Study Title

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

Test Guidelines

SANCO 3029/99 (2000)
OCSPP 850.6100 (2012)

Study Completed On

20 December 2018

1.0 INTRODUCTION

The purpose of this study was to validate an analytical method used to determine the content of tebuconazole in surface and ground water by liquid chromatography with tandem mass spectrometry detection (LC-MS/MS). The method was validated (5 to 21 September 2018) to quantify the concentrations of tebuconazole present in recovery samples prepared in surface and ground water. 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 surface and ground water by fortification with tebuconazole at concentrations of 0.100 (LOQ) and 1.00 (High) µg/L. The recovery samples were diluted into the calibration standard range with 20/80 acetonitrile/purified reagent water (v/v) prior to analysis. All samples were analyzed using 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 Aqueous Matrices by LC-MS/MS" (Appendix 1). The study was conducted under Good Laboratory Practice (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.	Balance:	Mettler Toledo XSE205DU
3.	Laboratory Equipment:	Positive displacement pipets, volumetric flasks, disposable glass vials, disposable glass pipets, graduated cylinders, Pasteur pipets, autosampler vials, and amber glass vials with Teflon 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 ground water and surface water.

Ground Water

Ground water used in the study was filtered well water, prepared by filtering to remove any potential organic contaminants. All documentation relating to the preparation, storage, and handling is maintained by Smithers Viscient.

Surface Water

The surface water used for this method validation analysis was collected from the Taunton River (SMV Lot No. 14Sep18 Wat-A, collected on 14 September 2018). The water was collected from an area of the river with approximately 30 to 60 cm of overlying water and was determined to have a pH of 6.43 (measured using a Yellow Springs Instrument, YSI, pH100 pH meter) and a dissolved oxygen concentration of 5.8 mg/L (measured using a YSI Pro 20 dissolved oxygen meter). All

documentation relating to the preparation, storage, and handling is maintained by Smithers Viscient.

2.6 Preparation of Liquid Reagents

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 300 mL of acetonitrile and 1200 mL of purified reagent water. The solution was mixed well using a stir bar and stir plate for five minutes.

An 18/10/72 acetonitrile/surface water/purified reagent water (v/v/v) liquid reagent solution was typically prepared by combining 90 mL of acetonitrile, 50 mL of surface water, and 360 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
9514J	0.0511	0.0502	Acetonitrile	50.0	1000	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	0 500	50.0	Acetonitrile	9514E-2	9.84	Sub-stock solutions
9514J	1000	0 500	50.0	Acetonitrile	9514J-1	10.0	Sub-stock solutions

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

Fortifying Stock ID	Fortifying Stock Concentration (mg/L)	Volume of Fortification (mL)	Final Volume (mL)	Stock Solvent	Stock ID	Stock Concentration (µg/L)	Stock Use
9514E-2	9.84	0.0510	50.0	Acetonitrile	Stk 1	10.0	Calibration standards, recovery samples, and sub-stock solution for ground water ECM
Stk 1	0.0100	1.00	10.0		Stk 2	1.00	Calibration standards for ground water ECM
9514J-1	10.0	0.0500	50.0		Stk 1	10.0	Calibration standards, recovery samples, and sub-stock solution for surface water ECM
Stk 1	0.0100	1.00	10.0		Stk 2	1.00	Calibration standards for surface water ECM

All primary and secondary stock solutions were stored refrigerated (2 to 8 ºC) 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

Solvent-based calibration standards were prepared for the ground water analysis, while matrix-matched calibration standards were prepared for the surface water analysis. Calibration standards were prepared in the following manner: solvent-based calibration standards were prepared in 20/80 acetonitrile/purified reagent water (v/v), for analysis with the ground water recovery samples. Matrix-matched calibration standards were prepared in 18/10/72 acetonitrile/surface water/purified reagent water (v/v/v), for analysis with the surface water recovery samples. Both sets of calibration standards were prepared in the same manner by fortifying 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, 0.250 µg/L.

2.9 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 as follows by fortifying matrix-matched and non matrix-matched diluent with a 1.00 µg/L sub-stock solution to yield a test substance concentration of 0.0100 µg/L.

Matrix-Matched Standards:

Fortifying Stock ID	Stock Concentration (µg/L)	Volume of Fortification (mL)	Final Volume (mL)	Standard Concentration (µg/L)	Sample ID
Stk 2	1.00	0.0500	5.00[a]	0.0100	MM-Std A-G
		0.0500	5.00[a]	0.0100	MM-Std B-G
		0.0500	5.00[a]	0.0100	MM-Std C-G
Stk 2	1.00	0.0500	5.00[b]	0.0100	MM-Std D-S
		0.0500	5.00[b]	0.0100	MM-Std E-S
		0.0500	5.00[b]	0.0100	MM-Std F-S

[a] Diluted with the final dilution of the matrix-matched control sample 14162.6113.03 (ground water).
[b] Diluted with the final dilution of the matrix-matched control sample 14162.6113.28 (surface water).

Non Matrix-Matched (Solvent-Based) Standards:

Fortifying Stock ID	Stock Concentration (mg/L)	Volume of Fortification (mL)	Final Volume (mL)[a]	Standard Concentration (µg/L)	Sample ID
Stk 2	1.00	0.0500	5.00	0.0100	SS-Std A
		0.0500	5.00	0.0100	SS-Std B
		0.0500	5.00	0.0100	SS-Std C
Stk 2	1.00	0.0500	5.00	0.0100	SS-Std D
		0.0500	5.00	0.0100	SS-Std E
		0.0500	5.00	0.0100	SS-Std F

[a] Dilution solvent: 20/80 acetonitrile/purified reagent water (v/v)

2.10 Sample Fortification and Preparation

The recovery samples were prepared in ground water and surface water with tebuconazole at concentrations of 0.100 (LOQ) and 1.00 (High) µg/L. Recovery samples were prepared separately ("de novo") at these concentrations. Five replicates were produced for each concentration level. Two samples were left unfortified to serve as controls and were diluted in the same fashion as the LOQ-level recovery samples. In addition, for each set of samples, one reagent blank was prepared without matrix (using purified reagent water only) and processed in the same manner as the control samples. The preparation procedure is outlined in the tables below.

Ground Water:

Sample ID 14162.6113-	Sample Type	Stock Concentration (µg/L)	Fortification Volume (mL)	Final Volume (mL)	Fortified Concentration (µg/L)
01	Reagent Blank	NA[a]	NA	5.00[b]	0.00
02 & 03	Control	NA	NA	5.00	0.00
06, 07, 08, 09, & 10	LOQ	10.0	0.0500	5.00	0.100
16, 17, 18, 19, & 20	High	10.0	0.500	5.00	1.00

[a] NA = Not Applicable
[b] Purified reagent water

Surface Water:

Sample ID 14162.6113-	Sample Type	Stock Concentration (mg/L)	Fortification Volume (mL)	Final Volume (mL)	Fortified Concentration (µg/L)
26	Reagent Blank	NA[a]	NA	5.00[b]	0.00
27 & 28	Control	NA	NA	5.00	0.00
29, 30, 31, 32, & 33	LOQ	10.0	0.0500	5.00	0.100
34, 35, 36, 37, & 38	High	10.0	0.500	5.00	1.00

[a] NA = Not Applicable
[b] Purified reagent water

2.11 Dilution of Samples

To minimize the potential for losses of the test substance during processing, the aqueous test samples were not sub-sampled prior to dilution. The recovery samples were diluted into the calibration standard range with 20/80 acetonitrile/purified reagent water (v/v) prior to analysis in the same vial in which the samples were fortified. The dilution procedures are outlined in the table below.

Ground Water:

Sample ID 14162.6113-	Sample Type	Fortified Concentration (µg/L)	Sample Volume (mL)	Final Volume[a] (mL)	Dilution Factor
01	Reagent Blank	0.00	5.00	50.0	10.0
02 & 03	Control	0.00	5.00	50.0	10.0
06, 07, 08, 09, & 10	LOQ	0.100	5.00	50.0	10.0
16, 17, 18, 19, & 20	High	1.00	5.00	50.0	10.0

[a] Dilution solvent: 20/80 acetonitrile/purified reagent water (v/v)

Surface Water:

Sample ID 14162.6113-	Sample Type	Fortified Concentration (µg/L)	Sample Volume (mL)	Final Volume[a] (mL)	Dilution Factor
26	Reagent Blank	0.00	5.00	50.0	10.0
27 & 28	Control	0.00	5.00	50.0	10.0
29, 30, 31, 32, & 33	LOQ	0.100	5.00	50.0	10.0
34, 35, 36, 37, & 38	High	1.00	5.00	50.0	10.0

[a] Dilution solvent: 20/80 acetonitrile/purified reagent water (v/v)

2.12 Analysis

2.12.1 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 (min.)	Flow rate (mL/min.)	Solvent A (%)	Solvent 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	8.00	20.0

Run Time:	5.0 minutes
Autosampler Wash Solution:	30/30/40 acetonitrile/methanol/reagent grade water (v/v/v)
Column Temperature:	40 °C
Sample Temperature:	10 °C
Injection Volume:	50 µL
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.

2.12.2 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.

2.13 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 times 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.

2.14 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.

2.15 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.

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

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

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

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

where:

x	=	analyte concentration
y	=	detector response (peak area) from the chromatogram
b	=	y-intercept from the regression analysis
m	=	slope from the regression analysis
DC (x)	=	detected concentration (µg/L) in the sample
DF	=	dilution factor (final volume of the sample divided by the original sample volume)
A	=	analytical result (µg/L), concentration in the original sample

The LOD was calculated using the following equation:

$$(4) \qquad LOD = ((3\times(SN_{ctl}))/Resp_{LS}) \times Conc_{LS} \times DF_{CNTL}$$

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., 10.0)
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.

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

where:

MDL_{LCAL}	=	lowest concentration calibration standard (0.00500 µg/L)
DF_{CNTL}	=	dilution factor of the control samples (smallest dilution factor used, 10.0)
MDL	=	method detection limit reported for the analysis (0.00500 µg/L × 10.0 = 0.0500 µg/L)

6.0 VALIDITY CRITERIA

The method validation in ground water with tebuconazole 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.	$r^2 = 1.00$	$r^2 = 0.999$
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. The matrix effect was <20% for ground water, therefore no significant matrix effect was observed and solvent-based 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 0.100 and 1.00 µg/L; 0.100 µg/L was set as the LOQ.	
Precision: Relative Standard Deviation (RSD)	Relative Standard Deviation (RSD) ≤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 (0.100 µg/L).	All blank sample values were <30% of the LOQ (0.100 µg/L).
Limit Of Detection (LOD)	The LOD will be calculated using three times the signal-to-noise value of the control samples.	0.0168 µg/L	0.0277 µg/L
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.	0.0500 µg/L	0.0500 µg/L
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 in surface water with tebuconazole 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. The matrix effect was >20% for surface water, therefore a significant matrix effect was observed and 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 0.100 and 1.00 µg/L; 0.100 µg/L was set as the LOQ.	
Precision: Relative Standard Deviation (RSD)	Relative Standard Deviation (RSD) ≤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 (0.100 µg/L).	All blank sample values were <30% of the LOQ (0.100 µg/L).
Limit Of Detection (LOD)	The LOD will be calculated using three times the signal-to-noise value of the control samples.	0.0539 µg/L	0.0583 µg/L
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.	0.0500 µg/L	0.0500 µg/L
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.

REFERENCES

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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.

APPENDIX 1 - STUDY PROTOCOL

Study No.: 14162.6113

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 surface and ground water 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

I. Ground water

Ground water used in the study will be filtered well water. This will be prepared by filtering to remove any potential organic contaminants. All documentation relating to the preparation, storage and handling will be maintained by Smithers Viscient.

II. Surface water

The surface water used for this method validation analysis will be collected from river water in Massachusetts. All documentation relating to the preparation, storage and handling will be maintained by Smithers Viscient.

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 two water matrices (surface & ground water) 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 water matrix. The validation study levels (approximate concentrations) for test substance are:

1.	Procedural blank-reagent blank	0.0 ppb
2.	Matrix blank-control matrix	0.0 ppb
3.	Control matrix fortified at LOQ	0.10 ppb
4.	Control matrix fortified at 10 x LOQ	1.0 ppb

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 ≤ 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 ± 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, r2 ≥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.

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.

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].