## 2. BACKGROUND

BY! 02960 is an insecticide currently being developed by Bayer CropScience with potential uses in several crops.

The purpose of this study was to conduct an independent laboratory validation in surface water for Bayer method RV-005-W12-01 "Analytical Method for the Determination of Residues of BY! 02960 and its Metabolites Difluoroacetic Acid (DFA), BY! 02960-Succinamide And BY! 02960-Azabicyclosuccinamide In Water Using LC/MS/MS" [1].

This study was performed in accordance with US EPA Residue Chemistry Test Guidelines, OPPTS 860.1340 Residue Analytical Method, August 1996 [2], and US EPA Ecological Effects Test Guidelines, OPPTS 850.7100 Data Reporting for Environmental Chemistry Method [3], Federal Insecticide, Fungicide and Rodenticide Act (FIFRA); Good Laboratory Practice Standards; Final Rule, 40 CFR, Part 160.[5].

## 3. EXPERIMENTAL DETAILS

Study initiation date: May 15, 2012
Experimental Completion Date: June 4, 2012

### 3.1 Test Substances

The test substances for this study were BY! 02960 and its metabolites DFA, BY! 02960-Succinamide and BY! 02960-Azabicyclosuccinamide. See Appendix 3 for complete nomenclature.

### 3.2 Analytical Reference Substances

The test substances also served as the analytical reference substances. See Appendix 3 for complete nomenclature and reference information for the reference substances as well as the internal standards. The test and reference substances were stored in a freezer until used to prepare fortification and calibration solutions. All stock solutions were stored in a freezer when not in use. All fortification and calibration solutions were stored in a refrigerator and given 3 months' expiration date.

### 3.3 Test System

The test system was surface water obtained from Upper Merion Township Park located at 174 W Valley Forage Road, King of Prussia, PA 19406. The sample was logged into the JRF sample system and stored in a refrigerator when not in use. The expiration date of 1 month was given to the surface water sample. The sample was used on the day it was collected.

### 3.4 Preparation of Standard Solutions

Stock Standard Solutions
Stock standards of 5 mg/mL for each analyte were prepared by dissolving all of the received reference material (purity adjusted) in acetonitrile. For example, 0.01048 g of BYI02960 with purity of 99.4% was received. The volume of ACN 2.083 mL was added to the standard vial. The resulting concentration of BYI 02960 was (0.01048 x 99.4%) x 1000/2.083 = 5.0 mg/ml.

The standard solution of 100 IJg/mL for each analyte was prepared by pipetting 0.2 mL of 5 mg/mL stock standard into a 20mL vial and mixing with 9.8 mL of ACN.

Fortification Standards
The fortification standards 0.1/0.5 and 1.0/5.0 IJg/mL were prepared as written in the method.

Calibration standards:
A total of 8 calibration standards were prepared as written in the method. Because of the instrument sensitivity to detect 0.1 and 0.05 ppb for DFA, an additional calibration standard 0.25/1.25 was prepared as suggested by the Sponsor.

### 3.5 Method Summary

The analytical set included a reagent blank, two unfortified control samples, five samples fortified at the LOQ and five samples fortified at 1Ox LOQ.

The LOQ was 1.0 ng/mL for BYI 02960 and DFA; and 5.0 ng/mL for BYI 02960-Succinamide and BYI 02960-Azabicyclosuccinamide.

The 20 mL water samples were amended with an isotopic internal standard and an aliquot analyzed by LC/MS/MS for BYI 02960, DFA, BYI 02960-Azabicyclosuccinamide and BYI 02960-Succinamide.

### 3.6 LC/MS/MS Conditions

Five separate analyses were performed on the LC/MS/MS for each sample:

1. BYI 02960 quantitation method and BYI 02960-Azabicyclosuccinamide quantitation/confirmatory methods (Luna column)
2. BYI 02960-Succinamide quantitation/confirmatory methods (Luna column)
3. DFA quantitation method (Allure column)
4. BYI 02960 confirmatory methods (HILIC Column)
5. DFA confirmatory methods (HILIC Column)

3.6.1 LC Conditions for BYI 02960 (Quantitation). BYI 02960-Azabicyclosuccinamide (Quantitation and Confirmatory) analysis

Mobile Phase A: 0.1% formic acid in water
Mobile Phase B: Acetonitrile.
HPLC column: Phenomenex Luna™2.51J C18(2) 50 x 2 mm Column
Injection volume: 40 lJL
Column Temperature: ambient

| Time (min) | Mobile Phase B % | Flow rate (A & B) ullmin |
|---|---|---|
| 0.0 | 5 | 300 |
| 0.5 | 5 | 300 |
| 4.0 | 40 | 300 |
| 6.0 | 95 | 300 |
| 6.1 | 5 | 300 |
| 9.0 | 5 | 300 |

BYI 02960 and BYI 2960-Azabicyclosuccinamide was analyzed with the MS/MS in positive polarity mode. The approximate retention times are below:

| Analyte | Approx. Retention Time (min) |
|---|---|
| BYI 2960-Azabicyclosuccinamide | 2.7 |
| BYI 02960 | 4.2 |

3.6.2 LC Conditions for BYI 02960-Succinamide (Quantitation and Confirmatory) analysis

Mobile Phase A: 0.1% formic acid in water
Mobile Phase B: Acetonitrile.
HPLC column: Phenomenex Luna™2.51J C18(2) 50 x 2 mm Column
Injection volume: 5 iJL
Column Temperature: ambient

| 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 | 5 | 700 |

BYI 2960-Succinamide was analyzed with the MS/MS in negative polarity mode. The approximate retention time is below:

| Anal te | Approx. Retention Time (min) |
|---|---|
| BYI 02960-Succinamide | 2.3 |

3.6.3 LC Conditions for DFA (Quantitationl analysis

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 11m particle size.
Injection volume: 50 iJL

| Time (min) | Mobile Phase B % | Flow rate (A &B) ul/min |
|---|---|---|
| 0.0 | 1 | 500 |
| 1.0 | 1 | 500 |
| 2.2 | 1 | 1000 |
| 3.0 | 80 | 1000 |
| 3.01 | 80 | 500 |
| 4.0 | 80 | 500 |
| 4.1 | 1 | 500 |
| 7.5 | 1 | 500 |

The approximate retention time is below:

| Analyte | Approx Retention Time (min) |
|---|---|
| DFA | 2.9 |

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

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 11m particle size
Injection volume: 10 iJL

| 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 | 95 | 400 |

| Analyte | Approx Retention Time (min) |
|---|---|
| BYI 02960 | 4.3 |
| DFA | 5.5 |

3.6.5 Mass Spectrometer Conditions

The following conditions were used on an API 4000 instrument.

Positive ion mode for BYI 02960 and BYI 02960-Azabicyclosuccinamide

| | |
|---|---|
| CUR: Curtain Gas | 30 |
| CAD: Collision Gas | 8 |
| GS1: ion Source Gas 1 | 60 |
| GS2: ion Source Gas 2 | 60 |
| TEM: Source Temp. | 700°C |
| IHE: Interface Heater | ON |
| IS: ion Transfer Voltage | 5500 |

Negative ion mode for BYI 02960-Succinamide and DFA

| | |
|---|---|
| CUR: Curtain Gas CAD: | 30 for SUCCA, 35 for DFA |
| Collision Gas GS1: ion | medium |
| Source Gas 1 | BYI 02960-Succinamide 60, DFA 70 |
| GS2: ion Source Gas 2 | BYI 02960-Succinamide 50, DFA 60 |
| TEM: Source Temp. | 700°C |
| IHE: Interface Heater | ON |
| IS: ion Transfer Voltage | BYI 02960-Succinamide (-4000), DFA (-4500) |

Mass Spectrometer Data Collection

The daughter ions used in this method were chosen due to their optimum sensitivity on the ABSciex API 4000 instrument used for this study.

| Analyte Name | Polarity | Q1 Mass (amu) | Q3 Mass (amu) | Dwell (msec) | DP | EP | CE | CXP |
|---|---|---|---|---|---|---|---|---|
| BYI02960 (Quant+Conf) | + | 289 | 126 | 50 | 71 | 10 | 30 | 22 |
| BYI02960 IS[1] | + | 295 | 130 | 50 | 66 | 10 | 29 | 6 |
| DFA (Quant+Conf) | - | 95 | 51 | 200 | -35 | -10 | -18 | -5 |
| DFAIS | - | 97 | 52 | 200 | -40 | -10 | -20 | -5 |
| BY! 02960-succinamide (MRM1)-Quant | - | 305 | 99 | 50 | -55 | -10 | -20 | -15 |
| BY! 02960-succinamide (MRM 2)-Conf | - | 305 | 285 | 50 | -55 | -10 | -14 | -1 |
| BY! 02960-succinamide IS | - | 309 | 289 | 50 | -50 | -10 | -14 | -5 |
| BY! 02960- Azabicyclosuccin-amide (MRM 1)-Quant | + | 289 | 108 | 50 | 51 | 10 | 25 | 18 |
| BY! 02960- Azabicyclosuccin-amide (MRM Z)-Conf | + | 289 | 189 | 50 | 51 | 10 | 11 | 10 |

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

### 3.7 Calculations

The standards were fit to the linear equation: $Y = MX + B$

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

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

The % recovery was calculated using the following equation:

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

Where: R = ng/ml of target analyte found in fortified sample
C = apparent residue in the control sample
T = theoretical ng/ml in fortified sample

An example calculation for BYI 02960 from sample UTC+LOQ-2 is shown below. This sample was fortified at 1 ng/mL for BYI 02960 and DFA; and at 5 ng/ml for

BYI 02960-Azabicyclosuccinamide and BYI 02960-Succinamide. The chromatogram used in this example is presented in Appendix 2 (Chromatogram 5). The example shown below is for the calculation of BYI 02960 residues. BYI 02960-Succinamide, BYI 02960-Azabicyclosuccinamide and DFA residues are calculated in a similar fashion.

The following data was obtained from the analyst software for the sample:

| Native Peak Area | IS Peak Area | y | M | B |
|---|---|---|---|---|
| 194271 | 524772 | 0.3702 | 0.3700 | $1.053 \times 10^{3}$ |

The slope and intercept were obtained from the calibration curve generated by Analyst, and is presented in Appendix 1 (Figure 1). The calibration points were weighted 1/x to provide better fit near the limit of detection

From the above equations:

$$\text{BYI 02960 found} = \frac{(0.3702 - 1.053 \times 10^{3})}{0.3700} = 0.9977 \text{ ng/ml}$$

Therefore sample UTC+LOQ-2 contains 0.998 ng/ml BYI 02960.

No residues of BYI 02960 were found in the control. Therefore, the % recovery was calculated using the following equation:

$$\%\text{Recovery} = \frac{(0.998-0.0) \times 100}{1.0} = 99.8\%$$

## 4. RESULTS

### 4.1 LC/MS/MS Verification

The API 4000 was optimized for each compound, and the conditions used for this study are recorded in the raw data and presented in Section 3.6 of this report. Calibration standards were injected prior to the method validation trial to determine the analyte retention times and instrumental sensitivity. Because an interference peak was detected during the standard test run, the LC conditions were modified to separate the interference peak from the BYI02960 peak in the analysis of BYI 02960 and BYI 02960-Azabicyclosuccinamide.

### 4.5 Problems Encountered with the Method

1) The calibration curve for BYI 02960-Succinamide did not fit a linear regression when the suggested calibration standards were used (from 0.25 to 100 ppb). Better linearity was obtained when the dynamic range was reduced (from 0.5 to 50 ppb).

2) It was difficult to achieve the sensitivity for DFA (0.05 ppb) as described in the method. The lowest standard of DFA that can be detected in the LC/MS/MS was 0.25 ppb, which was prepared additionally.

3) Due to the interference peak found in the analysis of BYI 02960, the LC conditions were modified to separate the interference peak from the BYI 02960.

### 4.6 Recommended changes to the method

It is recommend to use the secondary transition (289>90) for the confirmatory analysis for BYI 02960 when conducting the quantitative analysis.

### 4.7 Time requirement to perform method

It takes approximately 1 hour to prepare a set of 13 samples (surface water). Analysis on the LC/MS/MS takes about 3 days.

### 4.8 Contact with sponsor

The sponsor was contacted during the course of the study. The following critical issues were discussed with the sponsor:

1. Prior to the experiment, the sponsor was contacted to clarify the method, such as the instrument sequence, confirmatory data handling and LC flow rate, etc.

2. On 5/23/2012, the interference peak in the standard solution was reported to the sponsor and examples of the chromatograms were sent to the sponsor. The sponsor decided to ship a new standard. On 5/30/2012, the same interference peak was found in the new standard and reported to the sponsor. The decision was made to use the standard but modify the LC conditions for separation.

3. The poor linearity of BYI 02960-Succinamide was reported to the sponsor on 5/24/2012 during the standard test run on the LC/MS/MS system. The sponsor suggested using smaller injection volume and agreed that the highest standard could be eliminated.

4. The sponsor was informed on 5/24/2012 about the sensitivity of DFA. The LC/MS/MS was not able to detect the standard 0.1 and 0.05 ppb. The sponsor suggested eliminating 0.1 and 0.05 standards and adding a 0.25 ppb standard in the calibration curve.