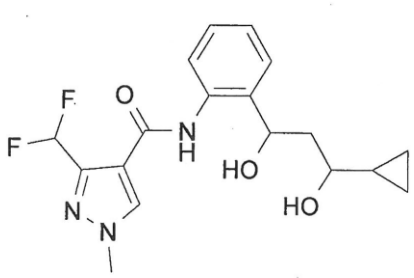
SYN548031:

Chemical Name: Unknown
IUPAC: Not Assigned
CAS: Unknown

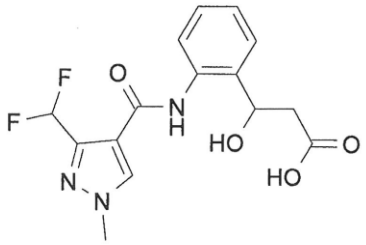
Structural Formula:



CAS No.: Not Assigned
Molecular Weight: 364.147 g/mol (Exact Mass)
Source: Syngenta Crop Protection, LLC
Purity: 85.4%
Batch ID: DAH-XXXV-62
Receipt Date: February 27, 2014
Expiration Date: November 30, 2015
Storage: Refrigerated (2 °C to 8°C)

SYN548032:

Chemical Name: Unknown
IUPAC: Not Assigned
CAS: Unknown
Structural Formula:



CAS No.: Not Assigned
Molecular Weight: 339.103 g/mol (Exact Mass)
Source: Syngenta Crop Protection, LLC
Purity: 93.6%
Batch ID: DAH-XXXV-63
Receipt Date: February 27, 2014
Expiration Date: October 31, 2015
Storage: Refrigerated (2 °C to 8°C)

3.2 Test System

The test system evaluated in this study was surface water collected from a rice paddy (Study No. 80290, Reference 4). This matrix was chosen because they are representative of the matrices for which the method was designed.

The control sample used in this study was provided by R & D Research at the request of the Sponsor. It was received at ABC Laboratories cold on March 28, 2014. Upon receipt, the sample was placed in refrigerated storage (typically 2 °C to 8 °C), where it remained pending analysis. The sample was logged in according to ABC Laboratories' SOPs.

3.3 Equipment and Reagents/Supplies

The equipment and reagents/supplies used for the method validation were as outlined in the method. Identical or equivalent equipment and materials were used, as permitted by the method. The equivalent equipment and reagents used were as follows:

3.3.1 Equipment

Balance:	Analytical balance capable of weighing to ±0.01 mg Model XP205DR (Mettler Toledo)
Pipets:	Gilson

General laboratory equipment, such as autosampler vials and caps, disposable Pasteur pipets, glass graduated pipets and glass culture tubes were used for the conduct of this study.

3.3.2 Reagents

Acetonitrile:	Fisher Scientific
Formic Acid:	99+% purity, Fisher
Methanol:	Fisher Scientific
Water:	HPLC, Fisher Scientific

3.3.3 Preparation of Reagents

Reagent solutions were prepared as specified in the method. Solutions were prepared as follows:

1. Formic Acid in Acetonitrile (0.1%)
    1. Prepared by mixing 4000 mL Acetonitrile and 4 mL Formic Acid (99+% purity).

2. Formic Acid in Water (0.1%)
    1. Prepared by mixing 20 L HPLC-grade Water and 20.0 mL Formic Acid (99+% purity).

3. Methanol:Acetonitrile:HPLC Water (1:1:1)
    1. Prepared by mixing 4000 mL HPLC-grade Water, 4000 mL Acetonitrile and 4000 mL Methanol.

4. Methanol:Acetonitrile:Water (1:1:2)
    1. Prepared by mixing 4000 mL Acetonitrile and 4000 mL Methanol and 8000 mL HPLC-grade Water.

5. Acetonitrile:Water Matrix 20:80 (v/v)
    1. Prepared by mixing 200-mL of HPLC water matrix and 50-mL Acetonitrile.

3.4 Preparation of Standard Solutions

The preparation of standard solutions used for this study is described below. The solutions were stored as recommended in the method when not in use (refrigerated, 2 °C to 8 °C).

3.4.1 Stock Standard Solutions

SYN548031

Approximately twelve milligrams (11.72 mg) of the analytical standard were accurately weighed, into a 14-mL glass amber vial, and 10 mL of acetonitrile was added and mixed well. The resulting concentration of the solution was 1.00 mg/mL (1000 µg/mL) (corrected for purity).

SYN548032

Approximately eleven milligrams (10.71 mg) of the analytical standard were accurately weighed into a 14-mL glass amber vial, and 10 mL of acetonitrile was added and mixed well. The resulting concentration of the solution was 1.00 mg/mL (1000 µg/mL)(corrected for purity).

3.4.2 Fortification/Intermediate Standard Solutions

100 µg/mL:	1.00 mL of each 1000 µg/mL stock standard solution was transferred to a 14-mL glass amber vial. The appropriate volume of acetonitrile was added to bring the final volume to 10 mL and mixed well.
10 µg/mL:	1.00 mL of the 100 µg/mL mixed standard solution was transferred to a 14-mL glass amber vial. The appropriate volume of acetonitrile was added to bring the final volume to 10 mL and mixed well.

1.0 µg/mL: 0.100 mL of the 100 µg/mL mixed standard solution was transferred to a 14-mL glass amber vial. The appropriate volume of acetonitrile was added to bring the final volume to 10 mL and mixed well.

0.10 µg/mL: 0.010 mL of the 100 µg/mL mixed standard solution was transferred to a 14-mL glass amber vial. The appropriate volume of acetonitrile was added to bring the final volume to 10 mL and mixed well.

0.010 µg/mL: 0.010 mL of the 10 µg/mL mixed standard solution was transferred to a 14-mL glass amber vial. The appropriate volume of acetonitrile was added to bring the final volume to 10 mL and mixed well.

3.4.3 HPLC (Calibration) Standard Solutions

2.50 ng/mL: 0.250 mL of a 0.10-µg/mL mixed standard solution were transferred to a 14-mL glass amber vial. The appropriate volume of acetonitrile:water matrix (20:80, v/v) was added to bring the final volume to 10 mL and mixed well.

1.00 ng/mL: 0.100 mL of a 0.10-µg/mL mixed standard solution were transferred to a 14 mL glass amber vial. The appropriate volume of acetonitrile:water matrix (20:80, v/v) was added to bring the final volume to 10 mL and mixed well.

0.500 ng/mL: 0.050 mL of a 0.10-µg/mL mixed standard solution were transferred to a 14-mL glass amber vial. The appropriate volume of acetonitrile:water matrix (20:80, v/v) was added to bring the final volume to 10 mL and mixed well.

0.250 ng/mL: 0.025 mL of a 0.10-µg/mL mixed standard solution were transferred to a 14-mL glass amber vial. The appropriate volume of acetonitrile:water matrix (20:80, v/v) was added to bring the final volume to 10 mL and mixed well.

0.100 ng/mL: 0.100 mL of a 0.010-µg/mL mixed standard solution were transferred to a 14-mL glass amber vial. The appropriate volume of acetonitrile:water matrix (20:80, v/v) was added to bring the final volume to 10 mL and mixed well.

0.075 ng/mL:	0.075 mL of a 0.010-µg/mL mixed standard solution were transferred to a 14-mL glass amber vial. The appropriate volume of acetonitrile:water matrix (20:80, v/v) was added to bring the final volume to 10 mL and mixed well.
0.050 ng/mL:	0.050 mL of a 0.010-µg/mL mixed standard solution were transferred to a 14-mL glass amber vial. The appropriate volume of acetonitrile:water matrix (20:80, v/v) was added to bring the final volume to 10 mL and mixed well.

3.5 Analytical Method

The analytical method independently validated in this study was Syngenta Analytical Method GRM023.13A, entitled "SYN548031 and SYN548032 – Residue Method (GRM023.13A) for the Determination of Degradates SYN548031 and SYN548032 in Ground and Surface Water", dated March 3, 2014. See Appendix1 for complete text of the method. The following is a summary of that method.

To summarize, a 10 mL sample of water was prepared for analysis by diluting with acetonitrile. Samples were analyzed by LC-MS/MS using ESI negative mode. Residue quantification was carried out using external standard calibrations. The limit of quantification of the method is 0.10 ppb for both analytes in surface water.

The method was used as written.

Residue calculations were performed as specified in the analytical method, using the multi-point calibration procedure. Calculations were conducted using an Applied BioSystems/ MDS Sciex Analyst Software, (version 1.5.1) to create a standard curve based on linear regression and Microsoft Excel® 2010. All standards injected were used to generate the standard curve. The regression functions were used to calculate a best-fit line (from a set of standard concentrations in ng/mL versus peak response) and to determine concentration of the analyte found during sample analysis from the calculated best-fit line. Weighting (1/x) was used in construction of the standard curve. Equations used for calculation of residues and example calculations can be found in Appendix 4. The calculation spreadsheets can be found in Appendix 5.

3.6 Instrumentation Conditions

All samples were analyzed by HPLC employing tandem mass spectrometric (MS/MS) detection. Optimum operating conditions were established prior to analysis (as permitted by the method). Typical conditions were as follows:

Operating Conditions

Instrument: AB Sciex API 5000 MS/MS detector System. The system was controlled and data processed by Applied BioSystem/MDS Sciex Analyst Software (Version 1.5.1).

Analytical column: 50-mm × 2.1-mm i.d., Waters XBridge C18, 2.5 µm particle size

Mobile phase: Fisher water, Fisher acetonitrile, EMD formic acid

Component A: 0.1% Formic Acid in water

Component B: 0.1% Formic Acid in acetonitrile

Gradient:

Time (min.)	% A	% B
0 - 3.50	95.0	5.0
3.50 - 4.10	0.0	100.0
4.10 - 5.00	95.0	5.0

Divert Valve: Not Applicable

Flow rate: 0.30 mL/minute

Interface: TIS (Turbo Ion Spray)

Ionization mode: Negative (

Acquisition mode: MRM

Source Temperature: 500 °C

Curtain Gas: Nitrogen @ setting of "40"

Collision Gas: Nitrogen @ setting of "5.00"

Injection volume: 10 µL

Column temperature: 40 °C

Resolution: Q1-Unit, Q3-Unit (Note: Unit is equivalent to medium)

Transitions Monitored:

	Ion, *m/z* Q1	Q3	Time, *ms*	CE,*v*	
SYN548031:	364	175	75	–27	(quantitation)
SYN548032:	338	278	75	–23	(quantitation)

Retention times:

Analyte	Retention Time
SYN548031	~2.6 minutes
SYN548032	~2.3 minutes

The detection method utilized was LC/MS/MS employing electrospray (TIS) interface in the negative mode on a triple quadrupole instrument. The instrument was tuned by infusing the analytes into a TIS (turbo ion spray) source, then creating a tune file to maximize the response of each analyte using the TIS source. The acquisition method was adjusted to maximize the response of the fragment ions detected. The ion transitions for each analyte are shown in the table below:

Additional detector settings are shown in the table below:

Parameter	**Setting**
Nebulizer (GS1):	60
Auxillary Gas (GS2):	40
Ion Spray Voltage:	4500 (-)

The instrument was operated in the MS/MS (MRM) negative ion mode for quantitative analysis. Single transition chromatograms for each analyte were integrated and the peak areas used for quantitation. Quantitation was performed using a single transition for each matrix.

Calibration/Sample Analysis

A standard curve consisting of both analytes was prepared by injecting constant volumes of calibration standards at specified concentrations. Constant volume injections were used for sample extracts as well. A standard was injected every 1-3 sample injections. All standard injections were used to construct the standard curve.

3.7 Fortification Procedures

Surface water samples were fortified for procedural recovery purposes with the addition of 0.1 mL of a 0.010-µg/mL mixed standard solution to a 10.0 mL aliquot of water sample which produced a 0.10 ppb fortification for both analytes, representative of the LOQ. Addition of 0.1 mL of a 0.10-µg/mL mixed standard solution to a 10.0 mL aliquot of water sample produced a 1.0 ppb fortification for both analytes, representative of 10 × LOQ.

Matrix	Sample Type	Fortifying Compounds	Fortification Level	No. of Samples
Surface Water	Reagent Blank	none	N/A	1
	Control	none	N/A	2
	Fortified control	SYN548031	0.10 ppb (LOQ)	5
			1.0 ppb (LOQ)	5
	Fortified control	SYN548032	0.10 ppb (LOQ)	5
			1.0 ppb (LOQ)	5

3.8 Modifications, Interpretations, and Critical Steps

The analytical method was run as written.

3.9 Statistics

Statistical methods used were limited to calculations of the mean, range, standard deviation relative standard deviation, and regression analysis. The software programs, Microsoft Excel® 2010 and Applied BioSystems/MDS Sciex Analyst software (version 1.5.1), were employed to develop all regression analysis and statistical data.

APPENDIX 4 Example Calculations

Equations

Calculations for instrumental analysis were conducted using a software application to create a standard curve based on linear regression. All standards injected were used in the generation of the standard curve. The regression functions were used to calculate a best fit line (from a set of standard concentrations in ng/mL versus peak response) and to determine concentrations of the analyte found during sample analysis from the calculated best fit line. For this study, the correlation coefficient for each calibration curve was equal to or greater than 0.990 (r^2 equal to or greater than 0.98). 1/x weighting was used.

The equation used for the least squares fit is:

$$y = mx + b$$

where:

y	=	peak response
m	=	slope
x	=	ng/mL found for peak of interest
b	=	y-intercept

The calculations used for ng /mL found was:

where:

(b)	=	y-intercept of the standard curve
(m)	=	slope of the curve obtained from standard curve

1. The calculations for ppb found and percent recovery (for fortified samples) were:

The amount of analyte (in ppb) found in the sample was calculated according to the following equation:

where:

Conversion Factors = 1000 mL/L conversion factor

Sample concentration = 0.0008 L/mL

a. The percent recovery in fortified control samples is calculated as follows:

Example Calculations

All analytes were calculated in an identical manner. Only examples of SYN548031 residue calculations were provided and thus serve to illustrate the calculations for both analytes in surface water.

1. ABC ID# 81081-002, Surface water, SYN548031, Set# 1, TK0069553.LA.Source.1,
   Control (Figure 1-2):

2. ABC ID# 81081-004, Surface water, SYN548031, Set# 1, TK0069553.LA.Source.1 + 0.100 ppm, (Figure 1-3):