INTRODUCTION

EAG Laboratories-Easton successfully conducted analytical trials to develop and to validate the performance of two separate methodologies, one for the analysis of Methiozolin and another for the analysis of its metabolite 2,6-difluorobenzyl alcohol (DFB Alcohol) in surface and ground water samples. Validation samples in each matrix were prepared and analyzed based upon methodologies developed by EAG Laboratories-Easton. The study was performed at EAG Laboratories analytical testing facility located in Easton, Maryland following U.S. Environmental Protection Agency Residue Chemistry Test Guideline, OPPTS 860.1340, entitled "*Residue Analytical Method*" (1). Due to differences in chemical properties between the analytes, two separate analytical processing and analysis methods were developed for validation. The analysis of processed Methiozolin validation samples was performed using High Performance Liquid Chromatography (HPLC) with Tandem Mass Selective Detection (MS/MS), and the analysis of processed DFB Alcohol validation samples was performed using Gas Chromatography (GC) with Mass Selective Detection (MSD). Raw data for all work performed and a copy of the final report are filed by project number in the archives located on the EAG Laboratories-Easton site. The original final report will be sent to the Sponsor.

OBJECTIVE

The purpose of this study was to develop and validate methods for the determination of Methiozolin and its metabolite 2,6-diflourobenzyl alcohol (DFB Alcohol) residues in surface and ground water matrices to provide support for environmental effects studies.

EXPERIMENTAL DESIGN

Separate untreated control samples of surface and ground water were fortified with Methiozolin at 0.0500 µg/L (LOQ) and 0.500 µg/L (10X LOQ), and with DFB Alcohol at 5.00 µg/L (LOQ) and 50.0 µg/L (10X LOQ), processed and analyzed according to methodologies developed for use by EAG Laboratories-Easton. Reagent and matrix blanks were also prepared and analyzed concurrently to evaluate the potential for analytical interferences. Solvent based calibration curves generated from the analysis of standard solutions of the reference substances were analyzed with each series of matrix validation samples. All samples were identified by project number and a unique sample identification number.

MATERIALS AND METHODS

This study was conducted according to the protocol "Analytical Method Validation for the Determination of Methiozolin and 2,6-diflourobenzyl alcohol (DFB Alcohol) in Surface and Ground Water".

Test System -Control Substrates

Both the control surface water and ground water method validation substrates were obtained locally. The surface water was collected from Tuckahoe Lake in Ridgely, Maryland on March 29, 2016 and was assigned the I.D. WI-TL-032916 upon receipt. The ground water was collected from a well located on the EAG Laboratories-Easton site in Easton, Maryland. The control substrates were stored under refrigerated conditions at the analytical testing facility when not in use. The surface water was characterized at EAG Laboratories-Easton by measuring the specific conductance, hardness, alkalinity, and pH following collection. The results of this characterization are summarized in Appendix IV. The ground water was also characterized at EAG Laboratories-Easton. The mean results of the specific conductance, hardness, alkalinity and pH of ground water measured during the 4-week period immediately preceding each applicable method validation are summarized in Appendix V.

Reference Substances

A reference substance of Methiozolin was received from Moghu on August 09, 2016. Upon receipt, the material was assigned EAG Laboratories-Easton substance number 13228 and stored under ambient conditions.

A reference substance of DFB Alcohol was received from Santa Cruz Biotechnology on January 03, 2017. Upon receipt the material was assigned EAG Laboratories-Easton substance number 13462, and stored under ambient conditions. Information received with the materials is summarized below:

Methiozolin

EAG Laboratories Number: 13228

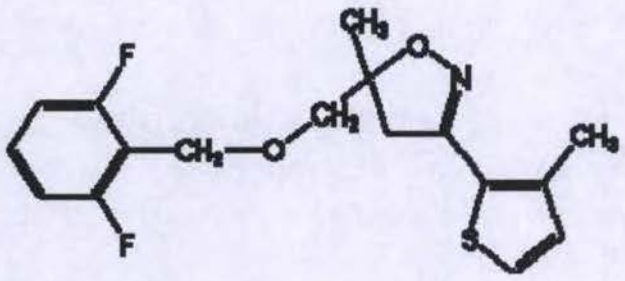
ISO Name: Methiozolin

CAS Number: 403640-27-7

Chemical Name:

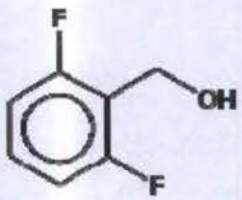
(5RS)-5-[2,6-difluorobenzloxy)]-4,5-dihydro-5-methyl-3(3-methyl-2-thienyl)-1,2-oxazole

Structural Formula:



Molecular Weight: 337.4
Appearance: White Powder
Lot Number: MRC111001
Purity: 99.75%
Expiration Date: July 03, 2021
Storage Conditions: Ambient

2,6-difluorobenzyl alcohol
EAG Laboratories Number: 13462
Code Name: DFB Alcohol
Chemical Name: (2,6-Difluorophenyl)methanol
Structural Formula:



Molecular Weight: 144.12
Appearance: Liquid
Lot Number: 12316
Purity: 99.6%
Manufacture Date: 2017
Re-test Date: 2020
Storage Conditions: Ambient

Chemical names, formulas, structures purity, lot number, expiration dates, and storage recommendations were obtained from the container label and/or COA.

These reference substances were used to prepare stocks for use in the preparation of calibration standard solutions for instrument calibration, and in the fortification of method validation samples. When not in use, the reference substance solutions were stored under refrigerated conditions at approximately 4°C in darkness.

Solvents

Solvent (acetonitrile and HPLC grade water) used for the preparation of stock solutions, both primary and secondary, and dilution solvents were prepared using HPLC-grade solvents, specifically Burdick and Jackson and/or Fisher Brand®, High Purity Solvent. Formic Acid used as an additive in the preparation of LC/MS/MS mobile phases was obtained from Sigma Aldrich. Dichloromethane (DCM) solvent was used as the extraction solvent for the DFB Alcohol method. Combinations of these solvents were used in the preparation of dilution solvents, and HPLC mobile phases as described below:

Dilution Solvent Preparation

Dilution Solvent - Acetonitrile: HPLC Grade Water (1:1, v/v): A 500 mL volume of acetonitrile was measured and combined with a 500 mL volume of water in a 1000 mL Erlenmeyer flask and mixed well. This solution was used for preparation of calibration standards of Methiozolin and for secondary dilutions of method validation samples, if necessary.

HPLC Mobile Phase Preparation

Mobile Phase A – HPLC Grade Water: 0.1% Formic Acid (v/v): 4000 mL of HPLC grade water was measured into a 4L amber glass bottle. 4.0 mL of formic acid was added to the amber glass bottle. The solution was mixed well and transferred to the HPLC reservoir as needed.

Mobile Phase B – Acetonitrile: 0.1% Formic Acid (v/v): 4000 mL of acetonitrile water was measured into a 4L amber glass bottle. 4.0 mL of formic acid was added to the amber glass bottle. The solution was mixed well and transferred to the HPLC reservoir as needed.

Equipment

- Laboratory Balance-capable of four decimal place accuracy
- Geno/Grinder Model 2010 SPEX SamplePrep Processor
- Bench Top Sample Vortexer
- THERMO Sorvall Legend XF Centrifuge or equivalent- capable of 4000 RPM
- Class A Volumetric Flasks – 10 and 50-mL sizes
- 50-mL Disposable Plastic Centrifuge Tubes
- Class A Volumetric Pipettes – 5 and 10-mL sizes
- Assorted Glass Beakers
- Graduated Cylinders – 1000-mL or equivalent

Assorted Gas-tight Syringes – 25, 50, 100, 250, 500 µL

Eppendorf 2500 Reference Pipettor and associated disposable tips or equivalent

Glass Culture Tubes - 15-mL

B&D Plastic Disposable Syringes - 5-mL

WHATMAN Puradisk 25 TF Syringe Filters (0.2µm)

Applied Biosystems/MDS Sciex API 5000 Mass Spectrometer with an Agilent Technologies 1200 Infinity Series HPLC (HPLC/MS/MS)

Alternative equipment may be substituted as long it is considered equivalent in function and generates successful method outcome.

