Title

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

-Analytical Method-

Test Substance

Diflufenican

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

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Completion Date

2018-02-06

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Bayer Method Number

DC-002-S18-01

Activity ID

RADC0091

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1.0 SUMMARY

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

Residues of diflufenican and its metabolites are extracted from soil and sediment using a mixture of 400:100:3 (v/v/v) acetonitrile/water/acetic acid with a microwave extraction. An isotopic internal standard is added to the sample. The samples are analyzed for 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 metabolites in soil and sediment at a target limit of quantification (LOQ) of 1.5 ng/g, but can be adjusted as required.

2.0 BACKGROUND

Diflufenican is a selective herbicide in the class phenoxynicotinanilides (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 soil and sediment 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.
- Microwave, Milestone Ethos-EX
- Phenomenex Luna C18(2)-HST 100 A, 50 mm x 2.0 mm 2.5 µm particle size (Part No: 00B-4446-B0)
- Vortex
- Sonicator
- Thermo Scientific TSQ Quantiva chromatograph/mass spectrometer (LC/MS/MS) equipped with LCQuan data collection software (Thermo Scientific).

4.0 REAGENTS AND CONSUMABLES

Functional equivalents may be substituted

- Acetic acid glacial (Fisher Part No. A38-500)
- 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)
- 400:100:3 (v/v/v) acetonitrile/water/acetic acid. Combine 400 mL acetonitrile, 100 mL water, and 3 mL of glacial acetic acid. Mix well.
- 9:1 Water:Methanol (v/v) with 10mM Ammonium Formate and 120uL per 1000mL Formic Acid. Mix 450 mL water, 50 mL methanol, 315 mg ammonium formate, and 60 uL of formic acid together.
- 1:9 Water:Methanol with 10mM Ammonium Formate and 120uL per 1000mL Formic Acid. Mix 50 mL water, 450 mL methanol, 315 mg ammonium formate, and 60 uL of formic acid together.
- Fisherbrand 125mL 4oz glass jars (Part No. 02-911-455)
- HPLC vials and caps (2-mL, National Scientific, Part Nos. C4011-5W and C4011-55)
- 60 mL Clear Vials Boro with Septa (Thermo Part No. S236-0060)

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) standard 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. Primary reference (native) standard solution preparation

Native Reference Standards	Weight (mg)	Volume (mL)	Solvent	Final Concentration (µg/mL)
Diflufenican	~10	50.0	ACN	~200
AE 0542291	~5	25.0	ACN	~200
AE B107137	~5	25.0	ACN	~200

Table 2. Primary internal standard solution preparation.

Isotopic Internal Standards	Weight (mg)	Volume (mL)	Solvent	Final Concentration (µg/mL)
Diflufenican -d_3	~5	50.0	ACN	~100
AE 0542291-d_3	~2.5	25.0	ACN	~100
AE B107137- d_3	~2.5	25.0	ACN	~100

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	~200	~1.25	25.0	10.0	ACN
AE 0542291	~200	~1.25			
AE B107137	~200	~1.25			
Diflufenican-d_3	~100	~2.5	25.0	10.0	ACN
AE 0542291-d_3	~100	~2.5			
AE B107137- d_3	~100	~2.5			

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	2.5	25.0	1.0	ACN
1.0	2.5	25.0	0.1	ACN
0.1	2.5	25.0	0.01	ACN

Table 5. Additional mixed internal standard solution preparation.

Concentration of Internal Standard Mixed Solution used for dilution (µg/mL)	Aliquot Taken (mL)	Dilution Volume (mL)	Final Concentration of Mixed Internal Standard Secondary Standard Solution (µg/mL)	Solvent
10.0	2.5	25.0	1.0	ACN

All secondary standards should be stored in a freezer 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 9:1 (v/v) water/methanol.

