Stock ID | Amount of Substance Weighed (g), Net Weight | Amount of Substance Weighed (g), as Active Ingredient | Stock Solvent | Final Volume (mL) | Primary Stock Concentration (mg/L) | Primary Stock Use |
|---|---|---|---|---|---|---|
| 8683-2A | 0.0256 | 0.02509 | Purified reagent water | 25.09 | 1000 | Secondary stock solutions |
| 8683-2H | 0.0256 | 0.02509 | Purified reagent water | 25.09 | 1000 | Secondary stock solutions |

Secondary stock solutions were prepared as per the table below:

| Fortifying Stock ID | Fortifying Stock Concentration (mg/L) | Volume of Fortification (mL) | Final Volume (mL) | Stock Solvent | Stock ID | Stock Concentration (mg/L) | Stock Use |
|---|---|---|---|---|---|---|---|
| 8683-2A | 1000 | 0.0500 | 50 | 50/50 Acetonitrile/purified reagent water | 8683-2A-1 | 1.00 | Tertiary stock solution |
| 8683-2A | 1000 | 0.500 | 50 | 50/50 Acetonitrile/purified reagent water | 8683-2A-2 | 10.0 | LOQ recovery samples |
| 8683-2H | 1000 | 0.0500 | 50 | 50/50 Acetonitrile/purified reagent water | 8683-2H-1 | 1.0 | Tertiary stock solution |
| 8683-2H | 1000 | 0.500 | 50 | 50/50 Acetonitrile/purified reagent water | 8683-2H-2 | 10.0 | LOQ recovery samples |
| 8683-2A | 1000 | 5.00 | 50 | 50/50 Acetonitrile/purified reagent water | 8683-2A-3 | 100 | 10X LOQ recovery samples |

Tertiary stock solutions were prepared as per the table below:

| Fortifying Stock ID | Fortifying Stock Concentration (mg/L) | Volume of Fortification (mL) | Final Volume (mL) | Stock Solvent | Stock ID | Stock Concentration (µg/L) | Stock Use |
|---|---|---|---|---|---|---|---|
| 8683-2A-1 | 1.00 | 0.100 | 10.0 | 80/20/0.1 Acetonitrile/purified reagent water/trifluoroacetic acid | 8683-2A-1-1 | 10.0 | Calibration standards |
| 8683-2H-1 | 1.00 | 0.100 | 10.0 | 80/20/0.1 Acetonitrile/purified reagent water/trifluoroacetic acid | 8683-2H-1-1 | 10.0 | Calibration standards |

All primary and secondary stock solutions were stored refrigerated (2 to 8 °C) in amber glass bottles fitted with Teflon-lined caps. Tertiary stock solutions were stored in a freezer.

## 2.8 Preparation of Calibration Standards

### 2.8.1 Calibration Standards – Recovery Samples

Calibration standards were prepared in 80/20/0.1 acetonitrile/purified reagent water/trifluoroacetic acid by fortifying with the 10.0 µg/L tertiary stock solution to yield test substance concentrations of 0.025, 0.05, 0.100, 0.500, 1.00, 1.50, and 2.50 µg/L. This procedure is detailed in the table below.

| Test Substance Stock ID | Stock Concentration (µg/L) | Fortification Volume (mL) | Final Volume (mL) | Standard Concentration (µg/L) | Sample ID |
|---|---|---|---|---|---|
| 8683-2A-1-1 or 8683-2H-1-1 | 10.0 | 0.025 | 10.0 | 0.025 | Std 1 |
| | | 0.05 | 10.0 | 0.05 | Std 2 |
| | | 0.10 | 10.0 | 0.10 | Std 3 |
| | | 0.50 | 10.0 | 0.50 | Std 4 |
| | | 1.0 | 10.0 | 1.0 | Std 5 |
| | | 1.5 | 10.0 | 1.5 | Std 6 |
| | | 2.5 | 10.0 | 2.5 | Std 7 |

### 2.8.2 Calibration Standards – Matrix Effects

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 (the LOQ).

Calibration standards used to assess possible matrix effects were prepared as follows by fortifying with the 10.0 µg/L tertiary stock solution to yield a test substance concentration of 0.0500 mg/L for chlormequat chloride. A portion of each standard was centrifuged at 13,000 rpm for five minutes and then transferred to clear vials prior to LC-MS/MS analysis.

### 2.8.2.1 Matrix-Matched Standards

**Prepared in KS Soil final fraction extract for analysis**

| Test Substance Stock ID | Stock Concentration (mg/L) | Fortification Volume (mL) | Final Volume (mL)[a] | Standard Concentration (mg/L) | Sample ID |
|---|---|---|---|---|---|
| 8683-2H-2 | 10.0 | 0.0500 | 10.0 | 0.0500 | F1017-255 |
| | | 0.0500 | 10.0 | 0.0500 | F1017-256 |
| | | 0.0500 | 10.0 | 0.0500 | F1017-257 |

[a] Samples were diluted with the final fraction of the Control A (Sample F0617-108) following dilution in 80/20/0.1 acetonitrile/purified reagent water/trifluoroacetic acid; (see Section 2.10 for extract preparation and dilution procedures).

**Prepared in CA Soil final fraction extract for analysis**

| Test Substance Stock ID | Stock Concentration (mg/L) | Fortification Volume (mL) | Final Volume (mL)[a] | Standard Concentration (mg/L) | Sample ID |
|---|---|---|---|---|---|
| 8683-2H-2 | 10.0 | 0.0500 | 10.0 | 0.0500 | F1017-252 |
| | | 0.0500 | 10.0 | 0.0500 | F1017-253 |
| | | 0.0500 | 10.0 | 0.0500 | F1017-254 |

[a] Samples were diluted with the final fraction of the Control A (Sample F0617-127) following dilution in 80/20/0.1 acetonitrile/purified reagent water/trifluoroacetic acid; (see Section 2.10 for extract preparation and dilution procedures).

### 2.8.2.2 Non-Matrix-Matched Standards

| Test Substance Stock ID | Stock Concentration (µg/L) | Fortification Volume (mL) | Final Volume (mL)[a] | Standard Concentration (µg/L) | Sample ID |
|---|---|---|---|---|---|
| 8683-2H-2 | 10.0 | 0.0500 | 10.0 | 0.0500 | F1017-258 |
| | | 0.0500 | 10.0 | 0.0500 | F1017-259 |
| | | 0.0500 | 10.0 | 0.0500 | F1017-260 |

[a] Samples were diluted with 80/20/0.1 acetonitrile/purified reagent water/trifluoroacetic acid.

## 2.9 Sample Fortification and Preparation

For each soil type, a total of 12 recovery samples (10.0 g dry weight) were weighed into individual 50-mL centrifuge tubes and were fortified with the appropriate test substance secondary stock solution at concentrations of 0.050 and 0.50 mg/kg. Five replicates were prepared for each concentration level. In addition, two samples were left unfortified to serve as controls (see Protocol Deviations) and were extracted in the same fashion as the 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.

**Recovery samples in KS Soil:**

| Sample ID F0617- | Sample Type | Stock Concentration (mg/L) | Fortification Volume (mL) | Dry Weight (g) | Fortified Concentration (mg/kg) |
|---|---|---|---|---|---|
| 107 | Reagent Blank | NA[a] | NA | NA | 0.00 |
| 108, 109 | Control | NA | NA | 10.0 | 0.00 |
| 110, 111, 112, 113, 114 | LOQ | 10.0 | 0.0500 | 10.0 | 0.050 |
| 115, 116, 117, 118, 119 | 10X LOQ | 100 | 0.0500 | 10.0 | 0.50 |

[a] NA = Not Applicable

**Recovery samples in CA Soil:**

| Sample ID F0617- | Sample Type | Stock Concentration (mg/L) | Fortification Volume (mL) | Dry Weight (g) | Fortified Concentration (mg/kg) |
|---|---|---|---|---|---|
| 126 | Reagent Blank | NA[a] | NA | NA | 0.00 |
| 127, 128 | Control | NA | NA | 10.0 | 0.00 |
| 129, 130, 131, 132, 133 | LOQ | 10.0 | 0.0500 | 10.0 | 0.050 |
| 134, 135, 136, 137, 138 | 10X LOQ | 100 | 0.0500 | 10.0 | 0.50 |

[a] NA = Not Applicable

## 2.10 Soil Extraction

A 30-mL aliquot of 50/50 methanol/1 M (pH 7) potassium carbonate was added to each soil recovery sample (10.0 g dry weight), samples were sonicated for 15 minutes, and placed on a shaker table for 30 minutes at 300 rpm. The samples were then centrifuged at 1500 rpm for 10 minutes and the extracts were transferred to graduated cylinders. The extraction and centrifugation procedures above were repeated three times, for a total of four extractions. The extracts were combined, taken to volume (120 mL) with 50/50 methanol/1 M (pH 7) potassium carbonate, transferred to a plastic container, and hand shaken to mix well. All samples were further diluted into the calibration standard range with 80/20/0.1 acetonitrile/purified reagent water/trifluoroacetic acid. All recovery samples were transferred to HPLC vials for analysis via LC-MS/MS. Secondary dilution volumes can be scaled up or down as necessary. The extraction and dilution procedures are detailed below.

**KS Soil:**

| Sample ID F0617- | Nominal Concentration (mg/kg) | Dry Weight (g) | Extract Volume[a] (mL) | Final Volume[b] (mL) | Secondary Volume (mL) | Final Volume[c] (mL) | Dilution Factor |
|---|---|---|---|---|---|---|---|
| 107 | 0.00 | NA[d] | 30 | 120 | 0.10 | 10.0 | 1200 |
| 108 | 0.00 | 10.0 | 30 | 120 | 1.0 | 100[e] | 1200 |
| 109 | 0.00 | 10.0 | 30 | 120 | 0.10 | 10.0 | 1200 |
| 110, 111, 112, 113, 114 | 0.050 | 10.0 | 30 | 120 | 0.10 | 10.0 | 1200 |
| 115, 116, 117, 118, 119 | 0.50 | 10.0 | 30 | 120 | 0.10 | 10.0 | 1200 |

[a] Extraction Solvent: 50/50 methanol/1 M (pH 7) potassium carbonate (performed four times).
[b] Dilution solvent: 50/50 methanol/1 M (pH 7) potassium carbonate.
[c] Dilution solvent: 80/20/0.1 acetonitrile/purified reagent water/trifluoroacetic acid.
[d] NA = Not Applicable
[e] Increased volume for matrix investigation diluent.

**CA Soil:**

| Sample ID F0617- | Nominal Concentration (mg/kg) | Dry Weight (g) | Extract Volume[a] (mL) | Final Volume[b] (mL) | Secondary Volume (mL) | Final Volume[c] (mL) | Dilution Factor |
|---|---|---|---|---|---|---|---|
| 126 | 0.00 | NA[d] | 30 | 120 | 0.10 | 10.0 | 1200 |
| 127 | 0.00 | 10.0 | 30 | 120 | 1.0 | 100[e] | 1200 |
| 128 | 0.00 | 10.0 | 30 | 120 | 0.10 | 10.0 | 1200 |
| 129, 130, 131, 132, 133 | 0.050 | 10.0 | 30 | 120 | 0.10 | 10.0 | 1200 |
| 134, 135, 136, 137, 138 | 0.50 | 10.0 | 30 | 120 | 0.10 | 10.0 | 1200 |

[a] Extraction Solvent: 50/50 methanol/1 M (pH 7) potassium carbonate (performed four times).
[b] Dilution solvent: 50/50 methanol/1 M (pH 7) potassium carbonate.
[c] Dilution solvent: 80/20/0.1 acetonitrile/purified reagent water/trifluoroacetic acid.
[d] NA = Not Applicable
[e] Increased volume for matrix investigation diluent.

