Title

An Analytical Method for the Determination of Residues of AE F088657 and its metabolites AE 0542291 and AE B107137 in Water Using LC/MS/MS

--Analytical Method--

Test Substance
AE F088657

Guideline Number

OECD Guidance Document on Pesticide Residue Analytical Methods, Series on Testing and Assessment Document 72 and Series on Pesticides: Document 39, August 2007 (OECD Guideline, ENV/JM/MONO (2007) 17, Aug 13, 2007)

PMRA Residue Chemistry Guidelines, Regulatory Directive 98-02, Section 3, Residue Analytical Method, June 1998

Completion Date

2019-02-20

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Bayer Method Number
DC-004-W19-01

Activity ID
RADC0097

Bayer Method DC-004-W19-01

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1.0 SUMMARY

An analytical method was developed to determine the residues of diflufenican (AE F088657) and its metabolites AE 0542291 and AE B107137 in water.

Residues of diflufenican and its metabolites are determined by amending an aliquot of water with isotopic internal standards followed by analysis of diflufenican, AE 0542291 and AE B107137 by LC/MS/MS. Quantification is based on a comparison of peak areas with those of known standards.

This method was developed to analyze residues of diflufenican and its metabolite in water at a target limit of quantification (LOQ) of 0.05 ng/mL (ppb), but can be adjusted as required.

2.0 BACKGROUND

Diflufenican is a selective herbicide in the class pyridinecarboxamide (F1 HRAC (Group 12)). The mode of action is inhibition of carotenoid biosynthesis at the phytoene desaturase step (PDS). The analytical method presented in this report is designed to measure residues of diflufenican and its metabolites AE 0542291 and AE B107137 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.
- Adjustable pipettors and tips.
- Phenomenex Luna C18(2)-HST 100 A, 50 mm x 2.0 mm 2.5 µm particle size (Part No: 00B-4446-B0)
- ABSciex 5500 chromatograph/mass spectrometer (LC-MS/MS) equipped with electrospray ionization (ESI) interface, Shimadzu HPLC pumps, Shimadzu oven and a PAL autosampler, and Analyst 1.6.2 data collection software (ABSciex)

4.0 REAGENTS AND CONSUMABLES

Functional equivalents may be substituted

- Acetonitrile (Optima Grade, Fisher Part No. A996-4)
- Ammonium formate (Acros Organics No: 401152500)

- Formic acid (Optima LC/MS; Fisher Part No. A117-50)
- Methanol (Optima grade; Fisher Part No. A-454-4)
- Water (Optima Grade; Fisher Part No. W7-4)
- 9:1 water:methanol (v/v) with 10 mM ammonium formate and 120 µL per 1000 mL formic acid. Mix 450 mL water, 50 mL methanol, 315 mg ammonium formate, and 60 µL of formic acid together.
- 1:9 water:methanol with 10 mM ammonium formate and 120 µL per 1000 mL formic acid. Mix 50 mL water, 450 mL methanol, 315 mg ammonium formate, and 60 µL of formic acid together.
- HPLC vials and caps (2 mL, National Scientific, Part Nos. C4011-5W and C4011-55)
- 120-mL graduated bottle with lined cap (Fisher Part Nos. 02911455A and 02911388)

5.0 PREPARATION OF STANDARD SOLUTIONS

The native reference standards used in this method are: diflufenican, AE 0542291 and AE B107137. The isotopic internal standards (IS) required for this method are: diflufenican-d_3, AE 0542291-d_3 and AE B107137-d_3. These standards may be obtained from Bayer CropScience, 2 T. W. Alexander Drive, Research Triangle Park, North Carolina, 27709. 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.

Volumetric glassware and calibrated pipets should be used in the preparation of all analytical standards. Corrections for standard purities should be applied when expressing standard concentrations.

5.1 Primary Standard Solutions

Native reference standards and internal standard primary solutions are prepared from the reference standards as shown below (Table 1 and 2).

