OBJECTIVE AND PURPOSE

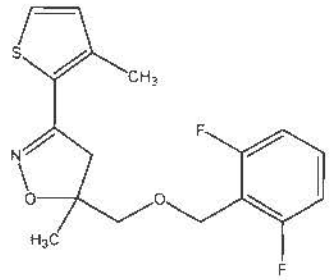
The study was designed to develop and validate the methodology for the extraction and quantification of residues of methiozolin and 2,6-difluorobenzoic acid (DFBA) in grass, turf (thatch), and soil. These methods were developed to use in terrestrial field dissipation studies and to assess freezer storage stability. In addition, this study also assessed the freezer storage stability of residues of methiozolin and DFBA in grass, turf (thatch), and soil.

The six analytical methods, used either in the storage stability study (Study Number 031775) or in the methiozolin terrestrial field dissipation (TFD) studies (Study Numbers 031786, 031812, 031813, and 031942), are listed below:

- Method 031775A – Methiozolin and/or DFBA in soil
  - Used for methiozolin and DFBA freezer storage stability study in soil (Study Number 031775)
  - Used for analysis of methiozolin and DFBA in New York, Georgia, California, and New Jersey TFD studies (Study Numbers 031786, 031812, 031813, and 031942, respectively)
- Method 031775B – Methiozolin in turf (thatch)
  - Used for methiozolin freezer storage stability study in turf (thatch) (Study Numher 031775)
- Method 031775C – Methiozolin and/or DFBA in turf (thatch)
  - Used for DFBA freezer storage stability study in turf (thatch) (Study Number 031775)
  - Used for analysis of methiozolin and DFBA in turf (thatch) in New York, Georgia, California, and New Jersey TFD studies (Study Numbers 031786, 031812, 031813, and 031942, respectively)
- Method 013775D – Methiozolin in grass
  - Used for methiozolin freezer storage stability study in grass (Study Number 031775)
- Method 031775E – Methiozolin in grass
  - Used for analysis of methiozolin in grass in New York, Georgia, California, and New Jersey TFD studies (Study Numbers 031786, 031812, 031813, and 031942, respectively)
- Method 031775F – DFBA in grass
  - Used for DFBA freezer storage stability study in grass (Study Number 031775)
  - Used for analysis of DFBA in grass in New York, Georgia, California, and New Jersey TFD studies (Study Numbers 031786, 031812, 031813, and 031942, respectively)

TEST SUBSTANCES

- **Methiozolin**



Information concerning the test substance is provided below:

Common Name:	Methiozolin
Chemical Name:	5-[[(2,6-difluorophenyl)methoxy]methyl]-4,5-dihydro-5-methyl-3-(3-methyl-2-thienyl)isoxazole
CAS No.:	403640-27-7
Molecular Formula:	$C_{17}H_{17}F_2NO_2S$
Molecular Weight:	337.38 g/mole
Source:	Moghu Research Center
Lot No.:	MRC111001
Purity:	99.71% (by HPLC)
Expiration Date:	September 30, 2016
Storage:	Room temperature

The identity of the test substance was confirmed by comparing the labeling on the material to the shipping paperwork and certificate of analysis. The certificate of analysis is provided in Appendix 2. Solutions made from the test substance were stored frozen at ≤-5 °C. Solubility and stability data for the test substance were the responsibility of the Sponsor. A sample of the test substance has been retained at Ricerca, as specified in 40 CFR 160.195.

- **2,6-Difluorobenzoic acid (DFBA)**

2,6-Difluorobenzoic acid

Information concerning the test substance is provided below:

Chemical Name:	2,6-Difluorobenzoic acid
CAS No.:	385-00-2
Molecular Formula:	$C_7H_4F_2O_2$
Molecular Weight:	158.1 g/mol
Source:	Sigma-Aldrich
Lot No.:	MKBQ3617V
Ricerca Sample Code:	CS_19165
Stated Purity:	94.86%
Storage:	Ambient temperature

This test substance was characterized under GLP by Ricerca. The certificate of analysis is provided in Appendix 2. Solutions made from the test substance were stored frozen at ≤-5 °C. Solubility and stability data for the test substance were the responsibility of the Sponsor. A sample of the test substance has been retained at Ricerca, as specified in 40 CFR 160.195.

TEST SYSTEMS

All test systems (soil, turf (thatch), and grass) used in this study are bulk soil, thatch, and grass control samples sampled at Ricerca study no. 031786 methiozolin terrestrial field dissipation (TFD) study at North Rose, New York test site on 10-28-13 and received at Ricerca on 10-30-13. The soil sample was screened through a 1-mm sieve to remove rocks, sticks, lumps, etc., before use in this study. The turf (thatch) and grass samples were homogenized with Dry Ice in a Robot Coupe Blixer, Model BX4V, and the Dry Ice was permitted to sublimate before samples were used in this study.

Two exceptions are 3 ppm methiozolin only validation in soil using a control soil sample (sample ID # -1 DAA1.UT.3-6) from Ricerca study no. 031786, and 4 ppm methiozolin only validation in turf (thatch) using a control turf (thatch) sample (sample ID # -1 DAA1.UT.0-3) from Ricerca study no. 031813 methiozolin TFD study at Woodland, California test site. All soil samples from the methiozolin TFD studies (Ricerca study no. 031786 New York, 031812 Georgia, 031813 California, and 031942 New Jersey) were homogenized with Dry Ice in a Hobart 8181D food cutter. All turf (thatch) samples from the methiozolin TFD studies were homogenized with Dry Ice in a Hobart 8181D food cutter or a Robot Coupe Blixer, Model BX4V or BX5V. All grass samples from the methiozolin TFD studies were homogenized with Dry Ice in a Robot Coupe Blixer, Model BX4V or BX5V.

ANALYTICAL PHASE MATERIALS AND METHODS

SUMMARY

Control and fortified soil, turf (thatch), and grass were extracted and analyzed using analytical methods developed at Ricerca Biosciences, LLC. The analytical methods for determination of methiozolin and 2,6-difluorobenzoic acid (DFBA) in soil, turf (thatch), and grass were as follows. Soil, turf (thatch), and grass samples were homogenized with Dry Ice before extraction, and the Dry Ice was permitted to sublimate before samples were weighed for analysis. Aliquots of control matrices (10 g soil, 5 g turf (thatch), and 5 g grass) were fortified with the appropriate amount of methiozolin (0.01 ppm to 3 ppm in soil, 0.01 ppm to 4 ppm in turf (thatch), and 0.01 ppm to 200 ppm in grass) and DFBA (0.01 ppm to 1 ppm in all three matrices). Residues of methiozolin and DFBA were extracted from soil, turf (thatch), and grass, cleaned up using solid phase extraction (SPE) or filtration (polytetrafluoroethylene (PTFE) syringe filters), and quantified by LC-MS/MS. A different extraction and clean-up procedure was used for each of the six methods and each is described separately in Appendices 3 to 8. The LC-MS/MS procedure for methiozolin is the same in each of the analytical methods that determine methiozolin. Likewise, the LC-MS/MS procedure for DFBA is the same in each of the analytical methods that determine DFBA. Complete details for each individual method, including standard solution preparation, description of the extraction and clean-up procedure, and LC-MS/MS analysis, are described in separate appendices (Appendices 3 to 8). Also, Protocol Amendment Two in Appendix 1 contains a description of all six analytical procedures in one document.

METHIOZOLIN AND DFBA STANDARD SOLUTION PREPARATION AND STABILITY

Over the course of the study, methiozolin and DFBA standard solutions (weighing to prepare stock solution(s), followed by dilution to prepare fortification and calibration solutions) were prepared freshly every 6 months, as the preparation dates shown in the following table. The dilution schemes from the 1st preparation (methiozolin only) are shown in the individual analytical methods 031775B and 031775D (Appendix 4 and Appendix 6) and were used for those method validations. The dilution schemes from the 2nd preparation (methiozolin and DFBA) are shown in the individual analytical methods 031775A, C, E, and F (Appendix 3, Appendix 5, Appendix 7, and Appendix 8) and were used for those method validations. The third and the fourth preparations were prepared in a similar way and were used for analysis of samples in the terrestrial field study nos. 031786, 031812, 031813 and 031942, and storage stability samples in this study. The reproducibility and stability of methiozolin and DFBA standard solutions were determined from comparison of fresh standard solutions with the previously prepared solutions (6 months old) by LC-MS/MS analysis.

	Methiozolin	DFBA
1st	10/25/13 two stock solutions (one for fortification solution and one for calibration solution preparation)	-
2nd	4/25/14 and 4/28/14 two stock solutions (one for fortification solution and one for calibration solution preparation)	4/25/14 and 4/28/14 two stock solutions (one for fortification solution and one for calibration solution preparation)
3rd	11/5/14 one stock solution (for both fortification and calibration solution preparation)	11/5/14 one stock solution (for both fortification and calibration solution preparation)
4th	5/1/15 one stock solution (for both fortification and calibration solution preparation)	5/4/15 one stock solution (for both fortification and calibration solution preparation)

METHOD VALIDATION SAMPLE PREPARATION AND ANALYSIS

Six analytical methods were validated. For the method validation, two untreated controls and fifteen to thirty treated samples, fortified at three to six levels for each matrix, were prepared, extracted, and analyzed by LC-MS/MS. A brief description, fortification levels, spiking and analysis procedures, and full copies of each method are detailed in the Appendices 3 to 8, as indicated below.

- **Method 031775A - Methiozolin and/or DFBA in Soil (Appendix 3)**
  In Method 031775A, residues of methiozolin and DFBA are extracted concomitantly from ten grams of soil with acetonitrile-HPLC water (80:20, v:v), filtered through 0.45 µm PTFE syringe filters, diluted with acetonitrile-HPLC water (30:70, v:v), then further diluted with acetonitrile-HPLC water (50:50, v:v) (if required), and quantified separately for methiozolin and DFBA by LC-MS/MS. For Method 031775A for methiozolin only in soil, the fortification levels were 0.01, 0.1, 1, and 3 ppm. For Method 031775A for methiozolin and DFBA in soil, the methiozolin and DFBA fortification levels were each 0.01, 0.1, and 1 ppm. The complete analytical procedure is provided in Appendix 3.

- **Method 031775B - Methiozolin in Turf (Thatch) (Appendix 4)**
  In Method 031775B, residues of methiozolin are extracted from five grams of turf (thatch) with acetonitrile-HPLC water (80:20, v:v), cleaned up with solid phase extraction, diluted with acetonitrile-HPLC water (50:50, v:v) (if required), and quantified for methiozolin by LC-MS/MS. For Method 031775B, the methiozolin fortification levels were 0.01, 0.1, and 4 ppm. The complete analytical procedure is provided in Appendix 4.

- **Method 031775C - Methiozolin and/or DFBA in Turf (Thatch) (Appendix 5)**
  In Method 031775C, residues of methiozolin and DFBA are extracted concomitantly from thatch with acetone, and then with acetonitrile-HPLC water (50:50, v:v), the extracts combined and then filtered through 0.45 µm PTFE syringe filters, diluted with acetonitrile-HPLC water (50:50, v:v) (if required), quantified separately for methiozolin and DFBA by LC-MS/MS. For Method 031775C, the methiozolin fortification levels

were 0.01, 0.1, 1, and 4 ppm, and the DFBA fortification levels were 0.01, 0.1, and 1 ppm. The complete analytical procedure is provided in Appendix 5.

- **Method 031775D - Methiozolin in Grass (Appendix 6)**
  In Method 031775D, residues of methiozolin are extracted from five grams of grass with acetone, and then with acetonitrile-HPLC water (80:20, v:v), the extracts combined and cleaned up with solid phase extraction, diluted with acetonitrile-HPLC water (50:50, v:v) (if required), and quantified for methiozolin by LC-MS/MS. For Method 031775D, the methiozolin fortification levels were 0.01, 0.1, and 1 ppm. The complete analytical procedure is provided in Appendix 6.

- **Method 031775E – Methiozolin in Grass (Appendix 7)**
  In Method 031775E, methiozolin in grass is extracted first with acetone, and then with acetonitrile-HPLC water (80:20, v:v), the extracts combined and then filtered through 0.45 µm PTFE syringe filters, diluted with acetonitrile-HPLC water (50:50, v:v), and quantified by LC-MS/MS. For Method 031775E, the methiozolin fortification levels were 0.01, 0.1, 1, 10, 49, and 200 ppm. The complete analytical procedure is provided in Appendix 7.

- **Method 031775F - DFBA in Grass (Appendix 8)**
  In Method 031775F, DFBA in grass was extracted with acetone-water (80:20, v:v), filtered through 0.45 µm PTFE syringe filters, diluted with acetonitrile-HPLC water-acetic acid (25:75:0.1, v:v:v), and quantified by LC-MS/MS. For Method 031775F, the DFBA fortification levels were 0.01, 0.1, and 1 ppm. The complete analytical procedure is provided in Appendix 8.

FREEZER STABILITY SAMPLE PREPARATION AND ANALYSIS

The freezer storage stability study was conducted on each compound separately. The analytical methods used for the freezer storage stability studies for methiozolin in soil, turf (thatch), and grass were Method 031775A, Method 031775B, and Method 031775D, respectively. The analytical methods used for the freezer storage stability studies for DFBA in soil, thatch and grass, are Method 031775A, Method 031775C, and Method 031775F, respectively.

Multiple replicate samples of soil, turf (thatch), and grass were fortified with methiozolin or DFBA at 0.1 ppm. One set of samples was extracted immediately (Day 0), and the rest of the samples were stored frozen at -22 °C to -18 °C. For methiozolin freezer stability, the time points were 0, 7, 32, 86, 121, and 528 days. For DFBA, the time points were 0, 7, 33, 90, 370, and 419 days.

For the freezer storage stability study at each time point, one analytical set for each matrix was extracted, and analyzed by LC-MS/MS. One analytical set consisted of one control, three freezer storage stability samples fortified with either methiozolin or DFBA at 0.1 ppm on Day 0, and two concurrent recovery samples fortified with either methiozolin or DFBA at 0.1 ppm at the time-point.

The spiking of samples at 0.1 ppm for freezer stability and for concurrent recovery determination with each analysis was done using the same methods as were used for the

method validation samples (refer to Appendix 3, Appendix 4, and Appendix 6 for spiking of methiozolin in soil, turf (thatch), and grass samples, respectively, and to Appendix 3, Appendix 5, and Appendix 8 for spiking of DFBA in soil, turf (thatch), and grass samples, respectively).

LIMITS OF QUANTITATION AND DETECTION

The Limit of Quantification was 0.01 ppm. The LOQ was defined as the lowest fortification level at which acceptable recovery data were obtained.

SAMPLE CALCULATIONS

Quantification of residues was made by injecting with the samples a series of calibration standards. The peak area measured by the MS/MS detector was collected by Analyst™ version 1.4.2. Calibration plots were generated by Analyst™ version 1.4.2 using the peak area responses of the external calibration standards injected with the samples. The sample concentration in ng/mL was determined automatically by Analyst™ version 1.4.2 from the peak area response of the sample and the slope and intercept of the linear plot of the standards.

The recoveries of methiozolin and DFBA from fortified freezer storage stability and concurrent samples were calculated using Microsoft® Excel 2010 as follows:

ppm (Methiozolin or DFBA) =

$$\frac{\text{Sample Conc. (ng/mL) x Extract Vol. (mL) x Dilution factor x 0.001 } \mu\text{g/ng}}{\text{Sample weight (grams)}}$$

where µg/g is equivalent to mg/kg and ppm.

$$\text{Percent Recovery} = \frac{\text{Conc. of Fortified Sample (ppm)} - \text{Conc. of Control (ppm)}}{\text{Fortification Level (ppm)}} \times 100\%$$

An example calculation for the recovery of methiozolin from turf (thatch) Day 528 freezer storage stability sample (sample # **Turf 0.1 ppm Storage Stability-2 Day 528** in Figure 37) and in Table 8 is shown below:

The ppm methiozolin in turf (thatch) was calculated as follows:

$$\text{ppm methiozolin} = \frac{4.812 \text{ ng/mL} \times 40 \text{ mL} \times 2.5 \times 0.001 \text{ } \mu\text{g/ng}}{5.0000 \text{ grams}} = 0.09624 \text{ ppm}$$

The percent recovery of methiozolin was calculated as follows:

$$\text{Methiozolin Percent Recovery} = \frac{0.09624 \text{ ppm} - 0 \text{ ppm}}{0.1 \text{ ppm}} \times 100\% = 96.2\%$$

Other descriptive statistics (average and standard deviation (SD)) were calculated using Excel.

Protocol Amendment One:

Validation of Methodology and Freezer Storage Stability of
Methiozolin and 2,6-Difluorobenzoic Acid in Grass, Turf and Soil

Ricerca Study Number: 031775
Ricerca Document Number: 031775-0-1

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ORIGINAL PROTOCOL SECTION

Title page, page 2, and page 5

STUDY TITLE

Validation of Methodology and Freezer Storage Stability of Methiozolin in Grass, Turf and Soil

CHANGE TO

STUDY TITLE

Validation of Methodology and Freezer Storage Stability of Methiozolin and 2,6-Difluorobenzoic Acid in Grass, Turf and Soil

ORIGINAL PROTOCOL SECTION

Page 5

INTRODUCTION

This protocol describes 1) development and validation of methodology for determination of residues of methiozolin in grass, turf, and soil, 2) freezer storage stability of residues of methiozolin in grass, turf, and soil.

CHANGE TO

INTRODUCTION

This protocol describes 1) development and validation of methodology for determination of residues of methiozolin in grass, turf, and soil, 2) freezer storage stability of residues of methiozolin in grass, turf, and soil. The validated methods for analysis of methiozolin in grass, turf, and soil are to be used for analysis of methiozolin storage stability samples.

2,6-Difluorobenzoic acid was identified as a transformation product of methiozolin in a soil photolysis study of methiozolin (Ricerca study no. 031689). The scope of this study is expanded to include: 1) development and validation of methodology for determination of residues of 2,6-difluorobenzoic acid in grass, turf, and soil, 2) freezer storage stability of residues of 2,6-difluorobenzoic acid in grass, turf, and soil.

ORIGINAL PROTOCOL SECTION

Page 6

OBJECTIVE

The study was designed to evaluate the methodology developed for the extraction and quantification of residues of methiozolin in grass, turf and soil. Once developed and validated these methods will be documented in a report so that a different scientist can re-

construct the analyses using the details provided. In addition, this study will also assess the freezer storage stability of residues of methiozolin on grass, turf and soil.

CHANGE TO

OBJECTIVE

The study was designed to evaluate the methodology developed for the extraction and quantification of residues of methiozolin and 2,6-difluorobenzoic acid in grass, turf and soil. Once developed and validated these methods will be documented in a report so that a different scientist can re-construct the analyses using the details provided. In addition, this study will also assess the freezer storage stability of residues of methiozolin and 2,6-difluorobenzoic acid on grass, turf and soil.

ORIGINAL PROTOCOL SECTION

Page 8

Analytical Method

Control and fortified grass, turf and soil will be extracted and analyzed using either an analytical method provided by the Study Sponsor or a method developed at Ricerca Biosciences, LLC. Two untreated controls and fifteen treated samples fortified at three levels for each matrix will be prepared, extracted, and analyzed by LC-MS/MS. Aliquots of the final solution may be diluted or concentrated as necessary with the appropriate solvent prior to LC-MS/MS analysis. The final analytical method will include the extraction and clean-up procedure, instrumentation, and LC-MS/MS parameters developed; any and all modifications will be fully described in the raw data and documented in the validation report.

CHANGE TO

Analytical Methods for Methiozolin in Grass, Turf and Soil

Control and fortified grass, turf and soil will be extracted and analyzed using either an analytical method provided by the Study Sponsor or a method developed at Ricerca Biosciences, LLC. Two untreated controls and fifteen treated samples fortified at three levels for each matrix will be prepared, extracted, and analyzed by LC-MS/MS. Aliquots of the final solution may be diluted or concentrated as necessary with the appropriate solvent prior to LC-MS/MS analysis. The final analytical method will include the extraction and clean-up procedure, instrumentation, and LC-MS/MS parameters developed; any and all modifications will be fully described in the raw data and documented in the validation report.

The analytical methods for determination of methiozolin in grass, turf, and soil are as follows. Grass and turf samples are homogenized with dry ice in a Robot Coupe Blixer, Model BX4V, and soil samples are homogenized with dry ice in a Hobart 8181D, a food cutter, before extraction. The dry ice is permitted to sublimate before samples are weighed for analysis. Residues of methiozolin are extracted from grass, turf, and soil using the

procedure described in the Extraction and Clean-up section below and quantified by LC-MS/MS.

EXTRACTION AND CLEAN-UP

Grass

Extraction

1. Aliquots (5 g each) of grass are weighed into 50-mL polypropylene centrifuge tubes. Acetone (40 mL) is added to each grass sample. Concurrent recovery samples are amended with the appropriate amount of methiozolin at this step.
2. The samples are shaken using a Burrell wrist-action shaker for 30 minutes.
3. The samples are centrifuged for 10 minutes at ~3000 rpm.
4. The supernatant is transferred to a new 125-mL polyethylene bottle.
5. The solid residue is re-extracted with 40 mL of acetonitrile-HPLC water (80:20 v:v), as for step 2 to 4, combining the supernatants in the 125-mL polyethylene bottle.
6. An aliquot of 4 mL of the combined extracts is removed from each sample and mixed with 20 mL of HPLC water for Solid Phase Extraction (SPE) clean-up.

SPE Cartridge Clean-up

1. A Waters Oasis HLB VAC (60 mg) SPE cartridge is placed on an SPE vacuum manifold and washed with 5 mL methanol followed by 5 mL HPLC water.
2. The cartridge is loaded with the sample from the extraction step # 6. The eluate is discarded.
3. Methiozolin is eluted with 5 mL of acetonitrile. The eluate is collected into a 15 mL polypropylene centrifuge tube.
4. The eluate is diluted to 10 mL with HPLC water and the sample is mixed.
5. Samples are transferred to autosampler vials for LC-MS/MS analysis. If required, samples may be diluted 10X with acetonitrile-HPLC water (50:50 v:v).

Turf

Extraction

1. Aliquots (5 g each) of turf are weighed into 50-mL polypropylene centrifuge tubes. Acetonitrile-HPLC water (80:20 v:v) (40 mL) is added to each sample. Concurrent recovery samples are amended with the appropriate amount of methiozolin at this step.
2. The samples are shaken using a Burrell wrist-action shaker for 30 minutes.
3. The samples are centrifuged for 10 minutes at ~3000 rpm.
4. An aliquot of 4 mL of the supernatant is removed from each sample and mixed with 20 mL of HPLC water for SPE clean-up.

SPE Cartridge Clean-up

1. A Waters Oasis HLB VAC (60 mg) SPE cartridge is placed on an SPE vacuum manifold and washed with 5 mL methanol followed by 5 mL HPLC water.
2. The cartridge is loaded with the sample from the extraction step # 4. The eluate is discarded.
3. Methiozolin is eluted with 5 mL of acetonitrile. The eluate is collected into a 15-mL polypropylene centrifuge tube.
4. The eluate is diluted to 10 mL with HPLC water and the sample is mixed.
5. Samples are transferred to autosampler vials for LC-MS/MS analysis. If required, samples may be diluted 10X with acetonitrile-HPLC water (50:50 v:v).

Soil

Extraction and Filtration

1. Aliquots (10 g each) of soil are weighed into 50-mL polypropylene centrifuge tubes. Acetonitrile-HPLC water (80:20 v:v) (40 mL) is added to each sample. Concurrent recovery samples are amended with the appropriate amount of methiozolin at this step.
2. The samples are shaken using a Burrell wrist-action shaker for 30 minutes.
3. The samples are centrifuged for 10 minutes at ~3000 rpm.
4. An aliquot of the supernatant is filtered through a 0.45-µm polytetrafluoroethylene (PTFE) syringe filter into a 15-mL polypropylene centrifuge tube.
5. A 4-mL aliquot of the filtrate is diluted to 10 mL with acetonitrile-HPLC water (30:70 v:v) and the sample is mixed.
6. Samples are transferred to autosampler vials for LC-MS/MS analysis. If required, samples may be diluted 20X with acetonitrile-HPLC water (50:50 v:v).

LC-MS/MS ANALYSIS

HPLC Method

Column: Luna 5 µ Phenyl-Hexyl, 150 mm x 2 mm
Column Temperature: Ambient
Solvent System:
Solvent A = 0.1% formic acid in HPLC water-methanol (90:10) + 10 mM ammonium formate
Solvent B = 0.1% formic acid in methanol
Wash solvent = HPLC water-acetonitrile (50:50)
Solvent program:

Time (minutes)	Flow Rate (mL/min)	%A	%B
0.0	0.4	35	65
3.0	0.4	5	95
5.0	0.4	5	95
5.5	0.4	35	65
8.0	0.4	35	65

The LC flow is diverted to the MS between 2.0 and 7.9 min, and to waste between 0.0 and 2.0 min and between 7.9 and 8.0 min.
Retention time of Methiozolin is approximately 5.0 min.

MS (SCIEX API4000) Parameters

Scan Type	MRM
Polarity	Positive
Ion Source	Turbo Spray
Resolution Q1	Unit
Resolution Q3	Unit
Ion Source Gas 1 (GS1)	50 psi
Ion Source Gas 2 (GS2)	50 psi
Curtain Gas (CUR)	12 psi
Collision Gas (CAD)	6 psi
IonSpray Voltage (IS)	5500 V
Temperature (TEM)	500 °C
Declustering Potential (DP)	101 V
Entrance Potential (EP)	10 V
Collision Energy (CE)	41 V
Collision Gas Exit Potential (CXP)	8 V

MRM transition	Analyte ID	Q1 Mass (amu)	Q3 Mass (amu)	Dwell Time (msec)
Primary	Methiozolin	338	127	200
Confirmatory	MethiozolinC	338	211	200

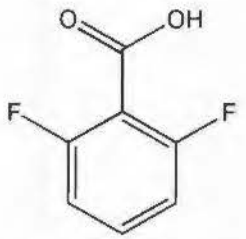
ADDITION TO PROTOCOL

Page 6

MATERIALS AND METHODS

TEST SUBSTANCE

- **2,6-Difluorobenzoic acid**



2,6-Difluorobenzoic acid

Information concerning the test substance is provided below:

Chemical Name:	2,6-Difluorobenzoic acid	
CAS No.:	385-00-2	
Molecular Formula:	$C_7H_4F_2O_2$	
Molecular Weight:	158.1 g/mol	
Source:	Sigma-Aldrich	Tokyo Chemical Industry Co., Inc.
Lot No.:	MKBQ3617V	OK23B
Ricerca Sample Code:	CS_19165	CS_18852
Stated Purity:	98%	≥98% (by neutralization titration)
Storage:	Ambient temperature	Refrigerated
Used for the method validation and storage stability studies:	Yes	No

Characterization of the 2,6-difluorobenzoic acid test substance, including HPLC analysis for purity and LC-MS for identity, will be determined by Ricerca. Two lots of 2,6-difluorobenzoic acid will be characterized and certificates of analysis issued. Only the lot from Sigma-Aldrich will be used for the method validation and storage stability studies. Solutions made from the test substance will be stored frozen at ≤-5 °C. Solubility and stability data for the test substance are the responsibility of the Sponsor. The Sponsor will assume the responsibility of retention of a sample of the test substance, as specified in 40 CFR 160.195.

Analytical Methods for 2,6-Difluorobenzoic Acid in Grass, Turf and Soil

Analytical methods for determination of 2,6-difluorobenzoic acid in grass, turf, and soil will be developed and validated at Ricerca Biosciences, LLC. The method either will analyze 2,6-difluorobenzoic acid only or will be a combined procedure that analyzes both the parent methiozolin and 2,6-difluorobenzoic acid as individual compounds. In either case, control samples will be fortified with both methiozolin and 2,6-difluorobenzoic acid. If a separate analytical procedure is developed for 2,6-difluorobenzoic acid, the fortified controls will be analyzed and the method validated for 2,6-difluorobenzoic acid only. If a combined analytical procedure is developed, the fortified controls will be analyzed and the method

validated for both compounds. The study designs, including fortification levels tested, will be the same as those of the methiozolin method validation. Once validated, the analytical method, either for 2,6-difluorobenzoic acid only or for both methiozolin and 2,6-difluorobenzoic acid, will be documented in a protocol amendment.

STORAGE STABILITY FOR 2,6-DIFLUOROBENZOIC ACID IN GRASS, TURF AND SOIL

For the 2,6-difluorobenzoic acid storage stability study, grass, turf, and soil will be spiked with 2,6-difluorobenzoic acid only. The study designs, including fortification levels tested, will be the same as those of methiozolin. The analytical methods for analysis of 2,6-difluorobenzoic acid storage stability samples will be documented in a protocol amendment.

REASON FOR CHANGE/ADDITIONS

1. To add one additional test substance, 2,6-difluorobenzoic acid, a transformation product of methiozolin in a soil metabolism study, for analytical method development and validation, and storage stability study in grass, turf, and soil.
2. To add the validated methods for analysis of methiozolin in grass, turf, and soil. The validated methods for analysis of methiozolin in grass, turf, and soil are to be used for analysis of methiozolin storage stability samples.
3. To develop and validate separate analytical methods for analysis of both methiozolin and 2,6-difluorobenzoic acid in grass, turf, and soil samples.

EFFECTIVE DATE

Date of Study Director's signature on the amendment.

PROTOCOL AMENDMENT ONE ACCEPTANCE

Study Title: Validation of Methodology and Freezer Storage Stability of Methiozolin and 2,6-Difluorobenzoic Acid in Grass, Turf and Soil

Ricerca Document Number: 031775-0-1

Testing Facility: AgChem Product Development
Ricerca Biosciences, LLC
7528 Auburn Road
Concord, OH 44077

Ling-Jen Ferguson
Ling-Jen Ferguson, Study Director
Ricerca Biosciences, LLC

11 Apr 2014
Date

Robert McClanahan, Management
Ricerca Biosciences, LLC

11 April 2014
Date

SPONSOR: Moghu Research Center Ltd

SPONSOR APPROVAL DATE: MARCH 26, 2014

PROTOCOL AMENDMENT TWO

Study Title:

Validation of Methodology and Freezer Storage Stability of Methiozolin and 2,6-Difluorobenzoic Acid in Grass, Turf and Soil

Ricerca Study Number: 031775
Ricerca Document Number: 031775-0-2

Data Requirement:

US EPA OPPTS Test Guidelines 860.1340
SANCO/3029/99 rev. 4 (11/07/00)
SANCO/825/00 rev. 8.1 (16/11/2010)
ENV/JM/MONO(2007)17 (13-Aug-2007)

Testing Facility:
AgChem Product Development
Ricerca Biosciences, LLC
7528 Auburn Road
Concord, OH 44077

Study Sponsor:
Moghu Research Center Ltd
BVC #311, KRIBB
52 Eoeun-dong, Yuseong
Daejeon, 305-33, Korea

Protocol Amendment Two:

Validation of Methodology and Freezer Storage Stability of
Methiozolin and 2,6-Difluorobenzoic Acid in Grass, Turf and Soil

Ricerca Study Number: 031775
Ricerca Document Number: 031775-0-2

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ORIGINAL PROTOCOL SECTION

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Analytical Method

Control and fortified grass, turf and soil will be extracted and analyzed using either an analytical method provided by the Study Sponsor or a method developed at Ricerca Biosciences, LLC. Two untreated controls and fifteen treated samples fortified at three levels for each matrix will be prepared, extracted, and analyzed by LC-MS/MS. Aliquots of the final solution may he diluted or concentrated as necessary with the appropriate solvent prior to LC-MS/MS analysis. The final analytical method will include the extraction and clean-up procedure, instrumentation, and LC-MS/MS parameters developed; any and all modifications will be fully described in the raw data and documented in the validation report.

CHANGE TO

ANALYTICAL METHODS FOR METHIOZOLIN AND 2,6-DIFLUOROBENZOIC ACID (DFBA) IN GRASS, TURF AND SOIL

INTRODUCTION

Control and fortified grass, turf and soil are extracted and analyzed using analytical methods developed at Ricerca Biosciences, LLC. The analytical methods for determination of methiozolin and 2,6-difluorobenzoic acid (DFBA) in grass, turf, and soil are as follows. Grass, turf (thatch) and soil samples are homogenized with Dry Ice before extraction, and the Dry Ice is permitted to sublimate before samples are weighed for analysis. Residues of methiozolin and DFBA are extracted with solvent(s) from grass, turf (thatch), and soil, cleaned up using solid phase extraction (SPE) or a polytetrafluoroethylene (PTFE) syringe filter and quantified by LC-MS/MS. A different extraction and clean-up procedure is used for each of the six methods and each is described separately in the Homogenization, Extraction and Clean-up section below. The LC-MS/MS procedure is the same for each compound and complete details are described.

Six analytical methods, used either in the storage stability study no. 031775 or in the methiozolin terrestrial field dissipation (TFD) study nos. 031786, 031812, 031786 or 031942, are validated:

- Method 031775A - Methiozolin and/or DFBA in soil
    - Used for methiozolin and DFBA freezer storage stability study in soil (Study No. 031775)
    - Used for analysis of methiozolin and DFBA in soil in New York, Georgia, California and New Jersey terrestrial field dissipation studies (Study Nos. 031786, 031812, 031813, and 031942, respectively).
- Method 031775B - Methiozolin in turf (thatch)

- Used for methiozolin freezer storage stability study in turf (thatch) (Study No. 031775)

- Method 031775C - Methiozolin and/or DFBA in turf (thatch)
  - Used for DFBA freezer storage stability study in turf (thatch) (Study No. 031775)
  - Used for analysis of methiozolin and DFBA in turf (thatch) in New York, Georgia, California and New Jersey terrestrial field dissipation studies (Study Nos. 031786, 031812, 031813, and 031942, respectively).
- Method 031775D - Methiozolin in grass
  - Used for methiozolin freezer storage stability study in grass (Study No. 031775)
- Method 031775E – Methiozolin in grass
  - Used for analysis of methiozolin in grass in New York, Georgia, California and New Jersey terrestrial field dissipation studies (Study Nos. 031786, 031812, 031813, and 031942, respectively).
- Method 031775F – DFBA in grass
  - Used for DFBA freezer storage stability study in grass (Study No. 031775)
  - Used for analysis of DFBA in grass in New York, Georgia, California and New Jersey terrestrial field dissipation studies (Study Nos. 031786, 031812, 031813, and 031942, respectively).

For the method validation, two untreated controls and **fifteen to thirty** treated samples fortified at **three to six** levels for each matrix were prepared, extracted, and analyzed by LC-MS/MS. The fortification levels (analytical method no.) are:

- Methiozolin in soil: 0.01 ppm, 0.1 ppm, 1 ppm, and 3 ppm (Method 031775A)
- Methiozolin and DFBA in soil: 0.01 ppm, 0.1 ppm, and 1 ppm (Method 031775A)
- Methiozolin in turf (thatch): 0.01 ppm, 0.1 ppm, and 1 ppm (Method 031775B)
- Methiozolin in turf (thatch): 4 ppm (Method 031775C)
- Methiozolin and DFBA in turf (thatch): 0.01 ppm, 0.1 ppm, and 1 ppm (Method 031775C)
- Methiozolin in grass: 0.01 ppm, 0.1 ppm, and 1 ppm (Method 031775D)
- Methiozolin in grass: 0.01 ppm, 0.1 ppm, 1 ppm, 10 ppm, 50 ppm, and 200 ppm (Method 031775E)
- DFBA in grass: 0.01 ppm, 0.1 ppm, and 1 ppm (Method 031775F)

The freezer storage stability studies associated with the present protocol amendment (Study No. 031775) were conducted on each compound separately. For these freezer storage stability studies at each time-point, one analytical set for each matrix was extracted, and analyzed by LC-MS/MS. One analytical set consisted of one control, three freezer storage stability samples fortified with either methiozolin or DFBA at 0.1 ppm on Day 0, and two concurrent recovery samples fortified with either methiozolin or DFBA at 0.1 ppm at the time-point. The analytical methods used for the freezer storage stability studies for methiozolin in soil, thatch and grass (Study No. 031775) are Method 031775A, Method 031775B, and Method 031775D, respectively. The analytical methods used for the freezer

storage stability studies for DFBA in soil, thatch and grass (Study No. 031775), are Method 031775A, Method 031775C, and Method 031775F, respectively.

CHEMICALS

- Acetic acid, glacial, Fisher Certified ACS Plus Grade
- Formic acid: Fisher Optima LC-MS Grade
- Ammonium formate, Fisher Optima LC-MS Grade
- Acetonitrile, Fisher Optima Grade
- Water, Fisher HPLC Grade
- Methanol, Fisher HPLC or Optima Grade
- Acetone, Fisher HPLC or Optima Grade

EQUIPMENT

- Hobart 8181D food cutter
- Robot Coupe Blixer, Model BX4V or BX5V
- Analytical electronic balance with 0.1-mg readability
- Benchtop electronic balance with 0.01-g readability
- Eppendorf micropipettes: 10-100 µL, 20-200 µL, and 100-1000 µL
- Eberbach Floor Shaker
- Burrell Wrist-Action Shaker
- Sorvall RC-5B Centrifuge
- 0.45-µm Polytetrafluoroethylene (PTFE) syringe filters (for Methods 031775A, 031775C, 031775E, and 031775F)
- Waters Oasis HLB VAC (60 mg) solid phase extraction (SPE) cartridges (for Methods 031775B and 031775D)
- Supelco Visiprep SPE vacuum manifolds (for Methods 031775B and 031775D)
- Glassware: Assorted beakers, bottles, graduated cylinders, pipettes, etc., which are routinely used for residue analysis.
- HPLC-MS/MS (see LC-MS/MS Instrumentation section for further information)

PREPARATION OF STOCK, FORTIFICATION, INTERMEDIATE, AND CALIBRATION SOLUTIONS

Methiozolin

The methiozolin stock, fortification, intermediate, and calibration solutions were prepared every six months during the course of the study. Once prepared, these solutions were assigned a 6-month expiry date and stored frozen at ≤ -5 °C when not in use. The representative preparation is shown below:

Preparation of Methiozolin Stock Solutions

- **Stock solution A** (1009 µg/mL Methiozolin in ACN, purity (99.71%) corrected, used for preparation of fortification solutions). Methiozolin (25.3 mg) was weighed in a 25-mL "Class A" volumetric flask and ACN was added to the mark.
- **Stock solution B** (1021 µg/mL Methiozolin in ACN, purity (99.71%) corrected, used for preparation of calibration solutions). Methiozolin (25.6 mg) was weighed in a 25-mL "Class A" volumetric flask and ACN was added to the mark.

Preparation of Methiozolin Fortification Solutions

The following fortification solutions were prepared by serial dilution of **Stock solution A** with ACN-water (50:50 v:v) in "Class A" volumetric flasks, as detailed below:

- **Stock solution A**: 1009 µg/mL Methiozolin in ACN (purity corrected)

Standard solution used	Volume taken (mL)	Final volume (mL)	Nominal concentration (µg/mL)
1009 µg/mL	4.96	50	100
100 µg/mL	5	50	10
10 µg/mL	5	50	1

Preparation of Methiozolin Intermediate and Calibration Solutions

The following intermediate and calibration solutions were prepared by serial dilution of **Stock solution B** with ACN-water (50:50 v:v) in "Class A" volumetric flasks, as detailed below:

- **Stock solution** B:1021 µg/mL Methiozolin in ACN (purity corrected)

Methiozolin Intermediate Solutions

Standard solution used	Volume taken (mL)	Final volume (mL)	Nominal concentration (ng/mL)
1021µg/mL	0.0980	100	1000
1 µg/mL	5	50	100
1 µg/mL	2.5	50	50
1 µg/mL	1	50	20

Methiozolin Calibration Solutions

Standard solution used	Volume taken (mL)	Final volume (mL)	Nominal concentration (ng/mL)
100 ng/mL	5	50	10
50 ng/mL	5	50	5
20 ng/mL	5	50	2
10 ng/mL	5	50	1
10 ng/mL	5	100	0.5
2 ng/mL	5	50	0.2
2 ng/mL	5	100	0.1
1 ng/mL	1	25	0.04

DFBA

The DFBA stock, fortification, intermediate, and calibration solutions were prepared every six months during the course of the study. Once prepared, these solutions were assigned a 6-month expiry date and stored frozen at ≤ -5 °C when not in use. The representative preparation is shown below:

Preparation of DFBA Stock Solutions

- **Stock solution A** (1396 µg/mL DFBA in ACN, purity (94.86%) corrected, used for preparation of fortification solutions). DFBA (36.8 mg) was weighed in a 25-mL "Class A" volumetric flask and ACN was added to the mark.
- **Stock solution B** (1032 µg/mL DFBA in ACN, purity (94.86%) corrected, used for preparation of calibration solutions). DFBA (27.2 mg) was weighed in a 25-mL "Class A" volumetric flask and ACN was added to the mark.

Preparation of DFBA Fortification Solutions

The following fortification solutions were prepared by serial dilution of **Stock solution A** with ACN-water (50:50 v:v) in "Class A" volumetric flasks, as detailed below:

- **Stock solution A**: 1396 µg/mL DFBA in ACN (purity corrected)

Standard solution used	Volume taken (mL)	Final volume (mL)	Nominal concentration (µg/mL)
1396 µg/mL	3.58	50	100
100 µg/mL	5	50	10
10 µg/mL	5	50	1

Preparation of DFBA Intermediate and Calibration Solutions

The following intermediate and calibration solutions were prepared by serial dilution of **Stock solution B** with ACN-water (50:50 v:v) in "Class A" volumetric flasks, as detailed below:

- **Stock solution** B:1032 µg/mL DFBA in ACN (purity corrected)

DFBA Intermediate Solutions

Standard solution used	Volume taken (mL)	Final volume (mL)	Nominal concentration (µg/mL)
1032 µg/mL	4.85	50	100
100 µg/mL	5	50	10
10 µg/mL	5	50	1
1 µg/mL	5	50	0.1

DFBA Calibration Solutions

Standard solution used	Volume taken (mL)	Final volume (mL)	Nominal concentration (ng/mL)
1 µg/mL	2.5	50	50
1 µg/mL	1	50	20
0.1 µg/mL	5	50	10
50 ng/mL	5	50	5
20 ng/mL	5	50	2
10 ng/mL	5	50	1
10 ng/mL	5	100	0.5
2 ng/mL	5	50	0.2
1 ng/mL	5	50	0.1

FORTIFICATION OF METHOD VALIDATION SAMPLES

Fortification Level	Fortification Solution, Volume (µL)	Extraction and Clean-up Method	LC-MS/MS Method
Methiozolin in Soil (10 g)			
Control	ACN:H_2O (50:50), 100 µL	031775A	Methiozolin
0.01 ppm	1 µg/mL methiozolin in ACN:H_2O (50:50), 100 µL	031775A	Methiozolin
0.1 ppm	10 µg/mL methiozolin in ACN:H_2O (50:50), 100 µL	031775A	Methiozolin
1 ppm	100 µg/mL methiozolin in ACN:H_2O (50:50), 100 µL	031775A	Methiozolin
3 ppm	100 µg/mL methiozolin in ACN:H_2O (50:50), 300 µL	031775A	Methiozolin
Methiozolin and DFBA in Soil (10 g)			
Control	ACN:H_2O (50:50), 100 µL	031775A	Methiozolin DFBA
0.01 ppm	1 µg/mL methiozolin in ACN:H_2O (50:50), 100 µL 1 µg/mL DFBA in ACN:H_2O (50:50), 100 µL	031775A	Methiozolin DFBA
0.1 ppm	10 µg/mL methiozolin in ACN:H_2O (50:50), 100 µL 10 µg/mL DFBA in ACN:H_2O (50:50), 100 µL	031775A	Methiozolin DFBA
1 ppm	100 µg/mL methiozolin in ACN:H_2O (50:50), 100 µL 100 µg/mL DFBA in ACN:H_2O (50:50), 100 µL	031775A	Methiozolin DFBA

FORTIFICATION OF METHOD VALIDATION SAMPLES (CONTINUED)

Fortification Level	Fortification Solution, Volume (µL)	Extraction and Clean-up Method	LC-MS/MS Method
Methiozolin in Turf (Thatch) (5 g)			
Control	ACN:H_2O (50:50), 50 µL	031775B	Methiozolin
0.01 ppm	1 µg/mL methiozolin in ACN:H_2O (50:50), 50 µL	031775B	Methiozolin
0.1 ppm	10 µg/mL methiozolin in ACN:H_2O (50:50), 50 µL	031775B	Methiozolin
1 ppm	100 µg/mL methiozolin in ACN:H_2O (50:50), 50 µL	031775B	Methiozolin
4 ppm	100 µg/mL methiozolin in ACN:H_2O (50:50), 200 µL	031775C	Methiozolin
Methiozolin and DFBA in Turf (Thatch) (5 g)			
Control	ACN:H_2O (50:50), 100 µL	031775C	Methiozolin DFBA
0.01 ppm	1 µg/mL methiozolin in ACN:H_2O (50:50), 50 µL 1 µg/mL DFBA in ACN:H_2O (50:50), 50 µL	031775C	Methiozolin DFBA
0.1 ppm	10 µg/mL methiozolin in ACN:H_2O (50:50), 50 µL 10 µg/mL DFBA in ACN:H_2O (50:50), 50 µL	031775C	Methiozolin DFBA
1 ppm	100 µg/mL methiozolin in ACN:H_2O (50:50), 50 µL 100 µg/mL DFBA in ACN:H_2O (50:50), 50 µL	031775C	Methiozolin DFBA
Methiozolin in Grass (5 g)			
Control	ACN:H_2O (50:50), 50 µL	031775D and 031775E	Methiozolin
0.01 ppm	1 µg/mL methiozolin in ACN:H_2O (50:50), 50 µL	031775D and 031775E	Methiozolin
0.1 ppm	10 µg/mL methiozolin in ACN:H_2O (50:50), 50 µL	031775D and 031775E	Methiozolin
1 ppm	100 µg/mL methiozolin in ACN:H_2O (50:50), 50 µL	031775D and 031775E	Methiozolin
10 ppm	100 µg/mL methiozolin in ACN:H_2O (50:50), 500 µL	031775E	Methiozolin
50 ppm	1029 µg/mL methiozolin in ACN, 243 µL	031775E	Methiozolin
200 ppm	5000 µg/mL methiozolin in ACN, 200 µL	031775E	Methiozolin
DFBA in Grass (5 g)			
Control	ACN:H_2O (50:50), 50 µL	031775F	DFBA
0.01 ppm	1 µg/mL DFBA in ACN:H_2O (50:50), 50 µL	031775F	DFBA
0.1 ppm	10 µg/mL DFBA in ACN:H_2O (50:50), 50 µL	031775F	DFBA
1 ppm	100 µg/mL DFBA in ACN:H_2O (50:50), 50 µL	031775F	DFBA

ACN = acetonitrile

FORTIFICATION OF FREEZER STORAGE STABILITY SAMPLES

Fortification Level	Fortification Solution, Volume (µL)	Extraction and Clean-up Method	LC-MS/MS Method
Methiozolin in Soil (10 g)			
Control	ACN:H_2O (50:50), 100 µL	031775A	Methiozolin
0.1 ppm	10 µg/mL methiozolin in ACN:H_2O (50:50), 100 µL	031775A	Methiozolin
DFBA in Soil (10 g)			
Control	ACN:H_2O (50:50), 100 µL	031775A	DFBA
0.1 ppm	10 µg/mL DFBA in ACN:H_2O (50:50), 100 µL	031775A	DFBA
Methiozolin in Turf (Thatch) (5 g)			
Control	ACN:H_2O (50:50), 50 µL	031775B	Methiozolin
0.1 ppm	10 µg/mL methiozolin in ACN:H_2O (50:50), 50 µL	031775B	Methiozolin
DFBA in Turf (Thatch) (5 g)			
Control	ACN:H_2O (50:50), 50 µL	031775C	DFBA
0.1 ppm	10 µg/mL DFBA in ACN:H_2O (50:50), 50 µL	031775C	DFBA
Methiozolin in Grass (5 g)			
Control	ACN:H_2O (50:50), 50 µL	031775D	Methiozolin
0.1 ppm	10 µg/mL methiozolin in ACN:H_2O (50:50), 50 µL	031775D	Methiozolin
DFBA in Grass (5 g)			
Control	ACN:H_2O (50:50), 50 µL	031775F	DFBA
0.1 ppm	10 µg/mL DFBA in ACN:H_2O (50:50), 50 µL	031775F	DFBA

ACN = acetonitrile

HOMOGENIZATION, EXTRACTION, AND CLEAN-UP

Method 031775A (for methiozolin and/or DFBA in soil)

1. Frozen soil samples are homogenized with Dry Ice in a Hobart 8181D food cutter, and the Dry Ice is permitted to sublimate before samples are weighed for analysis.
2. Aliquots (10 g each) of soil are weighed into 50-mL polypropylene centrifuge tubes and fortified.
3. Acetonitrile-HPLC water (80:20 v:v) (40 mL) is added to each sample.
4. The samples are shaken using a Burrell wrist-action shaker for 30 minutes.
5. The samples are centrifuged for 10 minutes at ~3000 rpm.
6. An aliquot of the supernatant is filtered through a 0.45-µm polytetrafluoroethylene (PTFE) syringe filter into a 15-mL polypropylene centrifuge tube.
7. A 4-mL aliquot of the filtrate is diluted to 10 mL with acetonitrile-HPLC water (30:70 v:v) and the sample is mixed.
8. The 10XLOQ (0.1 ppm) samples are further diluted 2X with ACN-HPLC water (50:50 v:v), 100XLOQ (1 ppm) samples diluted 20X, and 300XLOQ (3 ppm) samples diluted 50X.
9. Samples are transferred to autosampler vials for LC-MS/MS analysis.

Method 031775B (for methiozolin only in turf (thatch))

Extraction

1. Frozen turf (thatch) samples are homogenized with Dry Ice in a Hohart 8181D food cutter or a Robot Coupe Blixer, Model BX4V or BX5V, and the Dry Ice is permitted to sublimate before samples are weighed for analysis.
2. Aliquots (5 g each) of turf (thatch) are weighed into 50-mL polypropylene centrifuge tubes and fortified.
3. Acetonitrile-HPLC water (80:20 v:v) (40 mL) is added to each sample.
4. The samples are shaken using a Burrell wrist-action shaker for 30 minutes.
5. The samples are centrifuged for 10 minutes at ~3000 rpm.
6. An aliquot of 4 mL of the supernatant is removed from each sample and mixed with 20 mL of HPLC water for SPE clean-up.

SPE Cartridge Clean-up

1. A Waters Oasis HLB VAC (60 mg) SPE cartridge is placed on a Supelco Visiprep SPE vacuum manifold and washed with 5 mL methanol followed by 5 mL HPLC water.
2. The cartridge is loaded with the sample from the extraction step # 6. The eluate is discarded.
3. Methiozolin is eluted with 5 mL of acetonitrile. The eluate is collected into a 15-mL polypropylene centrifuge tube.

4. The eluate is diluted to 10 mL with HPLC water and the sample is mixed.
5. 100XLOQ (1 ppm) samples are diluted 10X with acetonitrile-HPLC water (50:50 v:v).
6. Samples are transferred to autosampler vials for LC-MS/MS analysis.

Method 031775C (for methiozolin and/or DFBA in turf (thatch))

1. Frozen turf (thatch) samples are homogenized with Dry Ice in a Hobart 8181D food cutter or a Robot Coupe Blixer, Model BX4V or BX5V, and the Dry Ice is permitted to sublimate before samples are weighed for analysis.
2. Aliquots (approximately 5 g each) of turf (thatch) are weighed into 50-mL polypropylene centrifuge tubes and fortified.
3. Acetone (40 mL) is added to each sample.
4. The samples are shaken using a Burrell wrist-action shaker for 30 minutes.
5. The samples are centrifuged for 10 minutes at ~3000 rpm.
6. The supernatant is transferred to a new 125-mL polyethylene bottle.
7. The solid residue is re-extracted with 40 mL of acetonitrile-HPLC water (50:50 v:v), as for step 3 to 5, combining the supernatants in the 125-mL polyethylene bottle.
8. An aliquot of the combined supernatants is filtered through a 0.45-µm PTFE syringe filter.
9. 100XLOQ (1 ppm) samples are diluted 10X with acetonitrile-HPLC water (50:50 v:v), and 400XLOQ (4 ppm) samples diluted 50X.
10. Samples are transferred to autosampler vials for LC-MS/MS analysis.

Method 031775D (for methiozolin only in grass)

Extraction

1. Frozen grass samples are homogenized with Dry Ice in a Robot Coupe Blixer, Model BX4V or BX5V, and the Dry Ice is permitted to sublimate before samples are weighed for analysis.
2. Aliquots (5 g each) of grass are weighed into 50-mL polypropylene centrifuge tubes and fortified.
3. Acetone (40 mL) is added to each grass sample.
4. The samples are shaken using a Burrell wrist-action shaker for 30 minutes.
5. The samples are centrifuged for 10 minutes at ~3000 rpm.
6. The supernatant is transferred to a new 125-mL polyethylene bottle.
7. The solid residue is re-extracted with 40 mL of acetonitrile-HPLC water (80:20 v:v), as for step 3 to 5, combining the supernatants in the 125-mL polyethylene bottle.
8. An aliquot of 4 mL of the combined extracts is removed from each sample and mixed with 20 mL of HPLC water for SPE clean-up.

SPE Cartridge Clean-up

1. A Waters Oasis HLB VAC (60 mg) SPE cartridge is placed on a Supelco Visiprep SPE vacuum manifold and washed with 5 mL methanol followed by 5 mL HPLC water.

2. The cartridge is loaded with the sample from the extraction step # 8. The eluate is discarded.
3. Methiozolin is eluted with 5 mL of acetonitrile. The eluate is collected into a 15 mL polypropylene centrifuge tube.
4. The eluate is diluted to 10 mL with HPLC water and the sample is mixed.
5. 100XLOQ (1 ppm) samples are diluted 10X with acetonitrile-HPLC water (50:50 v:v).
6. Samples are transferred to autosampler vials for LC-MS/MS analysis.

Method 031775E (for methiozolin only in grass)

1. Frozen grass samples are homogenized with Dry Ice in a Robot Coupe Blixer, Model BX4V or BX5V, and the Dry Ice is permitted to sublimate before samples are weighed for analysis.
2. Aliquots (approximately 5 g each) of grass are weighed into 50-mL polypropylene centrifuge tubes and fortified.
3. Acetone (40 mL) is added to each grass sample.
4. The samples are shaken using a Burrell wrist-action shaker for 30 minutes.
5. The samples are centrifuged for 10 minutes at ~3000 rpm.
6. The supernatant is transferred to a new 125-mL polyethylene bottle.
7. The solid residue is re-extracted with 40 mL of acetonitrile-HPLC water (80:20 v:v), as for step 3 to 5, combining the supernatants in the 125-mL polyethylene bottle.
8. An aliquot of the supernatant is filtered through a 0.45-µm PTFE syringe filter.
9. Samples are diluted with acetonitrile-HPLC water (50:50 v:v) according to the table below, before transferring to autosampler vials for LC-MS/MS analysis.

Fortification Level	Extraction volume (mL)	Dilution	Final Concentration (ng/mL)
Grass blank	80	10x	N/A
0.01 ppm	80	10x	0.0625
0.1 ppm	80	10x	0.625
1 ppm	80	50x	1.25
10 ppm	80	50x+50x*	0.25
50 ppm	80	50x+50x	1.25
200 ppm	80	50x+50x	5

**50x+50x: diluted 50x twice, equivalent to 2,500x dilution*

Method 031775F (for DFBA only in grass)

1. Frozen grass samples are homogenized with Dry Ice in a Robot Coupe Blixer, Model BX4V or BX5V, and the Dry Ice is permitted to sublimate before samples are weighed for analysis.
2. Aliquots (approximately 5 g each) of grass are weighed into 50-mL polypropylene centrifuge tubes and fortified.
3. Acetone-water (80:20 v:v, 40 mL) is added to the sample.
4. The samples are shaken using a Burrell wrist-action shaker for 30 minutes.

5. The samples are centrifuged for 10 minutes at ~3000 rpm.
6. An aliquot of the supernatant is filtered through a 0.45-µm PTFE syringe filter.
7. The samples are diluted with acetonitrile-HPLC water-acetic acid (25:75:0.1 v:v:v): control and LOQ (0.01 ppm) diluted 5X, 10XLOQ (0.1 ppm) diluted 10X, and 100XLOQ (1 ppm) diluted 20X.
8. Samples are transferred to autosampler vials for LC-MS/MS analysis.

LC-MS/MS Systems - Analysis of Methiozolin

Shimadzu HPLC-API4000 MS

- HPLC: Two Shimadzu LC-20AD pumps and a Shimadzu SIL-HTA Controller/Autosampler
- MS: SCIEX API4000
- Computer software: Analyst™ version 1.4.2

Flexar UHPLC-API4000 MS

- HPLC: PerkinElmer Flexar FX-15 UHPLC
- MS: SCIEX API4000
- Computer software: Analyst™ version 1.4.2

Methiozolin Calibration Standards

For turf (thatch) and soil analysis, a series of methiozolin calibration standards at 0.1, 0.2, 0.5, 1, 2, 5, and 10 ng/mL are prepared to quantify the observed methiozolin residues in spray pad, thatch, and soil samples.

For grass analysis, a series of methiozolin calibration standards at 0.04, 0.1, 0.2, 0.5, 1, 2, 5, and 10 ng/mL are prepared to quantify the observed methiozolin residues in grass samples.

HPLC Method - Methiozolin

Column: Phenomenex Luna 5 µ Phenyl-Hexyl 150 mm x 2 mm
Column Temperature: Ambient
Injection Volume: 5 µL (Shimadzu HPLC) or 20 µL (Flexar UHPLC)
Solvent System:
Solvent A = 0.1% formic acid in HPLC water-methanol (90:10) + 10 mM ammonium formate
Solvent B = 0.1% formic acid in methanol
Wash solvent = HPLC water-acetonitrile (50:50)

Solvent program (Shimadzu HPLC):

Time (minutes)	Flow Rate (mL/min)	%A	%B
0.0	0.4	35	65
3.0	0.4	5	95
5.0	0.4	5	95
5.5	0.4	35	65
8.0	0.4	35	65

The LC flow was diverted to the MS between 2.0 and 7.9 min, and to waste between 0.0 and 2.0 min and between 7.9 and 8.0 min.

Solvent program (Flexar UHPLC):

Time (minutes)	Flow Rate (mL/min)	%A	%B
0.0	0.4	35	65
3.5	0.4	5	95
5.0	0.4	5	95
5.5	0.4	35	65
8.0	0.4	35	65

The LC flow was diverted to the MS between 2.0 and 7.9 min, and to waste between 0.0 and 2.0 min and between 7.9 and 8.0 min.

MS Parameters - Methiozolin

Scan Type:	MRM
Polarity:	Positive
Ion Source:	Turbo Spray
Resolution Q1	Unit
Resolution Q3	Unit
Ion Source Gas 1 (GS1):	50 psi
Ion Source Gas 2 (GS2):	50 psi
Curtain Gas (CUR):	12 psi
Collision Gas (CAD):	6 psi
IonSpray Voltage (IS):	5500 V
Temperature (TEM):	500 °C
Declustering Potential (DP):	101 V
Entrance Potential (EP):	10 V
Collision Energy (CE):	41 V
Collision Gas Exit Potential (CXP):	8 V

MRM Transition	Analyte ID	Q1 Mass (amu)	Q3 Mass (amu)	Dwell Time (msec)
Primary	Methiozolin	338	127	200
Confirmatory	MethiozolinC	338	211	200

LC-MS/MS Systems - Analysis of DFBA

- HPLC: Two Shimadzu LC-20AD pumps and a Shimadzu SIL-HTA Controller/Autosampler
- MS: SCIEX API4000
- Computer software: Analyst™ version 1.4.2

DFBA Calibration Standards

For turf (thatch) and soil analysis, a series of DFBA calibration standards at 0.2, 0.5, 1, 2, 5, 10, 20, and 50 ng/mL are prepared to quantify the observed DFBA residues in all thatch and soil samples.

For grass analysis, a series of DFBA calibration standards at 0.1, 0.2, 0.5, 1, 2, 5, 10, and 20 ng/mL are prepared to quantify the observed DFBA residues in grass samples.

HPLC Method - DFBA

Column: Phenomenex Luna 5 µ Phenyl-Hexyl 150 mm x 2 mm
Column Temperature: Ambient
Injection Volume: 10 µL
Solvent System:

Solvent A = 0.1% acetic acid in HPLC water
Solvent B = 0.1% acetic acid in acetonitrile
Wash solvent = HPLC water:acetonitrile (50:50 v:v)

Solvent Program:

Time (minutes)	Flow Rate (mL/min)	%A	%B
0.0	0.3	75	25
1.0	0.3	75	25
4.0	0.3	5	95
6.0	0.3	5	95
6.1	0.3	75	25
10.0	0.3	75	25

The LC flow was diverted to the MS between 1.5 and 8.0 min, and to waste between 0.0 and 1.5 min and between 8.0 and 10.0 min.

MS Parameters - DFBA

Scan Type:	MRM
Polarity:	Negative
Ion Source:	Turbo Spray
Resolution Q1	Unit
Resolution Q3	Unit
Ion Source Gas 1 (GS1):	60 psi
Ion Source Gas 2 (GS2):	60 psi
Curtain Gas (CUR):	20 psi
Collision Gas (CAD):	8 psi
IonSpray Voltage (IS):	-4500 V
Temperature (TEM):	500° C
Declustering Potential (DP)	-25 V
Entrance Potential (EP):	-10 V
Collision Gas Exit Potential (CXP):	-23 V

MRM Transition	Analyte ID	Q1 Mass (amu)	Q3 Mass (amu)	Collision Energy (CE)	Dwell Time (msec)
Primary	DFBA	157	93	-35 V	500
Confirmatory	DFBA-C	157	73	-55 V	500

ORIGINAL PROTOCOL SECTION

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FREEZER STORAGE STABILITY

Once methodology has been validated a freezer storage stability study for grass, turf, and soil samples fortified with methiozolin will be conducted. Samples of the three matrices will be fortified with methiozolin at 10x the LOQ and will be stored frozen at ≤-5 °C. At 0, 7, 30, 90, 120 and 240 day intervals (approximate), three frozen and previously fortified samples will be removed and analyzed in a set that includes a control of the same matrix and two concurrent fortifications. Each interval will contain 6 samples for each of the three matrices (18 samples total). The total samples analyzed will equal 108 samples for analysis. Depending on stability, the intervals can be adjusted or the number of intervals can be reduced or expanded to meet the objectives of the study.

CHANGE TO

FREEZER STORAGE STABILITY

The sampling schedule for methiozolin and 2,6-difluorobenzoic acid (DFBA) freezer storage stability samples is changed as follows:

Methiozolin						
Original	Day 0	Day 7	Day 30	Day 90	Day 120	Day 240
Revised	Day 0	Day 7	Day 32	Day 86	Day 121	Day 528
Comment	No Change	No Change	Revised	Revised	Revised	Revised
DFBA						
Original	Day 0	Day 7	Day 30	Day 90	Day 120	Day 240
Revised	Day 0	Day 7	Day 33	Day 90	Day 370	Day 419
Comment	No Change	No Change	Revised	No Change	Revised	Revised

REASON FOR CHANGE/ADDITIONS

1. To describe the methods for analysis of methiozolin and 2,6-difluorobenzoic acid (DFBA) in soil, turf (thatch), and grass developed and validated in Study No. 031775 for analysis of methiozolin and DFBA in soil, turf (thatch), and grass freezer storage stability samples in Study No. 031775 and for analysis of soil, turf (thatch), and grass samples in New York, Georgia, California and New Jersey terrestrial field dissipation studies (Study Nos. 031786, 031812, 031813, and 031942, respectively).
2. The sampling schedule for methiozolin and 2,6-difluorobenzoic acid (DFBA) freezer storage stability samples is changed due to longer storage intervals of the field samples than originally estimated.

EFFECTIVE DATE

Date of Study Director's signature on the amendment.

PROTOCOL AMENDMENT TWO ACCEPTANCE

Study Title: Validation of Methodology and Freezer Storage Stability of Methiozolin and 2,6-Difluorobenzoic Acid in Grass, Turf and Soil

Ricerca Document Number: 031775-0-2

Testing Facility: AgChem Product Development
Ricerca Biosciences, LLC
7528 Auburn Road
Concord, OH 44077

Ling-Jen Ferguson — 17 August 2015

Ling-Jen Ferguson, Study Director
Ricerca Biosciences, LLC
Date

RMcClanahan — 17 August 2015

Robert McClanahan, Management
Ricerca Biosciences, LLC
Date

SPONSOR: Moghu Research Center Ltd

SPONSOR APPROVAL DATE: AUGUST 17, 2015

APPENDIX 3

Method 031775A:
LC-MS/MS Assay for the Determination of Methiozolin and/or DFBA in Soil

TITLE

Method 031775A: LC-MS/MS Assay for the Determination of Methiozolin and/or DFBA in Soil

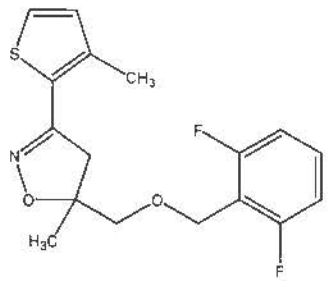
INTRODUCTION

In Method No. 031775A, residues of methiozolin and DFBA are extracted concomitantly from ten grams of soil with acetonitrile-HPLC water (80:20, v:v), filtered through 0.45 µm PTFE syringe filters, diluted with acetonitrile-HPLC water (30:70, v:v), then further diluted with acetonitrile-HPLC water (50:50, v:v) (if required), and quantified separately for methiozolin and DFBA by LC-MS/MS. This method was used for analysis of methiozolin and DFBA in soil in New York, Georgia, California, and New Jersey terrestrial field dissipation studies (Study Numbers 031786, 031812, 031813, and 031942, respectively).

MATERIALS AND METHODS

REFERENCE STANDARDS

- **Methiozolin**



Information concerning the reference standard is provided below:

Common Name:	Methiozolin
Chemical Name:	5-[[(2,6-difluorophenyl)methoxy]methyl]-4,5-dihydro-5-methyl-3-(3-methyl-2-thienyl)isoxazole
CAS No.:	403640-27-7
Molecular Formula:	$C_{17}H_{17}F_2NO_2S$
Molecular Weight:	337.38 g/mole
Source:	Moghu Research Center
Lot No.:	MRC111001
Purity:	99.71% (by HPLC)
Expiration Date:	September 30, 2016
Storage:	Room temperature

Solutions made from the reference standard were stored frozen at ≤ -5 °C.

- **2,6-Difluorobenzoic acid (DFBA)**

2,6-Difluorobenzoic acid

Information concerning the reference standard is provided below:

Chemical Name:	2,6-Difluorobenzoic acid
CAS No.:	385-00-2
Molecular Formula:	$C_7H_4F_2O_2$
Molecular Weight:	158.1 g/mol
Source:	Sigma-Aldrich
Lot No.:	MKBQ3617V
Ricerca Sample Code:	CS_19165
Stated Purity:	94.86%
Storage:	Ambient temperature

Solutions made from the reference standard were stored frozen at ≤ -5 °C.

Chemicals

- Acetic acid, glacial, Fisher Certified ACS Plus Grade
- Formic acid, Fisher Optima LC-MS Grade
- Ammonium formate, Fisher Optima LC-MS Grade
- Acetonitrile, Fisher Optima Grade
- Water, Fisher HPLC Grade
- Methanol, Fisher HPLC or Optima Grade

Equipment

- Hobart 8181D food cutter
- Analytical electronic balance with 0.1-mg readability
- Benchtop electronic balance with 0.01-g readability
- Eppendorf micropipettes: 10-100 µL, 20-200 µL, and 100-1000 µL
- Burrell Wrist-Action Shaker
- Sorvall RC-5B Centrifuge
- 0.45-µm Polytetrafluoroethylene (PTFE) syringe filters
- Glassware: Assorted beakers, bottles, graduated cylinders, pipettes, etc., which are routinely used for residue analysis
- HPLC-MS/MS (see LC-MS/MS Instrumentation section for further information)

PREPARATION OF METHIOZOLIN STOCK, FORTIFICATION, INTERMEDIATE, AND CALIBRATION SOLUTIONS

The methiozolin stock, fortification, intermediate, and calibration solutions were prepared every six months during the course of the study. Once prepared, these solutions were assigned a 6-month expiry date and stored frozen at ≤ -5 °C when not in use. The representative preparation is shown below:

Preparation of Methiozolin Stock Solutions on 4/25/14 and 4/28/14

- **Stock solution A** (1009 µg/mL Methiozolin in ACN, purity (99.71%) corrected, used for preparation of fortification solutions). Methiozolin (25.3 mg) was weighed in a 25-mL "Class A" volumetric flask and ACN was added to the mark.
- **Stock solution B** (1021 µg/mL Methiozolin in ACN, purity (99.71%) corrected, used for preparation of calibration solutions). Methiozolin (25.6 mg) was weighed in a 25-mL "Class A" volumetric flask and ACN was added to the mark.

Preparation of Methiozolin Fortification Solutions on 4/25/14

The following fortification solutions were prepared by serial dilution of **Stock solution A** with ACN-water (50:50, v:v) in "Class A" volumetric flasks, as detailed below:

- **Stock solution A**: 1009 µg/mL Methiozolin in ACN (purity corrected)

Standard solution used	Volume taken (mL)	Final volume (mL)	Nominal concentration (µg/mL)
1009 µg/mL	4.96	50	100
100 µg/mL	5	50	10
10 µg/mL	5	50	1

Preparation of Methiozolin Intermediate and Calibration Solutions on 4/28/14

The following intermediate and calibration solutions were prepared by serial dilution of **Stock solution B** with ACN-water (50:50, v:v) in "Class A" volumetric flasks, as detailed below:

- **Stock solution** B:1021 µg/mL Methiozolin in ACN (purity corrected)

Methiozolin Intermediate Solutions

Standard solution used	Volume taken (mL)	Final volume (mL)	Nominal concentration (ng/mL)
1021µg/mL	0.0980	100	1000
1 µg/mL	5	50	100
1 µg/mL	2.5	50	50
1 µg/mL	1	50	20

Methiozolin Calibration Solutions

Standard solution used	Volume taken (mL)	Final volume (mL)	Nominal concentration (ng/mL)
100 ng/mL	5	50	10
50 ng/mL	5	50	5
20 ng/mL	5	50	2
10 ng/mL	5	50	1
10 ng/mL	5	100	0.5
2 ng/mL	5	50	0.2
2 ng/mL	5	100	0.1

PREPARATION OF DFBA STOCK, FORTIFICATION, INTERMEDIATE, AND CALIBRATION SOLUTIONS

The DFBA stock, fortification, intermediate, and calibration solutions were prepared every six months during the course of the study. Once prepared, these solutions were assigned a 6-month expiry date and stored frozen at ≤ -5 °C when not in use. The representative preparation is shown below:

Preparation of DFBA Stock Solutions on 4/25/14 and 4/28/14

- **Stock solution A** (1396 µg/mL DFBA in ACN, purity (94.86%) corrected, used for preparation of fortification solutions). DFBA (36.8 mg) was weighed in a 25-mL "Class A" volumetric flask and ACN was added to the mark.
- **Stock solution B** (1032 µg/mL DFBA in ACN, purity (94.86%) corrected, used for preparation of calibration solutions). DFBA (27.2 mg) was weighed in a 25-mL "Class A" volumetric flask and ACN was added to the mark.

Preparation of DFBA Fortification Solutions on 4/28/14

The following fortification solutions were prepared by serial dilution of **Stock solution A** with ACN-water (50:50, v:v) in "Class A" volumetric flasks, as detailed below:

- **Stock solution A**: 1396 µg/mL DFBA in ACN (purity corrected)

Standard solution used	Volume taken (mL)	Final volume (mL)	Nominal concentration (µg/mL)
1396 µg/mL	3.58	50	100
100 µg/mL	5	50	10
10 µg/mL	5	50	1

Preparation of DFBA Intermediate and Calibration Solutions on 4/25/14

The following intermediate and calibration solutions were prepared by serial dilution of **Stock solution B** with ACN-water (50:50, v:v) in "Class A" volumetric flasks, as detailed below:

- **Stock solution** B:1032 µg/mL DFBA in ACN (purity corrected)

DFBA Intermediate Solutions

Standard solution used	Volume taken (mL)	Final volume (mL)	Nominal concentration (µg/mL)
1032 µg/mL	4.85	50	100
100 µg/mL	5	50	10
10 µg/mL	5	50	1
1 µg/mL	5	50	0.1

DFBA Calibration Solutions

Standard solution used	Volume taken (mL)	Final volume (mL)	Nominal concentration (ng/mL)
1 µg/mL	2.5	50	50
1 µg/mL	1	50	20
0.1 µg/mL	5	50	10
50 ng/mL	5	50	5
20 ng/mL	5	50	2
10 ng/mL	5	50	1
5 ng/mL	5	50	0.5
2 ng/mL	5	50	0.2

FORTIFICATION

The control soil samples (approximately 10 g each) are fortified with the following solutions:

Fortification Level	Fortification Solution and Volume (µL) Used
Methiozolin and DFBA in Soil (~10 g)	
Control	ACN:H_2O (50:50), 100 µL
0.01 ppm	1 µg/mL methiozolin in ACN:H_2O (50:50), 100 µL 1 µg/mL DFBA in ACN:H_2O (50:50), 100 µL
0.1 ppm	10 µg/mL methiozolin in ACN:H_2O (50:50), 100 µL 10 µg/mL DFBA in ACN:H_2O (50:50), 100 µL
1 ppm	100 µg/mL methiozolin in ACN:H_2O (50:50), 100 µL 100 µg/mL DFBA in ACN:H_2O (50:50), 100 µL
3 ppm	100 µg/mL methiozolin in ACN:H_2O (50:50), 300 µL

ACN = acetonitrile

ANALYSIS OF METHIOZOLIN AND DFBA

HOMOGENIZATION, EXTRACTION, AND CLEAN-UP

1. Frozen soil samples from terrestrial field dissipation studies are homogenized with Dry Ice in a Hobart 8181D food cutter, and the Dry Ice is permitted to sublimate before samples are weighed for analysis.
2. Aliquots (10 g each) of soil are weighed into 50-mL polypropylene centrifuge tubes and fortified with the appropriate amount of methiozolin and DFBA.
3. Acetonitrile-HPLC water (80:20, v:v) (40 mL) is added to each sample.
4. The samples are shaken using a Burrell wrist-action shaker for 30 minutes.
5. The samples are centrifuged for 10 minutes at ~3000 rpm.
6. An aliquot of the supernatant is filtered through a 0.45-µm PTFE syringe filter into a 15-mL polypropylene centrifuge tube.
7. A 4-mL aliquot of the filtrate is diluted to 10 mL with acetonitrile-HPLC water (30:70, v:v) and the sample is mixed.
8. Samples are diluted with ACN-HPLC water (50:50, v:v) as follows: 10XLOQ (0.1 ppm) diluted 2X, 100XLOQ (1 ppm) diluted 20X, and 300XLOQ (3 ppm) diluted 50X.
9. Samples are transferred to autosampler vials for LC-MS/MS analysis for methiozolin and/or DFBA.

LC-MS/MS SYSTEMS – ANALYSIS OF METHIOZOLIN

Shimadzu HPLC-API4000 MS

- HPLC: Two Shimadzu LC-20AD pumps and a Shimadzu SIL-HTA Controller/Autosampler
- MS: SCIEX API4000
- Computer software: Analyst™ version 1.4.2

Flexar UHPLC-API4000 MS

- HPLC: PerkinElmer Flexar FX-15 UHPLC
- MS: SCIEX API4000
- Computer software: Analyst™ version 1.4.2

Methiozolin Calibration Standards

For soil analysis, a series of methiozolin calibration standards at 0.1, 0.2, 0.5, 1, 2, 5, and 10 ng/mL are prepared to quantify the observed methiozolin residues in soil samples.

HPLC Method - Methiozolin

Column: Phenomenex Luna 5 µ Phenyl-Hexyl 150 mm x 2 mm
Column Temperature: Ambient
Injection Volume: 5 µL (Shimadzu HPLC) or 20 µL (Flexar HPLC)
Solvent System:

- Solvent A = 0.1% formic acid in HPLC water-methanol (90:10, v:v) + 10 mM ammonium formate
- Solvent B = 0.1% formic acid in methanol
- Wash solvent = HPLC water-acetonitrile (50:50, v:v)

Solvent program (Shimadzu HPLC):

Time (minutes)	Flow Rate (mL/min)	%A	%B
0.0	0.4	35	65
3.0	0.4	5	95
5.0	0.4	5	95
5.5	0.4	35	65
8.0	0.4	35	65

The LC flow is diverted to the MS between 2.0 and 7.9 min, and to waste between 0.0 and 2.0 min and between 7.9 and 8.0 min.

Solvent program (Flexar UHPLC):

Time (minutes)	Flow Rate (mL/min)	%A	%B
0.0	0.4	35	65
3.5	0.4	5	95
5.0	0.4	5	95
5.5	0.4	35	65
8.0	0.4	35	65

The LC flow is diverted to the MS between 2.0 and 7.9 min, and to waste between 0.0 and 2.0 min and between 7.9 and 8.0 min.

MS Parameters-Methiozolin

Scan Type:	MRM
Polarity:	Positive
Ion Source:	Turbo Spray
Resolution Q1	Unit
Resolution Q3	Unit
Ion Source Gas 1 (GS1):	50 psi
Ion Source Gas 2 (GS2):	50 psi
Curtain Gas (CUR):	12 psi
Collision Gas (CAD):	6 psi
IonSpray Voltage (IS):	5500 V
Temperature (TEM):	500 °C
Declustering Potential (DP):	101 V
Entrance Potential (EP):	10 V
Collision Energy (CE):	41 V
Collision Gas Exit Potential (CXP):	8 V

MRM Transition	Analyte ID	Q1 Mass (amu)	Q3 Mass (amu)	Dwell Time (msec)
Primary	Methiozolin	338	127	200
Confirmatory	MethiozolinC	338	211	200

LC-MS/MS SYSTEMS – ANALYSIS OF DFBA

Shimadzu HPLC-API4000 MS

- HPLC: Two Shimadzu LC-20AD pumps and a Shimadzu SIL-HTA Controller/Autosampler
- MS: SCIEX API4000
- Computer software: Analyst™ version 1.4.2

DFBA Calibration Standards

For soil analysis, a series of DFBA calibration standards at 0.2, 0.5, 1, 2, 5, 10, 20, and 50 ng/mL are prepared to quantify the observed DFBA residues in soil samples.

HPLC Method - DFBA

Column: Phenomenex Luna 5 µ Phenyl-Hexyl 150 mm x 2 mm
Column Temperature: Ambient
Injection Volume: 10 µL
Solvent System:

- Solvent A = 0.1% acetic acid in HPLC water
- Solvent B = 0.1% acetic acid in acetonitrile
- Wash solvent = HPLC water:acetonitrile (50:50, v:v)

Solvent Program:

Time (minutes)	Flow Rate (mL/min)	%A	%B
0.0	0.3	75	25
1.0	0.3	75	25
4.0	0.3	5	95
6.0	0.3	5	95
6.1	0.3	75	25
10.0	0.3	75	25

The LC flow was diverted to the MS between 1.5 and 8.0 min, and to waste between 0.0 and 1.5 min and between 8.0 and 10.0 min.

MS Parameters-DFBA

Scan Type:	MRM
Polarity:	Negative
Ion Source:	Turbo Spray
Resolution Q1	Unit
Resolution Q3	Unit
Ion Source Gas 1 (GS1):	60 psi
Ion Source Gas 2 (GS2):	60 psi
Curtain Gas (CUR):	20 psi
Collision Gas (CAD):	8 psi
IonSpray Voltage (IS):	-4500 V
Temperature (TEM):	500 °C
Declustering Potential (DP):	-25 V
Entrance Potential (EP):	-10 V
Collision Gas Exit Potential (CXP):	-23 V

MRM Transition	Analyte ID	Q1 Mass (amu)	Q3 Mass (amu)	Dwell Time (msec)
Primary	DFBA	157	93	500
Confirmatory	DFBA-C	157	73	500

LIMITS OF QUANTIFICATIION AND DETECTION

The Limit of Quantification (LOQ) is 0.01 ppm. The LOQ is defined as the lowest fortification level at which acceptable recovery data are obtained. The Limit of Detection (LOD) was set at 0.002 ppm.

APPENDIX 4

Method 031775B:
LC-MS/MS Assay for the Determination of Methiozolin in Turf (Thatch)

TITLE

Method 031775B: LC-MS/MS Assay for the Determination of Methiozolin in Turf (Thatch)

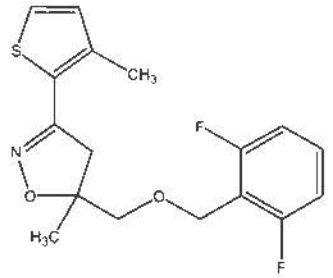
INTRODUCTION

In Method No. 031775B, residues of methiozolin are extracted from five grams of turf (thatch) with acetonitrile-HPLC water (80:20, v:v), cleaned up with solid phase extraction, diluted with acetonitrile-HPLC water (50:50, v:v) (if required), and quantified for methiozolin by LC-MS/MS.

MATERIALS AND METHODS

REFERENCE STANDARD

- **Methiozolin**



Information concerning the reference standard is provided below:

Common Name:	Methiozolin
Chemical Name:	5-[[(2,6-difluorophenyl)methoxy]methyl]-4,5-dihydro-5-methyl-3-(3-methyl-2-thienyl)isoxazole
CAS No.:	403640-27-7
Molecular Formula:	$C_{17}H_{17}F_2NO_2S$
Molecular Weight:	337.38 g/mole
Source:	Moghu Research Center
Lot No.:	MRC111001
Purity:	99.71% (by HPLC)
Expiration Date:	September 30, 2016
Storage:	Room temperature

Solutions made from the reference standard were stored frozen at ≤ -5 °C.

Chemicals

- Formic acid, Fisher Optima LC-MS Grade
- Ammonium formate, Fisher Optima LC-MS Grade
- Acetonitrile, Fisher Optima Grade
- Water, Fisher HPLC Grade
- Methanol, Fisher HPLC or Optima Grade

Equipment

- Hobart 8181D food cutter
- Robot Coupe Blixer, Model BX4V or BX5V
- Analytical electronic balance with 0.1-mg readability
- Benchtop electronic balance with 0.01-g readability
- Eppendorf micropipettes: 10-100 µL, 20-200 µL, and 100-1000 µL
- Burrell Wrist-Action Shaker
- Sorvall RC-5B Centrifuge
- Waters Oasis HLB VAC (60 mg) solid phase extraction (SPE) cartridges
- Supelco Visiprep SPE vacuum manifolds
- Glassware: Assorted beakers, bottles, graduated cylinders, pipettes, etc., which are routinely used for residue analysis
- HPLC-MS/MS (see LC-MS/MS Instrumentation section for further information)

PREPARATION OF METHIOZOLIN STOCK, FORTIFICATION, INTERMEDIATE, AND CALIBRATION SOLUTIONS

The methiozolin stock, fortification, intermediate, and calibration solutions were prepared every six months during the course of the study. Once prepared, these solutions were assigned a 6-month expiry date and stored frozen at ≤ -5 °C when not in use. The representative preparation is shown below:

Preparation of Methiozolin Stock Solutions on 10/25/13

- **Stock solution A** (1029 µg/mL Methiozolin in ACN, purity (99.71%) corrected, used for preparation of fortification solutions). Methiozolin (25.8 mg) was weighed in a 25-mL "Class A" volumetric flask and ACN was added to the mark.
- **Stock solution B** (1013 µg/mL Methiozolin in ACN, purity (99.71%) corrected, used for preparation of calibration solutions). Methiozolin (25.4 mg) was weighed in a 25-mL "Class A" volumetric flask and ACN was added to the mark.

