2 INTRODUCTION

The purpose of this study was to conduct an independent laboratory validation (ILV) for the determination of Methiozolin and its metabolite 2,6-Difluorobenzyl (DFB) Alcohol in surface and ground water. The analysis of the Methiozolin reference/test substance was performed by liquid chromatography coupled with positive-ion tandem mass spectrometry (LC-MS/MS); the analysis of DFB Alcohol was performed by gas chromatography with mass selective detection (GC/MSD). Both analyses were based on the method "Analytical Method Validation for the Determination of Methiozolin and 2,6-Difluorobenzyl Alcohol (DFB Alcohol) in Surface and Ground Water", EAG Laboratories-Easton, Method No. 716C-106, July 31, 2017, provided by the sponsor.

This study was designed to satisfy US EPA Guideline requirements described in OCSPP 850.6100. The study was initiated on September 8, 2017 at EAG Laboratories-Hercules, 625-B Alfred Nobel Drive, Hercules, CA 94547 under an approved protocol (Appendix A) according to the US EPA FIFRA Good Laboratory Practice Standards, 40 CFR §160.

3 MATERIAL AND METHODS

3.1 Test/Reference Substances

Methiozolin:

Common Name: Methiozolin

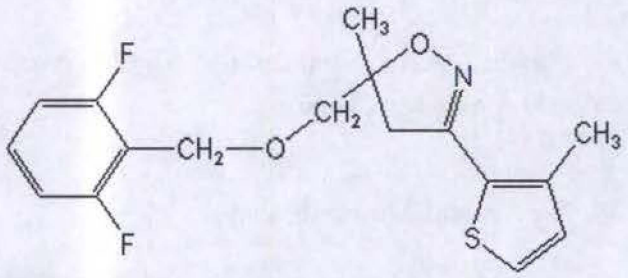
Chemical Name: 5-(2,6-difluorobenzyloxymethyl]-5-methyl-3-(3-methyl-thiophen-2-yl)-dihydro-isoxazole

CAS No.: 403640-27-7

Molecular Weight: 337.4 g/mol

Molecular Formula: $C_{17}H_{17}F_2NO_2S$

Structural Formula:



2,6-difluorobenzyl (DFB) alcohol:

Code Name: DFB Alcohol

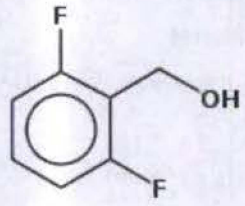
Chemical Name: (2,6-Difluorophenyl)methanol

CAS No.: 19064-18-7

Molecular Weight: 144.12 g/mol

Molecular Formula: $C_7H_6F_2O$

Structural Formula:



Methiozolin and DFB Alcohol standards were provided by EAG Laboratories – Easton on June 30, 2017. Upon receipt at EAG Laboratories-Hercules, the test/reference substances were assigned the inventory No. 2892W-001 (Methiozolin) and 2892W-002 (DFB Alcohol). The test/reference substances were stored at room temperature when not in use.

Inventory no.	Analyte	Lot No.	Purity (%)
2892W-001	Methiozolin	MRC111001	99.75
2892W-002	DFB Alcohol	I2316	99.6

The certificates of analysis are provided in Appendix B.

3.2 Reagents

HPLC grade water, acetonitrile (ACN), dichloromethane (DCM), and formic acid were obtained from Fisher Chemical.

3.3 Equipment/Materials List

Laboratory Balances
Glass weighing boats
Geno/Grinder 2010
Centrifuge
Vortex
Sonicator
Volumetric flasks and pipettes
Glass disposable tubes (15 mL capacity)
Plastic disposable centrifuge tubes (50 mL capacity)
Glass graduated disposable pipettes
Variable/adjustable volume pipettors with plastic disposable tips
Glass precision syringes
0.2 µm PTFE syringeless filters (Whatman)
Amber bottles and vials with Teflon® lined caps
Autosampler vials

AB Sciex API 5500 Series Triple Quad Mass Spectrometer with Agilent 1260 Series LC (LC-MS/MS), Analyst Data System Software

Agilent 6890 Gas Chromatograph (GC) equipped with Agilent 5973 Mass Selective Detector (MSD) and ATAS Combi-PAL Autosampler (GC/MSD # 3)
Mass Hunter Data System Software

3.4 Test Systems (Matrices)

3.4.1 Source of the Test Systems

Surface water was collected from Brandywine Creek, Chadds Ford, PA and collected on January 25, 2017. Upon arrival at EAG Laboratories-Hercules, the surface water was assigned the inventory No. 2706W-085.

Ground (well) water was collected from Northwood, ND and collected on August 7, 2017. Upon arrival at EAG Laboratories-Hercules, the well water was assigned the inventory No. 2706W-105.

Both matrices were stored refrigerated (typically between 4 °C and 10°C) in the dark when not in use.

3.4.2 Characterization of the Test Systems

The surface water and well water used in the study were characterized by Agvise Laboratories, Inc. (604 Highway 15 West, Northwood, North Dakota). The characterization reports are presented in Appendix C.

3.5 Test Methods

The analytical method for the analysis of Methiozolin in surface and ground (well) water was independently validated at EAG Laboratories-Hercules by LC-MS/MS. Analysis was based on the analytical method described in "Analytical Method Validation for the Determination of Methiozolin and 2,6-Difluorobenzyl Alcohol (DFB Alcohol) in Surface and Ground Water", EAG Laboratories-Easton, Method No. 716C-106, July 31, 2017 with the following modification: matrix-based calibration standard solutions were used for quantitation of Methiozolin in well water.

The method for the analysis of Methiozolin in surface and well water samples was based on the direct injection approach using liquid chromatography tandem mass spectrometry

LC-MS/MS with positive electrospray ionization. Extracts were filtered through 0.2 µm PTFE filters prior to analyzing by LC-MS/MS. The percent recovery of Methiozolin was determined using external standardization where a 1/x weighted linear curve of calibration standards was analyzed along with the samples.

The analytical method for the analysis of DFB Alcohol in surface and well water was independently validated at EAG Laboratories-Hercules by GC-MSD. Analysis was based on the analytical method described above with the following modification: the monitored fragment ion m/z 143 was used as the confirmation ion.

The method for the analysis of DFB Alcohol in surface and ground water samples was based on single DCM solvent liquid-liquid partition extraction with subsequent GC-MSD analysis. The percent recovery of DFB alcohol was determined using external standardization where a linear 1/x weighted curve of calibration standards was analyzed along with the samples.

3.6 Determination of Methiozolin in Surface and Ground Water by LC-MS/MS

3.6.1 Preparation of Stock Solution

One stock solution of Methiozolin was prepared by weighing an aliquot (50.92 mg) of Methiozolin test/reference substance in a weighing boat and transferring into a 50 mL volumetric flask with some ACN. Solution was briefly sonicated to ensure all solids have completely dissolved. Final solution was diluted to the mark with ACN. Additional solvent was added as necessary to achieve a nominal assay concentration of 1.0 mg/mL after adjusting for the purity of the reference substance as follows:

			Theoretical conc. [1]
Weight (mg)	Final volume (mL)	Purity (%)	Methiozolin (µg/mL)
50.92	50.80	99.75	999.9

[1]Theoretical conc. (µg/mL) = [weight (mg) x 1,000 µg/mg ÷ final volume (mL)] x [purity (%) ÷ 100]

The stock solution was transferred into an amber bottle and stored frozen (typically < -4°C) when not in use.

3.6.2 Preparation of Secondary and Fortification Solutions

Secondary and fortification solutions containing Methiozolin were prepared in serial dilution by volumetrically measuring aliquots (5 mL) of the source solution and transferring into separate 50 mL volumetric flasks. Final solutions were diluted to the mark with ACN and mixed. Final solutions were transferred into amber bottles and stored refrigerated (typically between 4°C and 10°C) when not in use.

Solution used (µg/mL)	Aliquot (mL)	Final volume (mL)	Theoretical conc. [1] Methiozolin (µg/mL)	Sample ID
999.86	5.0	50	99.99	SS-1A
99.986	5.0	50	9.999	SS-1B
9.999	5.0	50	1.000	SS-1C
1.000	5.0	50	0.100	High Fortification solution
0.100	5.0	50	0.010	Low Fortification solution

[1]Theoretical conc. (µg/mL) = {[theoretical conc. solution used x aliquot (mL)] ÷ final volume (mL)}

An additional standard solution was prepared by volumetrically measuring an aliquot (5 mL) of the 0.1 µg/mL solution prepared above and transferring into a 50 mL volumetric flask. The solution was diluted to the mark with ACN: HPLC water (1:1) v/v to yield a nominal assay concentration of 0.01 µg/mL. Standard solution was transferred into an amber bottle and stored refrigerated (typically between 4°C and 10°C) when not in use.

3.6.3 Preparation of Solvent-Based Calibration Standard Solutions for Surface Water Analysis

Six calibrants containing Methiozolin were prepared by measuring an appropriate volume of the 0.01 µg/mL standard solution in ACN: HPLC water (1:1) v/v and transferring into separate 10 mL volumetric flasks. Solutions were diluted to the mark with ACN: HPLC water (1:1) v/v. The concentration of Methiozolin ranged from 0.01 ng/mL to 0.5 ng/mL as shown below:

Solution used (µg/mL)	Aliquot (mL)	Final volume (mL)	Theoretical conc.[1] (ng/mL) Methiozolin	Level
0.0100	0.010	10	0.0100	1
0.0100	0.025	10	0.0250	2
0.0100	0.050	10	0.0500	3
0.0100	0.100	10	0.1000	4
0.0100	0.250	10	0.2500	5
0.0100	0.500	10	0.5000	6

[1]Theoretical conc. (ng/mL) = {[theoretical conc. solution used (µg/mL) x aliquot (mL)] ÷ final volume (mL)} x 1,000 ng/µg

Solvent-based calibration standard solutions were stored refrigerated (typically between 4°C and 10°C) when not in use.

3.6.4 Preparation of Matrix-Based Calibration Standard Solutions for Well Water Analysis

A standard solution containing Methiozolin was prepared by measuring an aliquot (0.5 mL) of the 1.0 µg/mL solution prepared in section 3.6.2 (Sample ID: SS-1C) and transferring into a 50 mL volumetric flask. The solution was diluted to the mark with ACN: well water (1:1) v/v to yield a nominal assay concentration of 0.01 µg/mL.

Note: solutions of well water samples became cloudy and precipitated with the addition of ACN. Final mixture ACN: well water (1:1) v/v was previously filtered thru a 0.2 µm PTFE filter prior to the preparation of the standard solution.

Standard solution was transferred into an amber bottle and stored refrigerated (typically between 4°C and 10°C) when not in use.

Six matrix-based calibration standard solutions containing Methiozolin were prepared by measuring an appropriate volume of the source solution and transferring into separate 5 mL volumetric flasks. Solutions were diluted to the mark with a mixture of filtered ACN: well water (1:1) v/v. The concentration of Methiozolin ranged from 0.01 ng/mL to 0.5 ng/mL as shown below:

Solution used	Aliquot (mL)	Final volume (mL)	Theoretical conc.[1] (ng/mL)	
			Methiozolin	Level
0.5 ng/mL	0.1000	5	0.0100	1
0.5 ng/mL	0.2500	5	0.0250	2
0.01 µg/mL	0.0250	5	0.0500	3
0.01 µg/mL	0.0500	5	0.1000	4
0.01 µg/mL	0.1250	5	0.2500	5
0.01 µg/mL	0.2500	5	0.5000	6

[1]Theoretical conc. (ng/mL) = {[theoretical conc. solution used (ng/mL) x aliquot (mL)] ÷ final volume (mL)}

Matrix-based calibration standard solutions were stored refrigerated (typically < 4°C) when not in use.

3.6.5 Preparation of Spiked Solutions for Matrix Effect Assessment

Samples in solvent were prepared by transferring 5 mL x 2 aliquots of HPLC water into separate 15 mL glass disposable tubes. An aliquot (5 mL) of ACN was added into each tube and vortexed to mix.

A 0.025 ng/mL spiked solvent sample was prepared by spiking 0.025 mL of the 0.01 µg/mL Methiozolin low fortification solution (section 3.6.2) via glass precision syringe into 10 mL of the mixture of ACN: HPLC water (1:1) v/v.

An additional spiked solvent sample was prepared at 0.25 ng/mL by spiking 0.025 mL of the Methiozolin 0.1 µg/mL high fortification solution (section 3.6.2) into 10 mL ACN: HPLC water (1:1) v/v. The amount of Methiozolin in solution of the spiked samples is equivalent to the LOQ (0.025 ng/mL) and 10XLOQ (0.25 ng/mL) respectively using the current methodology.

Similar procedure was conducted for matrix-based standard solutions for each surface and well water matrix except that 5 mL surface water or well water was used instead of HPLC water.

A small aliquot of each spiked sample was filtered thru a 0.2 µm PTFE filter prior to LC-MS/MS analysis. Spiked samples were stored refrigerated (typically between 4°C and 10°C) when not in use.

3.6.6 Fortification Procedure

Fortification of Methiozolin in untreated surface and well water samples was conducted at two fortification levels as shown below:

Test system (Matrix)	Fortification Level (µg/L)	Fortification volume (mL)	Solution used
Surface water and well water (10 mL)	0.05	0.05	0.01 µg/mL Low fortification solution
	0.5	0.05	0.1 µg/mL High fortification solution

Fortification was conducted to determine the percent recovery within the Independent Laboratory Validation. This procedure was performed in quintuplicate at each fortification level for each matrix.

3.6.7 Extraction Procedure for Methiozolin in Surface and Well Water

1. Measure 5 mL matrix (surface or ground water) into a 10 mL volumetric flask.
2. Fortify the samples as needed.
3. Dilute water sample to the mark with appropriate matrix and vortex to mix.
4. Combine 2 mL of water sample with 2 mL of ACN in a 4 mL amber bottle and vortex to mix. Dilution factor: 2X.
5. Filter a small aliquot of the 2X water sample thru a 0.2 µm PTFE filter into an autosampler vial and analyze by LC-MS/MS.

Note: Water samples were stored refrigerated (typically between 4°C and 10°C) when not in use.

3.6.8 Liquid Chromatography with Tandem Mass Spectrometry (LC-MS/MS) Analytical Method for Methiozolin

3.6.8.1 LC conditions

Column: Phenomenex Gemini C18, 3µm (50 mm x 2 mm)
Guard column: Phenomenex C18, 4 mm x 2 mm (AJO-7596)
Column Temperature: 40°C
Injection Volume: 25 µL
Needle rinse: 3 vials containing ACN: HPLC water (1:1) v/v

Mobile Phase: A) 0.1% formic acid in HPLC Water
B) 0.1% formic acid in ACN

Gradient Program:

Time (min.)	Flow Rate (µL/min)	% A	% B
0.0	250	50	50
1.0	250	50	50
4.0	250	5	95
5.0	250	5	95
5.1	250	50	50
8.0	250	50	50

Approximate retention time:

- Methiozolin: 4.7 minutes

3.6.8.2 MS conditions

Electrospray Ionization (ESI) in positive polarity mode
Scan mode: Multiple Reaction Monitoring (MRM)

Period 1 settings Experiment 1

For quantitation:

Compound	Molecular ion (m/z)	Product ion (m/z)	DP (V)	CE (V)	CXP (V)	Dwell (msec)
Methiozolin	338	127	40	47	16	500

For confirmation:

Compound	Molecular ion (m/z)	Product ion (m/z)	DP (V)	CE (V)	CXP (V)	Dwell (msec)
Methiozolin	338	211	40	25	22	500

	Period 1 Experiment 1
CUR:	25.0
CAD:	6.0
IS:	5500.0
TEM:	400.0
GS1:	35.0
GS2:	45.0
EP:	10.0

3.6.9 LC-MS/MS Analyses

For LC-MS/MS ILV surface water sample analysis, solvent-based calibrants were analyzed upfront from the lowest concentration to the highest concentration prior to the analysis of the surface water samples in single injection. All calibrants were reanalyzed interspersed among the samples and at the end of the sequence as quality control standards to ensure good chromatography and good instrument performance. ACN: HPLC water (1:1) v/v was analyzed as the solvent blank at the beginning of the sequence. Surface water samples were analyzed in single injection.

For LC-MS/MS ILV well water sample analysis, matrix-based calibrants were analyzed upfront from the lowest concentration to the highest concentration prior to the analysis of the well water samples in single injection. All calibrants were reanalyzed interspersed among the samples and at the end of the sequence as quality control standards to verify method performance. Well water samples were analyzed in single injection.

For LC-MS/MS matrix effects assessment analysis, each spiked solvent (LOQ and 10X LOQ) and each spiked matrix sample was analyzed in triplicate injection.

The stability of the signal was monitored by comparing the response (compound peak area) of a quality control standard injection with that of a comparable standard from the linear curve within the sequence.

3.7 Determination of DFB Alcohol in Surface and Ground Water by GC/MSD

3.7.1 Preparation of Stock Solution

One stock solution of DFB Alcohol was prepared by weighing an aliquot (50.48 mg) of DFB Alcohol test/reference substance in a weighing boat and transferring into a 50 mL volumetric flask with some ACN. Final solution was diluted to the mark with ACN. Additional solvent was added as necessary to achieve a nominal assay concentration of 1.0 mg/mL after adjusting for the purity of the reference substance as follows:

Weight (mg)	Final volume (mL)	Purity (%)	Theoretical conc. [1] DFB Alcohol (µg/mL)
50.48	50.278	99.6	1,000

[1]Theoretical conc. (µg/mL) = [weight (mg) x 1,000 µg/mg ÷ final volume (mL)] x [purity (%) ÷ 100]

The stock solution was transferred into an amber bottle and stored refrigerated (typically between 4°C and 10°C) when not in use.

3.7.2 Preparation of Secondary and Fortification Solutions

Secondary and fortification solutions containing DFB Alcohol were prepared in serial dilution by volumetrically measuring aliquots (5 mL) of the source solution and transferring into separate 50 mL volumetric flasks. Final solutions were diluted to the mark with ACN and mixed. Final solutions were transferred into amber bottles and stored refrigerated (typically between 4°C and 10°C) when not in use.

Solution used (µg/mL)	Aliquot (mL)	Final volume (mL)	Theoretical conc. [1] DFB Alcohol (µg/mL)	Sample ID
1,000	5.0	50	100	Intermediate 1
100	5.0	50	10.0	High Fortification solution
10.0	5.0	50	1.00	Low Fortification solution
1.00	5.0	50	0.100	Intermediate 2
0.100	5.0	50	0.010	Intermediate 3

[1]Theoretical conc. (µg/mL) = {[theoretical conc. solution used x aliquot (mL)] ÷ final volume (mL)}

3.7.3 Preparation of Solvent-Based Calibration Standard Solutions

Six calibrants containing DFB Alcohol were prepared by measuring an appropriate volume of the low fortification solution (1.00 µg/mL) and transferring into separate 10 mL volumetric flasks. Solutions were diluted to the mark with DCM. The concentration of DFB Alcohol ranged from 2.5 ng/mL to 50 ng/mL as shown below:

Solution used	Aliquot	Final volume	Theoretical conc. [1] (ng/mL)	
(µg/mL)	(mL)	(mL)	DFB Alcohol	Level
1.00	0.025	10	2.50	1
1.00	0.050	10	5.00	2
1.00	0.100	10	10.0	3
1.00	0.150	10	15.0	4
1.00	0.250	10	25.0	5
1.00	0.500	10	50.0	6

[1]Theoretical conc. (ng/mL) = {[theoretical conc. solution used (µg/mL) x aliquot (mL)] ÷ final volume (mL)} x 1,000 ng/µg

Solvent-based calibration standard solutions were stored refrigerated (typically between 4°C and 10°C) when not in use.

3.7.4 Preparation of Spiked Solutions for Matrix Effect Assessment

A 10 ng/mL solvent-based standard solution was prepared by spiking 0.010 mL of the 1.00 µg/mL low fortification solution (section 3.11.2) via adjustable volume pipettor into 0.990 mL of DCM. The amount of DFB Alcohol (10 ng/mL) in solution is equivalent to the LOQ using the current methodology.

Similar procedure was conducted for matrix-based standard solutions for each surface and well water matrix except that 0.990 mL surface water or well water sample extract was used instead of DCM.

3.7.5 Fortification Procedure

Fortification of DFB Alcohol in untreated surface and well water samples was conducted at two fortification levels as shown below:

Test system (Matrix)	Fortification Level (µg/L)	Fortification volume (mL)	Solution used
Surface water and well water (10 mL)	5.0	0.05	1.00 µg/mL Low fortification solution
	50	0.05	10.0 µg/mL High fortification solution

Fortification was conducted to determine the percent recovery within the Independent Laboratory Validation. This procedure was performed in quintuplicate at each fortification level for each matrix.

3.7.6 Extraction Procedure for DFB Alcohol in Surface and Well Water

1. Measure 10 mL matrix (surface or ground water) via 10 mL glass graduated disposable pipettes into separate 50 mL plastic centrifuge tubes.
2. Fortify the samples as needed.
3. Add 5 mL DCM via 5 mL glass graduated pipette to each sample.
4. Shake gently; open cap slowly to release pressure.
5. Place sample on GenoGrinder at 1,250 rpm for 5 minutes.
6. Centrifuge at 4,000 rpm for 5 minutes.
7. Remove lower DCM layer via a disposable pipette and transfer into an amber bottle (final extract).

Note: sample extracts were stored frozen (typically < -4°C) when not in use.

3.7.7 Gas Chromatography with Mass Spectral Detection (GC/MSD) Analytical Method for DFB Alcohol

3.7.7.1 GC Conditions

Column: Agilent DB-624, 30 m x 0.25 mm ID x 1.4 µm film thickness
Injection volume: 1 µL, splitless mode
Needle rinse: DCM x 2
Liner: single goose neck
Injector temperature: 120°C

Temperature program:

Initial conditions: 60°C (hold for 1 minute)

Ramp 1: 20°C/minute to 250°C (hold for 0 minutes)

Run time: 10.5 minutes

Gas flow rate:

Column flow: constant flow (He): 1.6 mL/min

Initial head pressure: 14.8 psi

Approximate retention time:

- DFB Alcohol: 6.4 min

3.7.7.2 MS Conditions

Electron Impact mode (EI)

Scan mode: Selected Ion Monitoring (SIM)

MSD transfer line temperature: 250°C

MS Quad temperature: 150°C

MS ion source temperature: 300°C

Solvent delay: 4 min

EM offset: 412

Resolution: high

For quantitation:

Compound	Monitored ion (m/z)	Dwell (msec)
DFB Alcohol	144	100

For confirmation:

Compound	Monitored ion (m/z)	Dwell (msec)
DFB Alcohol	143	100

Full scan parameters:

Full scan 100 -300 Da

Data acquisition: from 4 min to 10 min

3.7.8 GC/MSD Analyses

For GC/MSD ILV surface and well water sample analysis, solvent-based calibrants were analyzed upfront from the lowest concentration to the highest concentration prior to the analysis of the surface or well water samples in single injection. All calibrants were reanalyzed interspersed among the samples and at the end of the sequence as quality control standards to ensure good chromatography and good instrument performance. DCM was analyzed as the solvent blank at the beginning of the sequence. Water samples were analyzed in single injection.

For GC/MS matrix effects assessment analysis, the spiked solvent was analyzed in duplicate injection and each spiked matrix sample was analyzed in triplicate.

The stability of the signal was monitored by comparing the response (compound peak area) of a quality control standard injection with that of a comparable standard from the linear curve within the sequence.

3.8 Methods of Calculation

3.8.1 Quantitation of Methiozolin and DFB Alcohol

Methiozolin and DFB Alcohol were quantitated by the external standard method using separate six-point linear curve regression for each compound and for each matrix. Separation of Methiozolin was achieved by LC-MS/MS in MRM mode; separation of DFB Alcohol was achieved by GC/MS in SIM mode. Each compound was identified by the coincidence of retention time with their respective reference standard and MS characteristics. The quantitation of each compound was conducted by peak area relative to the theoretical concentration of the calibration standard solutions. The content of Methiozolin and DFB Alcohol in surface and well water samples was quantitated against separate 1/x weighted linear curves ($y = mx + b$) of Methiozolin and DFB Alcohol calibration standards respectively where:

y = peak area
x = ng/mL compound injected
m = slope
b = intercept

Weighting of the calibration curves was applied so as to provide better curve fit at the lower concentration levels of the compounds. The calculation of weighted curve equations (linear regression) and concentration (ng/mL) present in samples and calibration standards was conducted using Analyst® software for Methiozolin analysis and Mass Hunter for DFB Alcohol analysis.

Recoveries from samples were determined by averaging the found amount recovered (ng) of each compound (corrected for mean control contribution, if necessary) and dividing by the relevant theoretical fortified amount (ng).

3.8.2 Methiozolin and DFB Alcohol Residues in Surface and Well Water

% Recovery (%) = [(ng recovered – ng mean control) ÷ ng fortified)] x 100

Where:
ng recovered = calculated concentration (ng/mL) x dilution factor x final volume (mL)
ng fortified = sample volume (mL) x fortification level (µg/L)
Calculated concentration (ng/mL) was determined by Analyst® software (Methiozolin) and by Mass Hunter (DFB Alcohol)

Example 1:
Analyte: Methiozolin
Sample: F1A (m/z 127) surface water
Fortification level (µg/L) = 0.05 (equivalent to 0.05 ng/mL)
Calculated concentration (ng/mL) = 0.0229
Sample volume (mL) = 10
Final volume (mL) = 10
Dilution factor = 2

ng fortified = 10 mL x 0.05 ng/mL = 0.50
ng recovered = 0.0229 ng/mL x 2 x 10 mL = 0.458
% Recovery = [(0.458 ng - 0.0 ng) ÷ 0.50 ng] x 100 = 92%

No residues were found in the controls

Example 2:
Analyte: DFB Alcohol
Sample: F1A (m/z 144) surface water (reanalysis on October 4,2017)
Fortification level (µg/L) = 5.0 (equivalent to 5.0 ng/mL)
Calculated concentration (ng/mL) = 10.7931
Sample volume (mL) = 10
Final volume (mL) = 5
Dilution factor = 1

ng fortified = 10 mL x 5 ng/mL = 50.0
ng recovered = 10.7931 ng/mL x 1 x 5 mL = 54.0
% Recovery = [(54.0 ng -0.0 ng) ÷ 50.0 ng] x 100 = 108%

No residues were found in the controls

3.8.3 LOQ Theoretical/Expected Concentration (ng/mL) in Surface and Well Water Samples

LOQ (ng/mL) = (ng fortified ÷ final volume mL) ÷ dilution factor
Where:
ng fortified = sample volume (mL) x fortification level (µg/L)

Example 1:
Analyte = Methiozolin
Fortification level (µg/L) = 0.05 (equivalent to 0.05 ng/mL)
Sample volume (mL) = 10
Final volume (mL) = 10
Dilution factor = 2

Methiozolin fortified (ng) = 10 mL x 0.05 ng/mL = 0.5
LOQ (ng/mL) = (0.5 ng ÷ 10 mL) ÷ 2 = 0.025 ng/mL

Example 2:
Analyte: DFB Alcohol
Fortification level (µg/L) = 5.0 (equivalent to 5.0 ng/mL)
Sample volume (mL) = 10
Final volume (mL) = 5
Dilution factor = 1

DFB alcohol fortified (ng) = 10 mL x 5 ng/mL = 50.0
LOQ (ng/mL) = (50 ng ÷ 5 mL) ÷ 1 = 10 ng/mL

3.9 Calibration Range

The calibration curves for Methiozolin analysis were generated by Analyst® software for each matrix in each validation. The calibration range for Methiozolin in surface and well water was from 0.01 ng/mL to 0.5 ng/mL.

The calibration curves for DFB Alcohol analysis were generated by Mass Hunter software for each matrix in each validation; the calibration range for DFB Alcohol in surface and well water was from 2.5 ng/mL to 50 ng/mL.

3.10 Limit of Quantitation

The limit of quantitation (LOQ) was set at 0.05 µg/L for Methiozolin in surface and well water as validated in this study. The LOQ in surface and well water represented 0.025 ng/mL in calibration standard solution using the current methodology.

The limit of quantitation (LOQ) was set at 5.0 µg/L for DFB Alcohol in surface and well water as validated in this study. The LOQ in surface and well water represented 10 ng/mL in calibration standard solution using the current methodology.

3.11 Limit of Detection

The limit of detection (LOD) for Methiozolin analysis was defined as approximately 40% of LOQ. The calibration standard solutions were analyzed to confirm the desired LOD. The confirmed LOD in surface and well water was 0.02 µg/L. The LOD for Methiozolin in surface and well water represented 0.01 ng/mL in the calibration standard solution using the current methodology.

The limit of detection (LOD) for DFB Alcohol analysis was defined as approximately 25% of LOQ. The calibration standard solutions were analyzed to confirm the desired LOD. The confirmed LOD in surface and well water was 1.25 µg/L. The LOD for DFB Alcohol in surface and well water represented 2.5 ng/mL in the calibration standard solution using the current methodology.

3.12 Time Required for Completion of a Sample Set

A sample set can be completed in one set for efficient handling for each matrix. Each set consisted of a reagent blank, two controls (untreated samples) and five fortified samples at each level (LOQ and 10X LOQ).

Time required for one set from initiation of extraction until the completion of instrumental analysis and data evaluation for surface and well water matrix is as follows:

- Preparation of standard solutions takes approximately 10.5 hours
- Sample preparation and LC-MS/MS analysis for Methiozolin take approximately 10 hours

- Sample preparation and GC/MSD analysis for DFB Alcohol take approximately 9 hours
- Data processing for LC-MS/MS takes approximately 4 hours
- Data processing for GC/MSD takes approximately 4 hours

TOTAL = approximately 38 hours (5 calendar days) for one analyst to complete a sample set to satisfy the validation requirements for surface and well water matrix.

3.13 Statistical Methods

Means, standard deviation, relative standard deviation, and 1/x linear regression were the only statistical methods employed in this study.

3.14 Communication Pertaining to Independent Laboratory Validation

On September 19, 2017, the study director informed the sponsor about the signal enhancement of the well water samples by LC-MS/MS from the matrix effects assessment experiment and proposed the use of matrix-based calibrants for quantitation to mitigate such effect.

On September 28, 2017, the sponsor accepted the use of matrix-based calibrants for well water samples by LC-MS/MS.

On October 4, 2017, the study director informed the sponsor about a significant peak interference in the controls (> 30% LOQ) for both matrices in the confirmation ion (m/z 123) of the GC/MSD analysis and suggested to subtract this peak interference from the fortified water samples.

On October 10, the sponsor accepted the control subtraction approach for the GC/MSD analysis.

On October 10, 2017, the sponsor accepted ILV results for well water samples by LC-MS/MS.

On October 10, 2017, the study director updated the sponsor about the ILV results for both matrices by GC/MSD. Recoveries were not within acceptable range (70% -120%) for the

confirmation ion (m/z 123) even after the control subtraction approach was applied to the fortified samples.

On October 11, 2017, the sponsor expressed concern about variability of the GC/MSD confirmation ion (m/z 123). The study director had proposed to explore additional fragment ions as potential candidates based on the full scan of DFB Alcohol reference standard by analyzing selected extracts from the existing ILV sample set.

On October 12, 2017, the sponsor agreed to look into other fragment ions as GC/MSD confirmation ion.

On October 16, 2017, the study director informed the sponsor that among the GC/MSD fragment ions selected (m/z 115, 127, and 143), only m/z 143 did not show apparent interferences in representative controls samples of each matrix.

On October 17, 2017, the study director updated the sponsor of the reanalysis of ILV extracts for both matrices by GC/MSD using m/z 143 as confirmation ion.

On October 18, 2017, the sponsor accepted ILV results by GC/MSD.

3.15 Modifications of the Original Analytical Method

3.15.1 Methiozolin Analysis by LC-MS/MS

No modification of the original analytical method was necessary. The use of matrix-based calibrants is within the scope of the study protocol.

3.15.2 DFB Alcohol Analysis by GC/MSD

The original method validation used the fragment ion m/z 123 as the confirmatory ion for the determination of DFB Alcohol in surface and ground samples. Original ILV analysis, showed a significant peak interference in the controls (> 30% LOQ) that had an undesirable impact on the recoveries of the fortified samples. Confirmatory ion was replaced by m/z 143 since no apparent interferences were observed in the reanalysis of existing sample extracts and therefore, recoveries of samples significantly improved. Additional fragment ions (m/z 127 and 115) were explored as potential confirmation ion candidates based on the full scan spectrum of DFB Alcohol standard; however, these ions showed poor selectivity as high matrix interferences were observed in untreated water samples.

5 CONCLUSIONS

An independent laboratory validation for the analysis of Methiozolin and DFB Aalcohol has been successfully conducted at both defined LOQ and 10X LOQ levels in surface and ground (well) water by LC-MS/MS (Methiozolin) and GC/MSD (DFB Alcohol).

The limit of quantitation (LOQ) was targeted at 0.05 µg/L for Methiozolin and 5.0 µg/L for DFB Alcohol in surface and ground water as validated in this study.

Recovery data for the Independent Laboratory Validation of each compound in surface and ground water at the LOQ and 10X LOQ levels showed that the analytical method was acceptable as it demonstrated acceptable precision and accuracy. The mean recoveries were within the acceptable range (70 – 120%). The %RSD for each fortification level was within the acceptable range (< 20%).

This study meets the requirements outlined in EPA guideline OCSPP 850.6100. The study was also in compliance with Good Laboratory Practices (GLP) as stated in 40 CFR Part 160.