## 2.11 Analysis

### 2.11.1 Instrumental Conditions

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

**LC parameters:**

Column: Waters BEH Amide, 2.5 µm, 2.1 × 100 mm
Mobile Phase A: 50 mM (pH 3) ammonium formate
Mobile Phase B: acetonitrile
Gradient:

| Time (min.) | Flow rate (mL/min.) | Solvent A (%) | Solvent B (%) |
|---|---|---|---|
| 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 |
| 4.90 | 0.5 | 3 | 97 |
| 5.00 | 0.5 | 3 | 97 |

Run Time: 5.0 minutes
Column Temperature: 40 °C
Sample Temperature: 5 °C
Injection Volume: 10.0 µL
Retention Time: approximately 1.9 minutes

**MS parameters:**

| | |
|---|---|
| Instrument: | Sciex TripleTOF 5600 mass spectrometer |
| Ionization Mode: | Positive (+) ESI |
| Ion Spray Voltage: | 5500 V |
| Scan Type: | Product ion |
| Source Temperature: | 500 °C |
| Curtain Gas: | 25.0 |
| Ion Source – Gas 1 / Gas 2: | 50.0 / 50.0 |
| Collision Cell Entrance Potential: | 10.0 |
| Declustering Potential: | 100.0 |
| Resolution Q1: | Unit |
| Product of (Da): | 122.07 |
| Collison Energy: | 35.00 |
| Primary (Quantitative) Transition: | 122.07/58.0651 ± 0.0025 |
| Secondary (Confirmatory) Transition: | 122.07/62.9996 ± 0.0025 |

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 recovery sample set; one set prior to analysis of the recovery samples, and the second set immediately following the analysis of the recovery samples. Injection of samples and calibration standards onto the LC-MS/MS system was performed by programmed automated injection.

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

The accuracy was reported in terms of percent recovery of the fortified recovery samples. Recoveries of 70.0 to 120% (for the individual mean concentrations) are acceptable. The precision was reported in terms of the relative standard deviation (RSD) for the recovery samples. RSD values $\leq$ 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 chlormequat chloride which might interfere with the quantitation of the analyte peak of interest.

Linearity of the method was determined by the correlation coefficient (r) and the coefficient of determination ($r^2$).

## 2.13 Confirmation of the Test Substance

For the confirmatory method of chlormequat chloride by LC-MS/MS analysis, the second transition of the test substance was monitored and the ratio between the abundance of the primary (quantitative) and confirmatory transitions (qualitative) was calculated to assure greater confidence in the final result. In this procedure, the ratio of the area of the qualifier transition to the quantifier transition of the calibration standards was determined. Once the ratio is determined, unknown peaks in the samples are required to produce a transition ratio within ± 20% of that determined from the test substance in order for identification to be established.

## 2.14 Limit of Quantitation (LOQ)

The method was validated at the Limit of Quantitation (LOQ). This was defined as the lowest fortification level (0.050 mg/kg). Blank values (reagent blanks and untreated control samples) did not exceed 30% of the LOQ.

## 2.15 Limit of Detection (LOD) and Method Detection Limit (MDL)

The Limit of Detection (LOD) was calculated using three times the signal-to-noise value of the control samples. 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, 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 mass) |
| A | = | analytical result (mg/kg), concentration in the original sample |

The LOD was calculated using the following equation:

$$LOD = ((3 \times SN_{ctl})/(Resp_{LS} \times Conc_{LS})) \times DF_{CNTL}$$

where:

| | | |
|---|---|---|
| $SN_{ctl}$ | = | mean signal to noise in height of the control samples (or blanks) |
| $Resp_{LS}$ | = | mean response in height of the two low calibration standards |
| $Conc_{LS}$ | = | concentration of the low calibration standard |
| $DF_{CNTL}$ | = | dilution factor of the control samples (1200) |
| LOD | = | limit of detection for the analysis |

The MDL was calculated using the following equation.

$$MDL = MDL_{LCAL} \times DF_{CNTL}$$

where:

| | | |
|---|---|---|
| $MDL_{LCAL}$ | = | the lowest concentration calibration standard (0.0250 µg/L) |
| $DF_{CNTL}$ | = | dilution factor of the control samples (1200) |
| MDL | = | method detection limit reported for the analysis of chlormequat chloride recovery samples (0.030 mg/kg) |

The linearity was determined by preparing a calibration curve of seven standards of 0.0250, 0.0500, 0.100, 0.500, 1.00, 1.50, and 2.50 µg/L.

## 6.0 VALIDITY CRITERIA

The method validation in two soils utilizing solvent-based standards met the performance criteria as presented in the following table:

| Criterion | Acceptable Limits | Study Performance | |
|---|---|---|---|
| | | KS Soil | CA Soil |
| Specificity | Peaks attributable to the test substance should be sufficiently resolved from any peaks found in the samples of control matrix to enable quantification. | No extraneous peaks occurred which could interfere with quantification of the peak attributable to the test substance. | No extraneous peaks occurred which could interfere with quantification of the peak attributable to the test substance. |
| Linearity: Coefficient of Determination | The data should have a coefficient of determination ($r^2$) of not less than 0.990. | | |
| Matrix Effects | Possible effects of sample components will be evaluated. The effects of matrix enhancement or suppression will be evaluated through the assessment of solvent-based and matrix-matched LOQ calibration standards. | Matrix-matched and solvent-based calibration standards were prepared at the LOQ and analyzed with the recovery samples. Results of matrix-matched and solvent-based calibration standards met acceptance criteria and were not significantly different (< 5%). This indicates that there are likely no matrix effects for the soils tested. | |
| Accuracy: Mean Recoveries | Mean recoveries of 70.0 to 110% for each fortification level will be considered acceptable. | | |
| | | | |
| Accuracy: Test Concentrations | The study will be performed at two fortification levels which are set by anticipated testing levels, the lowest of which is the LOQ for this analysis and the high being 10X LOQ. | This study was performed at levels of 0.050 and 0.50 mg/kg; 0.050 mg/kg was set as the LOQ. | |
| Precision: Relative Standard Deviation (RSD) | Relative Standard Deviation (RSD) $\leq$ 20% for each fortification level will be considered acceptable while values $\leq$ 10% will be considered ideal. | | |
| | | | |
| Precision: Repeatability of Recovery | Five determinations will be made at each fortification level. | Five replicates were prepared and analyzed for each of the two fortification levels. | |
| Limit Of Quantitation (LOQ) | Blank values (reagent blanks and untreated control samples) should not exceed 30% of the LOQ. | All blank sample values were < 30% of the LOQ (0.050 mg/kg). | |

| Criterion | Acceptable Limits | Study Performance | |
|---|---|---|---|
| | | KS Soil | CA Soil |
| Limit Of Detection (LOD) | The LOD will be calculated using three times the signal-to-noise value of the control samples. | 0.00760 mg/kg | |
| Method Detection Limit (MDL) | The MDL will be set at the lowest concentration that can be detected in test solution samples. This value is calculated based on the concentration of the low calibration standard and the dilution factor of the control samples. | 0.030 mg/kg | |

# PROTOCOL DEVIATIONS

1. The protocol states that five replicates of the control matrix will be prepared for the validation. In this study, the controls were prepared in duplicate. Since there was no significant interference of chlormequat chloride in the LC-MS chromatograms of the control samples, this deviation has no impact on the results or interpretation of the study.

2. The protocol states that the method validation design will consist of a single soil matrix. In this study, two soil matrices were used. As this information provides additional information about the behavior of the test substance in soil, this deviation has no impact on the results or interpretation of the study.

# APPENDIX 1 - STUDY PROTOCOL

**Validation of the Analytical Method for the Determination of Chlormequat Chloride in Soil Matrix by LC-MS/MS**

## 1.0 INTRODUCTION

The purpose of this study is to validate an analytical method used to determine the content of chlormequat chloride in a single soil matrix by LC-MS/MS. The analytical method will be validated with regards to accuracy and precision, specificity, linearity, and limit of quantitation.

## 2.0 JUSTIFICATION OF THE TEST SYSTEM

This study is being conducted to support the registration of the test substance(s).