Preparation of Methiozolin Fortification Solutions on 10/25/13

The following fortification solutions were prepared by serial dilution of **Stock solution A** with ACN-water (50:50, v:v) in "Class A" volumetric flasks, as detailed below:

- **Stock solution A**: 1029 µg/mL Methiozolin in ACN (purity corrected)

Standard solution used	Volume taken (mL)	Final volume (mL)	Nominal concentration (µg/mL)
1029 µg/mL	4.86	50	100
100 µg/mL	5	50	10
10 µg/mL	5	50	1

Preparation of Methiozolin Intermediate and Calibration Solutions on 10/25/13

The following intermediate and calibration solutions were prepared by serial dilution of **Stock solution B** with ACN-water (50:50, v:v) in "Class A" volumetric flasks, as detailed below:

- **Stock solution** B: 1013 µg/mL Methiozolin in ACN (purity corrected)

Methiozolin Intermediate Solutions

Standard solution used	Volume taken (mL)	Final volume (mL)	Nominal concentration (ng/mL)
1013µg/mL	0.0988	100	1000
1 µg/mL	5	50	100
1 µg/mL	2.5	50	50
1 µg/mL	1	50	20

Methiozolin Calibration Solutions

Standard solution used	Volume taken (mL)	Final volume (mL)	Nominal concentration (ng/mL)
100 ng/mL	5	50	10
50 ng/mL	5	50	5
20 ng/mL	5	50	2
10 ng/mL	5	50	1
10 ng/mL	5	100	0.5
2 ng/mL	5	50	0.2
2 ng/mL	5	100	0.1

FORTIFICATION

The control soil samples (approximately 10 g each) are fortified with the following solutions:

Fortification Level	Fortification Solution	Volume (µL)
Methiozolin in Thatch		
Control	ACN:H_2O (50:50)	50
0.01 ppm	1 µg/mL methiozolin in ACN:H_2O (50:50)	50
0.1 ppm	10 µg/mL methiozolin in ACN:H_2O (50:50)	50
1 ppm	100 µg/mL methiozolin in ACN:H_2O (50:50)	50

ACN = acetonitrile

ANALYSIS OF METHIOZOLIN

HOMOGENIZATION, EXTRACTION, AND CLEAN-UP

Extraction

1. Frozen turf (thatch) samples from terrestrial field dissipation studies are homogenized with Dry Ice in a Hobart 8181D food cutter or a Robot Coupe Blixer, Model BX4V or BX5V, and the Dry Ice is permitted to sublimate before samples are weighed for analysis.
2. Aliquots (5 g each) of soil are weighed into 50-mL polypropylene centrifuge tubes and fortified with the appropriate amount of methiozolin.
3. Acetonitrile-HPLC water (80:20, v:v) (40 mL) is added to each sample.
4. The samples are shaken using a Burrell wrist-action shaker for 30 minutes.
5. The samples are centrifuged for 10 minutes at ~3000 rpm.
6. An aliquot of 4 mL of the supernatant is removed from each sample and mixed with 20 mL of HPLC water for SPE clean-up.

SPE Cartridge Clean-up

1. A Waters Oasis HLB VAC (60 mg) SPE cartridge is placed on a Supelco Visiprep SPE vacuum manifold and washed with 5 mL methanol followed by 5 mL HPLC water.
2. The cartridge is loaded with the sample from the extraction step # 6. The eluate is discarded.
3. Methiozolin is eluted with 5 mL of acetonitrile. The eluate is collected into a 15-mL polypropylene centrifuge tube.
4. The eluate is diluted to 10 mL with HPLC water and the sample is mixed.
5. 100XLOQ (1 ppm) samples are diluted 10X with acetonitrile-HPLC water (50:50, v:v).
6. Samples are transferred to autosampler vials for LC-MS/MS analysis.

LC-MS/MS SYSTEMS – ANALYSIS OF METHIOZOLIN

Shimadzu HPLC-API4000 MS

- HPLC: Two Shimadzu LC-20AD pumps and a Shimadzu SIL-HTA Controller/Autosampler
- MS: SCIEX API4000
- Computer software: Analyst™ version 1.4.2

Methiozolin Calibration Standards

For turf (thatch) analysis, a series of methiozolin calibration standards at 0.1, 0.2, 0.5, 1, 2, 5, and 10 ng/mL are prepared to quantify the observed methiozolin residues in turf (thatch) samples.

HPLC Method - Methiozolin

Column: Phenomenex Luna 5 µ Phenyl-Hexyl 150 mm x 2 mm
Column Temperature: Ambient
Injection Volume: 5 µL (Shimadzu HPLC)

Solvent System:

- Solvent A = 0.1% formic acid in HPLC water-methanol (90:10, v:v) + 10 mM ammonium formate
- Solvent B = 0.1% formic acid in methanol
- Wash solvent = HPLC water-acetonitrile (50:50, v:v)

Solvent program (Shimadzu HPLC):

Time (minutes)	Flow Rate (mL/min)	%A	%B
0.0	0.4	35	65
3.0	0.4	5	95
5.0	0.4	5	95
5.5	0.4	35	65
8.0	0.4	35	65

The LC flow is diverted to the MS between 2.0 and 7.9 min, and to waste between 0.0 and 2.0 min and between 7.9 and 8.0 min.

MS Parameters-Methiozolin

Scan Type:	MRM
Polarity:	Positive
Ion Source:	Turbo Spray
Resolution Q1	Unit
Resolution Q3	Unit
Ion Source Gas 1 (GS1):	50 psi
Ion Source Gas 2 (GS2):	50 psi
Curtain Gas (CUR):	12 psi
Collision Gas (CAD):	6 psi
IonSpray Voltage (IS):	5500 V
Temperature (TEM):	500 °C
Declustering Potential (DP):	101 V
Entrance Potential (EP):	10 V
Collision Energy (CE):	41 V
Collision Gas Exit Potential (CXP):	8 V

MRM Transition	Analyte ID	Q1 Mass (amu)	Q3 Mass (amu)	Dwell Time (msec)
Primary	Methiozolin	338	127	200
Confirmatory	MethiozolinC	338	211	200

LIMITS OF QUANTIFICATIION AND DETECTION

The Limit of Quantification (LOQ) is 0.01 ppm. The LOQ is defined as the lowest fortification level at which acceptable recovery data are obtained. The Limit of Detection (LOD) was set at 0.002 ppm.

APPENDIX 5

Method 031775C:
LC-MS/MS Assay for the Determination of Methiozolin and/or DFBA in Turf (Thatch)

TITLE

Method 031775C: LC-MS/MS Assau for the Determination of Methiozolin and/or DFBA in Turf (Thatch)

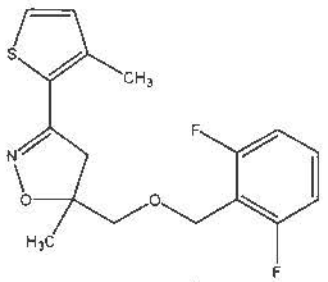
INTRODUCTION

In Method No. 031775C, residues of methiozolin and DFBA are extracted concomitantly from thatch with acetone, and then with acetonitrile-HPLC water (50:50, v:v), the extracts combined and then filtered through 0.45 µm PTFE syringe filters, diluted with acetonitrile-HPLC water (50:50, v:v) (if required), quantified separately for methiozolin and DFBA by LC-MS/MS. This method was used analysis of methiozolin and DFBA in turf (thatch) in New York, Georgia, California, and New Jersey terrestrial field dissipation studies (Study Numbers 031786, 031812, 031813, and 031942, respectively).

MATERIALS AND METHODS

REFERENCE STANDARDS

- **Methiozolin**



Information concerning the reference standard is provided below:

Common Name:	Methiozolin
Chemical Name:	5-[[(2,6-difluorophenyl)methoxy]methyl]-4,5-dihydro-5-methyl-3-(3-methyl-2-thienyl)isoxazole
CAS No.:	403640-27-7
Molecular Formula:	$C_{17}H_{17}F_2NO_2S$
Molecular Weight:	337.38 g/mole
Source:	Moghu Research Center
Lot No.:	MRC111001
Purity:	99.71% (by HPLC)
Expiration Date:	September 30, 2016
Storage:	Room temperature

Solutions made from the reference standard were stored frozen at ≤ -5 °C.

- **2,6-Difluorobenzoic acid (DFBA)**

2,6-Difluorobenzoic acid

Information concerning the reference standard is provided below:

Chemical Name:	2,6-Difluorobenzoic acid
CAS No.:	385-00-2
Molecular Formula:	$C_7H_4F_2O_2$
Molecular Weight:	158.1 g/mol
Source:	Sigma-Aldrich
Lot No.:	MKBQ3617V
Ricerca Sample Code:	CS_19165
Stated Purity:	94.86%
Storage:	Ambient temperature

Solutions made from the reference standard were stored frozen at ≤ -5 °C.

Chemicals

- Acetic acid, glacial, Fisher Certified ACS Plus Grade
- Formic acid, Fisher Optima LC-MS Grade
- Ammonium formate, Fisher Optima LC-MS Grade
- Acetonitrile, Fisher Optima Grade
- Water, Fisher HPLC Grade
- Methanol, Fisher HPLC or Optima Grade
- Acetone, Fisher HPLC or Optima Grade

Equipment

- Hobart 8181D food cutter
- Analytical electronic balance with 0.1-mg readability
- Benchtop electronic balance with 0.01-g readability
- Eppendorf micropipettes: 10-100 µL, 20-200 µL, and 100-1000 µL
- Burrell Wrist-Action Shaker
- Sorvall RC-5B Centrifuge
- 0.45-µm Polytetrafluoroethylene (PTFE) syringe filters
- Glassware: Assorted beakers, bottles, graduated cylinders, pipettes, etc., which are routinely used for residue analysis
- HPLC-MS/MS (see LC-MS/MS Instrumentation section for further information)

PREPARATION OF METHIOZOLIN STOCK, FORTIFICATION, INTERMEDIATE, AND CALIBRATION SOLUTIONS

The methiozolin stock, fortification, intermediate, and calibration solutions were prepared every six months during the course of the study. Once prepared, these solutions were assigned a 6-month expiry date and stored frozen at ≤ -5 °C when not in use. The representative preparation is shown below:

Preparation of Methiozolin Stock Solutions on 4/25/14 and 4/28/14

- **Stock solution A** (1009 µg/mL Methiozolin in ACN, purity (99.71%) corrected, used for preparation of fortification solutions). Methiozolin (25.3 mg) was weighed in a 25-mL "Class A" volumetric flask and ACN was added to the mark.
- **Stock solution B** (1021 µg/mL Methiozolin in ACN, purity (99.71%) corrected, used for preparation of calibration solutions). Methiozolin (25.6 mg) was weighed in a 25-mL "Class A" volumetric flask and ACN was added to the mark.

Preparation of Methiozolin Fortification Solutions on 4/25/14

The following fortification solutions were prepared by serial dilution of **Stock solution A** with ACN-water (50:50, v:v) in "Class A" volumetric flasks, as detailed below:

- **Stock solution A**: 1009 µg/mL Methiozolin in ACN (purity corrected)

Standard solution used	Volume taken (mL)	Final volume (mL)	Nominal concentration (µg/mL)
1009 µg/mL	4.96	50	100
100 µg/mL	5	50	10
10 µg/mL	5	50	1

Preparation of Methiozolin Intermediate and Calibration Solutions on 4/28/14

The following intermediate and calibration solutions were prepared by serial dilution of **Stock solution B** with ACN-water (50:50, v:v) in "Class A" volumetric flasks, as detailed below:

- **Stock solution** B:1021 µg/mL Methiozolin in ACN (purity corrected)

Methiozolin Intermediate Solutions

Standard solution used	Volume taken (mL)	Final volume (mL)	Nominal concentration (ng/mL)
1021µg/mL	0.0980	100	1000
1 µg/mL	5	50	100
1 µg/mL	2.5	50	50
1 µg/mL	1	50	20

Methiozolin Calibration Solutions

Standard solution used	Volume taken (mL)	Final volume (mL)	Nominal concentration (ng/mL)
100 ng/mL	5	50	10
50 ng/mL	5	50	5
20 ng/mL	5	50	2
10 ng/mL	5	50	1
10 ng/mL	5	100	0.5
2 ng/mL	5	50	0.2
2 ng/mL	5	100	0.1

PREPARATION OF DFBA STOCK, FORTIFICATION, INTERMEDIATE, AND CALIBRATION SOLUTIONS

The DFBA stock, fortification, intermediate, and calibration solutions were prepared every six months during the course of the study. Once prepared, these solutions were assigned a 6-month expiry date and stored frozen at ≤ -5 °C when not in use. The representative preparation is shown below:

Preparation of DFBA Stock Solutions on 4/25/14 and 4/28/14

- **Stock solution A** (1396 µg/mL DFBA in ACN, purity (94.86%) corrected, used for preparation of fortification solutions). DFBA (36.8 mg) was weighed in a 25-mL "Class A" volumetric flask and ACN was added to the mark.
- **Stock solution B** (1032 µg/mL DFBA in ACN, purity (94.86%) corrected, used for preparation of calibration solutions). DFBA (27.2 mg) was weighed in a 25-mL "Class A" volumetric flask and ACN was added to the mark.

Preparation of DFBA Fortification Solutions on 4/28/14

The following fortification solutions were prepared by serial dilution of **Stock solution A** with ACN-water (50:50, v:v) in "Class A" volumetric flasks, as detailed below:

- **Stock solution A**: 1396 µg/mL DFBA in ACN (purity corrected)

Standard solution used	Volume taken (mL)	Final volume (mL)	Nominal concentration (µg/mL)
1396 µg/mL	3.58	50	100
100 µg/mL	5	50	10
10 µg/mL	5	50	1

Preparation of DFBA Intermediate and Calibration Solutions on 4/25/14

The following intermediate and calibration solutions were prepared by serial dilution of **Stock solution B** with ACN-water (50:50, v:v) in "Class A" volumetric flasks, as detailed below:

- **Stock solution** B:1032 µg/mL DFBA in ACN (purity corrected)

DFBA Intermediate Solutions

Standard solution used	Volume taken (mL)	Final volume (mL)	Nominal concentration (µg/mL)
1032 µg/mL	4.85	50	100
100 µg/mL	5	50	10
10 µg/mL	5	50	1
1 µg/mL	5	50	0.1

DFBA Calibration Solutions

Standard solution used	Volume taken (mL)	Final volume (mL)	Nominal concentration (ng/mL)
1 µg/mL	2.5	50	50
1 µg/mL	1	50	20
0.1 µg/mL	5	50	10
50 ng/mL	5	50	5
20 ng/mL	5	50	2
10 ng/mL	5	50	1
5 ng/mL	5	50	0.5
2 ng/mL	5	50	0.2

FORTIFICATION

The concurrent recovery thatch samples from the control plot (approximately 5 g each) are fortified with the following solutions:

Fortification Level	Fortification Solution and Volume (µL) Used
Methiozolin and DFBA in Thatch (~5 g)	
Control	ACN:H_2O (50:50), 100 µL
0.01 ppm	1 µg/mL methiozolin in ACN:H_2O (50:50), 50 µL 1 µg/mL DFBA in ACN:H_2O (50:50), 50 µL
0.1 ppm	10 µg/mL methiozolin in ACN:H_2O (50:50), 50 µL 10 µg/mL DFBA in ACN:H_2O (50:50), 50 µL
1 ppm	100 µg/mL methiozolin in ACN:H_2O (50:50), 50 µL 100 µg/mL DFBA in ACN:H_2O (50:50), 50 µL
4 ppm	100 µg/mL methiozolin in ACN:H_2O (50:50), 200 µL

ACN = acetonitrile

ANALYSIS OF METHIOZOLIN AND DFBA

HOMOGENIZATION, EXTRACTION, AND CLEAN-UP

1. Frozen thatch samples are homogenized with Dry Ice in a Hobart 8181D food cutter, and the Dry Ice is permitted to sublimate before samples are weighed for analysis.

2. Aliquots (approximately 5 g each) of thatch are weighed into 50-mL polypropylene centrifuge tubes and fortified with the appropriate amount of methiozolin and DFBA.
3. Acetone (40 mL) is added to each sample.
4. The samples are shaken using a Burrell wrist-action shaker for 30 minutes.
5. The samples are centrifuged for 10 minutes at ~3000 rpm.
6. The supernatant is transferred to a new 125-mL polyethylene bottle.
7. The solid residue is re-extracted with 40 mL of acetonitrile-HPLC water (50:50, v:v), as for step 3 to 5, combining the supernatants in the 125-mL polyethylene bottle.
8. An aliquot of the combined supernatants is filtered through a 0.45-µm PTFE syringe filter.
9. Samples are diluted with ACN-HPLC water (50:50, v:v) as follows: 100XLOQ (1 ppm) diluted 10X, and 400XLOQ (4 ppm) diluted 50X.
10. Samples are transferred to autosampler vials for LC-MS/MS analysis for methiozolin and DFBA.

LC-MS/MS SYSTEMS – ANALYSIS OF METHIOZOLIN

Shimadzu HPLC-API4000 MS

- HPLC: Two Shimadzu LC-20AD pumps and a Shimadzu SIL-HTA Controller/Autosampler
- MS: SCIEX API4000
- Computer software: Analyst™ version 1.4.2

Flexar UHPLC-API4000 MS

- HPLC: PerkinElmer Flexar FX-15 UHPLC
- MS: SCIEX API4000
- Computer software: Analyst™ version 1.4.2

Methiozolin Calibration Standards

For thatch analysis, a series of methiozolin calibration standards at 0.1, 0.2, 0.5, 1, 2, 5, and 10 ng/mL are prepared to quantify the observed methiozolin residues in thatch samples.