Table 1. Primary reference (native) standard solution preparation

Native Reference Standards	Weight (mg)	Volume (mL)	Solvent	Final Concentration (µg/mL)
Diflufenican	~10	25.0	ACN	~400
AE 0542291	~10	25.0	ACN	~400
AE B107137	~10	25.0	ACN	~400

Table 2. Primary internal standard solution preparation

Isotopic Internal Standards	Weight (mg)	Volume (mL)	Solvent	Final Concentration (µg/mL)
Diflufenican-d_3	~5	25.0	ACN	~200
AE 0542291-d_3	~5	25.0	ACN	~200
AE B107137-d_3	~5	25.0	ACN	~200

Primary solutions should be stored in a freezer when not in use.

5.2 Secondary Standard Solutions

Mixed secondary reference and internal standard solutions are prepared from the primary standard solutions as shown below. Take the appropriate aliquot of each of the primary standard solutions to give the required mixed secondary standard concentration.

Table 3. Secondary mixed reference standard solution preparation

Compound	Primary Standard Concentration (µg/mL)	Aliquot from Primary Standard (mL)	Final Volume (mL)	Mixed Secondary Standard Final Concentration (µg/mL)	Solvent
Diflufenican	~400	~1.25	50.0	10.0	ACN
AE 0542291	~400	~1.25			
AE B107137	~400	~1.25			
Diflufenican-d_3	~200	~0.125	50.0	0.5	ACN
AE 0542291-d_3	~200	~0.125			
AE B107137- d_3	~200	~0.125			

Additional secondary standard solutions are prepared as shown below.

Table 4. Additional secondary mixed native secondary standard solution preparation

Concentration of Mixed Native Standard Solution used for dilution (µg/mL)	Aliquot Taken (mL)	Dilution Volume (mL)	Final Concentration of Mixed Native Secondary Standard Solution (µg/mL)	Solvent
10.0	5.0	50.0	1.0	ACN
1.0	5.0	50.0	0.1	ACN
0.1	5.0	50.0	0.01	ACN
0.1	0.2	50.0	0.0004	ACN

Table 5. Additional mixed internal standard solution preparation

Concentration of Mixed Internal Standard Solution used for dilution (µg/mL)	Aliquot Taken (mL)	Dilution Volume (mL)	Final Concentration of Mixed Native Secondary Standard Solution (µg/mL)	Solvent
0.5	0.4	50.0	0.004	ACN

All secondary standards should be stored in a refrigerator when not in use.

5.3 Calibration Standards

Note: Additional standards may be prepared when necessary; however, the concentration of internal standard must remain the same in all calibration standards. Calibration solutions are dissolved in 1:4 ACN/H_2O (v/v).

Table 6. Calibration standard solutions

Concentration of Native Standard Solution used for dilution (µg/mL)	Concentration of Internal Standard Solution used for dilution (µg/mL)	Aliquot Native Taken (mL)	Aliquot Internal Standard Taken (mL)	Dilution Volume (mL)	Concentration of Native in Calibration Solution (ng/mL)	Concentration of IS in Calibration Solution (ng/mL)
1.0	0.5	0.100	0.05	50	2.0	0.5
1.0	0.5	0.050	0.05	50	1.0	0.5
0.1	0.5	0.250	0.05	50	0.50	0.5
0.1	0.5	0.125	0.05	50	0.25	0.5
0.1	0.5	0.050	0.05	50	0.10	0.5
0.01	0.5	0.250	0.05	50	0.05	0.5
0.01	0.5	0.100	0.05	50	0.02	0.5

Calibration standards should be stored in a refrigerator when not in use.

6.0 EXTRACTION PROCEDURE

6.1 Sample Preparation

1. Shake the sample well.

2. Pour ~5 to 10 mL aliquot of each sample into a labeled scintillation vial; use this aliquoted sample for filing the LC vial. DO NOT put transfer pipets, micropipettes, etc. into the original sample bottle in order to minimize possible contamination of the original sample.

