1. SUMMARY

This method is suitable for the determination of the residues of BYI 08330 and BYI 08330-enol in drinking and raw surface water samples.

The test samples are diluted with acidified acetonitrile. The samples are fortified with isotopically labeled internal standards and analyzed by LC/MS/MS. Quantitation of residues in samples is performed by the use of internal standards method.

The data generated during the independent laboratory validation found that the method detection limit (MDL) fell below the targeted MDL of <0.02 µg/L for BYI 08330 and BYI 08330-enol[1]. Furthermore, the calculated limit of quantitation (LOQ) for both analytes was less than the targeted LOQ of 0.05 µg/L. Therefore, the practical MDL and LOQ of the method for both analytes are set at 0.02 µg/L and 0.05 µg/L, respectively.

The mean recovery and relative standard deviation (RSD) found for BYI 08330 and BYI 08330-enol based on multiple fortifications at 0.05 µg/L (LOQ) and 0.5 µg/L (10x LOQ) were all within the range of 70 to 120% and the precision values as measured by the relative standard deviation (RSD) were all less than 20%.

2. BACKGROUND

BYI 08330 is an insecticide currently being developed by Bayer CropScience with potential uses in several crops including vegetables and orchards.

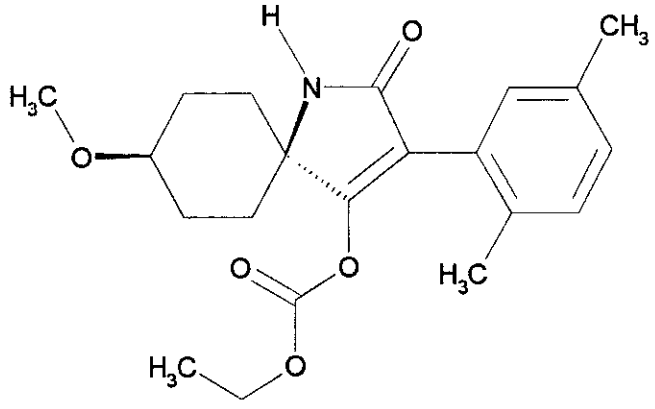
An analytical method was developed for the analysis of BYI 08330 and its associated metabolite BYI 08330-enol in water. The method was initially validated in Analytical Method 00836 (MR-131/03) for the Determination of BYI 08330 and BYI 08330-enol in Drinking and Surface Water by HPLC-MS/MS, 2004[2]. This method used matrix-matched calibration standards, and during the independent laboratory validation study[1] (ILV), the method 00836 was also validated using calibration solutions prepared in deionized water containing internal standards of BYI 08330 and BYI 08330-enol.

This method, based on analytical method 00836, was prepared on completion of the ILV study[1] and uses calibration solutions prepared in deionized water containing internal standards of BYI 08330 and BYI 08330-enol.

3. STRUCTURES OF ANALYTES

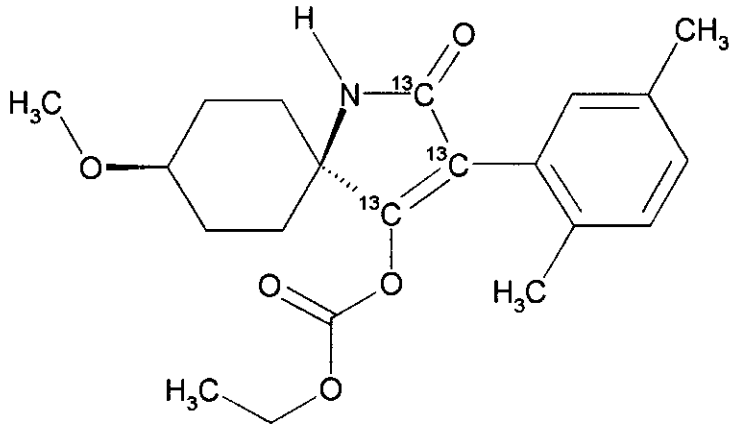
The structures of BYI 08330 and BYI 08330-enol and their isotopically labeled analogs are presented below.

BYI 08330 (Parent)



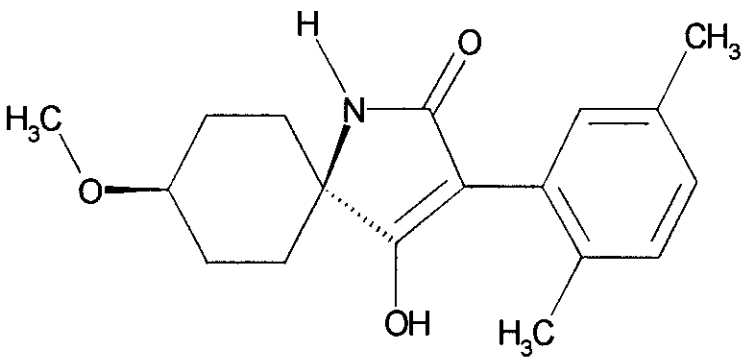
CAS Number:	203313-25-1
Common name:	Spirotetramat
Chemical name:	*cis*-3-(2,5-Dimethylphenyl)-8-methoxy-2-oxo-1-azaspiro[4.5]dec-3-en-4-yl ethyl carbonate
Empirical formula:	$C_{21}H_{27}NO_5$
Molecular weight:	373.45 g/mol

BYI 08330-cis-$^{13}C_3$ (Internal Standard)



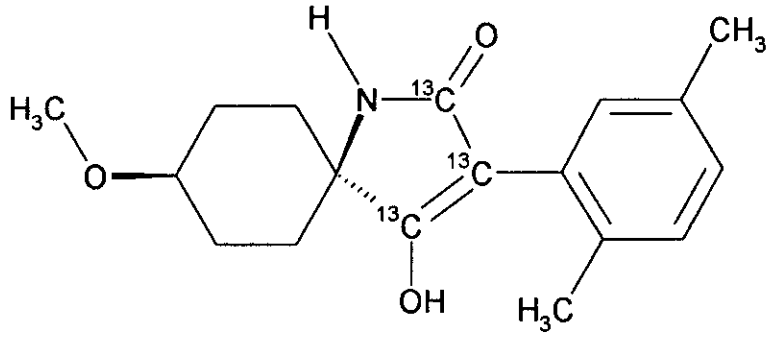
CAS Number: Unavailable
Chemical name: *cis*-3-(2,5-Dimethylphenyl)-8-methoxy-2-oxo-1-azaspiro[4,5] dec-3-en-4-yl -*2,3,4*-$^{13}C_3$ ethyl carbonate
Empirical formula: C_{21} H_{27} N O_5
Molecular weight: 376.41 g/mol

BYI 08330-enol (Metabolite)



CAS number: 203312-38-3
Chemical name: cis-3-(2,5-Dimethylphenyl)-4-hydroxy-8- methoxy-1-azaspiro[4,5]dec-3-en-2-one
Empirical formula: C_{18} H_{23} N O_3
Molecular weight: 301.38 g/mol

BYI 08330 enol-cis-$^{13}C_3$ (Internal Standard)



CAS number:	Unavailable
Chemical name:	*cis*-3-(2,5-Dimethylphenyl)-4-hydroxy-8-methoxy-1-azaspiro[4,5]dec-3-en-2-one-*2,3,4*-$^{13}C_3$
Empirical formula:	C_{18} H_{23} N O_3
Molecular weight:	304.35 g/mol

4. PRINCIPLE

Water samples are diluted with acidified acetonitrile. After dilution, isotopically labeled internal standards are added and the samples are analyzed by LC/MS/MS. The quantitation of residues in samples is based on the comparison of ratio of analyte to internal standards in sample extract against the ratio of analyte to internal standards in the calibration standards.

5. MATERIALS

5.1 Equipment

Use as a guide; where specified, equivalent apparatus and equipment may be substituted.

Balance for weighing analytical standards,
Accuracy ± 0.1 mg, Mettler AT 201 or equivalent

Amber glass bottles, various sizes to store solutions

Disposable Pasteur pipettes

Micropipette, Eppendorf brand, and pipette tips

Sonicator, Branson 5210 or equivalent

Graduated cylinders

Pipette bulb

Volumetric flasks

Volumetric pipettes

Glass containers for HPLC solvent delivery.

Autosampler vials

Synergi™ 4µ Fusion-RP 80, Length 150 mm x 4.6 mm i.d., Particle size 4µm, Part. No. 00F-4424-E0

Upchurch, ultra-low volume, inline pre-column filter, catalog # A-318, with A-102x, 0.5µm frits

MDS-Sciex API 4000 LC/MS/MS System ESP interface

Shimadzu SIL-20A autosampler

Two Shimadzu HPLC Pumps, LC-10AD$_{vp}$ (with low volume high pressure mixing)

Shimadzu Controller SCL10A$_{vp}$

Shimadzu Degasser DGU-14A

Eppendorf CH-3 Column Heater

5.2 Reagents

Use as a guide; equivalent or different manufacturers (brands) may be substituted.

- Acetonitrile, Fisher Scientific
- HPLC grade water, Fisher scientific
- Acetic Acid, Guaranteed Reagent, Fisher scientific
- Certified analytical reference standards of BYI 08330 and BYI 08330-enol and their isotopically labeled internal standards.

5.3 Reagent Solutions

Mobile Phase A: HPLC water / acetonitrile / acetic acid (900/100/0.1; v/v/v)

Transfer 900 mL HPLC grade water, 100 mL acetonitrile and 1 mL acetic acid to a 1000-mL mobile phase reservoir. Swirl to mix thoroughly, but do not shake, to prevent dissolving more air into the solution.

Place the container or reservoir in a sonicator bath and apply vacuum while sonicating for about 10 minutes or until air bubble formation or cavitation subsides to a minimum or use an in-line degasser.

Mobile phase is produced by high pressure mixing of the above with pure acetonitrile to produce the mobile phase gradient as outlined in the instrument conditions below. It has not been found necessary to sonicate acetonitrile.

Mobile Phase B: acetonitrile / acetic acid (1000/0.1; v/v)

Transfer 1000 mL acetonitrile and 1 mL acetic acid to a 1000-mL mobile phase reservoir. Swirl to mix thoroughly, but do not shake, to prevent dissolving more air into the solution.

6. FORTIFICATION AND CALIBRATION SOLUTIONS

6.1 Preparation

Use class "A" volumetric pipettes to prepare standards. The following is an example of a procedure to follow in preparing standard solutions. Alternate or additional standards of appropriate concentration and volume may be prepared as needed. The "~" symbol indicates approximately.

All standard solutions should be stored in amber glass bottles in a refrigerator set at below 10°C when not in use. Solutions should be allowed to warm to room temperature prior to use.

Note: All reusable glassware should be baked in a muffle oven at ~ 400°C for at least 2 hours to remove possible contamination before use.

6.2 Native Standard Solutions

Stock Solutions

Prepare individual 100 µg/mL stock solutions of native analytes, BYI 08330 and BYI 08330-enol, by placing 0.0100 grams of each analyte (corrected for purity) in separate 100-mL volumetric flasks. Dilute to volume with acetonitrile and mix well.

Note: Corrections for standard purities should be applied when expressing standard concentrations. For example, if an analytical standard material has 98.5%, then 0.0102 grams (0.0100 g / 0.985) would be required to prepare a 100 µg/mL stock solution.

Mixed Native Fortification Solutions

Prepare mixed fortification solutions of BYI 08330 and BYI 08330-enol by following the dilution scheme provided in the following table. All fortification solutions are prepared in 80:20:0.1 (v/v/v) denioized water:acetonitrile:acetic acid. After each dilution, the volumetric flask must be capped and the contents mixed by inversion. Each fortification solution contains both analytes at the same concentration.

Concentration of Original Solution (ng/mL)	Volume of Original Solution Taken (mL)	Final Dilution Volume (mL)	Concentration of New Mixed Native Solution (ng/mL)
100,000 (individual stock solutions)	2	50	4,000
4,000 (mixed solution)	10	50	800
800 (mixed solution)	6.25	50	100
100 (mixed solution)	5	50	10
10 (mixed solution)	5	50	1

6.3 Isotopically Labeled Internal Standard Solutions

Stock Solutions

Prepare individual 100 µg/mL stock solutions of isotopically labeled internal standards, BYI 08330-cis-$^{13}C_3$, and BYI 08330 enol-cis-$^{13}C_3$, by placing 0.0100 grams of each analyte (corrected for purity) in separate 100-mL volumetric flasks. Dilute to volume with acetonitrile and mix well.

Note: Corrections for standard purities should be applied when expressing standard concentrations. For example, if an analytical standard material has 98.5%, then 0.0102 grams (0.0100 g / 0.985) would be required to prepare a 100 µg/mL stock solution.

Mixed Internal Standard Fortification Solutions

Prepare mixed fortification solutions of BYI 08330-cis-$^{13}C_3$, and BYI 08330 enol-cis-$^{13}C_3$ by following the dilution scheme provided in the following table. All fortification solutions are prepared in 80:20:0.1 (v/v/v) denioized water:acetonitrile:acetic acid. After each dilution, the volumetric flask must be capped and the contents mixed by inversion. Each fortification solution contains both analytes at the same concentration.

Concentration of Original Solution (ng/mL)	Volume of Original Solution Taken (mL)	Final Dilution Volume (mL)	Concentration of New Mixed IS Solution (ng/mL)
100,000 (individual stock solutions)	2	50	4,000
4,000 (mixed solution)	10	50	800
800 (mixed solution)	6.25	50	100
100 (mixed solution)	5	50	10

6.4 Calibration Standards

Prepare calibration standards that contain both the native and isotopically labeled internal standards (IS) by following the dilution scheme provided in the table below. For example, to prepare a mixed calibration standard that contains 1.0 ng/mL native analytes and 0.2 ng/mL isotopically labeled internal standards (first solution in the table below), transfer 5 mL of a 10 ng/mL mixed native standard solution to a 50-mL volumetric flask. Then, transfer 1 mL of a 10 ng/mL mixed isotopically labeled internal standard (IS) solution to the same volumetric flask. Bring volume to the mark with 80:20:0.1 denioized water:acetonitrile:acetic acid. Cap volumetric flask and mix by inversion.

All calibration standards are prepared in 80:20:0.1 denioized water:acetonitrile:acetic acid.

Type of Standard	Concentration of Original Solution (ng/mL)	Volume of Original Solution Taken (mL)	Final Dilution Volume (mL)	Concentration of New Mixed Standard (ng/mL)
Mixed Native Mixed IS	10 10	5 1	50	1.0 0.2
Mixed Native Mixed IS	10 10	2.5 1	50	0.5 0.2
Mixed Native Mixed IS	10 10	1 1	50	0.2 0.2
Mixed Native Mixed IS	1 10	5 1	50	0.1 0.2
Mixed Native Mixed IS	1 10	2.5 1	50	0.05 0.2
Mixed Native Mixed IS	1 10	1.25 1	50	0.025 0.2

6.5 Stability of Standard Solutions

The standard solutions prepared in amber borosilicate bottles under refrigerated conditions below 10°C were stable for at least three months.

7. SAMPLE PREPARATION

Transfer 16 mL of test water sample (raw surface water or drinking water) to a 20-mL glass vial. Add 3.2 mL acetonitrile containing 0.05% acetic acid. Then, add a required amount of the BYI 08330 and BYI 08330-enol mixed internal standard solution to achieve 0.2 ng/mL. Mix the contents by shaking by hand.

To prepare the procedural recovery samples, transfer 16 mL of untreated control sample (raw surface water or drinking water) to a 20-mL vial. Fortify the samples using mixed solutions of native BYI 8330 and BYI 8330-enol to achieve a required fortification level. Add 3.2 mL of acetonitrile containing 0.05% acetic acid. Acetic acid concentration in the final sample volume is about 0.01%. Add a required amount of BYI 08330 and BYI 08330-enol mixed internal standard solution the final sample to achieve 0.2 ng/mL. Mix the contents by shaking by hand.

Transfer approximately 1 to 1.5 mL of sample to an autosampler vial and cap. Sample is now ready for analysis by LC/MS/MS.

8. LC/MS/MS ANALYSIS

Variations in equipment or sample characteristics may require different injection volumes or slight modifications in the chromatographic or detector conditions listed in order to obtain adequate chromatographic peak shapes or sensitivity.

It is often beneficial to make several priming injections of standards and/or samples prior to starting the LC/MS/MS analysis. Typically 4 to 6 priming injections are made. The results from these injections are not included in any calculations used in residue determinations. These injections help stabilize the LC/MS/MS response prior to running the analytical set.

8.1 Instrumentation

- Sciex API 4000 LC/MS/MS System (Applied Biosystems)
- Shimadzu LC-10AD$_{VP}$ HPLC Pumps (2) with a high pressure mixer and Shimadzu SCL-10A$_{VP}$ Pump Controller
- Shimadzu SIL-20A Autosampler
- Eppendorf CH-3 Column Heater

8.2 HPLC Conditions

Column: Synergi™ 4µ Fusion-RP 80, Length 150 mm x 4.6 mm i.d., Particle size 4µm, Part. No. 00F-4424-E0

Column oven temperature: 40 °C

Injection volume: 95 µL

Mobile phase: A: HPLC water / acetonitrile / acetic acid (900/100/0.1; v/v/v)
B: acetonitrile / acetic acid (1000/0.1; v/v)

Flow rate (column): 0.800 mL/min

Retention times: BYI 08330: ~7.7 min
BYI 08330-enol: ~5.8 min

8.3 HPLC Gradient Parameters

Time [min]	% A	% B
0.10	75	25
1	75	25
7	10	90
10	10	90
10.1	75	25
14.0	System Controller	Stop

8.4 Valco Valve Method Properties

Step	Total Time (min)	Position
1	0.0	B- To Waste
2	2.0	A- To MS

8.5 MS/MS Conditions

CAD Gas Setting [L/min]	5
Curtain Gas Setting [L/min]	11
GS1 Setting [L/min]	35
GS2 Setting [L/min]	50
Source Temperature [°C]	500
ihe	ON
Resolution of Q1 and Q3	Q1 Unit, Q3 Low
Scan type	MRM
Polarity	Positive
Ion Source	TurboIonSpray

Compound dependent:	BYI 08330	BYI 08330-enol	BYI 08330-$^{13}C_3$	BYI 08330-enol-cis-$^{13}C_3$
Q1 Mass [amu]	374.23	302.30	377.49	305.29
Q3 Mass [amu]	215.95	216.00	305.10	219.1
Dwell [msec]	500	600	500	500
Ionization Mode	Positive	Positive	Positive	Positive
Ion Spray Voltage (IS) [V]	5500	5500	5500	5500
Entrance Potential (EP) [V]	10	10	10	10
Declustering Potential (DP) [V]	76	75	71	96
Collision Energy (CE) [V]	47	39	23	39
Collision Cell Exit Potential (CXP) [V]	16	15	10	16

Detector Parameters:	
CEM	2200 V
DF	-50 V

9. CALCULATIONS

An example calculation for BYI 08330 for a surface water sample from the validation study[1] spiked at LOQ (sample ID: RAFNX019-034) is shown below. This sample was fortified with 0.05 ppb each of BYI 08330 and BYI 08330-enol and internal standards at 0.24 ppb each of BYI 08330-$^{13}C_3$ and BYI 08330-enol-$^{13}C_3$.

The equation for calculating residues in the samples is as follows.

$$X = \frac{(Y - B)}{M} \times IS$$

where

X = concentration of analyte in sample (µg/L)
Y = ratio of analyte response (area or height) to internal standard response (area or height)
B = intercept from linear regression analysis
M = slope from linear regression analysis (area ratio per conc. ratio)
IS = concentration of internal standard (µg/L) in the starting sample:

$$IS = \frac{V \times c}{S}$$

V = volume of internal standard solution added to sample, 0.0384 mL
c = concentration of internal standard solution, 100 µg/L
S = volume of starting sample, 16 mL

From above equation, $IS = \frac{0.0384 \times 100}{16}$

$= 0.24$ µg/L

$$\text{BYI 08330 Found (ppb)} = \frac{(0.175 - 0.011361)}{0.78244} \times 0.24$$

$= 0.503$ µg/L

Calculation of Percent Recovery

As the sample was fortified with known amounts of analyte prior to extraction, the percent recovery was determined using the following equation.

$$\% \text{ Recovery} = \frac{\text{analyte found (ppb) x 100}}{\text{analyte added (ppb)}}$$

Using the residue value for the example RAFNX019-034 above, the BYI 08330 recovery is calculated as follows.

$$\% \text{ Recovery} = \frac{(0.0503 \times 100)}{0.05} = 101\%$$

Remark: Example calculations shown above were performed using the LC/MS/MS software *Analyst (version 1.4.1)*. The example calculation was performed using the area values reported by the instrument. The instrument software carries additional figures not shown in the intermediate results. Therefore, instrument software calculated values will differ slightly from the results derived using a calculator.

10. REFERENCES

No.	Report No.	**Author(s).** Title. Year.
1	RAFNX019	**Nandihalli, U.B. and S. Boyle**. Independent Laboratory Validation of Analytical Method 00836 for the Determination of BYI 08330 and BYI 08330-enol in Drinking and Surface Water by HPLC-MS/MS and HPLC-UV, 2006
2	MR-131/03	**Brumhard, B.** Analytical Method 00836 (MR-131/03) for the Determination of BYI 08330 And BYI 08330-enol in Drinking and Surface Water by HPLC-MS/MS, 2004