Bayer Method RV-005-W12-01

Analytical Method For The Determination of Residues of BYI 02960 And Its Metabolites Difluoroacetic acid, BYI 2960-Succinamide And BYI 2960-Azabicyclosuccinamide In Water Using LC/MS/MS

1.0 SUMMARY

An analytical method was developed to determine the residues of BYI 02960, and its metabolites BYI 2960-Succinamide, BYI 02960-Azabicyclosuccinamide and Difluoroacetic acid (DFA) in water.

Residues of BYI 02960 and its metabolites are amended with an isotopic internal standard and analyzed by direct injection. The samples were analyzed for BYI 02960-Succinamide, BYI 02960-Azabicyclosuccinamide and Difluoroacetic acid (DFA) by LC/MS/MS with quantification based on a comparison of peak areas with those of known standards.

A confirmatory method is included for each analyte. BYI 02960 and DFA samples are analyzed using two separate sets of LC conditions and two MRM transitions are included.for BYI 02960-Succinamide and BYI 02960-Azabicyclosuccinamide.

The method limit of quantitation (LOQ) is 1.0ng/mL for BYI 02960 and DFA and 5.0ng/mL for BYI 02960-Succinamide and BYI 02960-Azabicyclosuccinamide.

2.0 BACKGROUND

BYI 02960 is an insecticide, currently under development by Bayer CropScience. The analytical method presented in this report is designed to measure residues of BYI 02960 and its metabolites BYI 02960-Succinamide, BYI 02960-Azabicyclosuccinamide and Difluoroacetic acid (DFA) 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 (M50, M250, and M1000).
- Eppendof pipettors and tips (1 mL and 5 mL).
- Eppendof repeater pipette and tips.
- Glass funnels
- SeQuant Zic-Hilic 150 mm X 4.6 mm 5 µm particle size (Part No: 150455.0001).
- Phenomenex Gemini C18 150 mm X 2.0 mm 5 µm particle size (Part No: 00F-4435-B0).
- RESTEK Allure Organic Acids 150x4.6mm 5 µm particle size (Part No: 9165565).
- Phenomenex Luna™ 2.5µ C18(2) 50 x 2 mm Column (Part No.: 008-4446-BO)

- API 4000 chromatograph/mass spectrometer (LC-MS/MS) equipped with electrospray ionization (ESI) interface, Shimadzu HPLC pumps and a CTC PAL autosampler, and Analyst 1.5.1 data collection software (ABSciex)

4.0 REAGENTS AND CONSUMABLES

(Functional equivalents may be substituted)

- Acetonitrile (HPLC Grade)
- Acetic Acid, 93% (Reagent Grade)
- Formic Acid 88% (Reagent Grade)
- Water (HPLC Grade; Fisher No. W7-4)
- 0.1% acetic acid in water. Add 1 mL acetic acid to 999 mL water. Mix well.
- 0.1% formic acid in water. Add 1 mL formic acid to 999 mL water. Mix well.
- 1.0% formic acid in water. Add 10 mL acetic acid to 990 mL water. Mix well.
- 0.5% formic acid in acetonitrile. Add 5 mL acetic acid to 995 mL acetonitrile. Mix well.
- Fisherbrand 125mL 4oz glass jars (Cat. No. 02-911-455)
- 60mL I-chem vials
- HPLC vials and caps (2-mL, National Scientific, Part Nos. C4011-5W and C4011-55)

5.0 PREPARATION OF STANDARD SOLUTIONS

Analytical Standards for BYI 02960 and its metabolites DFA, BYI 02960-Succinamide and BYI 02960-Azabicyclosuccinamide plus isotopic internal standards for BYI 02960-$^{13}C_5$ ^{15}N, BYI 02960-Succinamide-d_4, and difluoroacetic acid-$^{13}C_2$ are needed. BYI 02960-$^{113}C_5$ ^{15}N is used as a surrogate internal standard for BYI 02960-Azabicyclosuccinamide. These standards may be obtained from Bayer CropScience, 17745 South Metcalf, Stilwell, KS 66085. 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.

Class “A” volumetric glassware or calibrated pipets should be used in the preparation of all analytical standards. All standard solutions should be stored in a refrigerator in amber glass bottles when not in use. Solutions should be allowed to warm to room temperature prior to use. Corrections for standard purities should be applied when expressing standard concentrations.

5.1 Primary Stock Standard Solution

Prepare individual ~100µg/mL stock solutions of BYI 02960, BYI 02960-Succinamide, DFA and BYI 02960-Azabicyclosuccinamide. Standards used to prepare initial stock solutions should be weighed on an analytical balance capable of accurately weighing samples to ± 0.01mg. Standards are typically provided in 9 to 12mg aliquots for BYI 02960, BYI 2960-Succinamide and DFA and an 18 to 22mg aliquot for BYI 02960-Azabicyclosuccinamide which is supplied as a solution containing approximately 50% BYI 02960-Azabicyclosuccinamide in water.

The ~100µg/mL stock solutions of BYI 02960, BYI 02960-Succinamide and DFA standards are prepared by quantitatively transferring the standard aliquot into individual 100mL volumetric flasks using acetonitrile, and diluted to volume with acetonitrile.

The ~100µg/mL stock solution of BYI 02960-Azabicyclosuccinamide standard is prepared by quantitatively transferring the standard aliquot into a 100mL volumetric flask using deionized water, and diluted to volume with deionized water.

Pipet ~0.2mL of the BYI 02960 100ug/mL solution, ~0.2mL of the DFA 100ug/mL solution, ~1.0mL of the BYI 02960-Succinamide 100ug/mL solution and ~1.0mL of the 100ug/mL BYI 02960-Azabicyclosuccinamide solution into a 100mL volumetric flask and dilute to volume with acetonitrile/water (50/50) to give a solution containing 200ng/mL of BYI 02960 and DFA and 1000ng/mL BYI 02960-Succinamide and BYI 02960-Azabicyclosuccinamide

NOTE: Corrections for standard purities should be applied when expressing standard concentrations.

5.2 Fortification Standard Solutions

Mixed solution of BYI 02960 (1000ng/mL), DFA (1000ng/mL), BYI 02960-Succinamide (5000ng/mL) and BYI 02960-Azabicyclosuccinamide (5000ng/mL)

Pipet ~1.0mL of the BYI 02960 100ug/mL solution, ~1.0mL of the DFA 100ug/mL solution, ~5.0mL of the BYI 02960-Succinamide 100ug/mL solution and ~5.0mL of the 100ug/mL BYI 02960-Azabicyclosuccinamide solution into a 100mL volumetric flask and dilute to volume with acetonitrile/water (50/50) to give a solution containing 1000ng/mL of BYI 02960 and DFA and 5000ng/mL BYI 02960-Succinamide and BYI 02960-Azabicyclosuccinamide.

Mixed solution of BYI 02960 (100ng/mL), DFA (100ng/mL), BYI 02960-Succinamide (500ng/mL) and BYI 02960-Azabicyclosuccinamide (500ng/mL)

Pipet a 10.0mL aliquot of the mixed solution prepared above and dilute to 100mL with acetonitrile/water (50/50).

5.3 Isotopic Internal Standard Solutions

Prepare individual ~50µg/mL stock solutions of BYI 02960-$^{13}C_5$ ^{15}N, BYI 02960-Succinamide-d_4 and difluoroacetic acid-$^{13}C_2$. Standards used to prepare initial

stock solutions should be weighed on an analytical balance capable of accurately weighing samples to ± 0.01mg. Standards are typically provided in 4.0 to 6.0mg aliquots. The standards are quantitatively transferred to a 50mL volumetric flask using acetonitrile, and diluted to volume with acetonitrile.

Prepare a mixed 500ng/mL internal standard solution containing a mixture BYI 02960-$^{13}C_5{}^{15}N$, BYI 02960-Succinamide-d_4 and difluoroacetic acid-$^{13}C_2$ by taking an appropriate volume (~1.0mL) of each of the stock internal standard solutions and diluting to 100mL with 50/50 acetonitrile/water.