3. Transfer, by pipet, a 1.0 mL aliquot of sample into a HPLC vial.

   Remark: Add the corresponding amount of each analyte for the fortified recovery sample (e.g. 0.125 mL of a 0.0004 µg/mL analyte mixture at LOQ level).

4. Add 0.125 mL of the 0.004 µg/mL mixed internal standard solution to the HPLC vial.

5. Cap and mix sample. The sample is ready 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 a minimum of five 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.

Step 3. Repeat Step 1.

Step 4. When necessary analyze additional samples and calibration standard solutions. Always finish the procedure with a set of calibration solutions

7.2 HPLC Conditions

Note: The analyst should optimize chromatographic conditions to obtain satisfactory chromatography. As the HPLC column ages, the retention times of the analytes may change.

Mobile Phase A:	9:1 water:methanol (v/v) with 10 mM ammonium formate and 120 µL per 1000 mL formic acid
Mobile Phase B:	1:9 water:methanol (v/v) with 10 mM ammonium formate and 120 µL per 1000 mL formic acid
Oven:	40 ºC
Pre column (optional):	none
HPLC Column:	Phenomenex Luna C18(2)-HST 100A 50 mm x 2.0 mm 2.5 µm particle (Part # 00B-4446-B0)
Injection Volume:	90 µL (adjust as needed)

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

Analyte	Approx Retention Time (min)
AE B107137	3.2
AE 0542291	3.4
Diflufenican	4.0

7.3 Mass Spectrometer Conditions

The MS/MS instrument is operated in the multiple reaction monitoring mode (MRM). Precursor ions are selected and product ions created by collision-induced dissociation.
Two product ions per analyte are listed in Table 7, one product ion (MRM-transition) serving for quantitation and the second ion to be used, only if required, to confirm the presence of any detected residues.

Note: The following recommended conditions were used on an ABSciex 5500 instrument but the analyst should optimize the mass spectrometer conditions to obtain satisfactory system response prior to use.

Detector: AB Sciex 5500 Triple Quadrupole Tandem Mass Spectrometer

Scan Type:	MRM
Curtain Gas (CUR):	35
Collision Gas (CAD):	9
Ion Spray Voltage (IS):	5500
Temperature (TEM):	600
Ion Source Gas 1 (GS1):	70
Ion Source Gas 2 (GS2):	80

Table 7. MS/MS Parameters for the determination and confirmation of Diflufenican, AE B107137 and AE 0542291

Analyte Name[a]	Polarity	Q1 Mass (amu)	Q3 Mass (amu)	Time (msec)	CE	CXP	DP	EP
AE B107137-Q	Pos	284.2	266.0	35	27	20	65	10
AE B107137-C	Pos	284.2	238.0	35	37	20	65	10
AE B107137 IS-Q	Pos	287.2	268.0	35	27	20	65	10
AE B107137 IS-C	Pos	287.2	240.0	35	37	20	65	10
AE 0542291-Q	Pos	283.0	265.9	35	27	20	65	10
AE 0542291-C	Pos	283.0	246.0	35	33	20	65	10
AE 0542291 IS-Q	Pos	286.0	267.9	35	27	20	65	10
AE 0542291 IS-C	Pos	286.0	248.0	35	33	20	65	10
Diflufenican-Q	Pos	395.0	266.0	35	23	20	65	10
Diflufenican-C	Pos	395.0	246.0	35	57	20	65	10
Diflufenican-IS-Q	Pos	398.0	268.0	35	23	20	65	10
Diflufenican-IS-C	Pos	398.0	248.0	35	57	20	65	10

[a] Q is MRM transition for quantitation MRM and C for the confirmatory MRM transition

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 Analyst 1.6.2 software using linear regression with 1/x weighting.

The standards were fit to the linear equation:

Y = MX + B with 1/x 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/mL was determined using the following equation,

$$\text{Residue (ng/mL)} = \left(\frac{Y - B}{M}\right)$$

Analyst software was used to calculate the amount of diflufenican, AE 0542291 and AE B107137 in ng/mL for each sample and the percent recovery for the fortified samples.

