2.0 MATERIALS AND METHODS

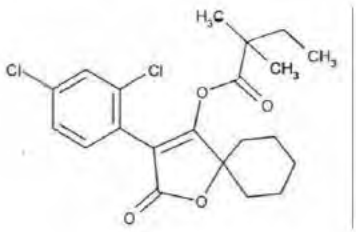
2.1 Protocol

Procedures used in this study followed those described in the Smithers protocol entitled "Environmental Chemistry Method: Validation of the Analytical Method for the Determination of Spirodiclofen and Spirodiclofen Enol in Aqueous Matrices by LC-MS/MS" (Appendix 1). The study was conducted under Good Laboratory Practice (GLP) regulations and principles as described in 40 CFR 160 (U.S. EPA, 1989) and the OECD principles on GLP (OECD, 1998), and followed the SANCO/3029/99 rev 4 guidance document (EC, 2000) and OCSPP 850.6100 guideline (U.S. EPA, 2012).

2.2 Test Substances

The test substance, spirodiclofen, was received on 12 August 2020 from Bayer CropScience, Chesterfield, Missouri. The Certificate of Analysis is presented in Appendix 2. The following information was provided:

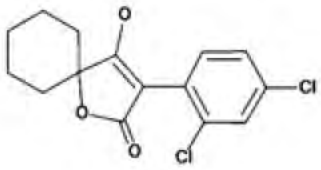
Name:	Spirodiclofen
Synonyms:	AE 1344097 technical substance; BAJ 2740-a.i.
Batch Code:	AE 1344097-01-05
Origin Batch No.:	EDFL055904
CAS No.:	148477-71-8
Customer Order No.:	TOX 21697-00
Certificate No.:	MZ 01849
Spec. No.:	102000008942
Purity:	99.3% w/w
Appearance:	Colorless to light brown solid
Date of Analysis:	6 August 2020
Expiry Date:	6 August 2022
Storage Conditions:	+10 to +30 °C
Structural Formula:	



Upon receipt at Smithers, the test substance (TMC No. 10773) was stored at room temperature in the original container in a dark, ventilated cabinet. Concentrations were adjusted for the purity of the test substance. This sample of test substance was used to prepare recovery samples and calibration standards during testing.

The test substance, spirodiclofen enol, was received on 30 September 2020 from Bayer Corporation, Kansas City, Missouri. The Certificate of Analysis is presented in Appendix 2. The following information was provided:

Name:	Spirodiclofen Enol
Lot No.	0218200501
CAS No.:	148476-22-6
Purity:	99.9%
Appearance:	Colorless to light brown solid
Date of Analysis:	24 June 2019
Expiry Date:	24 June 2027
Storage Conditions:	Freezer conditions when not in use.
Structural Formula:	



Upon receipt at Smithers, the test substance (TMC No. 10835) was stored in a freezer in the original container. Concentrations were adjusted for the purity of the test substance. This sample of test substance was used to prepare recovery samples and calibration standards during testing.

2.3 Reagents

1.	Acetonitrile:	EMD, reagent grade
3.	Methanol:	EMD, reagent grade
4.	Formic Acid:	BDH, reagent grade
5.	0.1% formic acid in water:	Fisher, reagent grade
6.	0.1% formic acid in acetonitrile:	Fisher, reagent grade
7.	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:	MDS Sciex API 5000 mass spectrometer equipped with an ESI Turbo V ion source Shimadzu SIL-20ACXR autoinjector Shimadzu DGU-20A5R vacuum degassers Shimadzu LC-20ADXR solvent delivery pumps Shimadzu CTO-20AC column compartment Shimadzu CBM-20A communications bus Analyst 1.6 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 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 Matrixes

The matrixes used during this method validation were ground water and surface water.

Ground water information:

Ground water consists of unadulterated water from a 100-meter bedrock well collected on site at Smithers, Wareham, MA prepared by filtering to remove any potential organic contaminants. Prior to use, the ground water was characterized by Agvise Laboratories, Northwood, North Dakota:

Parameter	Sediment
Smithers Viscient Batch No.:	GROUND WATER 2019
pH	7.6
Calcium	24 ppm
Magnesium	7.8 ppm
Sodium	92 ppm
Hardness	92 mg equivalent CaCO3/L
Conductivity	0.70 mmhos/cm
Sodium adsorption ratio (SAR)	4.19
Total dissolved solids	228 ppm
Turbidity	0.15 NTU

Surface water information:

The surface water used for this method validation analysis was collected from the Weweantic River, West Wareham, Massachusetts. The water was collected from an area of the river with approximately 30 to 60 cm of overlying water. Prior to use, the surface water was characterized by Agvise Laboratories, Northwood, North Dakota:

Parameter	Sediment
Smithers Viscient Batch No.:	28Dec20WAT-A WEWEANTIC WATER
pH	6.6
Calcium	3.0 ppm
Magnesium	1.4 ppm
Hardness	14 mg equivalent CaCO3/L
Conductivity	0.10 mmhos/cm
Total dissolved solids	52 ppm
Turbidity	1.47 NTU
Biological oxygen demand	0.8 ppm
Total organic carbon	9.2 ppm
Dissolve organic carbon	7.8 ppm
Nitrogen (total kjeldahl)	0.4 ppm
Nitrogen (nitrate)	0.1 ppm
Nitrogen (nitrile)	Below detection limit of 0.1 ppm
Nitrogen (ammoniacan distillation)	Below detection limit of 0.2 ppm
Total phosphorus (as PO_4^-)	0.1 ppm
Dissolved orthophosphate	Below detection limit of 0.1 ppm

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

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 50/50/0.1 acetonitrile/purified reagent water/formic acid (v/v/v) liquid reagent solution was typically prepared by adding 0.400 mL of formic acid to 200 mL of acetonitrile and 200 mL of purified reagent water. The solution was mixed well using a stir bar and stir plate for 5 minutes.

A 56/44/0.11 acetonitrile/purified reagent water/formic acid (v/v/v) liquid reagent solution was typically prepared by adding 1.65 mL of formic acid to 840 mL of acetonitrile and 660 mL of purified reagent water. The solution was mixed well using a stir bar and stir plate for 5 minutes.

A 50/10/40/0.1 acetonitrile/ground water/purified reagent water/formic acid (v/v/v/v) liquid reagent solution was typically prepared by adding 0.100 mL of formic acid to 50.0 mL of acetonitrile, 10.0 mL of ground water and 40.0 mL of purified reagent water. The solution was mixed well using a stir bar and stir plate for 5 minutes.

A 50/10/40/0.1 acetonitrile/surface water/purified reagent water/formic acid (v/v/v/v) liquid reagent solution was typically prepared by adding 0.100 mL of formic acid to 50.0 mL of acetonitrile, 10.0 mL of surface water and 40.0 mL of purified reagent water. The solution was mixed well using a stir bar and stir plate for 5 minutes.

A 90/10 acetonitrile/purified reagent water (v/v) liquid reagent solution was typically prepared by adding 45.0 mL of acetonitrile to 5.00 mL of purified reagent water. The solution was mixed well before use.

A 30/30/40 acetonitrile/methanol/purified reagent water (v/v/v) autosampler needle 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 each separate 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
10773H (Spirodiclofen)	0.0503	0.0500	Acetonitrile	50.0	1000	Secondary stock solution
10773I (Spirodiclofen)	0.0504	0.0500		50.0	1000	Secondary stock solution
10835E (Spirodiclofen Enol)	0.0250	0.0250	90/10 acetonitrile/purified reagent water	25.0	0.999	Secondary stock solution

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
10773H (Spirodiclofen)	1000	0.500	50.0	Acetonitrile	10773H-1	10.0	Sub-stock solutions
10773I (Spirodiclofen)	1000	0.500	50.0		10773I-1	10.0	Sub-stock solutions
10835E (Spirodiclofen Enol)	999	0.500	50.0		10835E-1	9.99	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 (mg/L)	Stock Use
10773H-1 (Spirodiclofen)	10.0	0.100	10.0	Acetonitrile	Mix-Tech Stk 1	0.100[a] / 0.0999[b]	10X LOQ-level recovery samples
10835E-1 (Spirodiclofen Enol)	9.99	0.100					
Mix-Tech Stk 1	0.100[a] / 0.0999[b]	1.00	10.0		Mix-Tech Stk 2	0.0100[a] / 0.00999[b]	LOQ-level recovery samples
10773I-1 (Spirodiclofen)	10.0	0.100	10.0		Mix-Ana Stk 1	0.100[a] / 0.0999[b]	Sub-stock solutions
10835E-1 (Spirodiclofen Enol)	9.99	0.100					
Mix-Ana Stk 1	0.100[a] / 0.0999[b]	1.00	10.0		Mix-Ana Stk 2	0.0100[a] / 0.00999[b]	Calibration standards and matrix effects investigation samples
Mix-Ana Stk 2	0.0100[a] / 0.00999[b]	1.00	10.0		Mix-Ana Stk 3	0.00100[a] / 0.000999 [b]	

[a] Concentration of spirodiclofen
[b] Concentration of spirodiclofen enol

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

2.8 Preparation of Calibration Standards

2.8.1 Calibration Standards

Solvent-based calibration standards used in the quantitation of ground water and surface water samples were prepared in 50/50/0.1 acetonitrile/purified reagent water/formic acid (v/v/v) by dosing with a mixed sub-stock solution of spirodiclofen and spirodiclofen enol at 0.00100 mg/L and 0.0100 mg/L for spirodiclofen and 0.000999 mg/L and 0.00999 mg/L for spirodiclofen enol

by fortification of both materials together into the same vessel to yield concentrations of 0.00200, 0.00500, 0.0100, 0.0200, 0.0300, 0.0500, 0.100, and 0.200 µg/L.

2.8.2 Matrix Effect Investigation

The effects of matrix enhancement or suppression were evaluated through the assessment of matrix-matched and solvent-based calibration standards in the following manner. Calibration standards used to assess possible matrix effects were prepared in triplicate in 50/10/40/0.1 acetonitrile/test matrix/purified reagent water/formic acid (v/v/v/v) control final extract and 50/50/0.1 acetonitrile/purified reagent water/formic acid (v/v/v) by fortifying with a mixed sub-stock solutions of spirodiclofen and spirodiclofen enol at 0.0100 mg/L and 0.00999 mg/L for spirodiclofen and spirodiclofen enol, respectively, as outlined in the following table by fortification of both materials together into the same vessel. Resulting standards had a concentration of 0.0100 µg/L and 0.00999 µg/L for spirodiclofen and spirodiclofen enol, respectively.

Spirodiclofen

Sample ID	Sample Type	Stock Concentration (mg/L)	Fortification Volume (mL)	Final Volume (mL)	Fortified Concentration (µg/L)
GW-MM-Std A, B, & C	Ground water matrix-matched calibration standard	0.0100[a] / 0.00999[b]	0.0200	20.0[c]	0.0100[a] / 0.00999[b]
SW-MM-Std A, B, & C	Surface water matrix-matched calibration standard	0.0100[a] / 0.00999[b]	0.0200	20.0[d]	0.0100[a] / 0.00999[b]
Sol-Std A, B, & C	Solvent-based calibration standard	0.0100[a] / 0.00999[b]	0.0200	20.0[e]	0.0100[a] / 0.00999[b]

[a] Concentration of spirodiclofen
[b] Concentration of spirodiclofen enol
[c] Diluted with matrix blank final extract 12791.6405-02, equivalent to 50/10/40/0.1 acetonitrile/ground water/purified reagent water/formic acid (v/v/v/v)
[d] Diluted with matrix blank final extract 12791.6405-15, equivalent to 50/10/40/0.1 acetonitrile/surface water/purified reagent water/formic acid (v/v/v/v)
[e] Diluted with 50/50/0.1 acetonitrile/purified reagent water/formic acid (v/v/v)

2.9 Sample Fortification and Preparation

The recovery samples were prepared in two different matrixes (ground water and surface water) by fortification with mixed sub-stock solution of spirodiclofen and spirodiclofen enol at concentrations of 0.0100 (LOQ) and 0.100 (10X LOQ) µg/L for spirodiclofen and 0.00999 (LOQ) and 0.0999 (10X LOQ) µg/L for spirodiclofen enol, together into the same vessel. Recovery samples for both matrixes were prepared separately ("de novo") at these concentrations. Five replicates were produced for each concentration level. Two samples of each matrix were left unfortified to serve as controls and were processed in the same fashion as the LOQ concentration recovery samples. In addition, one reagent blank of purified reagent water was prepared for each sample set and processed in the same manner as the control samples in order to assess any potential interferences from the test matrix or dilution process. The preparation procedure for each separate matrix is outlined in the tables below.

Ground water recovery samples

Sample ID 12791-6405-	Sample Type	Stock Concentration (mg/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	10.0[c]	0.00
04, 05, 06, 07, & 08	LOQ	0.0100[d] / 0.00999[e]	0.0500	5.00	0.100[d] / 0.0999[e]
09, 10, 11, 12, & 13	10X LOQ	0.100[d] / 0.0999[e]	0.0500	5.00	1.00[d] / 0.999[e]

[a] NA = Not Applicable
[b] Volume increased for use in matrix effects assessment.
[c] Dilution solvent: purified reagent water
[d] Concentration of spirodiclofen
[e] Concentration of spirodiclofen enol

Surface water recovery samples

Sample ID 12791-6405-	Sample Type	Stock Concentration (mg/L)	Fortification Volume (mL)	Final Volume (mL)	Fortified Concentration (µg/L)
14	Reagent Blank	NA[a]	NA	5.00[b]	0.00
15 & 16	Control	NA	NA	10.0[c]	0.00
17, 18, 19, 20, & 21	LOQ	0.0100[d] / 0.00999[e]	0.0500	5.00	0.100[d] / 0.0999[e]
22, 23, 24, 25, & 26	10X LOQ	0.100[d] / 0.0999[e]	0.0500	5.00	1.00[d] / 0.999[e]

[a] NA = Not Applicable
[b] Volume increased for use in matrix effects assessment.
[c] Dilution solvent: purified reagent water
[d] Concentration of spirodiclofen
[e] Concentration of spirodiclofen enol

2.10 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 first dilution with 56/44/0.11 acetonitrile/purified reagent water/formic acid (v/v/v) was performed by the addition of the reagent to the entire volume of the aqueous sample in the container in which it was fortified to a final composition of 50/10/40/0.1 acetonitrile/test matrix/purified reagent water/formic acid (v/v/v/v). The High-level recovery samples were subsequently diluted into the calibration standard range with 50/10/40/0.1 acetonitrile/test matrix/purified reagent water/formic acid (v/v/v/v) prior to analysis. The dilution procedures are outlined in the tables below.

Ground water recovery samples

Sample ID 12791-6405-	Sample Type	Fortified Concentration (µg/L)	Sample Volume (mL)	Final Volume[a] (mL)	Sample Volume (mL)	Final Volume[b] (mL)	Dilution Factor
01	Reagent Blank	0.00	5.00	50.0	NA[c]	NA	10.0
02 & 03	Control	0.00	10.0[d]	100[d]	NA	NA	10.0
04, 05, 06, 07, & 08	LOQ	0.100[e] / 0.0999[f]	5.00	50.0	NA	NA	10.0
09, 10, 11, 12, & 13	10X LOQ	1.00[e] / 0.999[f]	5.00	50.0	2.50	5.00	20.0

[a] Diluted with 56/44/0.11 acetonitrile/purified reagent water/formic acid (v/v/v)
[b] Diluted with 50/10/40/0.1 acetonitrile/ground water/purified reagent water/formic acid (v/v/v/v)
[c] NA = Not Applicable
[d] Volume increased for use in matrix effects assessment.
[e] Concentration of spirodiclofen
[f] Concentration of spirodiclofen enol

Surface water recovery samples

Sample ID 12791-6405-	Sample Type	Fortified Concentration (µg/L)	Sample Volume (mL)	Final Volume[a] (mL)	Sample Volume (mL)	Final Volume[b] (mL)	Dilution Factor
14	Reagent Blank	0.00	5.00	50.0	NA[c]	NA	10.0
15 & 16	Control	0.00	10.0[d]	100[d]	NA	NA	10.0
17, 18, 19, 20, & 21	LOQ	0.100[e] / 0.0999[f]	5.00	50.0	NA	NA	10.0
22, 23, 24, 25, & 26	10X LOQ	1.00[e] / 0.999[f]	5.00	50.0	2.50	5.00	20.0

[a] Diluted with 56/44/0.11 acetonitrile/purified reagent water/formic acid (v/v/v)
[b] Diluted with 50/10/40/0.1 acetonitrile/surface water/purified reagent water/formic acid (v/v/v/v)
[c] NA = Not Applicable
[d] Volume increased for use in matrix effects assessment.
[e] Concentration of spirodiclofen
[f] Concentration of spirodiclofen enol

2.11 Analysis

2.11.1 Instrumental Conditions

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

LC parameters:

Column: Phenomenex Kinetex Biphenyl, 2.6 µm, 3 × 50 mm
Mobile Phase A: 0.1% formic acid in reagent grade 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	95.0	5.00
0.60	0.500	50.0	50.0
4.00	0.500	0.00	100
5.00	0.500	0.00	100
5.10	0.500	95.0	5.00
6.00	0.500	95.0	5.00

Run Time: 6.0 minutes
Autosampler Wash Solvent: 30/30/40 acetonitrile/methanol/reagent grade water (v/v/v)
Column Temperature: 40 °C
Sample Temperature: 10 °C
Injection Volume: 50.0 µL
Retention Times: approximately 3.6 minutes (spirodiclofen)
approximately 2.5 minutes (spirodiclofen enol)

MS parameters:

Instrument: AB MDS Sciex API 5000 mass spectrometer
Ionization Mode: Positive (+) ESI
Ion Spray Voltage: 5500 V
Scan Type: MRM
Source Temperature: 400 °C
Curtain Gas: 20.0
Ion Source – Gas 1 / Gas 2: 70.0 / 70.0
Collision Gas: 6.00
Collision Cell Entrance Potential: 10.0
Collision Cell Exit Potential: 10.0
Declustering Potential: 50.0
Resolution Q1/Q3: Unit/Low

Test Substance	Spirodiclofen		Spirodiclofen enol	
Instrument Parameter	**Primary Transition**	**Confirmatory Transition**	**Primary Transition**	**Confirmatory Transition**
Q1/Q3 Masses (Da):	411.3/313.0	411.3/71.1.0	313.2/212.9	313.2/184.8
Collision Energy:	18.0	27.0	35.0	43.0
Dwell Time:	150	150	200	200

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 Accuracy, Precision, Specificity, and Linearity

The accuracy was reported in terms of percent recovery of the fortified recovery samples. Recoveries of 70.0 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 spirodiclofen and spirodiclofen enol 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 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 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 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 analysis with 1/x weighting, 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

NOTE: A 1/x weighting was used for calibration curves and sample quantitation using Analyst software, version 1.6.

The LOD was calculated using the following equation:

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

where:

N_{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 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 (0.00200 µg/L)
DF_{CNTL}	=	dilution factor of the control samples (smallest dilution factor used, i.e., 10.0)
MDL	=	method detection limit reported for the analysis (0.00200 µg/L × 10.0= 0.0200 µg/L)

Study No.: 12791.6405

Environmental Chemistry Method: Validation of the Analytical Method for the Determination of Spirodiclofen and Spirodiclofen Enol 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 Spirodiclofen and its metabolite Spirodiclofen Enol in two aqueous matrices. The analytical method will be validated with regards to specificity, linearity, accuracy, precision, limit of quantitation (LOQ), limit of detection (LOD), method detection limit (MDL), and confirmation of 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 40 CFR 160 and the OECD principles of GLP.

3.0 TEST SUBSTANCE

3.1 Test Substance and Analytical Standard

Upon arrival at Smithers, the test substances and analytical standard was 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 was recorded and any damage noted. The packaging was removed, the primary storage container inspected for leakage or damage, and the condition recorded. Any damage was reported to the Sponsor and/or manufacturer.

The test substance and analytical standard was 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, SDS, and safe handling procedures, and a verified expiration or reanalysis date.

3.1.1 Spirodiclofen Technical

Test Substance Name: Spirodiclofen
Purity: 99.3%
Batch or Lot #: EDFL055904

3.1.2 Spirodiclofen Analytical Standard

Analytical Standard Name: Spirodiclofen
Purity: 99.7%
Batch or Lot #: 0128200401

3.1.3 Spirodiclofen Enol Analytical Standard

Test Substance Name (also the analytical standard): Spirodiclofen Enol
Purity: 99.9%
Batch or Lot #: 0218200501

3.2 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.

3.3 Validation Matrices

The water used for the method validations will be two types of aqueous matrices (i.e. groundwater & surface water). Water characterization data such as pH, hardness, conductivity, total dissolved solids, and turbidity will be determined. All documentation relating to the preparation, storage and handling will be maintained by Smithers.

3.3.1 Ground Water

Ground water used in the study will be filtered Town of Wareham well water and 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.

3.3.2 Surface Water

The surface water used for this method validation analysis will be collected from river water in Massachusetts. The water will be collected from an area of the river with approximately 30 to 60 cm of overlying water. All documentation relating to the preparation, storage and handling will be maintained by Smithers.

4.0 VALIDATION DESIGN

The test design will consist of two aqueous matrices (i.e. groundwater & surface water) fortified with each test substance at two concentrations with five replicates for each fortification level. The control matrices for the validation will be the appropriate untreated aqueous matrix. The validation study levels (approximate concentrations) for each test substance are:

1.	Procedural blank-reagent blank	0.0 µg/L
2.	Matrix blank-control matrix	0.0 µg/L
3.	Control matrix fortified at LOQ	0.100 µg/L
4.	Control matrix fortified at 10 x LOQ	1.00 µg/L

4.1 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). 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(s) will 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.

4.2 Linearity

The analytical calibration will extend over a range appropriate to the lowest and highest nominal concentration of the analyte in relevant analytical solutions ± at least 20%. A minimum of five concentrations will be utilized in the determination of the calibration line. The calibration data will be subjected to a regression analysis. The equation of the calibration line and a regression parameter, e.g., the coefficient of determination (r^2), will be reported and a typical calibration plot submitted. The data should have a coefficient of determination (r^2) of not less than 0.990 or a correlation coefficient (r) of not less than 0.995.

4.3 Matrix Effects Determination

Determination of matrix effects will be assessed as outlined in the analytical methods for both primary and confirmatory transitions. Matrix effects will 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. In the event that there are no matrix effects observed, matrix matched standards may also be used if deemed appropriate.

4.4 Accuracy

The accuracy of the method will be reported as the mean recovery ± the relative standard deviation (see Section 4.5 below) for each test substance in the sample matrix. Recovery determinations will be made on representative samples prepared by fortification of the control matrix with a known quantity of each analyte. Recovery data will be reported for two fortification levels appropriate to the proposed LOQ (see Section 4.6 below) and 10X LOQ. The samples described above will be prepared '*de novo*'. Mean recoveries in the range 70 – 110% of nominal concentrations of the target analyte in the fortified samples will be considered acceptable.

4.5 Precision

The precision of the method will be reported as repeatability of recovery at each fortification level. The number of determinations made at each fortification level for each validation is outlined in Section 4.0 above. The precision will be calculated for the fortified samples in terms of the standard deviation (SD), overall relative standard deviation (RSD), and/or coefficient of variation (CV). RSD values should be ≤ 20% for each fortification level. The overall RSD will also be reported. RSD values outside of the ranges detailed above will be discussed with the Sponsor prior to proceeding.

4.6 Limits of Quantitation (LOQ)

The method will be validated at the limit of quantitation (LOQ) for each analyte. 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.

4.8 Confirmation of Analyte Identification

A chromatographic confirmatory method will be used to determine test solution concentrations during validation. Utilizing an LC-MS/MS system, an example of a confirmatory method would be where the primary product ion and one confirmatory (secondary) product ion will be used for identification/quantification. Additional modes of confirmation may include different stationary phase types, or types of detection. The confirmatory method analysis will also adhere to the aforementioned method specifications (Sections 4.1 – 4.7 above).

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 SAMPLE DISPOSAL

All study specimens, and/or samples collected during the study, and test materials and reference standards, etc., provided by the Sponsor, client, or customer will either be returned to the originator, shipped to a third party archival facility on behalf of the Study Sponsor who will incur the costs of shipping and archival, or disposed of according to Smithers SOPs.

8.0 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. The location of original raw data, protocol, final report and other related materials will be noted in the study report.

9.0 REPORTING

The raw data generated at Smithers 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 and to confirm adherence with the study protocol. A single copy of the draft report will be submitted to the Sponsor for review. The report will be finalized according to standard operating procedures. The final report will meet the formatting requirements of EPA's PR Notice 2011-3. All reports will include, but will not be limited to, the following information:

- The report and project numbers from Smithers 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 substances 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 specificity, linearity, accuracy, precision, LOQ, LOD and MDL, and confirmation of 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.
- Statement of claims for non-confidentiality and that the method contains no trade secrets or proprietary data.
- Location of raw data and report.
- A copy of the study protocol and study amendments, if any.

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10.0 PROTOCOL CHANGES

Protocol amendments are defined as planned permanent changes in the study design of an approved protocol. 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. Deviations are defined as unplanned excursions or unforeseen circumstances from the approved protocol. The Study Sponsor and Study Monitor will be informed of any deviation from the test protocol in a timely manner and any necessary amendments will be discussed with the Study Sponsor before finalization. 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.

11.0 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).

12.0 ARCHIVAL

At the study closure, all original raw data (original protocol and amendments, correspondence, all study data, study documentation) and the final report will be sent for archival to:

Maria Jauregui
Gowan Company
370 South Main Street
Yuma, AZ 85364

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 A, 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. 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. 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.