HPLC Method - Methiozolin

Column: Phenomenex Luna 5 µ Phenyl-Hexyl 150 mm x 2 mm
Column Temperature: Ambient
Injection Volume: 5 µL (Shimadzu HPLC) or 20 µL (Flexar HPLC)

Solvent System:

- Solvent A = 0.1% formic acid in HPLC water-methanol (90:10, v:v) + 10 mM ammonium formate
- Solvent B = 0.1% formic acid in methanol
- Wash solvent = HPLC water-acetonitrile (50:50, v:v)

Solvent program (Shimadzu HPLC):

Time (minutes)	Flow Rate (mL/min)	%A	%B
0.0	0.4	35	65
3.0	0.4	5	95
5.0	0.4	5	95
5.5	0.4	35	65
8.0	0.4	35	65

The LC flow is diverted to the MS between 2.0 and 7.9 min. and to waste between 0.0 and 2.0 min and between 7.9 and 8.0 min.

Solvent program (Flexar UHPLC):

Time (minutes)	Flow Rate (mL/min)	%A	%B
0.0	0.4	35	65
3.5	0.4	5	95
5.0	0.4	5	95
5.5	0.4	35	65
8.0	0.4	35	65

The LC flow is diverted to the MS between 2.0 and 7.9 min, and to waste between 0.0 and 2.0 min and between 7.9 and 8.0 min.

MS Parameters-Methiozolin

Scan Type:	MRM
Polarity:	Positive
Ion Source:	Turbo Spray
Resolution Q1	Unit
Resolution Q3	Unit
Ion Source Gas 1 (GS1):	50 psi
Ion Source Gas 2 (GS2):	50 psi
Curtain Gas (CUR):	12 psi
Collision Gas (CAD):	6 psi
IonSpray Voltage (IS):	5500 V
Temperature (TEM):	500 °C
Declustering Potential (DP):	101 V
Entrance Potential (EP):	10 V
Collision Energy (CE):	41 V
Collision Gas Exit Potential (CXP):	8 V

MRM Transition	Analyte ID	Q1 Mass (amu)	Q3 Mass (amu)	Dwell Time (msec)
Primary	Methiozolin	338	127	200
Confirmatory	MethiozolinC	338	211	200

LC-MS/MS SYSTEMS – ANALYSIS OF DFBA

Shimadzu HPLC-API4000 MS

- HPLC: Two Shimadzu LC-20AD pumps and a Shimadzu SIL-HTA Controller/Autosampler
- MS: SCIEX API4000
- Computer software: Analyst™ version 1.4.2

DFBA Calibration Standards

For thatch analysis, a series of DFBA calibration standards at 0.2, 0.5, 1, 2, 5, 10, 20, and 50 ng/mL are prepared to quantify the observed DFBA residues in thatch samples.

HPLC Method - DFBA

Column: Phenomenex Luna 5 µ Pbenyl-Hexyl 150 mm x 2 mm
Column Temperature: Ambient
Injection Volume: 10 µL

Solvent System:

- Solvent A = 0.1% acetic acid in HPLC water
- Solvent B = 0.1% acetic acid in acetonitrile
- Wash solvent = HPLC water:acetonitrile (50:50, v:v)

Solvent Program:

Time (minutes)	Flow Rate (mL/min)	%A	%B
0.0	0.3	75	25
1.0	0.3	75	25
4.0	0.3	5	95
6.0	0.3	5	95
6.1	0.3	75	25
10.0	0.3	75	25

The LC flow was diverted to the MS between 1.5 and 8.0 min, and to waste between 0.0 and 1.5 min and between 8.0 and 10.0 min.

MS Parameters-DFBA

Scan Type:	MRM
Polarity:	Negative
Ion Source:	Turbo Spray
Resolution Q1	Unit
Resolution Q3	Unit
Ion Source Gas 1 (GS1):	60 psi
Ion Source Gas 2 (GS2):	60 psi
Curtain Gas (CUR):	20 psi
Collision Gas (CAD):	8 psi
IonSpray Voltage (IS):	-4500 V
Temperature (TEM):	500 °C
Declustering Potential (DP):	-25 V
Entrance Potential (EP):	-10 V
Collision Gas Exit Potential (CXP):	-23 V

MRM Transition	Analyte ID	Q1 Mass (amu)	Q3 Mass (amu)	Dwell Time (msec)
Primary	DFBA	157	93	500
Confirmatory	DFBA-C	157	73	500

LIMITS OF QUANTIFICATIION AND DETECTION

The Limit of Quantification (LOQ) is 0.01 ppm. The LOQ is defined as the lowest fortification level at which acceptable recovery data are obtained. The Limit of Detection (LOD) was set at 0.002 ppm.

APPENDIX 6

Method 031775D:
LC-MS/MS Assay for the Determination of Methiozolin in Grass

TITLE

Method 031775D: LC-MS/MS Assay for the Determination of Methiozolin in Grass

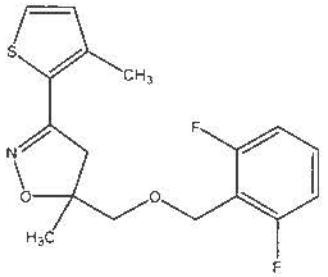
INTRODUCTION

In Method No. 031775D, residues of methiozolin are extracted from five grams of grass with acetone, and then with acetonitrile-HPLC water (80:20, v:v), the extracts combined and cleaned up with solid phase extraction, diluted with acetonitrile-HPLC water (50:50, v:v) (if required), and quantified for methiozolin by LC-MS/MS.

MATERIALS AND METHODS

REFERENCE STANDARD

- **Methiozolin**



Information concerning the reference standard is provided below:

Common Name:	Methiozolin
Chemical Name:	5-[[(2,6-difluorophenyl)methoxy]methyl]-4,5-dihydro-5-methyl-3-(3-methyl-2-thienyl)isoxazole
CAS No.:	403640-27-7
Molecular Formula:	$C_{17}H_{17}F_2NO_2S$
Molecular Weight:	337.38 g/mole
Source:	Moghu Research Center
Lot No.:	MRC111001
Purity:	99.71% (by HPLC)
Expiration Date:	September 30, 2016
Storage:	Room temperature

Solutions made from the reference standard were stored frozen at ≤ -5 °C.

Chemicals

- Formic acid, Fisher Optima LC-MS Grade
- Ammonium formate, Fisher Optima LC-MS Grade
- Acetonitrile, Fisher Optima Grade
- Water, Fisher HPLC Grade
- Methanol, Fisher HPLC or Optima Grade
- Acetone, Fisher HPLC or Optima Grade

Equipment

- Hobart 8181D food cutter
- Robot Coupe Blixer, Model BX4V or BX5V
- Analytical electronic balance with 0.1-mg readability
- Benchtop electronic balance with 0.01-g readability
- Fisher Isotemp Oven, 200 Series, Model 230G
- Eppendorf micropipettes: 10-100 µL, 20-200 µL, and 100-1000 µL
- Burrell Wrist-Action Shaker
- Sorvall RC-5B Centrifuge
- Waters Oasis HLB VAC (60 mg) solid phase extraction (SPE) cartridges
- Supelco Visiprep SPE vacuum manifolds
- Glassware: Assorted beakers, bottles, graduated cylinders, pipettes, etc., which are routinely used for residue analysis
- HPLC-MS/MS (see LC-MS/MS Instrumentation section for further information)

PREPARATION OF METHIOZOLIN STOCK, FORTIFICATION, INTERMEDIATE, AND CALIBRATION SOLUTIONS

The methiozolin stock, fortification, intermediate, and calibration solutions were prepared every six months during the course of the study. Once prepared, these solutions were assigned a 6-month expiry date and stored frozen at ≤ -5 °C when not in use. The representative preparation is shown below:

Preparation of Methiozolin Stock Solutions on 10/25/13

- **Stock solution A** (1029 µg/mL Methiozolin in ACN, purity (99.71%) corrected, used for preparation of fortification solutions). Methiozolin (25.8 mg) was weighed in a 25-mL "Class A" volumetric flask and ACN was added to the mark.
- **Stock solution B** (1013 µg/mL Methiozolin in ACN, purity (99.71%) corrected, used for preparation of calibration solutions). Methiozolin (25.4 mg) was weighed in a 25-mL "Class A" volumetric flask and ACN was added to the mark.

Preparation of Methiozolin Fortification Solutions on 10/25/13

The following fortification solutions were prepared by serial dilution of **Stock solution A** with ACN-water (50:50, v:v) in "Class A" volumetric flasks, as detailed below:

- **Stock solution A**: 1029 µg/mL Methiozolin in ACN (purity corrected)

Standard solution used	Volume taken (mL)	Final volume (mL)	Nominal concentration (µg/mL)
1029 µg/mL	4.86	50	100
100 µg/mL	5	50	10
10 µg/mL	5	50	1

Preparation of Methiozolin Intermediate and Calibration Solutions on 10/25/13

The following intermediate and calibration solutions were prepared by serial dilution of **Stock solution B** with ACN-water (50:50, v:v) in "Class A" volumetric flasks, as detailed below:

- **Stock solution** B:1013 µg/mL Methiozolin in ACN (purity corrected)

Methiozolin Intermediate Solutions

Standard solution used	Volume taken (mL)	Final volume (mL)	Nominal concentration (ng/mL)
1013µg/mL	0.0988	100	1000
1 µg/mL	5	50	100
1 µg/mL	2.5	50	50
1 µg/mL	1	50	20

Methiozolin Calibration Solutions

Standard solution used	Volume taken (mL)	Final volume (mL)	Nominal concentration (ng/mL)
100 ng/mL	5	50	10
50 ng/mL	5	50	5
20 ng/mL	5	50	2
10 ng/mL	5	50	1
10 ng/mL	5	100	0.5
2 ng/mL	5	50	0.2
2 ng/mL	5	100	0.1

FORTIFICATION

The control grass samples (approximately 5 g each) are fortified with the following solutions:

Fortification Level	Fortification Solution	Volume (µL)
Methiozolin in Thatch		
Control	ACN:H_2O (50:50)	50
0.01 ppm	1 µg/mL methiozolin in ACN:H_2O (50:50)	50
0.1 ppm	10 µg/mL methiozolin in ACN:H_2O (50:50)	50
1 ppm	100 µg/mL methiozolin in ACN:H_2O (50:50)	50

ACN = acetonitrile

ANALYSIS OF METHIOZOLIN

HOMOGENIZATION, EXTRACTION, AND CLEAN-UP

Extraction

1. Frozen grass samples are homogenized with Dry Ice in a Robot Coupe Blixer, Model BX4V or BX5V, and the Dry Ice is permitted to sublimate before samples are weighed for analysis.
2. Aliquots (5 g each) of grass are weighed into 50-mL polypropylene centrifuge tubes and fortified with the appropriate amount of methiozolin.
3. Acetone (40 mL) is added to each sample.
4. The samples are shaken using a Burrell wrist-action shaker for 30 minutes.
5. The samples are centrifuged for 10 minutes at ~3000 rpm.
6. The supernatant is transferred to a new 125-mL polyethylene bottle.
7. The solid residue is re-extracted with 40 mL of acetonitrile-HPLC water (80:20, v:v) as for step 3 to 5, combining the supernatants in the 125-mL polyethylene bottle.
8. An aliquot of 4 mL of the supernatant is removed from each sample and mixed with 20 mL of HPLC water for SPE clean-up.

SPE Cartridge Clean-up

1. A Waters Oasis HLB VAC (60 mg) SPE cartridge is placed on a Supelco Visiprep SPE vacuum manifold and washed with 5 mL methanol followed by 5 mL HPLC water.
2. The cartridge is loaded with the sample from the extraction step # 8. The eluate is discarded.
3. Methiozolin is eluted with 5 mL of acetonitrile. The eluate is collected into a 15-mL polypropylene centrifuge tube.
4. The eluate is diluted to 10 mL with HPLC water and the sample is mixed.
5. 100XLOQ (1 ppm) samples are diluted 10X with acetonitrile-HPLC water (50:50, v:v).
6. Samples are transferred to autosampler vials for LC-MS/MS analysis.

LC-MS/MS SYSTEMS – ANALYSIS OF METHIOZOLIN

Shimadzu HPLC-API4000 MS

- HPLC: Two Shimadzu LC-20AD pumps and a Shimadzu SIL-HTA Controller/Autosampler
- MS: SCIEX API4000
- Computer software: Analyst™ version 1.4.2

Methiozolin Calibration Standards

A series of methiozolin calibration standards at 0.1, 0.2, 0.5, 1, 2, 5, and 10 ng/mL are prepared to quantify the observed methiozolin residues in grass samples.

HPLC Method - Methiozolin

Column: Phenomenex Luna 5 µ Phenyl-Hexyl 150 mm x 2 mm
Column Temperature: Ambient
Injection Volume: 5 µL (Shimadzu HPLC)

Solvent System:

- Solvent A = 0.1% formic acid in HPLC water-methanol (90:10, v:v) + 10 mM ammonium formate
- Solvent B = 0.1% formic acid in methanol
- Wash solvent = HPLC water-acetonitrile (50:50, v:v)

Solvent program (Shimadzu HPLC):

Time (minutes)	Flow Rate (mL/min)	%A	%B
0.0	0.4	35	65
3.0	0.4	5	95
5.0	0.4	5	95
5.5	0.4	35	65
8.0	0.4	35	65

The LC flow is diverted to the MS between 2.0 and 7.9 min, and to waste between 0.0 and 2.0 min and between 7.9 and 8.0 min.

MS Parameters-Methiozolin

Scan Type:	MRM
Polarity:	Positive
Ion Source:	Turbo Spray
Resolution Q1	Unit
Resolution Q3	Unit
Ion Source Gas 1 (GS1):	50 psi
Ion Source Gas 2 (GS2):	50 psi
Curtain Gas (CUR):	12 psi
Collision Gas (CAD):	6 psi
IonSpray Voltage (IS):	5500 V
Temperature (TEM):	500 °C
Declustering Potential (DP):	101 V
Entrance Potential (EP):	10 V
Collision Energy (CE):	41 V
Collision Gas Exit Potential (CXP):	8 V

MRM Transition	Analyte ID	Q1 Mass (amu)	Q3 Mass (amu)	Dwell Time (msec)
Primary	Methiozolin	338	127	200
Confirmatory	MethiozolinC	338	211	200

LIMITS OF QUANTIFICATIION AND DETECTION

The Limit of Quantification (LOQ) is 0.01 ppm. The LOQ is defined as the lowest fortification level at which acceptable recovery data are obtained. The Limit of Detection (LOD) was set at 0.002 ppm.

APPENDIX 7

Method 031775E:
LC-MS/MS Assay for the Determination of Methiozolin in Grass

TITLE

Method 031775E: LC-MS/MS Assay for the Determination of Methiozolin in Grass

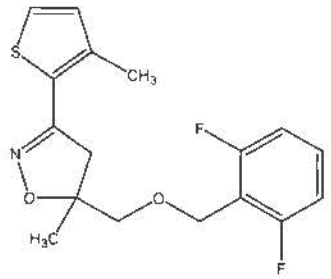
INTRODUCTION

In Method No .031775E, methiozolin in five grams of grass is extracted first with acetone, and then with acetonitrile-HPLC water (80:20, v:v), the extracts combined and then filtered through 0.45 µm PTFE syringe filters, diluted with acetonitrile-HPLC water (50:50, v:v), and quantified by LC-MS/MS. This method was used for analysis of methiozolin in grass in New York, Georgia, California, and New Jersey terrestrial field dissipation studies (Study Numbers 031786, 031812, 031813, and 031942, respectively).

MATERIALS AND METHODS

REFERENCE STANDARD

- **Methiozolin**



Information concerning the reference standard is provided below:

Common Name:	Methiozolin
Chemical Name:	5-[[(2,6-difluorophenyl)methoxy]methyl]-4,5-dihydro-5-methyl-3-(3-methyl-2-thienyl)isoxazole
CAS No.:	403640-27-7
Molecular Formula:	$C_{17}H_{17}F_2NO_2S$
Molecular Weight:	337.38 g/mole
Source:	Moghu Research Center
Lot No.:	MRC111001
Purity:	99.71% (by HPLC)
Expiration Date:	September 30, 2016
Storage:	Room temperature

Solutions made from the reference standard were stored frozen at ≤ -5 °C.

- **Chemicals**
  - Formic acid, Fisher Optima LC-MS Grade
  - Ammonium formate, Fisher Optima LC-MS Grade
  - Acetonitrile, Fisher Optima Grade
  - Water, Fisher HPLC Grade
  - Methanol, Fisher HPLC or Optima Grade
  - Acetone, Fisher HPLC or Optima Grade

Equipment

- Robot Coupe Blixer, Model BX4V or BX5V
- Analytical electronic balance with 0.1-mg readability
- Benchtop electronic balance with 0.01-g readability
- Eppendorf micropipettes: 10-100 µL, 20-200 µL, and 100-1000 µL
- Burrell Wrist-Action Shaker
- Sorvall RC-5B Centrifuge
- 0.45-µm Polytetrafluoroethylene (PTFE) syringe filters
- Glassware: Assorted beakers, bottles, graduated cylinders, pipettes, etc., which are routinely used for residue analysis
- HPLC-MS/MS (see LC-MS/MS Instrumentation section for further information)

PREPARATION OF METHIOZOLIN STOCK, FORTIFICATION, INTERMEDIATE, AND CALIBRATION SOLUTIONS

The methiozolin stock, fortification, intermediate, and calibration solutions were prepared every six months during the course of the study. Once prepared, these solutions were assigned a 6-month expiry date and stored frozen at ≤ -5 °C when not in use. The representative preparation is shown below:

Preparation of Methiozolin Stock Solutions on 4/25/14 and 4/28/14

- **Stock solution A** (1009 µg/mL Methiozolin in ACN, purity (99.71%) corrected, used for preparation of fortification solutions). Methiozolin (25.3 mg) was weighed in a 25-mL "Class A" volumetric flask and ACN was added to the mark.
- **Stock solution B** (1021 µg/mL Methiozolin in ACN, purity (99.71%) corrected, used for preparation of calibration solutions). Methiozolin (25.6 mg) was weighed in a 25-mL "Class A" volumetric flask and ACN was added to the mark.