5.4 Calibration Standard Solutions

Prepare working calibration solutions for BYI 02960 and its metabolites diluting to 100mL with deionized water/acetonitrile (99/1). Before bringing the calibration solutions to volume, add by pipet 0.5mL of the 500ng/mL internal standard solution prepared in acetonitrile/water to each of the calibration solutions. (see Section 5.3 Isotopic Internal Standard Solutions)

Concentration of Standard Solution used for dilution (ng/mL)	Concentration of Internal Standard Solution used for dilution (ng/mL)	Aliquot Native mix Taken (mL)	Aliquot Internal Standard Taken (mL)	Dilution Volume (mL)	BYI 02960 and DFA concentration in Solution (ng/mL)	BYI 02960-succ. and BYI 02960-azabis concentration in Solution (ng/mL)	Concentration of Internal Standard (ng/mL)
1000/5000	500	2.0	0.5	100	20	100	2.5
1000/5000	500	1.0	0.5	100	10	50	2.5
1000/5000	500	0.5	0.5	100	5	25	2.5
1000/5000	500	0.2	0.5	100	2	10	2.5
1000/5000	500	0.1	0.5	100	1	5	2.5
1000/5000	500	0.05	0.5	100	0.5	2.5	2.5
100/500	500	0.1	0.5	100	0.1	0.5	2.5
100/500	500	0.05	0.5	100	0.05	0.25	2.5
---	500	---	0.5	100	0	0	2.5

Further calibration solutions may be prepared as needed. Depending on the analytical range for the water samples, at least six calibration standards are needed.

6.0 PROCEDURE

6.1 Sample extraction

Appendix 2 shows the analytical scheme for the analysis of BYI 02960, DFA, BYI 02960-Succinamide and BYI 02960-Azabicyclosuccinamide in water. The detailed stepwise procedure is as follows:

1. Transfer 10mL of water into a suitable stoppered container.

2. Fortify the recovery samples at the desired fortification level with the appropriate mixed standard solution prepared in acetonitrile/water. For fortifications at the LOQ add, by pipet, 0.10mL of the BYI 02960 (100ng/mL), DFA (100ng/mL), BYI 02960-Azabicyclosuccinamide (500ng/mL), BYI 02960-Succinamide (500ng/mL) mixed fortification solution.

3. Add, by pipet, 50uL of the 500ng/mL internal standard solution. Stopper the container and shake well.

4. Transfer an aliquot to a LC vial 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 an aliquot of each of the 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. In the case of over 20 samples, extra standard solutions could be added between sample analyses.

Step 3. Again, analyze an aliquot of each of the calibration standard solutions (and, if necessary, additional standard solutions).

7.2 LC/MS/MS Conditions

Notes: The analyst should optimize chromatographic conditions to obtain satisfactory chromatography. The following recommended conditions were used on an ABSciex API 4000 instrument.

Quantitiation and confirmatory conditions are described for each of the analytes. Alternate LC conditions are shown for BYI 02960 and DFA. Two sets of MRM transitions are shown for BYI 2960-Succinamide and BYI 2960-Azabicyclosuccinamide.

The presence of formic acid on the HILIC column used for the BYI 02960 and DFA confirmatory analyses in Section 7.2.3 will suppress the DFA signal. Purge the LC system of all solvents containing formic acid prior to installing the HILIC column.

7.2.1 LC Conditions for BYI 02960 (Quantitation), BYI 02960-Succinamide (Quantitation and Confirmatory) and BYI 02960-Azabicyclosuccinamide (Quantitation and Confirmatory) analysis

Inject a sample aliquot from Section 6.1, Step 4.

Mobile Phase A: 0.1% formic acid in water
Mobile Phase B: Acetonitrile.
HPLC column: Phenomenex Luna™ 2.5µ C18(2) 50 x 2 mm Column
Injection volume: 40 µL (Adjust for LC/MS/MS system being used)

Time (min)	Mobile Phase B %	Flow rate (A &B) uL/min
0.0	5	700
0.5	5	700
6.0	95	700
6.1	5	700
9.0	Controller	Stop

Analyte	Approx Retention Time (min)
BYI 2960-Azabicyclosuccinamide	1.7
BYI 02960	2.2
BYI 2960-Succinamide	2.2

7.2.2 LC Conditions for DFA (Quantitation) analysis

Inject a sample aliquot from Section 6.1, Step 4.

Mobile Phase A: 1.0% formic acid in water
Mobile Phase B: 0.5% formic acid in acetonitrile.
HPLC column: RESTEK Allure Organic Acids 150x4.6mm 5 µm particle size.

Injection volume: 25 µL (Adjust for LC/MS/MS system being used)

Start condition: Binary Gradient Total Flow: 0.5 mL/min.

Time (min)	Module	Events	Flow rate (A &B) uL/min
0.0	Pumps	% B	99%
1.0	Pumps	% B	99%
2.2	Pumps	Total Flow	1mL/min
3.0	Pumps	% B	20%
3.01	Pumps	Total Flow	0.5mL/min
4.0	Pumps	% B	20%
4.1	Pumps	% B	99%
7.5	Controller	Stop	

Analyte	Approx Retention Time (min)
DFA	2.6

7.2.3 LC Conditions for BYI 02960 (Confirmatory) and DFA (Confirmatory) analysis

Inject a sample aliquot from Section 6.1, Step 4.

Mobile Phase A: Aqueous 0.1% acetic acid in water
Mobile Phase B: Acetonitrile

HPLC column: SeQuant Zic-Hilic 150 mm X 4.6 mm 5 µm particle size
Injection volume: 3-10 µL (Adjust for LC/MS/MS system being used)

Time (min)	Mobile Phase B %	Flow rate (A &B) uL/min
0.0	95	400
0.01	95	400
4.0	75	400
4.1	75	700
4.2	10	700
6.5	10	400
7.8	10	400
8.0	95	400
12.0	Controller	Stop

Analyte	Approx Retention Time (min)
BYI 02960	2.7
DFA	3.0

7.3 Mass Spectrometer Conditions

Note: The analyst should optimize the mass spectrometer conditions to obtain satisfactory system response. The following conditions were used on an API 4000 instrument. If the analysis involves switching between polarities, as in Section 7.2.1 and Section 7.2.3, the analyst may either inject each sample twice or set up two separate experiments within a single method.

Positive ion mode for BYI 02960 and BYI 02960-Azabicyclosuccinamide

CUR: Curtain Gas	30
CAD: Collision Gas	8
GS1: Ion Source Gas 1	45
GS2: Ion Source Gas 2	60
TEM: Source Temp.	700 ºC
IHE: Interface Heater	ON
IS: Ion Transfer Voltage	5500

Negative ion mode for BYI 02960-Succinamide and DFA

CUR: Curtain Gas	30
CAD: Collision Gas	8
GS1: Ion Source Gas 1	45
GS2: Ion Source Gas 2	60
TEM: Source Temp.	700ºC
IHE: Interface Heater	ON
IS: Ion Transfer Voltage	-4000

7.4 Mass Spectrometer Data Collection

Note: The analyst should optimize the mass spectrometer data collection to obtain satisfactory system response. As the HPLC column ages, the retention times of the analytes will change. The time between the ion mode transition from negative mode to positive mode may change with the retention time change. A standard solution should be analyzed before each set of samples to confirm the data collection parameters.

The daughter ions used in this method were chosen due to their optimum sensitivity on the ABSciex API 4000 instrument used for this study. The following recommended ion transitions and conditions were example conditions used on an ABSciex API 4000 instrument:

Analyte Name	Polarity	Q1 Mass (amu)	Q3 Mass (amu)	Dwell (msec)	DP	EP	CE	CXP
BYI02960	+	289	126	50	45	11	40	25
BYI02960 IS[1]	+	295	130	50	45	11	40	25
DFA	-	95.2	51.1	50	-39	-9	-17	-8
DFA IS	-	97.2	52.1	50	-39	-9	-17	-8
BYI 02960-succinamide (MRM1)	-	305	99	50	-120	-4	-32	-5
BYI 02960-succinamide (MRM 2)	-	305	285	50	-40	-2	-12	-5
BYI 02960-succinamide IS	-	309	289	50	-40	-2	-12	-5
BYI 02960-Azabicyclosuccinamide (MRM 1)	+	289	108	50	41	6	24	6
BYI 02960-Azabicyclosuccinamide (MRM 2)	+	289	189	50	41	6	24	6

[1] Used as a surrogate internal standard for BYI 02960- Azabicyclosuccinamide

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 quantitation software Analyst (Version 1.4.1) 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,

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

Analyst software was used to calculate the amount of BYI 02960 and its metabolites in ng/mL for each sample and the percent recovery for the spiked samples.

8.1 Fortification Experiments

Note: 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. Fortification experiments are intended to be used for data collection methods or establishing and validating method efficiency.

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

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

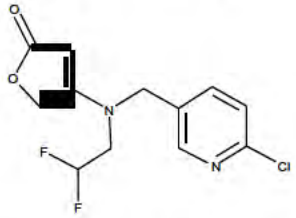
Where: R = ppb of target analyte found in fortified sample
S = ppb of target analyte found in control sample, real or apparent
T = theoretical ppb in fortified sample

Recoveries are determined by analyzing fortified control samples alone or in conjunction with a sample set. Samples may be fortified prior to extraction at the LOQ of 5ng/g or other appropriate level with fortification solutions. Calculate the final residue for the control (S) and fortified control (R) samples.

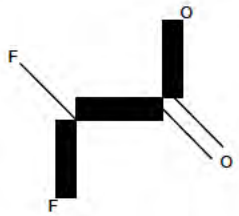
Appendix 1 Test and Reference Substances

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.

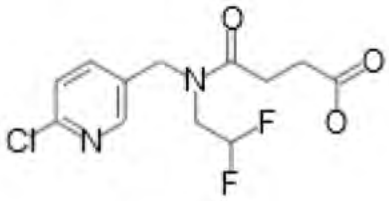
Code Name: BYI 02960
Molecular Formula: $C_{12}H_{11}ClF_2N_2O_2$
Molecular Weight: 289 g/mol



Code Name: Difluoroacetic acid (DFA)
Molecular Formula: $C_2H_2F_2O_2$
Molecular Weight: 96 g/mol

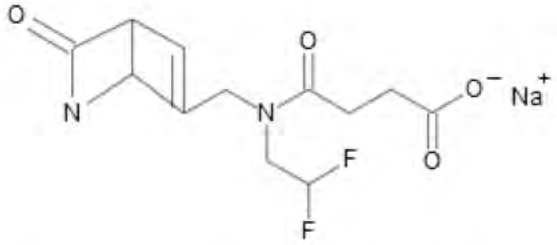


Code Name: BYI 02960-succinamide (BCS-CR74729)
Molecular Formula: $C_{12}H_{13}ClF_2N_2O_3$
Molecular Weight: 307 g/mol

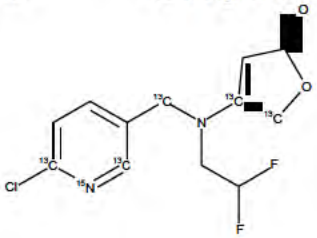


Appendix 1 Test and Reference Substances (Cont'd)

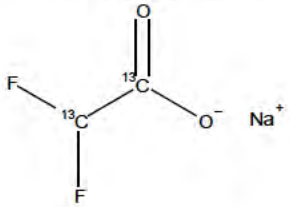
Code Name: BYI 02960-azabicyclosuccinamide-Na salt (BCS-CU93236)
Molecular Formula: $C_{12}\ H_{13}\ F_2\ N_2\ O_4\ .\ Na$
Molecular Weight: 307 g/mol



Code Name: BYI 02960 $^{13}C_5{}^{15}N$
Molecular Formula: $C_{12}\ H_{11}\ Cl\ F_2\ N_2\ O_2$
Molecular Weight: 294.6 g/mol

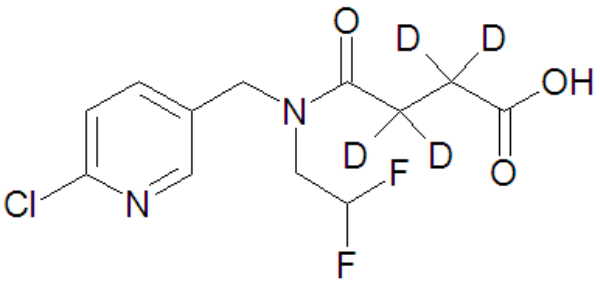


Code Name: Difluoroacetate-$^{13}C_2$ (BCS-AB60481-$^{13}C_2$)
Molecular Formula: $C_2\ H\ F_2\ O_2\ .\ Na$
Molecular Weight: 120.0 g/mol



Appendix 1 Test and Reference Substances (Cont'd)

Code Name:	BYI 02960-succinamide-d_4
Molecular Formula:	C_{12} H_{13} Cl F_2 N_2 O_3
Molecular Weight:	311 g/mol



Appendix 2 Extraction Scheme for Water Samples

Transfer 10mL of water into a suitable stoppered container.

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Fortify the recovery samples at the desired fortification level with the appropriate mixed standard solution prepared in acetonitrile/water

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Add, by pipet, 50uL of the 500ng/mL internal standard solution

↓

Analyze by LC/MS/MS analysis.