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

1.0 SUMMARY

An analytical method was developed to determine the residues of spiromesifen (BSN 2060) and its degradates spiromesifen-enol, spiromesifen-enol acid, spiromesifen-cyclobutyl photoisomer, and spiromesifen-enol photoisomer in water.

Isotopic internal standards are added to the water sample prior to analysis. Residues of spiromesifen and its degradates are analyzed by direct injection with LC/MS/MS. Quantification is based on a comparison of peak areas with those of known standards.

This method was developed to analyze residues of spiromesifen and its degradates in water at a target limit of quantitation (LOQ) of 0.5 ng/mL, and this LOQ can be adjusted as required.

2.0 BACKGROUND

Spiromesifen is an insecticide registered by Bayer CropScience. The analytical method presented in this report is designed to measure residues of spiromesifen and its degradates: BSN2060-enol, BSN2060 enol acid, BSN2060-cyclobutyl photoisomer, and BSN2060-enol photoisomer in water using isotopically labeled internal standards and LC/MS/MS detection.

3.0 APPARATUS

Functional equivalents may be substituted

- Various general laboratory glassware and utensils
- MicroMan pipettors and tips (M250, M50, and M1000)
- Phenomenex Kinetex C18 50 mm x 2.1 mm 1.7 µm particle size (Part No:00B-4475-AN)
- ABSciex 6500 chromatograph/mass spectrometer (LC-MS/MS) equipped with electrospray ionization (ESI) interface, Shimadzu HPLC pumps, Shimadzu oven and a CTC PAL autosampler, and Analyst 1.6.2 data collection software (ABSciex)

4.0 REAGENTS AND CONSUMABLES

Functional equivalents may be substituted

- Acetonitrile (Optima Grade, Fisher Part No. A996-4)
- Formic acid 99% (Acros, Part no. 14793-0010)
- Water (Optima Grade; Fisher Part No. W7-4)

- 80:20 (v/v) acetonitrile/water with 0.05% acetic acid. Combine 1200 mL acetonitrile, 300 mL water. Add 750 uL formic acid. Mix well.
- 0.05% formic acid in water. Add 0.5 mL formic acid to 1000 mL water. Mix well.
- 0.1% formic acid in water. Add 1 mL formic acid to 1000 mL water. Mix well.
- HPLC vials and caps (2-mL, National Scientific, Part Nos. C4011-5W and C4011-55)
- Disposable Glass pipet, 5 mL (Fisher Part No. 13-678-31H)
- 50 mL conical tubes (Fisher Part No. 352070)
- 60 mL Clear Vials Boro with Septa (Thermo Part No. S236-0060)

5.0 PREPARATION OF STANDARD SOLUTIONS

The native reference standards used in this method are: Spiromesifen, BSN2060-enol, BSN2060 enol acid, BSN2060-cyclobutyl photoisomer, and BSN2060-enol photoisomer. The isotopic internal reference standards (IS) required for this method are: BSN2060-enol-d_3, BSN2060-d_3, and 4-carboxy-degradate-spirononyl-d_4. These standards may be obtained from Bayer CropScience, 2 T.W. Alexander Drive, Research Triangle Park, North Carolina, 27709. Additional details about these chemicals are given in Appendix 1.

The toxicities of these chemicals have not been precisely determined. Thus, each chemical must be treated as a potential health hazard. Exposure to these chemicals should be reduced to the lowest reasonable level.

NOTE: The following procedure is an example description of how these standard solutions may be prepared. Alternate or additional standards of appropriate weight and volume may be prepared as needed.

Volumetric glassware and calibrated pipets should be used in the preparation of all analytical standards. Corrections for standard purities should be applied when expressing standard concentrations.

5.1 Primary Standards

Native reference standards and internal standard primary solutions are prepared from the reference standards as shown below.

Table 1: Primary reference (native) standard solution preparation

Reference Standard	Weight (mg)	Volume (mL)	Solvent	Final Concentration (µg/mL)
Spiromesifen	~10	100.0	80:20 ACN/H_2O w/ 0.05% Formic Acid	~100
BSN2060-Enol	~10	100.0	80:20 ACN/H_2O w/ 0.05% Formic Acid	~100
BSN2060-Enol Acid	~10	100.0	80:20 ACN/H_2O w/ 0.05% Formic Acid	~100
BSN2060-Cyclobutyl Photoisomer	~5	50.0	80:20 ACN/H_2O w/ 0.05% Formic Acid	~100
BSN2060-Enol Photoisomer	~5	50.0	80:20 ACN/H_2O w/ 0.05% Formic Acid	~100

Table 2: Primary internal standard solution preparation

BSN2060-d_3	~5	50.0	80:20 ACN/H_2O w/ 0.05% Formic Acid	~100
BSN2060-Enol-d_3	~5	50.0	80:20 ACN/H_2O w/ 0.05% Formic Acid	~100
4-Carboxy-metabolite-Spirononyl-d_4	~5	50.0	80:20 ACN/H_2O w/ 0.05% Formic Acid	~100

Primary solutions should be stored in a freezer when not in use.

5.2 Secondary Standards

Mixed secondary reference and internal standard solutions are prepared from the primary solutions as shown below. Take the appropriate aliquot of each of the primary standard solutions to give the required mixed secondary standard concentration.

Table 3: Secondary mixed standard solution preparation

Compound	Primary Standard Concentration (µg/mL)	Aliquot (mL) from Primary Standard	Final Volume (mL)	Mixed Secondary Standard Final Concentrations (µg/mL)	Solvent
Spiromesifen	~100	~5	50.0	10.0	80:20 ACN/H_2O w/ 0.05% Formic Acid
BSN2060-Enol	~100	~5			
BSN2060-Enol Acid	~100	~5			
BSN2060-Cyclobutyl Photoisomer	~100	~5			
BSN2060-Enol Photoisomer	~100	~5			
BSN2060-d_3	~100	~5	100.0	5.0	80:20 ACN/H_2O w/ 0.05% Formic Acid
BSN2060-Enold_3	~100	~5			
4-Carboxy-metabolite-Spirononyl-d_4	~100	~5			

Additional secondary reference standard solutions are prepared as shown below.

Table 4: Additional mixed native secondary standard solution preparation

Concentration of Mixed Native Standard Solution used for dilution (µg/mL)	Aliquot Taken (mL)	Dilution Volume (mL)	Final Concentration of Mixed Native Secondary Standard Solution (µg/mL)	Solvent
10	5.0	50.0	1.0	80:20 ACN/H_2O w/ 0.05% Formic Acid
1.0	5.0	50.0	0.10	80:20 ACN/H_2O w/ 0.05% Formic Acid
0.1	5.0	50.0	0.01	80:20 ACN/H_2O w/ 0.05% Formic Acid

Additional secondary internal standard solutions are prepared as shown below

Table 5: Additional mixed internal standard solution preparation

Concentration of Internal Standard Mixed Solution used for dilution (µg/mL)	Aliquot Taken (mL)	Dilution Volume (mL)	Final Concentration of Mixed Internal Standard Secondary Standard Solution (µg/mL)	Solvent
5.0	20.0	50.0	2.0	80:20 ACN/H_2O w/ 0.05% Formic Acid
5.0	10.0	50.0	1.0	80:20 ACN/H_2O w/ 0.05% Formic Acid
1.0	5.0	50.0	0.1	80:20 ACN/H_2O w/ 0.05% Formic Acid

All secondary standards should be stored in a refrigerator when not in use.

5.3 Calibration Standards

Note: Additional standards may be prepared when necessary; however, the concentration of the internal standard must remain the same in all calibration standards. Calibration solutions are dissolved in 0.05% formic acid in water.

Table 6: Calibration standard solutions

Concentration of Native Standard Solution used for dilution (µg/mL)	Concentration of Internal Standard Solution used for dilution (µg/mL)	Aliquot Native Taken (mL)	Aliquot Internal Standard Taken (mL)	Dilution Volume (mL)	Concentration of Native in Calibration Solution (ppb)	Concentration of IS in Calibration Solution (ppb)
10.0	0.1	0.10	0.25	50.0	20	0.5
10.0	0.1	0.05	0.25	50.0	10	0.5
1.0	0.1	0.25	0.25	50.0	5	0.5
1.0	0.1	0.05	0.25	50.0	1	0.5
0.1	0.1	0.25	0.25	50.0	0.5	0.5
0.1	0.1	0.125	0.25	50.0	0.25	0.5

Calibration standards should be stored in a refrigerator when not in use.

6.0 EXTRACTION PROCEDURE

6.1 Sample Extraction

1. Aliquot 50 ± 0.05 mL of water into a 50-mL conical tube.
2. Fortify the recovery samples (Section 8.1) at the desired fortification level with the appropriate standard solution. Mix well.
3. Add 0.250 mL of the 0.1 µg/mL of the mixed internal standard solution Table 5.
4. Transfer an aliquot to a HPLC to the vial. Cap and mix sample. The sample is ready for LC/MS/MS analysis.

7.0 ANALYSIS BY LC/MS/MS

7.1 Analytical Procedure

Step 1 Using the recommended procedures listed below; analyze a minimum of five calibration standard solutions (if necessary, additional standard solutions may be added).

Step 2 Analyze an aliquot of each of the analytical samples.

Note: Up to 20 sample analyses can be made after the analysis of the standard solutions.

Step 3 Repeat Step 1.

Step 4 When necessary analyze additional samples and standard solutions. Always finish the procedure with a set of calibration solutions

7.2 HPLC Conditions

Note: The analyst should optimize chromatographic conditions to obtain satisfactory chromatography. As the HPLC column ages, the retention times of the analytes may change.

Mobile Phase A:	0.1% Formic Acid in Water
Mobile Phase B:	ACN
Oven:	40 °C
HPLC column:	Phenomenex Kinetex C18 50 mm x 2.1 mm 1.7 µm particle size (Part No: 00B-4475-AN)
Injection volume:	20 µL (adjust as needed)

Time (min)	Mobile Phase B%	Flow rate µL/min
0.10	10	400
1.25	99	400
2.50	99	400
2.51	10	400
4.00	STOP	

Analyte	Approx Retention Time (min)
Spiromesifen	2.0
BSN2060-Enol	1.5
BSN2060 Enol Photoisomer	1.5
BSN2060 Cyclobutyl Photoisomer	1.9
BSN2060 Enol Acid	1.3

7.3 Mass Spectrometer Conditions

The MS/MS instrument is operated in the Multiple Reaction Monitoring mode (MRM). Precursor ions are selected and product ions created by collision-induced dissociation.
Two product ions per analyte are listed below: one product ion (MRM-transition) serving for quantitation and the second to be used, only if required, to confirm the presence of any detected residues.

Note: The following recommended conditions were used on an ABSciex 6500 instrument but the analyst should optimize the mass spectrometer conditions to obtain satisfactory system response prior to use.

Ionization Mode:	Electrospray ionization (ESI) interface
Curtain Gas (CUR)	40
Collision Gas (CAD)	10
Ion Spray Voltage (IS)	5000
Temperature (TEM)	300
Ion Source Gas 1 (GS1)	90
Ion Source Gas 2 (GS2)	65

Table 7: MS/MS Parameters for the determination of Spiromesifen, BSN2060-enol, BSN2060 enol acid, BSN2060-cyclobutyl photoisomer, and BSN2060-enol photoisomer

Analyte Name	Polarity	Q1 Mass (amu)	Q3 Mass (amu)	Dwell Time (msec)	CE	CXP	DP	EP
Spiromesifen	Pos	371.3	273.0	25	15	15	21	10
Spiromesifen (Confirmatory MRM)	Pos	371.3	255.0	25	31	14	21	10
BSN2060-Enol	Pos	273.2	254.9	25	19	14	1	10
BSN2060-Enol (Confirmatory MRM)	Pos	273.2	227.0	25	25	12	1	10
BSN2060-Cyclobutyl Photoisomer	Pos	371.2	209.0	25	27	14	27	10
BSN2060-Cyclobutyl Photoisomer (Confirmatory MRM)	Pos	371.2	169.0	25	37	12	27	10
BSN2060-Enol Photoisomer	Pos	255.1	209.0	25	21	12	66	10
BSN2060-Enol Photoisomer (Confirmatory MRM)	Pos	255.1	179.0	25	49	10	66	10
BSN2060-Enol-d_3[1]	Pos	276.1	258.1	25	19	14	81	10
BSN2060-d_3[2]	Pos	374.2	275.9	25	15	14	51	10

[1] Used as a surrogate internal standard for BSN2060-Enol Photoisomer
[2] Used as a surrogate internal standard for BSN2060-Cyclobutyl Photoisomer

Ionization Mode:	Electrospray ionization (ESI) interface
Curtain Gas (CUR)	40
Collision Gas (CAD)	10
Ion Spray Voltage (IS)	-4500
Temperature (TEM)	400
Ion Source Gas 1 (GS1)	60
Ion Source Gas 2 (GS2)	60

Table 8: MS/MS Parameters for the determination of BSN2060-Enol Acid

Analyte Name	Polarity	Q1 Mass (amu)	Q3 Mass (amu)	Dwell Time (msec)	CE	CXP	DP	EP
BSN2060-Enol Acid	Neg	301.0	257.0	25	-34	-13	-5	-10
BSN2060-Enol Acid (Confirmatory MRM)	Neg	301.0	239.0	25	-32	-13	-5	-10
4-Carboxy-metabolite-Spirononyl-d_4	Neg	305.0	198.1	25	-40	-13	-5	-10

8.0 CALCULATION OF RESULTS

The example calculation displayed below was used by the laboratory developing this method. Alternate calculation procedures appropriate to the reporting requirements may be substituted.

Residue concentrations were determined using calibration curves which were generated after each analysis using ABSciex Analyst 1.6.2 software using linear regression with 1/x weighting.

The standards were fit to the linear equation:

$$Y = MX + B \text{ with } 1/x \text{ weighting.}$$

where: X is the concentration of the reference standard in ng/mL
M is the calibration line slope
B is the calibration line intercept
Y is the native peak area: isotopic peak area ratio

After regression coefficients were calculated, the residue in ng/g was determined using the following equation,

$$\text{Residue found (ng/mL)} = \frac{(Y-B)}{M}$$

Analyst software was used to calculate the amount of spiromesifen (BSN 2060) and its degradates: BSN2060-Enol, BSN2060 Enol Acid, BSN2060-Cyclobutyl photoisomer, and BSN2060-Enol photoisomer in ng/mL for each sample and the percent recovery for the fortified control samples.

Residue levels beyond the calibration curve: In some cases, an unknown sample contains residues at a level above the calibration curve. If so, the preferred strategy is to extend the calibration curve to cover the unknown sample, if this is not an option contact the development laboratory for instructions on how to proceed

8.1 Recovery Experiments

Note: Recovery (fortification) experiments may be performed as needed to monitor method efficiency and reproducibility, but are not required when analysis of samples is performed for tolerance enforcement. Fortified experiments are intended to be used for data collection methods or establishing and validating method efficiency and are prepared by adding a known amount of native standard solution to a sample aliquot and preparing the sample for analysis as described in Section 6.

With each sample set, analyze an untreated control sample and one or more fortified control samples Calculate recoveries using the following equation:

$$\text{Recovery (\%)} = \frac{(R - S)}{T} \times 100$$

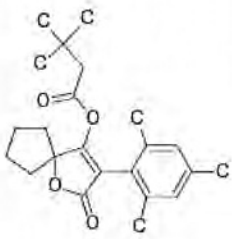
Where: R = ng/mL of target analyte found in fortified (recovery) sample
S = ng/mL of target analyte found in control sample, real or apparent
T = theoretical ng/mL in fortified sample

Recoveries are determined by analyzing fortified control samples alone or in conjunction with a sample set. Recovery samples are be fortified prior to extraction at the LOQ of 0.5 ng/g or other appropriate level with fortification solutions. Calculate the final residue for the control (S) and fortified control (R) samples.

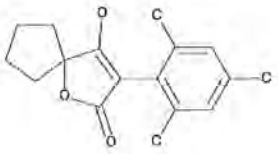
Appendix 1 Reference Substances

The toxicities of these chemicals have not been precisely determined. Thus, each chemical must be treated as a potential health hazard. Exposure to these chemicals should be reduced to the lowest reasonable level.

Code Name:	Spiromesifen
Synonyms:	BSN 2060; spiromesifin; AE 0952850
CAS Name:	2-oxo-3-(2,4,6-trimethylphenyl)-1-oxaspiro[4.4]non-3-en-4-yl 3,3-dimethylbutanoate
CAS Number:	283594-90-1
Molecular Formula:	C_{23} H_{30} O_4
Molecular Weight:	370.48 g/mol
Chemical Structure:	

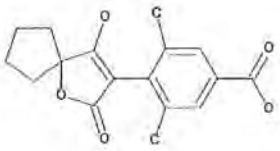


Code Name:	Spiromesifen enol
Synonyms	BSN 2060 enol; spiromesifin enol; BSN 0546; AE 0920586
CAS Name:	4-hydroxy-3-(2,4,6-trimethylphenyl)-1-oxaspiro[4.4]non-3-en2-one
CAS Number:	148476-30-6
Molecular Formula:	C_{17} H_{20} O_3
Molecular Weight:	272.34 g/mol
Chemical Structure:	

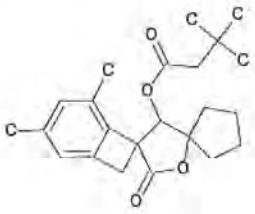


Appendix 1 (Continued)

Code Name:	Spiromesifen enol acid ()
Synonyms:	BSN 2060 enol acid; spiromesifin enol acid; 4-carboxy-BSN 0546; BAY BSN 2060 phenol acid; BSN2060-4-carboxy; KTS 9439
CAS Name:	4-(4-hydroxy-2-oxo-1-oxaspiro[4.4]non-3-en-3-yl)-3,5-dimethylbenzoic acid
CAS Number:	Not available
Molecular Formula:	C_{17} H_{18} O_5
Molecular Weight:	302.32 g/mol
Chemical Structure:	

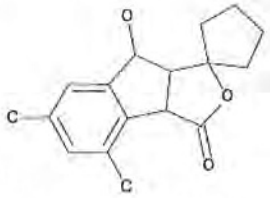


Code Name:	Spiromesifen cyclobutyl photoisomer
Synonym:	BSN 2060 cyclobutyl photoisomer; spiromesifin cyclobutyl photoisomer
CAS Name:	3,5-dimethyl-5'-oxodispiro[bicyclo[4.2.0]octa-1,3,5-triene-7,4'(5',H)-furan-2'(3'H),1-cyclpentan]-3'-yl 3,3-dimethylbutanoate
CAS Number:	Not available
Molecular Formula:	C_{23} H_{30} O_4
Molecular Weight:	370.48 g/mol
Chemical Structure:	

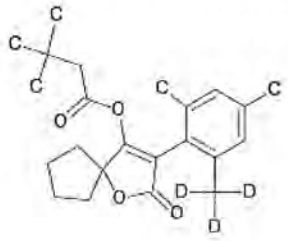


Appendix 1 (Continued)

Code Name:	Spiromesifen enol photoisomer
Synonyms:	BSN 2060 enol photoisomer; spiromesifen enol photoisomer
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 Number;	Not available
Molecular Formula:	$C_{17}H_{20}O_3$
Molecular Weight:	272.34 g/mol
Chemical Structure:	

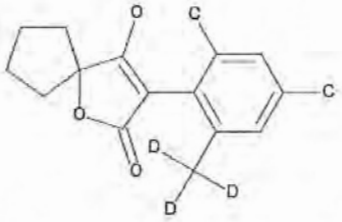


Code Name:	Spiromesifen-d_3
Synonyms:	BSN 2060-d_3; spiromesifin-d_3
CAS Name:	3-[2,4-dimethyl-6-(methyl-d3)phenyl]-2-oxo-1-oxaspiro[4.4]non-3-en-4-yl 3,3-dimethylbutanoate
CAS Number:	Not available
Molecular Formula:	$C_{23}H_{27}D_3O_4$
Molecular Weight:	373.50 g/mol
Chemical Structure:	



Appendix 1 Continued)

Code Name:	Spiromesifen enol-d_3
Synonyms:	Spiromesifin enol-d_3; BSN 2060 enol-d_3
CAS Name:	3-[2,4-dimethyl-6-(methyl-d3)phenyl]-4-hydroxy-1-oxaspiro[4.4]non-3-en-2-one
CAS Number:	Not available
Molecular Formula:	C_{17} H_{17} D_3 O_3
Molecular Weight:	275.36 g/mol
Chemical Structure:	



Code Name:	Spiromesifen enol acid-d_4
Synonyms:	d4-Bay BSN2060 phenol acid; 4-Carboxy metabolite-spirononyl-d_4; Spiromesifen enol acid-d_4
CAS Name:	4-(4-Hydroxy-2-oxo-1-oxaspiro[4.4]non-3-en-3-yl-6,6,9,9-d4)-3,5-dimethylbenzoic acid
CAS Number:	Not available
Molecular Formula:	C_{17} H_{14} D_4 O_5
Molecular Weight:	306.35 g/mol
Chemical Structure: