

Study Title

Environmental Chemistry Method: Validation of the Analytical Method for the Determination of Tebuconazole in Sediment/Soil Matrices by LC-MS/MS

Test Guidelines

SANCO 3029/99 (2000)
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Laboratory Project ID

Smithers Viscient Study No. 14162.6114

1.0 INTRODUCTION

The purpose of this study was to validate an analytical method used to determine the content of tebuconazole in two soil matrices, loamy sand and sandy loam (see [Protocol Deviation](#)). The method was validated (6 to 14 September 2018) to quantify the concentrations of tebuconazole in two soil matrices. The analytical method was validated with regards to specificity, linearity, accuracy, precision, limit of quantitation (LOQ), limit of detection (LOD), method detection limit (MDL), and confirmation of analyte identification.

The method was validated in soil by fortification with tebuconazole at concentrations of 50.0 (LOQ) and 500 (High) $\mu\text{g}/\text{kg}$. The recovery samples were extracted with acetonitrile and were diluted into the calibration standard range with 20/80 acetonitrile/purified reagent water (v/v). 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 “Environmental Chemistry Method: Validation of the Analytical Method for the Determination of Tebuconazole in Sediment/Soil Matrices by LC-MS/MS” ([Appendix 1](#)). The study was conducted under Good Laboratory Practices (GLP) Standards and principles as described in

40 CFR 160 (U.S. EPA, 1989) and as compatible with 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, tebuconazole technical, was received on 2 July 2018 from United Phosphorus Inc., Audubon, Pennsylvania. The following information was provided:

| | |
|-----------------------|--|
| Name: | Tebuconazole technical |
| Lot No.: | 201706010 |
| CAS No.: | 107534-96-3 |
| Purity: | 98.2% (Certificate of Analysis, Appendix 2) |
| Recertification Date: | 9 August 2020 |

Upon receipt at Smithers Viscient, the test substance (SMV No. 9514) 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. 0.1% Formic acid in water: Fisher, reagent grade
2. 0.1% Formic acid in acetonitrile: Fisher, reagent grade
3. Methanol: EMD reagent grade
4. Acetonitrile: EMD, reagent grade
5. Purified reagent water: Prepared from a Millipore MilliQ Direct 8 water purification system (meets ASTM Type II requirements)

2.4 Instrumentation and Laboratory Equipment

1. Instrument: AB MDS Sciex API 4000 mass spectrometer equipped with an AB MDS Sciex ESI Turbo V source
Shimadzu LC-20AD binary pumps
Shimadzu DGU-20A3 vacuum degasser
Shimadzu DGU-20A5R vacuum degasser
Shimadzu SIL-20ACHT autosampler
Shimadzu CTO-20AC column oven
Shimadzu CBM-20A communications bus
Analyst version 1.6.3 software for data acquisition
2. Balances: Mettler Toledo PG-2002-S, Mettler Toledo XSE205DU
3. Moisture Balance: Mettler Toledo HB43-S
4. Shaker Table: VWR 3500
5. Centrifuges: Thermo Scientific Sorvall Legend XFR, Eppendorf Centrifuge 5417C, Beckman Coulter 367160
6. Laboratory Equipment: Positive displacement pipets, volumetric flasks, disposable glass vials, disposable glass pipets, Nalgene 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 Matrices

The matrices used during this method validation were two soil matrices (referred to as soil #1 and soil #2). Characterization of soil was performed by Agvise Laboratories, Northwood, North Dakota. Soil characterization data is provided below.

Soil #1 validation:

| Parameter | Soil |
|--|---------------------------------|
| Smithers Viscient Batch No.: | 012616A |
| Collection location: | Rochester, Massachusetts |
| Percent organic carbon: | 4.9% |
| USDA textural class: | Loamy sand |
| Particle size distribution: | 78% sand 18% silt 4% clay |
| pH (1/1 soil/water ratio): | 6.8 |
| Percent water holding capacity (at 1/3 bar): | 17.8% |
| Percent Moisture: | 24.93% |

Soil #2 validation:

| Parameter | Soil |
|--|---------------------------------|
| Smithers Viscient Batch No.: | 041917B |
| Collection location: | Rochester, Massachusetts |
| Percent organic carbon: | 7.9% |
| USDA textural class: | Loamy Sand |
| Particle size distribution: | 83% sand 16% silt 1% clay |
| pH (1/1 soil/water ratio): | 6.6 |
| Percent water holding capacity (at 1/3 bar): | 31.1% |
| Percent Moisture: | 31.98% |

2.6 Preparation of Liquid Reagent Solutions

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

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

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

2.7 Preparation of Stock Solutions

The volumes and masses listed in this section 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 typically prepared as described in the table below:

| Primary Stock ID | Amount Weighed (g), Net Weight | Amount Weighed (g), as Active Ingredient | Stock Solvent | Final Volume (mL) | Primary Stock Concentration (mg/L) | Primary Stock Use |
|------------------|--------------------------------|--|---------------|-------------------|------------------------------------|---------------------------|
| 9514E | 0.0501 | 0.0492 | Acetonitrile | 50.0 | 984 | Secondary stock solutions |

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

| Fortifying Stock ID | Fortifying Stock Concentration (mg/L) | Volume of Fortification (mL) | Final Volume (mL) | Stock Solvent | Stock ID | Stock Concentration (mg/L) | Stock Use |
|---------------------|---------------------------------------|------------------------------|-------------------|---------------|----------|----------------------------|--------------------|
| 9514E | 984 | 5.00 | 50.0 | Acetonitrile | 9514E-1 | 98.4 | Sub-stock solution |
| 9514E | 984 | 0.500 | 50.0 | | 9514E-2 | 9.84 | Sub-stock solution |

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

| Fortifying Stock ID | Fortifying Stock Concentration (mg/L) | Volume of Fortification (mL) | Final Volume (mL) | Stock Solvent | Stock ID | Stock Concentration (µg/L) | Stock Use |
|---------------------|---------------------------------------|------------------------------|-------------------|---------------|------------|----------------------------|--|
| 9514E-1 | 98.4 | 0.510 | 5.00 | Acetonitrile | Tech Stk 1 | 10,000 | Recovery samples and sub-stock solution |
| Tech Stk 1 | 10.0 | 0.500 | 5.00 | | Tech Stk 2 | 1000 | Recovery samples |
| 9514E-2 | 9.84 | 0.0510 | 50.0 | | Ana Stk 1 | 10.0 | Calibration standards and sub-stock solution |
| Ana Stk 1 | 0.0100 | 1.00 | 10.0 | | Ana Stk 2 | 1.00 | Calibration standards |

All primary and secondary stock solutions were stored refrigerated (2 to 8 °C) in amber glass bottles fitted with Teflon-lined caps until use. Sub-stock solutions were prepared fresh on the day of use and discarded after use.

2.8 Preparation of Calibration Standards

2.8.1 Calibration Standards

Matrix-matched calibration standards were prepared in final fraction soil diluent for both soil types by dosing with the 10.0 µg/L sub-stock solution to yield concentrations of 0.00500, 0.00800, 0.0125, 0.0200, 0.0300, 0.0450, 0.0650, 0.100, 0.180, and 0.250 µg/L.

2.8.2 Matrix Effect Investigation

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 solvent-based standards fortified at the same concentration. Calibration standards used to assess possible matrix effects were prepared with a 1.00 µg/L sub-stock solution to yield a final test substance concentration of 0.0150 µg/L.

2.8.2.1 Matrix-Matched Standards

| Fortifying Stock ID | Stock Concentration (µg/L) | Volume of Fortification (mL) | Final Volume (mL) | Standard Concentration (µg/L) | Sample ID |
|---------------------|----------------------------|------------------------------|-------------------|-------------------------------|-----------|
| Ana Stk 2 | 1.00 | 0.150 | 10.0 ^a | 0.0150 | MM-Std A |
| | | 0.150 | 10.0 ^a | 0.0150 | MM-Std B |
| | | 0.150 | 10.0 ^a | 0.0150 | MM-Std C |
| | | 0.150 | 10.0 ^b | 0.0150 | MM-Std D |
| | | 0.150 | 10.0 ^b | 0.0150 | MM-Std E |
| | | 0.150 | 10.0 ^b | 0.0150 | MM-Std F |

^a Diluted with the final dilution of the matrix-matched control sample 14162.6114.16 (soil #2)

^b Diluted with the final dilution of the matrix-matched control sample 14162.6114.03 (soil #1)

2.8.2.2 Non-Matrix-Matched (Solvent-Based) Standards

| Fortifying Stock ID | Stock Concentration (µg/L) | Volume of Fortification (mL) | Final Volume (mL) ^a | Standard Concentration (µg/L) | Sample ID |
|---------------------|----------------------------|------------------------------|--------------------------------|-------------------------------|-----------|
| Ana Stk 2 | 1.00 | 0.150 | 10.0 | 0.0150 | SS-Std A |
| | | 0.150 | 10.0 | 0.0150 | SS-Std B |
| | | 0.150 | 10.0 | 0.0150 | SS-Std C |
| | | 0.150 | 10.0 | 0.0150 | SS-Std D |
| | | 0.150 | 10.0 | 0.0150 | SS-Std E |
| | | 0.150 | 10.0 | 0.0150 | SS-Std F |

^a Diluted with 20/80 acetonitrile/purified reagent water (v/v)

2.9 Sample Fortification and Preparation

The recovery samples were prepared in soil (5.00 g dry weight), which were weighed into individual 50-mL centrifuge tubes and were fortified with the appropriate test substance sub-stock solution at concentrations of 50.0 (LOQ) and 500 (High) µg/kg (dry weight). Five replicates were prepared for the 50.0 µg/kg (LOQ) concentration level and five replicates were prepared for the 500 µg/kg concentration level in each soil type. In addition, two samples were left unfortified to serve as controls for each soil type and were extracted in the same fashion as the LOQ-level 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.

Soil #1:

| Sample ID 14162.6114. | Sample Type | Stock Concentration (µg/L) | Fortification Volume (mL) | Dry Weight (g) | Fortified Concentration (µg/kg) |
|--------------------------|---------------|----------------------------------|---------------------------------|----------------------|---------------------------------------|
| 01 | Reagent Blank | NA ^a | NA | NA | 0.00 |
| 02 & 03 | Control | NA | NA | 5.00 | 0.00 |
| 04, 05, 06, 07, & 08 | LOQ | 1000 | 0.250 | 5.00 | 50.0 |
| 09, 10, 11, 12, & 13 | High | 10,000 | 0.250 | 5.00 | 500 |

^a NA = Not Applicable

Soil #2:

| Sample ID 14162.6114. | Sample Type | Stock Concentration (µg/L) | Fortification Volume (mL) | Dry Weight (g) | Fortified Concentration (µg/kg) |
|--------------------------|---------------|----------------------------------|---------------------------------|----------------------|---------------------------------------|
| 14 | Reagent Blank | NA ^a | NA | NA | 0.00 |
| 15 & 16 | Control | NA | NA | 5.00 | 0.00 |
| 17, 18, 19, 20, & 21 | LOQ | 1000 | 0.250 | 5.00 | 50.0 |
| 22, 23, 24, 25, & 26 | High | 10,000 | 0.250 | 5.00 | 500 |

^a NA = Not Applicable

2.10 Soil Extraction and Dilution

A 20.0-mL aliquot of acetonitrile was added to each soil sample directly after preparation and samples were then placed on a shaker table at 150 rpm for 30 minutes. Following shaking, the samples were then centrifuged at 3000 rpm for 10 minutes. The extraction and mixing procedures were repeated with an additional 20-mL aliquot of acetonitrile. The extracts were combined, taken to volume (50.0 mL) with acetonitrile, and mixed well. The samples were further diluted into the calibration standard range with 20/80 acetonitrile/purified reagent water (v/v) as needed and were then centrifuged at 13,000 rpm for five minutes. All recovery samples were transferred to autosampler vials for analysis. The extraction and dilution procedures are detailed below.

Soil #1:

| Sample ID 14162.6114. | Sample Type | Nominal Concentration (µg/kg) | Dry Weight (g) | Final Volume ^a (mL) | Sample Volume (mL) | Final Volume ^b (mL) | Dilution Factor |
|--------------------------|---------------|----------------------------------|-------------------|-----------------------------------|-----------------------|-----------------------------------|-----------------|
| 01 | Reagent Blank | 0.00 | NA ^c | 50.0 | 0.0300 | 10.0 | 3330 |
| 02 | Control | 0.00 | 5.00 | 50.0 | 0.750 ^d | 250 ^d | 3330 |
| 03 | Control | 0.00 | 5.00 | 50.0 | 0.300 ^e | 100 ^e | 3330 |
| 04, 05, 06, 07, & 08 | LOQ | 50.0 | 5.00 | 50.0 | 0.0300 | 10.0 | 3330 |
| 09, 10, 11, 12, & 13 | High | 500 | 5.00 | 50.0 | 0.0300 | 10.0 | 3330 |

^a Dilution solvent: acetonitrile^b Dilution solvent: 20/80 acetonitrile/purified reagent water (v/v)^c NA = Not Applicable^d Final volume increased to accommodate preparation of matrix-matched standards.^e Final volume increased to accommodate use for matrix effect investigation.**Soil #2:**

| Sample ID 14162.6114. | Sample Type | Nominal Concentration (µg/kg) | Dry Weight (g) | Final Volume ^a (mL) | Sample Volume (mL) | Final Volume ^b (mL) | Dilution Factor |
|--------------------------|---------------|----------------------------------|-------------------|-----------------------------------|-----------------------|-----------------------------------|-----------------|
| 14 | Reagent Blank | 0.00 | NA ^c | 50.0 | 0.0300 | 10.0 | 3330 |
| 15 | Control | 0.00 | 5.00 | 50.0 | 0.0300 ^d | 10.0 ^d | 3330 |
| 16 | Control | 0.00 | 5.00 | 50.0 | 0.300 ^e | 100 ^e | 3330 |
| 17, 18, 19, 20, & 21 | LOQ | 50.0 | 5.00 | 50.0 | 0.0300 | 10.0 | 3330 |
| 22, 23, 24, 25, & 26 | High | 500 | 5.00 | 50.0 | 0.0300 | 10.0 | 3330 |

^a Dilution solvent: acetonitrile^b Dilution solvent: 20/80 acetonitrile/purified reagent water (v/v)^c NA = Not Applicable^d An additional 1.50-mL aliquot was diluted to 500 mL with 20/80 acetonitrile/purified reagent water (v/v) for an additional matrix effects investigation.^e Final volume increased to accommodate use for matrix effect investigation.**2.11 Analysis****2.11.1 Instrumental Conditions**

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

LC parameters:

Column: Waters XBridge C18 BEH, 2.5 µm, 2.1 × 50 mm

Mobile Phase A: 0.1% formic acid in water

| | | | | |
|----------------------------|---|-----------|---------|---------|
| Mobile Phase B: | 0.1% formic acid in acetonitrile | | | |
| Gradient: | Time | Flow rate | Solvent | Solvent |
| | (min.) | (mL/min.) | A (%) | B (%) |
| | 0.50 | 0.500 | 80.0 | 20.0 |
| | 3.00 | 0.500 | 0.00 | 100 |
| | 3.50 | 0.500 | 0.00 | 100 |
| | 3.51 | 0.500 | 80.0 | 20.0 |
| | 5.00 | 0.500 | 80.0 | 20.0 |
| Run Time: | 5.00 minutes | | | |
| Autosampler Wash Solution: | 30/30/40 acetonitrile/methanol/purified reagent water (v/v/v) | | | |
| Column Temperature: | 40 °C | | | |
| Sample Temperature: | 10 °C | | | |
| Injection Volume: | 50.0 µL (soil #1), 20.0 µL (soil #2) | | | |
| Retention Time: | approximately 2.8 minutes | | | |

MS parameters:

| | |
|------------------------------------|---|
| Instrument: | AB MDS Sciex API 4000 mass spectrometer |
| Ionization Mode: | Positive (+) ESI |
| Ion Spray Voltage: | 5000 V |
| Scan Type: | MRM |
| Dwell Time: | 75.0 milliseconds |
| Source Temperature: | 500 °C |
| Curtain Gas: | 20.0 |
| Ion Source – Gas 1 / Gas 2: | 60.0 / 60.0 |
| Collision Gas: | 12.0 |
| Collision Cell Entrance Potential: | 10.0 |
| Declustering Potential: | 61.00 |
| Resolution Q1/Q3: | Unit/Unit |

| | Primary Transition | Confirmatory Transition |
|--------------------------------|-----------------------|----------------------------|
| Q1/Q3 Masses (amu): | 308.2/70.2 | 308.2/125.2 |
| Collision Energy: | 57.0 | 51.0 |
| Collision Cell Exit Potential: | 12.0 | 10.0 |

Other instrumentation may be used but may require optimization to achieve the desired separation and sensitivity. It is important to note that the parameters above have been established for this particular instrumentation and may not be applicable for other similar equipment that may be used.

2.11.2 Preparation of Calibration Standard Curve

Two sets of calibration standards were analyzed with each sample set. Calibration standards were interspersed among analysis of the recovery samples, every two to six injections. Injection of recovery samples and calibration standards onto the chromatographic system was performed by programmed automated injection.

2.12 Evaluation of Precision, Accuracy, Specificity, and Linearity

The accuracy was reported in terms of percent recovery of the fortified recovery samples. Recoveries of 70 to 110% (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 less than or equal to 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 time as tebuconazole, which might interfere with the quantitation of the analytes. Linearity of the method was determined by the coefficient of determination (r^2), y-intercept, and slope of the regression line.

2.13 Limit of Quantitation (LOQ)

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

2.14 Limit of Detection (LOD) and Method Detection Limit (MDL)

The Limit of Detection (LOD) was calculated using three times the signal-to-noise value of the control samples.

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.

3.0 STATISTICAL METHODS

A calibration curve was constructed by plotting the analyte concentration ($\mu\text{g/L}$) of the calibration standards against the peak area of the analyte in the calibration standards. The equation of the line (equation 1) was algebraically manipulated to give equation 2. The concentration of test substance in each recovery sample was calculated using the slope and intercept from the linear regression analysis, the detector response, and the dilution factor of the recovery sample. Equations 2 and 3 were then used to calculate measured concentrations and analytical results.

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

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

$$(3) \quad A = \text{DC} \times \text{DF}$$

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 ($\mu\text{g/L}$) in the sample |
| DF | = | dilution factor (final volume of the sample divided by the original sample volume) |
| A | = | analytical result ($\mu\text{g/kg}$), concentration in the original sample |

The LOD was calculated using the following equation:

$$(4) \quad \text{LOD} = ((3 \times (\text{SN}_{\text{ctl}})) / \text{Resp}_{\text{LS}}) \times \text{Conc}_{\text{LS}} \times \text{DF}_{\text{CNTL}}$$

where:

| | | |
|---------------------------|---|--|
| SN_{ctl} | = | mean 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 (smallest dilution factor used, i.e., 3330) |
| LOD | = | limit of detection for the analysis |

The method detection limit (MDL) is defined as the lowest concentration that can be detected by this method in test solution samples. The MDL is calculated (equation 5) based on the concentration of the low calibration standard and the dilution factor of the control samples.

$$(5) \quad \text{MDL} = \text{MDL}_{\text{LCAL}} \times \text{DF}_{\text{CNTL}}$$

where:

| | | |
|---------------------|---|--|
| MDL _{LCAL} | = | lowest concentration calibration standard (i.e., 0.00500 µg/L) |
| DF _{CNTL} | = | dilution factor of the control samples (smallest dilution factor used, i.e., 3330) |
| MDL | = | method detection limit reported for the analysis (i.e., 0.00500 µg/L × 3330 = 16.7 µg/kg) |

Limit of detection and method detection limits were also appropriately determined through the calculations previously presented in [Section 3.0](#). Confirmation of analyte identification was established through the confirmatory analysis adhering to the aforementioned method specifications.

6.0 VALIDITY CRITERIA

The method validation for tebuconazole in soil #1 met the performance criteria as presented in the following table:

| Criterion | Acceptable Limits | Study Performance | |
|--|--|--|--|
| | | Primary | Confirmatory |
| 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. | | |
| | 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 calibration standards. | Matrix-matched and solvent-based calibration standards were prepared and analyzed with the recovery samples. Since the matrix effect was <20%, a significant matrix effect was not observed. As a conservative measure, matrix-matched calibration standards were used for quantitation. | |
| Accuracy: Mean Recoveries | Mean recoveries of 70 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 the highest predicted level to be used during testing. | This portion of the study was performed at levels of 50.0 and 500 $\mu\text{g}/\text{kg}$; 50.0 $\mu\text{g}/\text{kg}$ was set as the LOQ. | |
| Precision: Relative Standard Deviation (RSD) | Relative Standard Deviation (RSD) $\leq 20\%$ for each fortification level. | | |
| 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 (50.0 $\mu\text{g}/\text{kg}$). | All blank sample values were <30% of the LOQ (50.0 $\mu\text{g}/\text{kg}$). |
| Limit Of Detection (LOD) | The LOD will be calculated using three times the signal-to-noise value of the control samples. | 6.71 $\mu\text{g}/\text{kg}$ | 7.66 $\mu\text{g}/\text{kg}$ |
| 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. | 16.7 $\mu\text{g}/\text{kg}$ | 16.7 $\mu\text{g}/\text{kg}$ |
| Confirmation of Analyte Identification | A chromatographic confirmatory method will be used to determine test solution concentrations during validation. | Primary ion: 308.2/70.2 Meets all method and guideline specifications outlined in this table. | Confirmatory ion: 308.2/125.2 Meets all method and guideline specifications outlined in this table. |

The method validation for tebuconazole in soil #2 met the performance criteria as presented in the following table:

| Criterion | Acceptable Limits | Study Performance | |
|--|--|--|--|
| | | Primary | Confirmatory |
| 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. | | |
| Linearity: 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 calibration standards. | Matrix-matched and solvent-based calibration standards were prepared and analyzed with the recovery samples. Since the matrix effect was <20%, a significant matrix effect was not observed. As a conservative measure, matrix-matched calibration standards were used for quantitation. | |
| Accuracy: Mean Recoveries | Mean recoveries of 70 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 the highest predicted level to be used during testing. | This portion of the study was performed at levels of 50.0 and 500 $\mu\text{g}/\text{kg}$; 50.0 $\mu\text{g}/\text{kg}$ was set as the LOQ. | |
| Precision: Relative Standard Deviation (RSD) | Relative Standard Deviation (RSD) $\leq 20\%$ for each fortification level will be considered acceptable. | | |
| 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 (50.0 $\mu\text{g}/\text{kg}$). | All blank sample values were <30% of the LOQ (50.0 $\mu\text{g}/\text{kg}$). |
| Limit Of Detection (LOD) | The LOD will be calculated using three times the signal-to-noise value of the control samples. | 11.4 $\mu\text{g}/\text{kg}$ | 22.8 $\mu\text{g}/\text{kg}$ |
| 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. | 16.7 $\mu\text{g}/\text{kg}$ | 16.7 $\mu\text{g}/\text{kg}$ |
| Confirmation of Analyte Identification | A chromatographic confirmatory method will be used to determine test solution concentrations during validation. | Primary ion: 308.2/70.2 Meets all method and guideline specifications outlined in this table. | Confirmatory ion: 308.2/125.2 Meets all method and guideline specifications outlined in this table. |

PROTOCOL DEVIATION

The protocol states that the soils used for the method validation will be two types of soil matrices (loamy sand and sandy loam soil). It is standard practice to validate more than one soil, even though it is not required. SANCO 3029 and SANCO 850.6100 do not specify the use of two soil matrices. SANCO 825, which is also a commonly referenced guideline for Environmental Chemistry Methods (ECMs) also does not specify the use of two soil matrices. At the time the ECM was performed, it was believed that two different soil compositions were available. However, the soil characterization sheets demonstrated the two soil matrices were closer in soil type than was anticipated. Even though there were slight differences in characterization, both soil types were classified as loamy sand. Since the method met acceptance criteria for both soils, validity of the ECM method was achieved. Therefore, there is no significant impact on the validity of the execution of the ECM under protocol.

REFERENCES

- European Commission, 2000. Residues: Guidance for the generating and reporting methods of analysis in support of pre-registration data requirements for Annex II (part V, section 4) and Annex III (part A, section 5) of Directive 91/414, SANCO/3029/99 rev.4.
- OECD, 1998. OECD Series on Principles of Good Laboratory Practice and Compliance Monitoring. Number 1. OECD Principles on Good Laboratory Practice (as revised in 1997). Environment Directorate Chemicals Group and Management Committee. ENV/MC/CHEM(98)17. OECD Paris, France. 41 pp.
- U.S. EPA, 1989. Federal Insecticide, Fungicide and Rodenticide Act (FIFRA); Good Laboratory Practice Standards; Final Rule (40 CFR, Part 160); FR: 8/17/89; pp. 34052. U.S. Environmental Protection Agency, Washington, D.C.
- U.S. EPA, 2012. Office of Chemical Safety and Pollution Prevention. Ecological Effects Guideline, OCSPP 850.6100. Environmental Chemistry Methods and Associated Independent Laboratory Validation. EPA 712-C-001. January 2012. U.S. Environmental Protection Agency, Washington, D.C.

Study No.: 14162.6114

Validation of the Analytical Method for the Determination of Tebuconazole in Aqueous Matrices by LC-MS/MS

1.0 INTRODUCTION

The purpose of this study is to validate an analytical method used to determine the content of Tebuconazole in loamy sand and sandy loam by LC-MS/MS. The analytical method will be validated with regards to accuracy and precision, specificity, linearity, limit of detection, method detection limit, limit of quantitation, and confirmation of analyte identification.

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 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 40CFR160 and the OECD principles on GLP.

3.0 TEST SUBSTANCE

3.1 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 water, MSDS, and safe handling procedures, and a verified expiration or reanalysis date.

3.2 Test Matrices

The soils used for the method validation will be two types of soil matrices (loamy sand & sandy loam soil). Prior to testing, soil moisture content will be determined using a moisture analyzer. Soil characterization data such as % sand, silt clay, bulk density and % organic matter will be determined. All documentation relating to the preparation, storage and handling will be maintained by Smithers Viscient.

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3.3 Reagents

Highly pure reagents will be used throughout the study. The actual reagent grade will be depending on the manufacturer's designation. Generally these reagents will have grades, such as high purity solvent, ACS grade, or Select. The reagents used are recorded along with test chemical information at the time of preparation.

4.0 VALIDATION DESIGN

The test design will consist of both a sediment and soil matrix (loamy sand & sandy loam) fortified with each test substance at two concentrations with five replications for each fortification level. The control matrices for the validation will be the appropriate untreated sediment or soil matrix. 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 – 110% 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 $\leq 20\%$. 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=2). Chromatogram 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 Regression Analysis

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 \pm 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

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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.

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 IDENTIFICATION 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.

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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. The final report will meet the formatting requirements of EPA's PR Notice 2011-3. 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, method detection limit, limit of quantitation, and confirmation of analyte identification.
- 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.

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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

- European Commission, Directorate General Health and Consumer Protection, (SANCO/3029/99 rev.4) 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, 11/07/2000.
- OECD, 1998. OECD Series on Principles of Good Laboratory Practice and Compliance Monitoring. Number 1. OECD Principles on Good Laboratory Practice (as revised in 1997). Environment Directorate Chemicals Group and Management Committee. ENV/MC/CHEM(98)17. OECD Paris. France. 41 pp.
- U.S. EPA. 1989. Federal Insecticide, Fungicide and Rodenticide Act (FIFRA); Good Laboratory Practice Standards; Final Rule (40 CFR, Part 160); FR: 8/17/89; pp. 34052. U.S. Environmental Protection Agency, Washington, D.C.
- U.S. EPA, 2011. Pesticide Registration (PR) Notice 2011-3 Standard Format for Data Submitted Under the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) and Certain Provisions of the Federal Food, Drug, and Cosmetic Act (FFDCA). US Environmental Protection Agency Office of Pesticide Programs. November 30, 2011
- U.S. EPA, January 2012. OCSPP 850.6100: Environmental Chemistry Methods and Associated Independent Laboratory Validation [EPA 712-C-001]. U.S. EPA, January 2012. OCSPP 850.6100: Environmental Chemistry Methods and Associated Independent Laboratory Validation [EPA 712-C-001].



PROTOCOL AMENDMENT

Amendment No.: 1

Protocol Title: Environmental Chemistry Method: Validation of the Analytical Method for the Determination of Tebuconazole in Sediment/Soil Matrices by LC-MS/MS

Study Sponsor: Generic Tebuconazole DCI Task Force c/o United Phosphorous, Inc.

Test Substance: Tebuconazole technical

Study No.: 14162.6114

Amendment:

Cover Page

Test Substance: Name: Tebuconazole technical
Purity: 98.2%
Batch or Lot #: 201706010

Reason for change:
Addition of purity information.

Title on Page 2

Protocol currently states:

Validation of the Analytical Method for the Determination of Tebuconazole in Aqueous Matrices by LC-MS/MS

Protocol updated to:

Environmental Chemistry Method: Validation of the Analytical Method for the Determination of Tebuconazole in Sediment/Soil Matrices by LC-MS/MS

Reason for change:
Correcting title to match the cover page.

3.2 Test Matrices

Protocol currently states:

The soils used for the method validation will be two types of soil matrices (loamy sand & sandy loam soil). Prior to testing, soil moisture content will be determined using a moisture analyzer. Soil characterization data such as % sand, silt clay, bulk density and % organic matter will be determined. All documentation relating to the preparation, storage and handling will be maintained by Smithers Viscient.