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	1.0	0.50	0.05	10.0	50	5.0
1.0	1.0	0.20	0.05	10.0	20	5.0
0.1	1.0	0.50	0.05	10.0	5.0	5.0
0.1	1.0	0.20	0.05	10.0	2.0	5.0
0.01	1.0	0.50	0.05	10.0	0.50	5.0
0.01	1.0	0.20	0.05	10.0	0.20	5.0
0.01	1.0	0.10	0.05	10.0	0.10	5.0

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

6.0 EXTRACTION PROCEDURE

6.1 Sample Preparation

Soil samples from each horizon should be milled and thoroughly mixed prior to analysis. Add about 2kg of dry ice to each frozen soil sample and transfer the whole sample into a hammer mill. Collect the milled sample in a 5 gallon bucker or other suitable container.

1. . Mix the contents of the bucket on a bucket tumber for ~5 minutes. Transfer an aliquot of the homogenized soil into doubled plastic bags or other suitable container, and store the sample at -20 ± 5°C until the last traces of dry ice
have sublimed.

2. Seal and label the bags or containers appropriately.

3. Maintain the homogenized soil under freezer conditions, -20 ± 5°C.

6.2 Sample Extraction

Fortified recovery samples are prepared by spiking a known level of the appropriate standard(s) into a sample. A control sample should also be prepared, using the same samples as the fortified sample, to determine the residue levels, real or apparent, found in this sample and the % recovery calculated as described in Section 8.1.

1. Weigh 25 ± 0.25 grams of the homogenized matrix into a 4 ounce jar.

   Remark : Add the corresponding amount of each analyte for the fortified recovery sample (e.g. 0.375 mL of a 100 µg/L analyte mixture at LOQ level).

2. Add ~50 mL of 400:100:3 (v/v/v) acetonitrile/water/acetic acid to each sample.

3. Cap the jar, but do not fully close to avoid pressure build up.

4. Place samples into the microwave extractor. If you have more than 10 samples, adjust the power used in step 6.

5. Switch on the magnetic stirrer or confirm that samples will be stirred during extraction.

6. Extract for 15 minutes: From 0-3 minutes at 400 W (for 10 samples; ambient temperature to approx. 70°C) and from 3-15 minutes at 110 W (approx. 70°C). If more than 10

samples are extracted in the microwave, increase the wattage in the 0-3 minutes phase by 40 W for each additional sample.

7. Add 0.250 mL of the 1.0 µg/mL mixed internal standard solution and mix.

8. Transfer approximately 1-2 mL aliquot of supernatant to a micro-centrifuge tube and centrifuge for 5 minutes at >12000 g to remove fine particles. Transfer aliquot to an LC vial.

9. Analyze by 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 10mM Ammonium Formate and 120uL per 1000mL Formic Acid
Mobile Phase B: 1:9 Water:Methanol (v/v) with 10mM Ammonium Formate and 120uL per 1000mL Formic Acid
Oven: 60 ºC
Pre column (optional): none
HPLC Column: Phenomenex Luna C18(2)-HST 100A 50 mm x 2.0 mm 2.5 µm particle
Injection Volume: 10-20 µL (adjust as needed)

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

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

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 a Finnigan TSQ Quantiva instrument but the analyst should optimize the mass spectrometer conditions to obtain satisfactory system response prior to use.

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

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)	Resolution for Q1MS (v)	Resolution for Q3MS (v)	Source Fragmen-tation (v)	Collision Energy (v)
AE B107137`Q	Pos	284.274	246.018	0.7	0.4	5	28
AE B107137-C	Pos	284.275	266.014	0.7	0.4	5	20
AE B107137 IS-Q	Pos	287.243	248	0.7	0.4	5	29
AE B107137 IS-C	Pos	284.244	268	0.7	0.4	5	21
AE 0542291-Q	Pos	283.243	255.929	0.7	0.4	5	20
AE 0542291-C	Pos	283.244	246	0.7	0.4	5	28
AE 0542291 IS-Q	Pos	286.304	268.014	0.7	0.4	5	20
AE 0542291 IS-C	Pos	286.306	248	0.7	0.4	5	28
Diflufenican-Q	Pos	395.02	266	0.7	0.4	5	23
Diflufenican-C	Pos	395.021	246	0.7	0.4	5	32
Diflufenican-IS-Q	Pos	398.05	268	0.7	0.4	5	25
Diflufenican-IS-C	Pos	398.051	248	0.7	0.4	5	32

[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 LCQuan 3.0 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/g was determined using the following equation,

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

Where Dilution Factor (D) = $\frac{\text{Initial volume } (V_1)}{\text{Initial sample wt. (W)}}$

Where: W = 25 g
V_1 = 50 mL

LCQuan software was used to calculate the amount of diflufenican, AE 0542291 and AE B107137 in ng/mL and ng/g 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 will 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/g of target analyte found in fortified (recovery) sample
S = ng/g of target analyte found in control sample, real or apparent
T = theoretical ng/g 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 1.5 ng/g or other appropriate level with fortification solutions. Calculate the final residue for the control (S) and fortified control (R) samples.

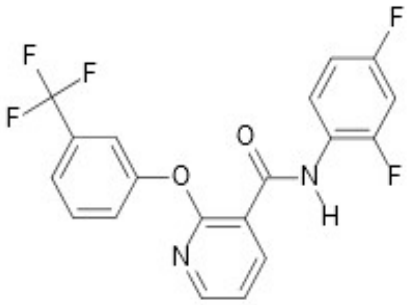
9.0 REFERENCES

No.	Doc. No.	Report No.	**Author(s).** Title. Year.
1	Method-No. 00830	MR-112/03	**Brumhard, B**. Method 00830 for the Determination of Residues of Diflufenican, MB38181, and MB 43625 in soil by HPLC-MS/MS, 2005

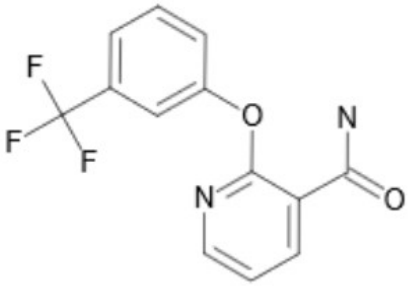
Appendix 1 Reference Substances

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.

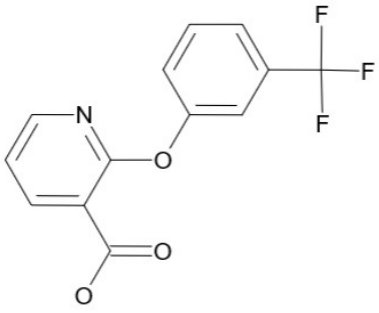
Code Name: Diflufenican
Molecular Formula: $C_{19}H_{11}F_5N_2O_2$
Molecular Weight: 394.29 g/mol
Chemical Structure:



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

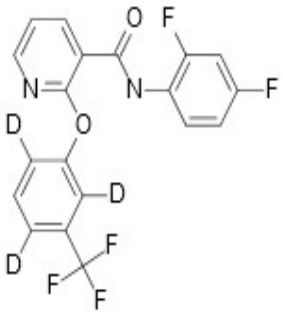


Code Name: AE B107137
Molecular Formula: $C_{13}H_8F_3NO_3$
Molecular Weight: 283.20 g/mol
Chemical Structure:

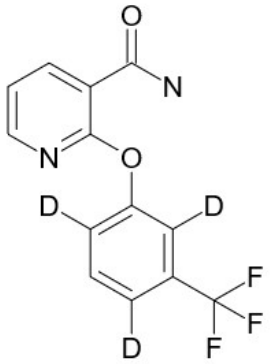


````**Appendix 1 Continued**

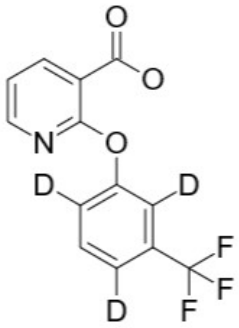
Code Name: Diflufenican-d3
(Isotopic Internal Standard of parent molecule)
Molecular Formula: $C_{19}H_8D_3F_5N_2O_2$
Molecular Weight: 397.32 g/mol
Chemical Structure:



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



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