TITLE

In House Laboratory Validation of Analytical Method for the Determination of AE F088657 and Its Metabolites: AE 0542291 and AE B107137 in Soil and Sediment by LC/MS/MS

- Final Report -

Test Substance
AE F088657, AE 0542291 and AE B107137

Data Requirement
US EPA Residue Chemistry Test Guidelines
OPPTS 860.1340 Residue Analytical Method

US EPA Ecological Effects Test Guidelines OCSPP 850.6100 Data Reporting for Environmental Chemistry Methods

PMRA: DACO 7.2.2 Residue Analytical Method

Guidance document on residue analytical methods; SANCO/825/00 rev. 7

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2019-09-10

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Study ID
RADC0091

Activity ID
RADC0091

GENERAL INFORMATION

Study Title:	In House Laboratory Validation of Analytical Method for the Determination of AE F088657 and Its Metabolites: AE 0542291 and AE B107137 in Soil and Sediment by LC/MS/MS
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Bayer CropScience Study No.:	RADC0091
Test Item/ Reference Standards:	AE F088657, AE 0542291 and AE B107137
Test Systems:	Soil and sediment
Study Initiation Date: Experimental Start Date: Experimental Completion Date	23 February 2018 01 March 2018 24 March 2018
Personnel Involved In Study:	Tehane Ali Scientist I

1.0 SUMMARY

The purpose of this study was to perform an In House Laboratory Validation (IHLV) of analytical method DC-002-S18-01 entitled "Analytical Method for the Determination of Diflufenican and Its Metabolites: AE 0542291 and AE B107137 in Soil and Sediment by LC/MS/MS" [1].

The in house laboratory validation of this method was be performed by Bayer CropScience Laboratories at 2 T.W. Alexander Drive, Research Triangle Park, NC 27709.

Bayer CropScience validated the method according to the guidelines issued by The US EPA - Residue Chemistry Test Guidelines OPPTS 860.1340 [2], US EPA Ecological Effects Test Guidelines OCSPP 850.6100 [3], PMRA: DACO 7.2.2 Residue Analytical Method [4] and Guidance document on residue analytical methods; SANCO/825/00 rev. 7 [5]. This study was conducted to satisfy guideline requirements and in compliance with EPA FIFRA Good Laboratory Practice Standards, 40 CFR Part 160 [6].

The method was validated using sub-samples of bulk untreated soil from two AE F088657 terrestrial field dissipation studies. The soils selected were from New York (Study Number MEDC0006 [7]) and Georgia (Study Number MEDC0007 [8]).
The sediment validation was performed on one sediment sample, obtained from Louisburg, North Carolina (Study Number MEDC0014 [9]).
Samples from each test system were stored at room temperature prior to fortification. After fortification, the soil extracts were stored in a refrigerator until they were prepared for analysis using Analytical Method DC-002-S18-01.

In brief, the method used for analysis was as follows:

An analytical method was developed to determine the residues of AE F088657 and its metabolites AE 0542291 and AE B107137 in soil and sediment.

AE F088657 residues were extracted from soil and sediment using a mixture of 400:100:3 (v/v/v) acetonitrile/water/acetic acid with microwave extraction. An isotopic internal standard was added to the sample. The samples were analyzed for AE F088657, AE 0542291 and AE B107137 by LC/MS/MS. Quantification was based on a comparison of peak areas with those of known standards.

This method was developed to analyze residues of AE F088657 and its metabolites in soil and sediment at a target limit of quantification (LOQ) of 1.5 ng/g

Identification and quantitation of the active substance was performed by high performance liquid chromatography using tandem MS/MS detection (LC-MS/MS) in the Multiple Reaction Monitoring (MRM) mode using the following MRM transitions:

Analyte	Quantitation MRM	Confirmation MRM
AE F088657	395.0 → 266.0	395.0 → 246.0
AE 0542291	283.2 → 255.9	283.2 → 246.0
AE B107137	284.3 → 246.0	284.3 → 266.0

The limit of quantitation (LOQ) for each single analyte was 1.5 ng/g in soil.

Apparent residues in control samples were below the method LOD. The correlation between the injected amount of substance and the detector response was linear for standards ranging from 0.1 ng/mL to 50 ng/mL, with correlation coefficients of >0.99 for both the soil and sediment samples for the three test items.

Samples were fortified at the 1.5 ng/g (LOQ) and 10ng/g (10x LOQ) for AE F088657 and its metabolites AE 0542291 and AE B107137.

2.0 INTRODUCTION

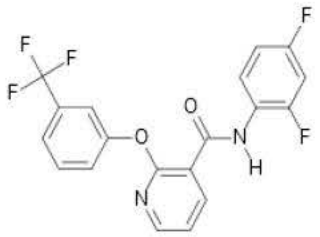
The purpose of this study was to validate analytical method DC-002-S18-01 entitled "Analytical Method for the Determination of AE F088657 and Its Metabolites: AE 0542291 and AE B107137 in Soil and Sediment by LC/MS/MS" [1].
This study was conducted to satisfy guideline requirements described in the US EPA - Residue Chemistry Test Guidelines OPPTS 860.1340 [2], US EPA Ecological Effects Test Guidelines OCSPP 850.6100 [3], PMRA: DACO 7.2.2 Residue Analytical Method [4] and Guidance document on residue analytical methods; SANCO/825/00 rev. 7 [5]. In addition, this study will be conducted in compliance with EPA FIFRA Good Laboratory Practice Standards, 40 CFR Part 160 [6].

3.0 MATERIALS AND METHODS

3.1 TEST ITEMS/REFERENCE SUBSTANCES

Only sufficiently characterized and certified substances were used as reference items.

AE F088657:

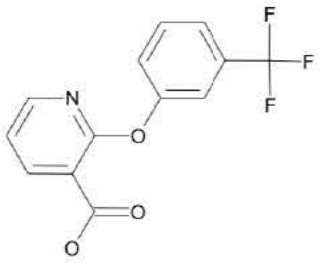


Common Name:	Diflufenican
CAS Name:	N-(2,4-Difluorophenyl)-2-[3-(trifluoromethyl)phenoxy]-3-pyridinecarboxamide
Molecular Formula:	$C_{19}H_{11}F_5N_2O_2$
Molecular Weight:	394.29 g/mol
CAS No.:	83164-33-4
ID No.:	K-1412
Purity:	99.8%
Expiration Date:	04/21/2024
Storage Conditions:	Frozen

AE 0542291:

Code Name:	AE 0542291
CAS Name:	2-(3-Trifluoromethoxy) phenoxy-3-pyridinecarboxamide
Molecular Formula:	$C_{13}H_9F_3N_2O_2$
Molecular Weight:	282.22 g/mol
ID No.:	K-2230
Purity:	99.0%
Expiration Date:	07/18/2022
Storage Conditions:	Frozen

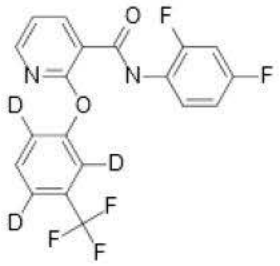
AE B107137:



Common Name:	Diflufenican acid
CAS Name:	2-[3-(Trifluoromethyl) phenoxy]-3-pyridinecarboxylic acid
Molecular Formula:	$C_{13}H_8F_3NO_3$
Molecular Weight:	283.20 g/mol
ID No.:	K-2238
Purity:	98.9%
Expiration Date:	07/12/2022
Storage Conditions:	Frozen

3.2 INTERNAL STANDARDS

AE F088657-d_3:

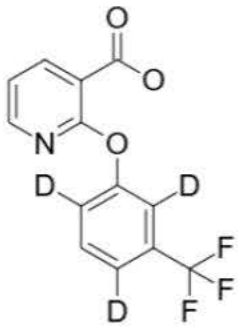


Connon Name:	Diflufenican-d_3
Molecular Formula:	$C_{19}H_8D_3F_5N_2O_2$
Molecular Weight:	397.32 g/mol
ID No.:	K-2236
Purity:	99.9%
Expiration Date:	01/03/2027
Storage Conditions:	Frozen

AE 0542291-d_3:

Code Name:	AE 0542291-d_3
Molecular Formula:	$C_{13}D_3H_6F_3N_2O_2$
Molecular Weight:	285.24 g/mol
ID No.:	K-2256
Purity:	100.0%
Expiration Date:	06/20/2027
Storage Conditions:	Frozen

AE B107137-d_3:



Common Name:	Diflufenican acid-d_3
Molecular Formula:	$C_{13}D_3H_5F_3NO_3$
Molecular Weight:	286.22 g/mol
ID No.:	K-2257
Purity:	100.0%
Expiration Date:	06/20/2027
Storage Conditions:	Frozen

Characterization data for the reference substances (analytical standards) and internal standards are maintained by Bayer CropScience.

The test/reference substances (analytical standards) and internal standard used in this study were procured from Bayer CropScience and stored as directed on "Analytical Standards Chain of Custody" and "Certificate of Analysis" documents. All solutions made from the reference substances (analytical standards) were stored according to the method.

3.3 TEST SYSTEMS

The analytical method DC-002-S18-01 was evaluated in two different soils and one sediment sample all of which originated in the United States. The two soils were collected as sub-samples of bulk untreated samples from soil dissipation studies MEDC0006 (New York) and MEDC0007 (Georgia). The sediment sample was collected in MEDC0014 (North Carolina) in an aerobic aquatic metabolism study. The samples were classified according to USDA specifications and the characteristics are summarized below:

Description	New York Soil (Study MEDC0006)	Georgia Soil (Study MEDC0007)	North Carolina Sediment (Study MEDC0014)
pH ($CaCl_2$ solution)	4.5	6.2	5.3
pH (aqueous solution)	4.8	6.6	5.9
Organic carbon (%)[a]	2.1	0.56	1.3
Organic matter (%)	3.6	0.96	2.2
Cation Exchange Capacity (CEC) (meq/100 g dry soil)	7.6	4.2	3.6
Maximum water holding capacity (g water/100 g dry soil)	NR	NR	36.7
USDA Textural Description- Hydrometer method (Fraction %)			
Clay (<0.002 mm)	11	5	6.5
Silt (0.002 – 0.050 mm)	56	10	9.3
Sand (0.050 - 2.000 mm)	33	85	84.2
Soil type	Slit Loam	Sandy Loam	Sand

[a] Organic carbon = Organic matter ÷ 1.72
NR = Not reported

3.4 SAMPLE PREPARATION AND EXTRACTION

Bayer Analytical Method DC-002-S18-01 was used for the analysis of AE F088657, 0542291 and AE B107137 in soil and sediment. Soil and sediment samples were prepared as follows:

Samples were weighed into glass jars and were then taken up in an extraction solvent mixture of acetonitrile/water/acetic acid (400/100/3, v/v/v). The samples were then extracted for 15 minutes using a microwave extractor. Once the extraction was complete, an internal standard solution was added and the sample mixed. The samples were cooled to ambient temperatures and centrifuged for five minutes at >12000 rpm to remove fine particles of soil. An aliquot of the supernatant was then analyzed by LC/MS/MS.

Two product ions per analyte were selected for monitoring; one ion mass transition served as quantitation and the other ion mass transition was used for confirmation. The

mass transitions for both the quantitation as well as the confirmation for all substances are presented below:

Analyte	Quantitation[a]	Confirmation[a]
AE F088657	395.0 → 266.0	395.0 → 246.0
AE 0542291	283.2 → 255.9	283.2 → 246.0
AE B107137	284.3 → 246.0	284.3 → 266.0

[a] All masses reported in amu

3.5 INSTRUMENTATION

Standard Method for all Analytes

The HPLC conditions employed were as follows:

HPLC Systems: Thermo Vanquish Pumps UHPLC
Thermo Vanquish Column Compartment UHPLC
Thermo Vanquish sampler UHPLC

HPLC analytical Column: Phenomenex Luna C18 (2)-HST 100A 50 mm x 2.0 mm 2.5 µm particle

Mobile phase:

Mobile Phase A: 9:1 Water: Methanol (v/v) with 10mM Ammonium Formate and 120uL formic acid per liter
Mobile Phase B: 1:9 Water: Methanol (v/v) with 10mM Ammonium Formate and120uL formic acid per liter

Gradient and flow rate:

Time (min)	Mobile Phase B%	Flow rate µL/min
0.00	0	500
0.50	0	500
3.00	100	500
3.50	100	500
3.51	0	500
5.00	0	500
5.00	Stop Run	500

Divert Valve: Programmed to divert LC flow from column to waste (bypassing detector) from 0 to 0.5 minutes and again from 4 to end. LC flow is directed to detector during the 0.5 to 4 minute window.

Column Temperature: 60 °C

Injection Volume: 10 µL
Retention Times:

Analyte	Approx Retention Time (min)
AE B107137	2.6
AE 0542291	2.8
AE F088657	3.5

The MS/MS conditions employed were as follows:

MS System: Thermo Scientific TSQ Quantiva LC/MS/MS System with LCQuan Software (ver. 3.0.26.0)

Ionization Mode: API interface-electrospray ionization mode
Sheath/Aux/Ion Sweep Gas: Nitrogen
Collision Gas: Argon
Spray Voltage (V): 4000 (positive)
Vaporizer Temp(C): 400
Sheath Gas Pressure: 35
Aux Gas Pressure: 25
Ion Sweep Gas Pressure: 3.0
Ion Transfer Tube Temp(C): 350
Q2 Collision Gas (mT): 1.5

MS/MS Parameters for the determination and confirmation of AE F088657, AE B107137 and AE 0542291 were as follows:

Analyte Name	Polarity	Q1 Mass (amu)	Q3 Mass (amu)	Resolution for Q1MS (v)	Resolution for Q3MS (v)	Source Fragmentation (v)	Collision Energy (v)
AE B107137	Pos	284.3	246.0	0.7	0.4	5	28
AE B107137-2	Pos	284.3	266.0	0.7	0.4	5	20
AE B107137 IS	Pos	287.2	248.0	0.7	0.4	5	29
AE B107137 IS-2	Pos	284.2	268.0	0.7	0.4	5	21
AE 0542291	Pos	283.2	255.9	0.7	0.4	5	20
AE 0542291-2	Pos	283.2	246.0	0.7	0.4	5	28
AE 0542291 IS	Pos	286.3	268.0	0.7	0.4	5	20
AE 0542291 IS-2	Pos	286.3	248.0	0.7	0.4	5	28
AE F088657	Pos	395.0	266.0	0.7	0.4	5	23
AE F088657-2	Pos	395.0	246.0	0.7	0.4	5	32
AE F088657-IS	Pos	398.0	268.0	0.7	0.4	5	25
AE F088657-IS-2	Pos	398.0	248.0	0.7	0.4	5	32

Example LC/MS/MS conditions are presented in Appendix 4.

3.6 CALCULATIONS

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 LCQuan Software (ver. 3.0.26.0) using linear regression with 1/x weighting.

Residue concentrations were determined using calibration curves which were generated after each analysis using LCQuan 3.0 software using linear regression with 1/x weighting.

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

The example shown below (Sample No. 18DC-GA-SLLOQ-001) is for the calculation of AE F088657 residues. AE 0542291 and AE B107137 residues are calculated in a similar fashion.

After regression coefficients were calculated, the residue in ng/g was determined. The ng/g of AE F088657 in soil was calculated using the following equation,

$$\text{AE F088657 found (ng/g)} = \frac{(Y-B) \times D}{M}$$

$$\text{Where Dilution Factor (D)} = \frac{\text{Initial volume } (V_1)}{\text{Initial sample wt. (W)}} \times \frac{\text{Final dilution volume } (V_3)}{\text{Aliquot taken } (V_2)}$$

Where: $W = 25$ g
$V_1 = 50$ mL
$V_2 = 1$ mL
$V_3 = 1$ mL

Native Peak Area	IS Peak Area	Y	M	B
434372	1860042	0.234	0.329	-0.0197

The slope and intercept were obtained from the calibration curve generated by LCQuan, and example is presented in Appendix 3. The calibration points were weighted 1/x to provide better fit near the limit of detection

From the above equations:

$$\text{AE F088657 found} = \frac{(0.234 + 0.0197) \times 2}{0.329} = 1.54 \text{ ng/g}$$

Therefore sample 18DC-GA-SLLOQ-001contains 1.54 ng/g AE F088657. This result, using rounded numbers, agrees with the result shown in the raw data.

The percent recovery was calculated as follows:

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

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

Therefore, for sample 18DC-GA-SLLOQ-001,

R = 1.54 ng/g
C = 0.0 ng/g
T = 1.5 ng/g

$$\text{\% Recovery} = \frac{(1.54 - 0.0)}{1.5} \times 100 = 103\%$$

Remark: Example calculations shown above were performed using the LC/MS/MS software LCQuan (3.0.26.0). 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.

4.8 TIME REQUIREMENTS

A single analyst completed sample sets consisting of 15 samples in two to three hours with HPLC-MS/MS analysis performed overnight.

4.9 PROTOCOL/SOP/METHOD DEVIATIONS

Protocol deviation 1 was generated during the study.

5.0 CONCLUSION

Bayer CropScience in the U.S. successfully independently validated Bayer CropScience analytical method "Analytical Method for the Determination of AE F088657 and Its Metabolites: AE 0542291 and AE B107137 in Soil and Sediment by LC/MS/MS "[1]. The method was validated on the first validation set.

The method was demonstrated to be suitable for the determination of all targeted analytes in two separate soils collected from sites in New York (NY), Georgia (GA) and one sediment sample from North Carolina (NC). A LOQ of at least 1.5 ng/g was demonstrated for each analyte in each matrix evaluated. In all of the samples tested, none of the parent compound or any of the metabolite were found in the untreated control samples.

6.0 ARCHIVING

All raw data associated with this study are retained along with the protocol, protocol amendments and the final report under Notebook Number RADC0091 at Bayer CropScience, 2 T. W. Alexander Drive, Research Triangle Park, NC 27709.

7.0 REFERENCES

1. DC-002-S18-01, Murphy, I., An Analytical Method for the Determination of Residues of Diflufenican and its metabolites AE 0542291 and AE B107137 in Soil and Sediment Using LC/MS/MS, 2018
2. US EPA Residue Chemistry Test Guidelines OPPTS 860.1340, Residue Analytical Method, August 1996
3. US EPA Ecological Effects Test Guidelines OCSPP 850.6100 Environmental Chemistry Methods and Associated Independent Laboratory Validation, Jan 2012
4. PMRA: DACO 7.2.2 Residue Analytical Method
5. Guidance document on residue analytical methods; SANCO/825/00 rev. 7, European Commission, Directorate General Health and Consumer Protection, 2004-03-17.

6. U.S. Environmental Protection Agency. 1989. Federal Insecticide, Fungicide and Rodenticide Act (FIFRA); Good Laboratory Practice Standards; Final Rule, 40 CFR, Part 160.
7. MEDC0006, Yang, Y., Terrestrial Field Dissipation of AE F088657 in New York Bare Ground Soil, 2017
8. MEDC0007, Yang, Y., Terrestrial Field Dissipation of AE F088657 in Georgia Bare Ground Soil, 2017
9. MEDC0014, Shrestha, S., [Difluorophenyl-UL-^{14}C] AE F088657: Aerobic Aquatic Metabolism in Two US Water/Sediment Systems, Ongoing Study