Preparation of Methiozolin Fortification Solutions on 4/25/14

The following fortification solutions were prepared by serial dilution of **Stock solution A** with ACN-water (50:50, v:v) in "Class A" volumetric flasks, as detailed below:

- **Stock solution A**: 1009 µg/mL Methiozolin in ACN (purity corrected)

Standard solution used	Volume taken (mL)	Final volume (mL)	Nominal concentration (µg/mL)
1009 µg/mL	4.96	50	100
100 µg/mL	5	50	10
10 µg/mL	5	50	1

Preparation of Methiozolin Intermediate and Calibration Solutions on 4/28/14

The following intermediate and calibration solutions were prepared by serial dilution of **Stock solution B** with ACN-water (50:50, v:v) in "Class A" volumetric flasks, as detailed below:

- **Stock solution** B:1021 µg/mL Methiozolin in ACN (purity corrected)

Methiozolin Intermediate Solutions

Standard solution used	Volume taken (mL)	Final volume (mL)	Nominal concentration (ng/mL)
1021µg/mL	0.0980	100	1000
1 µg/mL	5	50	100
1 µg/mL	2.5	50	50
1 µg/mL	1	50	20

Methiozolin Calibration Solutions

Standard solution used	Volume taken (mL)	Final volume (mL)	Nominal concentration (ng/mL)
100 ng/mL	5	50	10
50 ng/mL	5	50	5
20 ng/mL	5	50	2
10 ng/mL	5	50	1
10 ng/mL	5	100	0.5
2 ng/mL	5	50	0.2
2 ng/mL	5	100	0.1
1 ng/mL	1	25	0.04

FORTIFICATION

The control grass samples (approximately 5 g each) are fortified with the following solutions:

Fortification Level	Fortification Solution and Volume (µL) Used
Methiozolin in Grass (~5 g)	
Control	ACN:H_2O (50:50), 50 µL
0.01 ppm	1 µg/mL methiozolin in ACN:H_2O (50:50), 50 µL
0.1 ppm	10 µg/mL methiozolin in ACN:H_2O (50:50), 50 µL
1 ppm	100 µg/mL methiozolin in ACN:H_2O (50:50), 50 µL
10 ppm	100 µg/mL methiozolin in ACN:H_2O (50:50), 500 µL
49 ppm	1009 µg/mL methiozolin in ACN:H_2O (50:50), 243 µL
200 ppm	Methiozolin 5 mg/mL in ACN, 200 µL*

ACN = acetonitrile

*Methiozolin 5 mg/mL in ACN was prepared by weighing 125.4 mg of methiozolin (purity 99.71%) in a 25-mL "Class A" volumetric flask and ACN was added to the mark.

ANALYSIS OF METHIOZOLIN

HOMOGENIZATION, EXTRACTION, AND CLEAN-UP

1. Frozen grass samples are homogenized with a Robot Coupe Blixer, Model BX4V or BX5V, and the Dry Ice is permitted to sublimate before samples are weighed for analysis.
2. Aliquots (approximately 5 g each) of grass are weighed into 50-mL polypropylene centrifuge tubes and fortified with the appropriate amount of methiozolin.
3. Acetone (40 mL) is added to each sample.
4. The samples are shaken using a Burrell wrist-action shaker for 30 minutes.
5. The samples are centrifuged for 10 minutes at ~3000 rpm.
6. The supernatant is transferred to a new 125-mL polyethylene bottle.
7. The solid residue is re-extracted with 40 mL of acetonitrile-HPLC water (80:20, v:v) as for step 3 to 5, combining the supernatants in the 125-mL polyethylene bottle.
8. An aliquot of the supernatant is filtered through a 0.45-µm PTFE syringe filter.
9. Samples are diluted with ACN-water (50:50, v:v) according to the table below, before transferring to autosampler vials for LC-MS/MS analysis for methiozolin.

Samples	Extraction volume (mL)	Dilution	Final Concentration (ng/mL)
Grass blank	80	10X	N/A
0.01 ppm	80	10X	0.0625
0.1 ppm	80	10X	0.625
1 ppm	80	50X	1.25
10 ppm	80	50X+50X*	0.25
50 ppm	80	50X+50X*	1.25
200 ppm	80	50X+50X	5

*50X+50X: diluted 50X twice, equivalent to 2,500X dilution

LC-MS/MS SYSTEMS – ANALYSIS OF METHIOZOLIN

Shimadzu HPLC-API4000 MS

- HPLC: Two Shimadzu LC-20AD pumps and a Shimadzu SIL-HTA Controller/Autosampler
- MS: SCIEX API4000
- Computer software: Analyst™ version 1.4.2

Flexar UHPLC-API4000 MS

- HPLC: PerkinElmer Flexar FX-15 UHPLC
- MS: SCIEX API4000
- Computer software: Analyst™ version 1.4.2

Methiozolin Calibration Standards

For grass analysis, a series of methiozolin calibration standards at 0.04, 0.1, 0.2, 0.5, 1, 2, 5, and 10 ng/mL are prepared to quantify the observed methiozolin residues in grass samples.

HPLC Method - Methiozolin
Column: Phenomenex Luna 5 µ Phenyl-Hexyl 150 mm x 2 mm
Column Temperature: Ambient
Injection Volume: 5 µL (Shimadzu HPLC) or 20 µL (Flexar HPLC)
Solvent System:

- Solvent A = 0.1% formic acid in HPLC water-methanol (90:10, v:v) + 10 mM ammonium formate
- Solvent B = 0.1% formic acid in methanol
- Wash solvent = HPLC water-acetonitrile (50:50, v:v)

Solvent program (Shimadzu HPLC):

Time (minutes)	Flow Rate (mL/min)	%A	%B
0.0	0.4	35	65
3.0	0.4	5	95
5.0	0.4	5	95
5.5	0.4	35	65
8.0	0.4	35	65

The LC flow is diverted to the MS between 2.0 and 7.9 min, and to waste between 0.0 and 2.0 min and between 7.9 and 8.0 min.

Solvent program (Flexar UHPLC):

Time (minutes)	Flow Rate (mL/min)	%A	%B
0.0	0.4	35	65
3.5	0.4	5	95
5.0	0.4	5	95
5.5	0.4	35	65
8.0	0.4	35	65

The LC flow is diverted to the MS between 2.0 and 7.9 min, and to waste between 0.0 and 2.0 min and between 7.9 and 8.0 min.

MS Parameters-Methiozolin

Scan Type:	MRM
Polarity:	Positive
Ion Source:	Turbo Spray
Resolution Q1	Unit
Resolution Q3	Unit
Ion Source Gas 1 (GS1):	50 psi
Ion Source Gas 2 (GS2):	50 psi
Curtain Gas (CUR):	12 psi
Collision Gas (CAD):	6 psi
IonSpray Voltage (IS):	5500 V
Temperature (TEM):	500 °C
Declustering Potential (DP):	101 V
Entrance Potential (EP):	10 V
Collision Energy (CE):	41 V
Collision Gas Exit Potential (CXP):	8 V

MRM Transition	Analyte ID	Q1 Mass (amu)	Q3 Mass (amu)	Dwell Time (msec)
Primary	Methiozolin	338	127	200
Confirmatory	MethiozolinC	338	211	200

LIMITS OF QUANTIFICATIION AND DETECTION

The Limit of Quantification (LOQ) is 0.01 ppm. The LOQ is defined as the lowest fortification level at which acceptable recovery data are obtained. The Limit of Detection (LOD) was set at 0.002 ppm.

APPENDIX 8

Method 031775F:
LC-MS/MS Assay for the Determination of DFBA in Grass

TITLE

Method 031775F: LC-MS/MS Assay for the Determination of DFBA in Grass

INTRODUCTION

In Method No. 031775F, DFBA in five grams of grass was extracted with acetone-water (80:20, v:v), filtered through 0.45 µm PTFE syringe filters, diluted with acetonitrile-HPLC water-acetic acid (25:75:0.1, v:v:v), and quantified by LC-MS/MS. This method was used for analysis of DFBA in grass in New York, Georgia, California, and New Jersey terrestrial field dissipation studies (Study Numbers 031786, 031812, 031813, and 031942, respectively).

MATERIALS AND METHODS

REFERENCE STANDARD

- **2,6-Difluorobenzoic acid (DFBA)**

2,6-Difluorobenzoic acid

Information concerning the reference standard is provided below:

Chemical Name:	2,6-Difluorobenzoic acid
CAS No.:	385-00-2
Molecular Formula:	$C_7H_4F_2O_2$
Molecular Weight:	158.1 g/mol
Source:	Sigma-Aldrich
Lot No.:	MKBQ3617V
Ricerca Sample Code:	CS_19165
Stated Purity:	94.86%
Storage:	Ambient temperature

Solutions made from the reference standard were stored frozen at ≤ -5 °C.

Chemicals

- Acetic acid, glacial, Fisher Certified ACS Plus Grade
- Acetonitrile, Fisher Optima Grade
- Water, Fisher HPLC Grade
- Acetone, Fisher HPLC or Optima Grade

Equipment

- Robot Coupe Blixer, Model BX4V or BX5V
- Analytical electronic balance with 0.1-mg readability
- Benchtop electronic balance with 0.01-g readability
- Eppendorf micropipettes: 10-100 µL, 20-200 µL, and 100-1000 µL
- Burrell Wrist-Action Shaker
- Sorvall RC-5B Centrifuge
- 0.45-µm Polytetrafluoroethylene (PTFE) syringe filters
- Glassware: Assorted beakers, bottles, graduated cylinders, pipettes, etc., which are routinely used for residue analysis
- HPLC-MS/MS (see LC-MS/MS Instrumentation section for further information)

PREPARATION OF DFBA STOCK, FORTIFICATION, INTERMEDIATE, AND CALIBRATION SOLUTIONS

The DFBA stock, fortification, intermediate, and calibration solutions were prepared every six months during the course of the study. Once prepared, these solutions were assigned a 6-month expiry date and stored frozen at ≤ -5 °C when not in use. The representative preparation is shown below:

Preparation of DFBA Stock Solutions on 4/25/14 and 4/28/14

- **Stock solution A** (1396 µg/mL DFBA in ACN, purity (94.86%) corrected, used for preparation of fortification solutions). DFBA (36.8 mg) was weighed in a 25-mL "Class A" volumetric flask and ACN was added to the mark.
- **Stock solution B** (1032 µg/mL DFBA in ACN, purity (94.86%) corrected, used for preparation of calibration solutions). DFBA (27.2 mg) was weighed in a 25-mL "Class A" volumetric flask and ACN was added to the mark.

Preparation of DFBA Fortification Solutions on 4/28/14

The following fortification solutions were prepared by serial dilution of **Stock solution A** with ACN-water (50:50, v:v) in "Class A" volumetric flasks, as detailed below:

- **Stock solution A**: 1396 µg/mL DFBA in ACN (purity corrected)

Standard solution used	Volume taken (mL)	Final volume (mL)	Nominal concentration (µg/mL)
1396 µg/mL	3.58	50	100
100 µg/mL	5	50	10
10 µg/mL	5	50	1

Preparation of DFBA Intermediate and Calibration Solutions on 4/25/14

The following intermediate and calibration solutions were prepared by serial dilution of **Stock solution B** with ACN-water (50:50, v:v) in "Class A" volumetric flasks, as detailed below:

- **Stock solution** B:1032 µg/mL DFBA in ACN (purity corrected)

DFBA Intermediate Solutions

Standard solution used	Volume taken (mL)	Final volume (mL)	Nominal concentration (µg/mL)
1032 µg/mL	4.85	50	100
100 µg/mL	5	50	10
10 µg/mL	5	50	1
1 µg/mL	5	50	0.1
Standard solution used	**Volume taken (mL)**	**Final volume (mL)**	**Nominal concentration (ng/mL)**
1 µg/mL	2.5	50	50

DFBA Calibration Solutions

Standard solution used	Volume taken (mL)	Final volume (mL)	Nominal concentration (ng/mL)
1 µg/mL	1	50	20
0.1 µg/mL	5	50	10
50 ng/mL	5	50	5
20 ng/mL	5	50	2
10 ng/mL	5	50	1
5 ng/mL	5	50	0.5
2 ng/mL	5	50	0.2
1 ng/mL	5	50	0.1

FORTIFICATION

The control grass samples (approximately 5 g each) are fortified with the following solutions:

Fortification Level	Fortification Solution and Volume (µL) Used
DFBA in Grass (~5 g)	
Control	ACN:H_2O (50:50), 50 µL
0.01 ppm	1 µg/mL DFBA in ACN:H_2O (50:50), 50 µL
0.1 ppm	10 µg/mL DFBA in ACN:H_2O (50:50), 50 µL
1 ppm	100 µg/mL DFBA in ACN:H_2O (50:50), 50 µL

ACN = acetonitrile

ANALYSIS OF DFBA

HOMOGENIZATION, EXTRACTION, AND CLEAN-UP

1. Frozen grass samples are homogenized with Dry Ice in a Robot Coupe Blixer, Model BX4V or BX5V, and the Dry Ice is permitted to sublimate before samples are weighed for analysis.
2. Aliquots (approximately 5 g each) of grass are weighed into 50-mL polypropylene centrifuge tubes and fortified with the appropriate amount of DFBA.
3. Acetone-water (80:20, v:v, 40 mL) is added to the sample.

4. The samples are shaken using a Burrell wrist-action shaker for 30 minutes.
5. The samples are centrifuged for 10 minutes at ~3000 rpm.
6. An aliquot of the supernatant is filtered through a 0.45-µm PTFE syringe filter.
7. Samples are diluted with ACN-HPLC water-acetic acid (25:75:0.1, v:v:v) as follows: control and LOQ (0.01 ppm) diluted 5X, 10XLOQ (0.1 ppm) diluted 10X, and 100XLOQ (1 ppm) diluted 20X.
8. Samples are transferred to autosampler vials for LC-MS/MS analysis for DFBA.

LC-MS/MS SYSTEMS – ANALYSIS OF DFBA

Shimadzu HPLC-API4000 MS

- HPLC: Two Shimadzu LC-20AD pumps and a Shimadzu SIL-HTA Controller/Autosampler
- MS: SCIEX API4000
- Computer software: Analyst™ version 1.4.2

DFBA Calibration Standards

For grass analysis, a series of DFBA calibration standards at 0.1, 0.2, 0.5, 1, 2, 5, 10, and 20 ng/mL are prepared to quantify the observed DFBA residues in grass samples.

HPLC Method - DFBA

Column: Phenomenex Luna 5 µ Phenyl-Hexyl 150 mm x 2 mm
Column Temperature: Ambient
Injection Volume: 10 µL
Solvent System:

- Solvent A = 0.1% acetic acid in HPLC water
- Solvent B = 0.1% acetic acid in acetonitrile
- Wash solvent = HPLC water:acetonitrile (50:50, v:v)

Solvent Program:

Time (minutes)	Flow Rate (mL/min)	%A	%B
0.0	0.3	75	25
1.0	0.3	75	25
4.0	0.3	5	95
6.0	0.3	5	95
6.1	0.3	75	25
10.0	0.3	75	25

The LC flow was diverted to the MS between 1.5 and 8.0 min, and to waste between 0.0 and 1.5 min and between 8.0 and 10.0 min.

MS Parameters-DFBA

Scan Type:	MRM
Polarity:	Negative
Ion Source:	Turbo Spray
Resolution Q1	Unit
Resolution Q3	Unit
Ion Source Gas 1 (GS1):	60 psi
Ion Source Gas 2 (GS2):	60 psi
Curtain Gas (CUR):	20 psi
Collision Gas (CAD):	8 psi
IonSpray Voltage (IS):	-4500 V
Temperature (TEM):	500 °C
Declustering Potential (DP):	-25 V
Entrance Potential (EP):	-10 V
Collision Gas Exit Potential (CXP):	-23 V

MRM Transition	Analyte ID	Q1 Mass (amu)	Q3 Mass (amu)	Dwell Time (msec)
Primary	DFBA	157	93	500
Confirmatory	DFBA-C	157	73	500

LIMITS OF QUANTIFICATION AND DETECTION

The Limit of Quantification (LOQ) is 0.01 ppm. The LOQ is defined as the lowest fortification level at which acceptable recovery data are obtained. The Limit of Detection (LOD) was set at 0.002 ppm.