Residue levels beyond the calibration curve: In some cases, an unknown sample contains residues at a level above the calibration curve. If so, the preferred strategy is to extend the calibration curve to cover the unknown sample, if this is not an option contact the development laboratory for instructions on how to proceed

8.1 Recovery Experiments

Recovery (fortification) experiments may be performed as needed to monitor method efficiency and reproducibility. Recovery experiments are intended to be used for data collection methods or establishing and validating method efficiency and are prepared by adding a known amount of native standard solution to a sample aliquot and preparing the sample for analysis as described in Section 6.

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

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

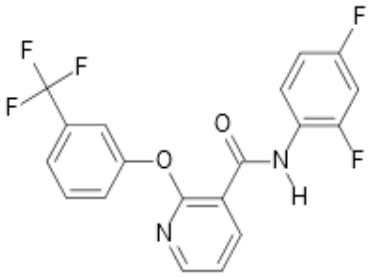
Where: R = ng/mL of target analyte found in fortified (recovery) sample
S = ng/mL of target analyte found in control sample, real or apparent
T = theoretical ng/mL in fortified sample

Recoveries are determined by analyzing fortified control samples alone or in conjunction with a sample set. Recovery samples are fortified prior to extraction at the LOQ of 0.05ng/mLor other appropriate level with fortification solutions. Calculate the final residue for the control (S) and fortified control (R) samples.

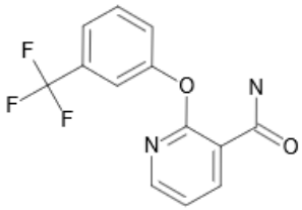
Appendix 1 Reference Substances

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.

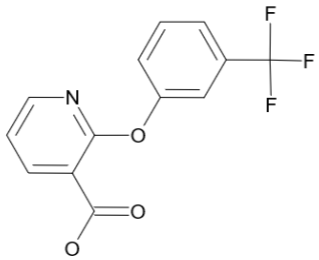
Code Name:	Diflufenican
Synonym:	AE F088657
Mol-ID	156
Molecular Formula:	$C_{19}H_{11}F_5N_2O_2$
Molecular Weight:	394.29 g/mol
Chemical Structure:	



Code Name:	AE 0542291
Molecular Formula:	$C_{13}H_9F_3N_2O_2$
Mol-ID	5251
Molecular Weight:	282.22 g/mol
Chemical Structure:	

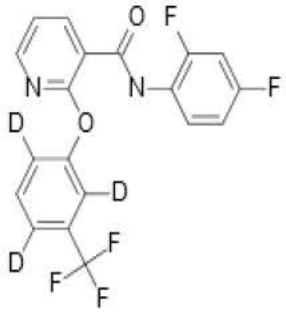


Code Name:	AE B107137
Synonym:	MB 38181
Mol-ID	5252
Molecular Formula:	$C_{13}H_8F_3NO_3$
Molecular Weight:	283.20 g/mol
Chemical Structure:	

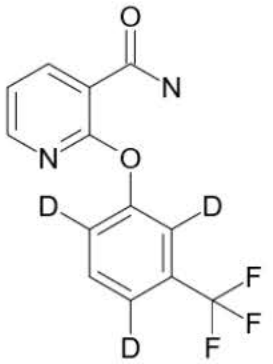


Appendix 1 **Continued**

Code Name: Diflufenican-d3
Mol-ID 27729
(Isotopic Internal Standard of parent molecule)
Molecular Formula: $C_{19}H_8D_3F_5N_2O_2$
Molecular Weight: 397.32 g/mol
Chemical Structure:



Code Name: AE 0542291-d_3
Mol-ID 27855
Molecular Formula: $C_{13}D_3H_6F_3N_2O_2$
Molecular Weight: 285.24 g/mol
Chemical Structure:



Code Name: AE B107137-d_3
Mol-ID 27856
Molecular Formula: $C_{13}D_3H_5F_3NO_3$
Molecular Weight: 286.22 g/mol
Chemical Structure: