2.0 INTRODUCTION

The objective of this study was to validate Bayer Method BS-003-W17-01, "An Analytical Method for the Determination of Residues of Spiromesifen (BSN 2060) and its Degradates: Spiromesifen-Enol, Spiromesifen-Enol Acid, Spiromesifen-Cyclobutyl Photoisomer, and Spiromesifen-Enol Photoisomer in Water Using LC/MS/MS" [1].

This study was designed to fulfill the requirements of the US EPA Test Guidelines OCSPP 850.6100 [2] and OPPTS 860.1340 [3]. In addition, this study was conducted in compliance with US EPA FIFRA (40 CFR Part 160) GLP standards [4].

3.0 MATERIALS AND METHODS

3.1 Test Substance and Internal Standard

Test Substance

Standard name:	Spiromesifen
Standard no.:	K-1725
CAS name:	2-Oxo-3-(2,4,6-trimethylphenyl)-1-oxaspiro[4.4]non-3-en-4-yl 3,3-dimethylbutanoate
CAS no.:	283594-90-1
GLP purity:	98.7%
Expiration date:	05 Aug 2019
Storage conditions:	Refrigerator

Standard name:	Spiromesifen enol
Standard no.:	K-1961
CAS name:	4-Hydroxy-3-(2,4,6-trimethylphenyl)-1- oxaspiro[4.4]non-3-en-2-one
CAS no.:	148476-30-6
GLP purity:	99.0%
Expiration date:	04 Jun 2018
Storage conditions:	Freezer

Standard name:	Spiromesifen phenol acid
Standard no.:	K-912
CAS name:	4-(4-Hydroxy-2-oxo-1-oxaspiro[4.4]non-3-en-3-yl)-3,5-dimethylbenzoic acid
CAS no.:	Not available
GLP purity:	97.7%
Expiration date:	27 Sep 2023
Storage conditions:	Freezer

Standard name:	Spiromesifen cyclobutyl photoisomer
Standard no.:	K-957
CAS name:	3,5-Dimethyl-5′-oxodispiro[bicyclo[4.2.0]octa-1,3,5-triene-7,4′(5′,*H*)-furan-2′(3′*H*), 1-cyclpentan]-3′-yl 3,3-dimethylbutanoate
CAS no.:	Not available
GLP purity:	99.8%
Expiration date:	26 Sep 2021
Storage conditions:	Freezer

Standard name:	Spiromesifen enol photoisomer
Standard no.:	K-966
CAS name:	8′,8′a-Dihydro-8′-hydroxy-4′,6′-dimethylspiro[cyclopentane-1,1′-[1*H*]indeno[1,2-c] furan]-3′(3′a*H*)-one
CAS no.:	Not available
GLP purity:	97.5%
Expiration date:	27 Sep 2023
Storage conditions:	Freezer

Internal Standard (IS)

Standard name:	d_3-Spiromesifen
Standard no.:	K-926
CAS name:	3-[2,4-Dimethyl-6-(methyl-d_3)phenyl]-2-oxo-1-oxaspiro[4.4]non-3-en-4-yl 3,3-dimethylbutanoate
CAS no.:	Not available
GLP purity:	98.9%
Expiration date:	30 Sep 2025
Storage conditions:	Freezer

Standard name:	*d_3*-Spiromesifen phenol
Standard no.:	K-925
CAS name:	3-[2,4-Dimethyl-6-(methyl-*d_3*)phenyl]-4-hydroxy-1-oxaspiro[4.4]non-3-en-2-one
CAS no.:	Not available
GLP purity:	99.0%
Expiration date:	30 Sep 2025
Storage conditions:	Freezer

Standard name:	*d_4*-Spiromesifen phenol acid
Standard no.:	K-920
CAS name:	4-(4-Hydroxy-2-oxo-1-oxaspiro[4.4]non-3-en-3-yl-*6,6,9,9-d_4*)-3,5-dimethylbenzoic acid
CAS no.:	Not available
GLP purity:	98.7%
Expiration date:	29 Jul 2021
Storage conditions:	Freezer

3.2 Test System

The test system used for the validation was a water sample (CPS ID GS-17-27-1) provided by Bayer CropScience. The water sample was held in a refrigerator at 2 to 8°C (CPS Equipment ID REF.1.14) until needed for analysis.

3.3 Equipment and Reagents

The equipment and reagents used for the independent laboratory validation (ILV) were as outlined in Bayer Method BS-003-W17-01 (Appendix 4; Section 3.0: Apparatus and Section 4.0: Reagents and Consumables). Identical or equivalent apparatuses and materials were used.

3.3.1 Equipment and Apparatus

- Volumetric flasks, glass class A (assorted volumes)
- Electronic pipettors, various volumes (Eppendorf)
- Manual pipettors, various volumes (VWR, Brand)
- Pasteur pipettes (VWR part no. 414004-007)
- HPLC vials and caps, 2 mL (VWR part no. 46610-722)
- Vortex mixer (VWR)
- LC-MS/MS—Shimadzu Nexera X2 Modular UHPLC system with a PAL® autosampler (CTC Analytics) coupled to a Sciex® Triple Quad™ 6500 tandem mass

spectrometer with an electrospray ionization interface and Analyst® 1.6.2 data collection software
- Kinetex®, 1.7 µm XB-C18, 50 × 2.1 mm (Phenomenex® part no. 00D-4498-AN)
- Various general laboratory glassware and utensils

3.3.2 Reagents

- Milli-Q® Water, Millipore Direct-Q5 (CPS ID WPS.1.10)
- Acetonitrile (ACN; BDH, HPLC grade, Lot# 16G181241)
- Formic acid 99% (Amresco®, Lot# 2515C292)
- 80:20 (v/v) acetonitrile:water with 0.05% formic acid; prepared by combining 400 mL acetonitrile, 100 mL water, and 250 µL formic acid
- 0.05% formic acid in water; prepared by adding 0.0500 mL formic acid to 100 mL water
- 0.1% formic acid in water; prepared by adding 1.00 mL formic acid to 1000 mL water

3.4 Experimental Design

3.4.1 Establishment of the Method

Prior to performing the ILV, the analyte retention times, instrument detection sensitivity, and linearity of instrument responses to a range of analyte concentrations were determined.

3.4.2 Sample Validation Sets, Fortification, and Extraction Procedure

Sample validation sets
The analytical set consisted of 13 samples: one reagent blank, two untreated controls, five untreated controls fortified with test substances at the LOQ, and five untreated controls fortified with test substances at 10× LOQ.

Data are summarized in Table 1 and Table 2. Residue data sheets are included in Appendix 1.

Calibration standard solutions (0.250 to 20.0 ng/mL and 0.500 ng/mL for IS) and a solvent blank were also included in each sample analysis batch.

Fortification
The water control LOQ and 10× LOQ samples were fortified with 250 µL of the fortification standard solutions, FS-0300-03 (100 ng/mL) for LOQ and FS-0300-02 (1000 ng/mL) for 10× LOQ.

Extraction and workup for water samples
The following extraction steps were followed for each water sample.

1. Measure 50-mL sample into a 50-mL centrifuge tube.
2. Fortify the recovery samples at the desired fortification level with the appropriate mixed standard solution (see Section 3.4.4).
3. Add 250 µL of the 0.100 µg/mL IS solution IS-0300-03 to each sample, and mix well.
4. Transfer a portion of sample to an HPLC vial for analysis by LC-MS/MS.

3.4.3 Sample Processing and Analysis

The samples were processed and analyzed as described in Bayer Method BS-003-W17-01. The mass spectrometer, Sciex® Triple Quad™ 6500 with an electrospray ionization interface, was optimized for the best sensitivities for the analytes.

3.4.4 Fortification and Calibration Standard Solutions Preparation

Fortification and calibration standard solutions were prepared following the methods below.

Primary stock reference (native) standard solutions of spiromesifen and its degredates
Prepare individual stock solutions of spiromesifen and its degredates. Prepare the primary stock solution for each reference standard separately by pipetting 5.00 mL 80:20 (v/v) acetonitrile:water with 0.05% formic acid into each pre-weighed standard vial. Mix well.

Secondary mixed standard solutions (100 µg/mL) of spiromesifen and its degredates
Pipette an appropriate volume of each of the primary stock solutions, and dilute to 10.0 mL with 80:20 (v/v) acetonitrile:water with 0.05% formic acid. Mix well.

Mixed fortification standard (FS-300-01 10.0 µg/mL) of spiromesifen and its degredates
Transfer 1.00 mL of the 100 µg/mL secondary mixed standard solutions into a 10-mL volumetric flask. Dilute to volume with 80:20 (v/v) acetonitrile:water with 0.05% formic acid. Mix well.

Mixed fortification standard (FS-300-02 1.00 µg/mL) of spiromesifen and its degredates
Transfer 1.00mL of the 10.0 µg/mL mixed standard solution into a 10-mL volumetric flask. Dilute to volume with 80:20 (v/v) acetonitrile:water with 0.05% formic acid. Mix well.

Mixed fortification standard (FS-300-03 0.100 µg/mL) of spiromesifen and its degredates
Transfer 1.00mL of the 1.00 µg/mL mixed standard solution into a 10-mL volumetric flask. Dilute to volume with 80:20 (v/v) acetonitrile:water with 0.05% formic acid. Mix well.

Mixed fortification standard (FS-300-04 0.0100 µg/mL) of spiromesifen and its degredates
Transfer 1.00mL of the 0.100 µg/mL mixed standard solution into a 10-mL volumetric flask. Dilute to volume with 80:20 (v/v) acetonitrile:water with 0.05% formic acid. Mix well.

Primary stock internal standard (IS) solutions of spiromesifen and its degredates
Prepare IS stock solutions of ~1 mg/mL of spiromesifen and its degredates by pipetting 5.00 mL 80:20 (v/v) acetonitrile:water with 0.05% formic acid into each pre-weighed standard (approximately 0.005 g) vial. Mix well.

Secondary mixed IS solutions (100 µg/mL) of spiromesifen and its degredates
Pipette an appropriate volume of each of the primary stock IS solutions, and dilute to 10.0 mL with 80:20 (v/v) acetonitrile:water with 0.05% formic acid. Mix well.

Mixed IS fortification solution (IS-300-01 5.00 µg/mL) of spiromesifen and its degredates
Transfer 0.500 mL of the 100 µg/mL secondary mixed IS solutions into a 10-mL volumetric flask. Dilute to 10 mL with 80:20 (v/v) acetonitrile:water with 0.05% formic acid. Mix well.

Mixed IS fortification solution (IS-300-02 1.00 µg/mL) of spiromesifen and its degredates
Transfer 2.00 mL of the 5.00 µg/mL secondary mixed IS solution into a 10-mL volumetric flask. Dilute to 10 mL with 80:20 (v/v) acetonitrile:water with 0.05% formic acid. Mix well.

Mixed IS fortification solution (IS-300-03 0.100 µg/mL) of spiromesifen and its degredates
Transfer 1.00 mL of the 1.00 µg/mL secondary mixed IS solution into a 10-mL volumetric flask. Dilute to 10 mL with 80:20 (v/v) acetonitrile:water with 0.05% formic acid. Mix well.

Calibration standard solutions
Prepare working calibration solutions consisting of 0.250, 0.500, 1.00, 5.00, 10.0, and 20.0 ppb of spiromesifen and its degredates by diluting to 25.0 mL with 0.05% formic acid in water. Before bringing the calibration solutions to volume, pipette 125 µL of the 0.100 µg/mL IS solution into each of the calibration solutions.

Concentration of Standard Solution Used for Dilution (µg/mL)	Concentration of IS Solution Used for Dilution (µg/mL)	Aliquot Native Mix Taken (mL)	Aliquot IS Taken (mL)	Concentration of Calibration Solution (ppb)	Concentration of IS (ppb)
10.0	0.100	0.0500	0.25	20.0	0.500
10.0	0.100	0.0250	0.25	10.0	0.500
1.00	0.100	0.125	0.25	5.00	0.500
1.00	0.100	0.0250	0.25	1.00	0.500
0.100	0.100	0.125	0.25	0.500	0.500
0.100	0.100	0.0625	0.25	0.250	0.500

The primary standard solutions were stored in a freezer, and all other standard solutions were stored in a refrigerator when not in use.

3.5 LC-MS/MS Instrumentation

Shimadzu Nexera X2 Modular UHPLC system with a PAL® autosampler (CTC Analytics)
Sciex® Triple Quad™ 6500 LC-MS/MS
Software: Sciex® Analyst® 1.6.2
HPLC column: Phenomenex® Kinetex®, 1.7 µm C18, 50 × 2.1 mm (part no. 00D-4498-AN)

3.6 Data Acquisition and Reporting

Peak integration was performed by Analyst® software version 1.6.2. The MS detector responses (peak area) for various injected standard concentrations were used to generate an external calibration curve for the analytes of interest. The overall purpose of the external calibration curve was to display acceptable linearity ($r^2 \geq 0.99$) of the assigned calibration range. The recoveries of the analyte from the fortified samples were calculated by multi-point calibration.

Recovery results were computed for each sample. The equation used for quantification is presented in Appendix 2. A statistical treatment of the data includes the calculation of means, standard deviations (SD), and RSDs as percentages. All statistics were calculated using Microsoft® Excel® 2010.