The method validations described in this protocol are designed to conform to SANCO 3029/99 rev.4: guidance for generating and reporting methods of analysis in support of pre-registration data requirements for Annex II and EPA guideline OCSPP 850.6100: Environmental Chemistry Methods and Associated Independent Laboratory Validation. The study will be conducted under Good Laboratory Practices (GLP) regulations and principles as described in 40 CFR 160 and the OECD principles on GLP.

## 3.0 TEST SUBSTANCE

Upon arrival at Smithers Viscient, the test substance (also the reference substance) will be received by the Test Material Center. Records will be maintained in accordance with GLP requirements, and a Chain-of-Custody established. The condition of the external packaging of the test substance will be recorded and any damage noted. The packaging will be removed, the primary storage container inspected for leakage or damage, and the condition recorded. Any damage will be reported to the Sponsor and/or manufacturer.

Each test and reference substance will be given a unique sample ID number and stored under the conditions specified by the Sponsor or manufacturer. The following information should be provided by the Study Sponsor, if applicable: test substance lot or batch number, test substance purity, water solubility (pH and temperature of solubility determination), vapor pressure, storage stability, methods of analysis of the test substance in soil, MSDS, and safe handling procedures, and a verified expiration or reanalysis date.

## 4.0 VALIDATION DESIGN

The method validation design will consist of a single soil matrix (considered to be worst-case for the program) fortified with test substance at two concentrations with five replications for each fortification level. The control matrix for the validation will be the appropriate untreated soil matrix with five replications. The validation study levels (approximate concentrations) for test substance are:

| | | |
|---|---|---|
| 1. | Procedural blank-reagent blank | 0.0 mg/kg |
| 2. | Matrix blank-control matrix | 0.0 mg/kg |
| 3. | Control matrix fortified at LOQ | 0.050 mg/kg |
| 4. | Control matrix fortified at 10 x LOQ | 0.50 mg/kg |

### 4.1 Accuracy and Precision

The accuracy of the analytical method will be determined by applying the method to five samples at the LOQ and five samples at 10X LOQ. Accuracy will be reported as the mean recovery at each fortification level. Mean recoveries in the range 70 – 120% of nominal concentrations of the target analyte in the fortified samples will be considered acceptable.

The precision of the method will be calculated and reported as the Relative Standard Deviation (RSD, %) of the accuracy data set at each fortification level (n = 5 per level). The RSD at each fortification level should be ≤ 20%. The overall RSD will also be reported.

### 4.2 Specificity

The specificity of the method will be determined by applying the method to the appropriate number of reagent blank (n=1) and control matrix samples (n=5). Chromatograms will be obtained for the control samples and examined for peaks that might interfere with the quantitation of the analyte peak of interest. Peaks attributable to the test substance should be sufficiently resolved from any peaks found in the samples of control matrix to enable quantification. Unequivocal identification of the target analyte will be achieved by LC-MS/MS primary and confirmatory analysis.

### 4.3 Calibration and linearity

Quantitative analysis will be achieved with the aid of a calibration curve. The calibration curve will be constructed using a minimum of five analytical standards and will extend over a range appropriate to the lowest and highest nominal concentrations of the target analyte in relevant analytical solutions ± at least 20%.

The calibration data will be subjected to regression analysis; a plot of analyte concentration versus detector response will be included in the report along with the correlation coefficient (r) and the equation describing the curve. The linearity of the detector response will be assessed according to the strength of the correlation coefficient: this should be ≥ 0.995 (or coefficient of determination, $r^2 \geq 0.990$). If non-linear calibration is used an explanation will be provided.

### 4.4 Confirmatory Analyses

All of the required elements need to be met for this confirmatory method with full method validation results generated for both ions. The confirmation method is including a confirmatory ion in the method; whereas the primary ion is used as primary method. Quantitation with confirmatory methods only needs to occur in the validation and does not need to continue in testing.

### 4.5 Matrix Effects Determination

Determination of LC-MS/MS matrix effects should be assessed as outlined in the analytical methods for both primary and confirmatory transitions. Matrix effects should be evaluated at the LOQ level for each test substance. Only if experiments clearly demonstrate that matrix effects are not significant (i.e. <20%), calibration with standards in solvent may be used.

**4.6 Limits of Quantitation (LOQ)**

The method will be validated at the limit of quantitation (LOQ). This will be defined as the lowest fortification level. Blank values (reagent blanks and untreated control samples) should not exceed 30% of the LOQ. If this is exceeded, it will be discussed with the Sponsor and detailed justification provided prior to processing.

**4.7 Limits of Detection (LOD) and Method Detection Limit (MDL)**

The limit of detection (LOD) will be calculated using three times the signal-to-noise value of the control samples. The method detection limit (MDL) will be set at the lowest concentration that can be detected in test solutions samples. The value is calculated based on the concentration of the low calibration standard and the dilution factor of the control samples.

## 5.0 PROCEDURE FOR THE INDENTIFICATION OF THE TEST SYSTEM

The test system will be defined as the fortified recovery samples. The fortified recovery samples will be labeled as defined in section 4.0 and each sample replicate will be assigned a unique identifier. Processing of fortified recovery samples will be performed at a lab station labeled with the study number.

## 6.0 CONTROL OF BIAS

Bias will be effectively controlled through techniques such as, but not limited to, preparation of replicate samples and replicate analysis.

## 7.0 RECORDS TO BE MAINTAINED

Records to be maintained will include, but will not be limited to, correspondence and other documents relating to the interpretation and evaluation of data as well as all raw data and documentation generated as a result of the study.

## 8.0 REPORTING

The validation of the analytical method will be fully reported according to the requirements of SANCO/3029/99 rev. 4. The raw data generated at Smithers Viscient will be peer-reviewed and the final report will be reviewed by the Study Director. All values will be reported to various levels of significance depending on the accuracy of the measuring devices employed during any one process. The Quality Assurance Unit will inspect the final report to confirm that the methods, procedures, and observations are accurately and completely described, that the reported results accurately and completely reflect the raw data generated at Smithers Viscient and to confirm adherence with the study protocol. A copy of the draft report will be submitted to the Sponsor for review. Upon acceptance by the Sponsor, a copy of the final report will be submitted. All reports will include, but will not be limited to, the following information:

- The report and project numbers from Smithers Viscient and Sponsor Study number (if any).
- Laboratory and site, dates of testing and personnel involved in the study, i.e., Program

Coordinator (if applicable), Study Director and Principal Investigator.

- Identification of the test substance including chemical name, additional designations (e.g., trade name), chemical designation (CAS number), empirical formula, molecular structure, manufacturer, lot or batch number, degree of purity of test substance (percent test chemical) (Sponsor supplied, if available).
- A full description of the experimental design and procedures followed and a description of the test equipment used.
- The determined accuracy, precision, specificity, linearity and limit of detection.
- The mathematical equations and statistical methods used in generating and analyzing the data as well as calculations using these equations. Tabular and graphical representations (if appropriate) of the data.
- Description of any problems experienced and how they were resolved.
- Good Laboratory Practice (GLP) Compliance Statement signed by the Study Director.
- Date(s) of Quality Assurance reviews, and dates reported to the Study Director and management, signed by the Quality Assurance Unit.
- Location of raw data and report.
- A copy of the study protocol and study amendments, if any.

## 9.0 PROTOCOL AMENDMENTS

All amendments to the approved protocol must be documented in writing and signed by both the Study Director and the Sponsor's contact or representative. Protocol amendments and deviations must include the reasons for the change and the predicted impact of the change on the results of the study, if any.

## 10.0 GOOD LABORATORY PRACTICES

All test procedures, documentation, records and reports will comply with the U.S. Environmental Protection Agency's Good Laboratory Practices as set forth under the Federal Insecticide, Fungicide and Rodenticide Act (40 CFR, Part 160) and as compatible with OECD Principles of Good Laboratory Practice (OECD, 1998).

## REFERENCES

U.S. EPA, January 2012. OCSPP 850.6100: Environmental Chemistry Methods and Associated Independent Laboratory Validation [EPA 712-C-001].

European Commission, 2000. Residues: 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. SANCO/3029/99 rev.4.