Bayer Method SE-001-S15-02

An Analytical Method for the Determination of Residues of Metribuzin and its metabolites Metribuzin DADK (AE F149970), Metribuzin DK (AE1344183) and Metribuzin DA (AE B142111) in Soil and Water Using LC/MS/MS

1.0 SUMMARY

An analytical method was developed to determine the residues of Metribuzin and its metabolites Metribuzin DA (DA-Metribuzin), Metribuzin DK (DK-Metribuzin), and Metribuzin DADK (DADK-Metribuzin) in soil and water.

Residues of metribuzin and its metabolites are extracted from soil using a mixture of acetonitrile/water (50/50 v/v) with microwave extraction. The sample is amended with an isotopic internal standard and an aliquot centrifuged, diluted with water is to achieve 4:1 water:acetonitrile and an aliquot analyzed by LC/MS/MS

Residues of Metribuzin and its metabolites are extracted from water by amending the sample with an isotopic internal standard, combining equal parts water sample and methylene chloride. The organic layer is transferred to a culture tube, dried to completeness, reconstituted in 4:1 water:acetonitrile and an aliquot analyzed by LC/MS/MS

The method limit of quantitation (LOQ) in soil samples for Metribuzin and its metabolites DA-Metribuzin, DK-Metribuzin, and DADK-Metribuzin is 10 ng/g.

The method limit of quantitation (LOQ) in water samples for Metribuzin and its metabolites DA-Metribuzin, DK-Metribuzin, and DADK-Metribuzin is 0.5 ng/g.

2.0 BACKGROUND

The analytical method presented in this report is designed to measure residues of Metribuzin and its metabolites DA-Metribuzin, DK-Metribuzin, and DADK-Metribuzin in soil and 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.
- MicroMan pipettors and tips (M250, M50, and M1000).
- Eppendorf 5810 Centrifuge
- Milestone Ethos E Microwave Labstation, equipped with a Terminal 640 Touch Screen Controller and automatic temperature control with fiber optic sensor.
- Phenomenex Kinetex C8 100 mm x 3.0 mm 2.6 µm particle size(Part No: 00D-4497-Y0).
- TurboVap LV
- Vortex
- Sonicator
- ABSciex 6500 chromatograph/mass spectrometer (LC-MS/MS) equipped with electrospray ionization (ESI) interface, Shimadzu HPLC pumps and column oven and a CTC 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)
- Water (Optima Grade; Fisher Part No. W7-4)
- Methylene Chloride (Fisher Part No. D151-4)
- Acetic Acid Glacial (Fisher Part No. A38-500)
- 50/50 v/v acetonitrile/water. Combine 500 mL acetonitrile, 500 mL water. Mix well.
- 4:1 water:acetonitrile. Combine 800 mL water and 200 mL acetonitrile. Mix well.
- 0.2% acetic acid in acetonitrile. Add 2 mL acetic acid to 1000 mL acetonitrile. Mix well.
- 0.2% acetic acid in 9/1 water/acetonitrile. Combine 900 mL water, 100 mL acetonitrile, and 2mL acetic acid. Mix well.
- 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)
- Microcentrifuge Tubes, 2mL (Fisher Part No. 02-681-266)
- Pasture pipets (Fisher Part No. 13-678-20A)
- Culture tubes 13 x 100mm (Fisher Part No. 14-961-27)
- Culture tubes 20 x 150mm (Fisher Part No. 14-961-33)
- Disposable stir bars, 1 x 5/16 (Fisher Part No. 1451394)
- 50 mL conical tubes (Falcon Part No. 352070)

5.0 PREPARATION OF STANDARD SOLUTIONS

Metribuzin, DA-Metribuzin, DK-Metribuzin, and DADK-Metribuzin analytical standards and the isotopic internal standards Metribuzin-D_9, DA-Metribuzin-D_9, DK-Metribuzin-D_9, and DADK-Metribuzin-D_9 are needed. 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.

Class “A” volumetric glassware or calibrated pipets should be used in the preparation of all analytical standards. Primary standards should be stored in a freezer when not in use and all other standard solutions should be stored in a refrigerator when not in use. Solutions should be allowed to warm to room temperature prior to use. Corrections for standard purities should be applied when expressing standard concentrations.

5.1 Primary Standards

Table 1: Representative Scheme for Preparation of Reference Standards and Internal Standard Stock Solutions

No.	Reference Standard	Weight (mg)	Volume (mL)	Solvent	Final Concentration (µg/mL)
1	Metribuzin	~10	100	ACN	100
2	DA-Metribuzin	~10	100	ACN	100
3	DK-Metribuzin	~10	100	ACN	100
4	DADK-Metribuzin	~10	100	ACN	100
5	Metribuzin-IS	~5	50	ACN	100
6	DA-Metribuzin-IS	~5	50	ACN	100
7	DK-Metribuzin-IS	~5	50	ACN	100
8	DADK-Metribuzin-IS	~5	50	ACN	100

NOTE: Corrections for standard purities should be applied when expressing standard concentrations.

5.2 Secondary Standards

Secondary standard solutions were prepared from the stock solutions by appropriate dilution with 50/50 acetonitrile/water (v/v) mixtures. These standards were used for fortification of control samples and preparation of the calibration standards. A representative scheme for preparation of secondary standard solutions is shown in Table 2.

Table 2: Scheme for Preparation of Representative Secondary Standard Solutions

No	Reference Standard	Final Concentration (µg/mL)	Aliquot (mL)	No. of Diluted Standard	Volume (mL)	Solvent
A1	[Metribuzin] [DA-Metribuzin] [DK-Metribuzin] [DADK-Metribuzin]	10.0	5.0 5.0 5.0 5.0	1 2 3 4	50	1:1 ACN:H_2O
A2	[Metribuzin] [DA-Metribuzin] [DK-Metribuzin] [DADK-Metribuzin]	1.0	5.0	A1	50	1:1 ACN:H_2O
A3	[Metribuzin] [DA-Metribuzin] [DK-Metribuzin] [DADK-Metribuzin]	0.10	5.0	A2	50	1:1 ACN:H_2O

Table 3: Scheme for Preparation of Representative Internal Standard Solutions

No	Reference Standard	Final Concentration (µg/mL)	Aliquot (mL)	No. of Diluted Standard	Volume (mL)	Solvent
B1	[Metribuzin-IS] [DA-Metribuzin-IS] [DK-Metribuzin-IS] [DADK-Metribuzin-IS]	10.0	5.0 5.0 5.0 5.0	5 6 7 8	50	1:1 ACN:H_2O
B2	[Metribuzin-IS] [DA-Metribuzin-IS] [DK-Metribuzin-IS] [DADK-Metribuzin-IS]	1.0	5.0	B1	50	1:1 ACN:H_2O
B3	[Metribuzin-IS] [DA-Metribuzin-IS] [DK-Metribuzin-IS] [DADK-Metribuzin-IS]	0.10	5.0	B2	50	1:1 ACN:H_2O

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 4:1 water:ACN.

Table 4: Scheme for Preparation of Representative Calibration Standard Solutions

No.	Reference Standard	Final Concentration (ppb)	Aliquot (mL)	No. of diluted Standard	Diluted to (mL)
C1	[Metribuzin] [DA-Metribuzin] [DK-Metribuzin] [DADK-Metribuzin] / [Metribuzin-IS] [DA-Metribuzin-IS] [DK-Metribuzin-IS] [DADK-Metribuzin-IS]	100 10	0.50 0.05	A1 B1	50
C2	[Metribuzin] [DA-Metribuzin] [DK-Metribuzin] [DADK-Metribuzin] / [Metribuzin-IS] [DA-Metribuzin-IS] [DK-Metribuzin-IS] [DADK-Metribuzin-IS]	50 10	0.25 0.05	A1 B1	50
C3	[Metribuzin] [DA-Metribuzin] [DK-Metribuzin] [DADK-Metribuzin] / [Metribuzin-IS] [DA-Metribuzin-IS] [DK-Metribuzin-IS] [DADK-Metribuzin-IS]	20 10	0.10 0.05	A1 B1	50
C4	[Metribuzin] [DA-Metribuzin] [DK-Metribuzin] [DADK-Metribuzin] / [Metribuzin-IS] [DA-Metribuzin-IS] [DK-Metribuzin-IS] [DADK-Metribuzin-IS]	10 10	0.05 0.05	A1 B1	50
C5	[Metribuzin] [DA-Metribuzin] [DK-Metribuzin] [DADK-Metribuzin] / [Metribuzin-IS] [DA-Metribuzin-IS] [DK-Metribuzin-IS] [DADK-Metribuzin-IS]	5 10	0.25 0.05	A2 B1	50
C6	[Metribuzin] [DA-Metribuzin] [DK-Metribuzin] [DADK-Metribuzin] / [Metribuzin-IS] [DA-Metribuzin-IS] [DK-Metribuzin-IS] [DADK-Metribuzin-IS]	2 10	0.10 0.05	A2 B1	50
C7	[Metribuzin] [DA-Metribuzin] [DK-Metribuzin] [DADK-Metribuzin] / [Metribuzin-IS] [DA-Metribuzin-IS] [DK-Metribuzin-IS] [DADK-Metribuzin-IS]	1 10	0.05 0.05	A2 B1	50

6.0 PROCEDURE

6.1 Sample Extraction and Clean-up for Soil

Appendix 2 shows the analytical scheme for the extraction of metribuzin and its metabolites in soil and water. The detailed stepwise procedure is as follows:

1. Weigh 10 ±0.10 grams of soil into a 125 mL glass jar.

2. Fortify the recovery samples at the desired fortification level with the appropriate mixed standard solution. Allow to sit for ~ 10 minutes.

3. Add 40 mL of 1:1 acetonitrile:water to each sample.

4. Add a magnetic stirrer to each sample and loosely attach the lid in order to minimize the possibility of a pressure build up inside the jar during the microwave extraction.

   Note: The microwave extraction system monitors the reaction temperature using an automatic fiber optic temperature control system. The temperature sensor and Teflon sleeve are directly inserted into one of samples by piercing a hole in one of the lids. It is recommended that the reaction temperature is monitored using the UTC sample.

5. Load the samples onto the microwave carousel. Insert the fiber optic temperature control probe and Teflon sleeve into the UTC sample, and manually rotate the carousel to check that the temperature control probe cable does not catch on any of the samples.

6. Close the microwave door, and program the microwave with the following method:

Step Number	Time Duration	T1 Temperature set point at end of step	E Power Limit (to maintain/ control temperature)	Comments
1	5 min.	70 ℃	≤350 W	Ramp from ambient to 70℃
2	10 min.	70 ℃	≤250 W	Maintain at 70 ℃

   Once the samples have cooled, remove them from the microwave.

7. Add 0.650 mL of the 1 µg/mL metribuzin internal standard solution containing all analytes to each sample (standard solution B2). Reattach the lid and mix well.

8. Take ~1.5 mL aliquot and place in a micro centrifuge tube. Centrifuge at 12,000 RPM for ~2 minute.

9. Aliquot ~1 mL of the supernatant into an HPLC vial and add ~0.625 mL of water.

10. Vortex to mix sample for LC/MS/MS analysis.

6.2 Sample Extraction and Clean-up for Water

1. Weigh 10 ± 0.10 grams of water sample into 50 mL conical tube.

2. Fortify the recovery samples at the desired fortification level with the appropriate mixed standard solution.

3. Add 0.100 mL of the 0.10 µg/mL metribuzin internal standard solution containing all analytes to each sample (standard solution B3) and mix well.

4. Add ~10 mL of methylene chloride and stopper the tube.

5. Mix thoroughly and allow phases to separate.

6. With a pasture pipet, transfer ~10 mL of the bottom organic layer to a culture tube.

7. Dry sample to completeness on a TurboVap set at 50C.

8. Reconstitute in ~1 mL 4:1 water: acetonitrile.

9. Mix thoroughly and transfer to an HPLC vial 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 an aliquot of each of the 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. In the case of over 20 samples, extra standard solutions could be added between sample analyses.

Step 3. Again, analyze an aliquot of each of the calibration standard solutions (and, if necessary, additional standard solutions).

7.2 HPLC Conditions

Note: The analyst should optimize chromatographic conditions to obtain satisfactory chromatography. The following recommended conditions were used on a ABSciex 6500 with a Shimadzu HPLC system.

7.2.1 HPLC Conditions for Metribuzin and Metabolites

Inject a sample aliquot from Section 6.1, Step 10 and Section 6.2, Step 9

Mobile Phase A: 9:1 Water/ACN with 0.2% acetic acid
Mobile Phase B: Acetonitrile with 0.2% acetic acid
Oven: 40 ºC
HPLC column: Phenomenex Kinetex C8 100 mm X 3.0 mm 2.6 µm particle size
Pre column (optional): SecurityGuard ULTRA UHPLC C8
Injection volume: 50 µL (Adjust for LC/MS/MS system being used)

Time (min)	Mobile Phase B %	Flow rate (A &B) µL/min
0.0	20	500
0.20	20	500
6.00	90	500
7.00	90	500
7.10	20	500
8.00	20	500

Analyte	Approx Retention Time (min)
DK-Metribuzin	2.0
DADK-Metribuzin	2.1
DA-Metribuzin	2.7
Metribuzin	2.9

7.3 Mass Spectrometer Conditions

7.3.1 Mass Spectrometer Conditions for Metribuzin and Metabolites

Note: The analyst should optimize the mass spectrometer conditions to obtain satisfactory system response. The following conditions were used on an ABSciex 6500 instrument.

Positive ion mode

Ion Spray Voltage	4000
Temperature (℃)	400
Curtain Gas	20
Collision Gas	9
Ion Source Gas 1	40
Ion Source Gas 2	60

7.4 Mass Spectrometer Data Collection

Note: The analyst should optimize the mass spectrometer data collection to obtain satisfactory system response. As the HPLC column ages, the retention times of the analytes will change. A standard solution should be analyzed before each set of samples to confirm the data collection parameters.

The daughter ions used in this method were chosen due to their optimum sensitivity on the ABSciex 6500 instrument used for this study. The following recommended ion transitions and conditions were example conditions used on an ABSciex 6500 instrument:

Analyte Name	Polarity	Q1 Mass (amu)	Q3 Mass (amu)	EP	Dwell (msec)	CE	CXP	DP
Metribuzin	Pos	215.1	187.1	10	50	25	12	96
Metribuzin IS	Pos	224.1	196.2	10	50	25	14	111
DA-Metribuzin	Pos	200.1	172.1	10	50	25	10	106
DA-Metribuzin IS	Pos	209.1	181.2	10	50	25	18	111
DK-Metribuzin	Pos	185.1	155.9	10	50	23	18	121
DK-Metribuzin IS	Pos	194.1	166.2	10	50	21	12	96
DADK-Metribuzin	Pos	170.1	141.4	10	50	23	24	126
DADK-Metribuzin IS	Pos	179.1	150.1	10	50	25	14	106
Metribuzin Confirmatory	Pos	215.1	145.1	10	50	25	12	96
DA-Metribuzin Confirmatory	Pos	200.1	116.0	10	50	31	12	106
DK-Metribuzin Confirmatory	Pos	185.1	110.5	10	50	27	12	121
DADK-Metribuzin Confirmatory	Pos	170.1	125.7	10	50	25	6	126

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 software (version 1.6.2) using linear regression with 1/x weighting.

The standards were fit to the linear equation:

$$Y = MX + B \text{ with } 1/x \text{ weighting.}$$

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,

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

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

For Soil Where:
W = 10 g
V_1 = 40 mL
V_2 = 1 mL
V_3 = 1.625 mL

For Water Where:
W = 10 g
V_1 = 10 mL
V_2 = 10 mL
V_3 = 1 mL

Analyst software was used to calculate the amount of metribuzin, DA-metribuzin, DK-metribuzin, and DADK-metribuzin in ng/g for each sample and the percent recovery for the fortified samples.

8.1 Fortification Experiments

Note: Fortification experiments may be performed as needed to monitor method efficiency and reproducibility, but are not required when analysis of samples is performed for tolerance enforcement. Fortification experiments are intended to be used for data collection methods or establishing and validating method efficiency.

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

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

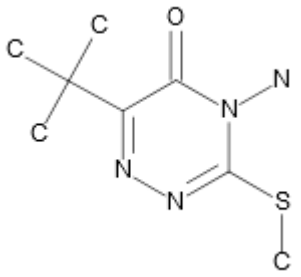
Where: R = ppb of target analyte found in fortified sample
S = ppb of target analyte found in control sample, real or apparent
T = theoretical ppb in fortified sample

Recoveries are determined by analyzing fortified control samples alone or in conjunction with a sample set. Samples may be fortified prior to extraction at the LOQ of 10 ng/g for soil and at the LOQ of 0.5 ng/g for water or other appropriate level with fortification solutions. Calculate the final residue for the control (S) and fortified control (R) samples.

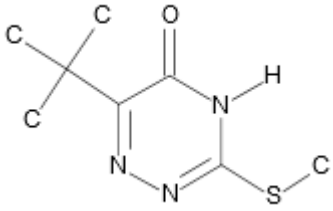
Appendix 1 Test and Reference Substances

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.

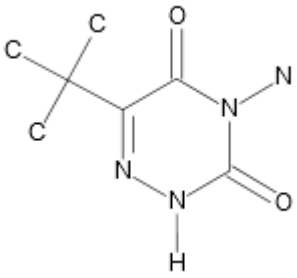
Code Name: Metribuzin
Molecular Formula: $C_8H_{14}N_4OS$
Molecular Weight: 214.29 g/mol



Code Name: Metribuzin DA (AE B142111, Desamino-Metribuzin)
Molecular Formula: $C_8H_{13}N_3OS$
Molecular Weight: 199.27 g/mol

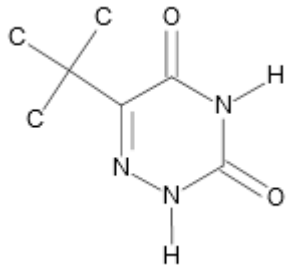


Code Name: Metribuzin DK (AE 1344183, Diketo-Metribuzin)
Molecular Formula: $C_7H_{12}N_4O_2$
Molecular Weight: 184.20 g/mol

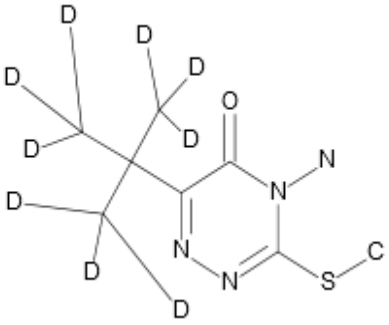


Appendix 1 Test and Reference Substances (Cont'd)

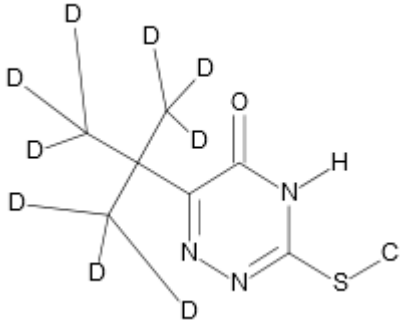
Code Name: Metribuzin DADK (Desamino-diketo-Metribuzin, AE F149970)
Molecular Formula: $C_7 H_{11} N_3 O_2$
Molecular Weight: 169.18 g/mol



Code Name: D_9-Metribuzin
Molecular Formula: $C_8 H_5 D_9 N_4 O S$
Molecular Weight: 223.34 g/mol

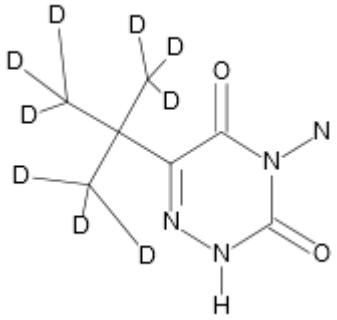


Code Name: D_9-DA-Metribuzin (D_9-DA Sencor)
Molecular Formula: $C_8 H_4 D_9 N_3 O S$
Molecular Weight: 208.33 g/mol

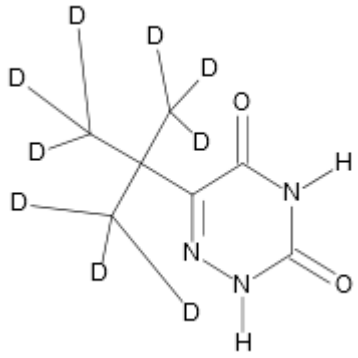


Appendix 1 Test and Reference Substances (Cont'd)

Code Name: D_9-DK-Metribuzin (D_9-DK Sencor)
Molecular Formula: $C_7 H_3 D_9 N_4 O_2$
Molecular Weight: 193.25 g/mol



Code Name: D_9-DADK-Metribuzin (D_9-DADK-Sencor)
Molecular Formula: $C_7 H_2 D_9 N_3 O_2$
Molecular Weight: 178.24 g/mol



Appendix 2A Extraction Scheme for Soil Samples

Weigh a 10 g aliquot of soil into a 125mL glass jar.

↓

Add ~40 mL of acetonitrile:water (1:1).

↓

Microwave extraction. Ramp temperature to 70°C over 5 minutes
then hold at 70°C for 10 minutes.

↓

Add 0.650 mL of the 1000 ng/mL mixed internal standard and mix well.

↓

Transfer ~1.5 mL to a micro centrifuge tube and centrifuge at 12,000 RPM for ~2 minutes.

↓

Transfer ~1 mL of the supernatant to an HPLC vial and add 0.625 mL of water.

↓

Cap and mix sample vial for LC/MS/MS analysis

Appendix 2B Extraction Scheme for Water Samples

Weigh a 10 g aliquot of water into a 50 mL conical tube.

↓

Add 0.100 mL of the 100 ng/mL mixed internal standard and mix well.

↓

Add 10 mL methylene chloride, vortex, and allow phase separation.

↓

Transfer 10 mL of the bottom organic layer to a culture tube.

↓

Dry sample in a TurboVap at 50°C to dryness.

↓

Reconstitute in 1 mL of 4:1 water:acetonitrile.

↓

Mix and transfer to an HPLC vial for LC/MS/MS analysis.