Reference Substance Primary Stock Preparation

A primary stock solution of methiozolin was prepared by weighing 0.0501 grams (weight corrected for purity) of the reference substance on an analytical balance. The reference substance was transferred to a 50-mL class A volumetric flask and brought to final volume using acetonitrile to achieve a 1000 µg/mL primary stock solution.

A primary stock solution of DFB Alcohol metabolite was prepared by weighing 0.0502 grams (weight corrected for purity) of the reference substance on an analytical balance. The reference substance was transferred to a 50-mL class A volumetric flask and brought to final volume using acetonitrile to achieve a 1000 µg/mL primary stock solution.

Methiozolin Secondary Fortification/Calibration Stock Solutions Preparation

Secondary working fortification stock solutions of methiozolin were prepared in acetonitrile by serial dilution of the primary stock to achieve concentrations of 100, 10.0, 1.00, 0.100, and 0.0100 µg/mL. An additional calibration stock solution was also prepared from dilution of the 0.100 µg/mL acetonitrile stock solution using acetonitrile: HPLC Grade Water (1:1, v/v) to achieve a 0.0100 µg/mL for use in the preparation of calibration standard solutions for methiozolin HPLC/MS/MS analysis. The dilution scheme for the preparation of these secondary fortification/calibration stock solutions is shown below:

Secondary Fortification Stock Solutions (Acetonitrile)

Primary Stock Concentration (µg/mL)	Aliquot (mL)	Final Volume (mL)	Fortification Stock Concentration (µg/mL)
1000	5.00	50.0	100
100	5.00	50.0	10.0
10.0	5.00	50.0	1.00
1.00	5.00	50.0	0.100
0.100	5.00	50.0	0.0100

Calibration Stock Solution (Acetonitrile:HPLC Grade Water, 1:1, v/v)

Primary Stock Concentration (µg/mL)	Aliquot (mL)	Final Volume (mL)	Calibration Stock Concentration (µg/mL)
0.100	5.00	50.0	0.0100

The 0.0100 and 0.100 µg/mL secondary fortification stocks were used to prepare the method validation samples for this study. The 0.0100 µg/mL secondary calibration stock was used to prepare the calibration standards for methiozolin HPLC/MS/MS analysis.

Methiozolin Calibration Standards Preparation

Methiozolin calibration standard solutions ranging from 0.0100 to 0.500 µg/L were prepared by dilution of the above 0.0100 µg/mL calibration stock solution in acetonitrile: HPLC Grade Water (1:1, v/v) dilution solvent. The calibrations standards were prepared in 10-mL class A volumetric flasks using gas-tight syringes to measure fortification volumes. The final prepared calibrations standards were mixed well. The following shows the dilution scheme for a set of methiozolin calibration standards:

Calibration Stock Concentration (µg /mL)	Aliquot (mL)	Final Volume (mL)	Calibration Standard Concentration (µg /L)
0.0100	0.0100	10.0	0.0100
0.0100	0.0250	10.0	0.0250
0.0100	0.0500	10.0	0.0500
0.0100	0.100	10.0	0.100
0.0100	0.250	10.0	0.250
0.0100	0.500	10.0	0.500

All stocks and standards were stored refrigerated at approximately 4°C when not in use.

DFB Alcohol Secondary Fortification/Calibration Stock Solutions Preparation

Secondary working fortification/calibration stock solutions of DFB Alcohol were prepared in acetonitrile by serial dilution of the primary stock to achieve concentrations of 100, 10.0, 1.00, 0.100, and 0.0100 µg/mL.

The dilution scheme for the preparation of these secondary fortification/calibration stock solutions is shown below:

Secondary Fortification Stock Solutions (Acetonitrile)

Stock Concentration (µg/mL)	Aliquot (mL)	Final Volume (mL)	Secondary Stock Concentration (µg/mL)
1000	5.00	50.0	100
100	5.00	50.0	10.0
10.0	5.00	50.0	1.00
1.00	5.00	50.0	0.100
0.100	5.00	50.0	0.0100

The 1.00 and 10.0 µg/mL secondary stocks were used to prepare the method validation samples for this study. The 1.00 µg/mL secondary stock was used to prepare the calibration standards for DFB Alcohol GC/MSD analysis.

DFB Alcohol Calibration Standards Preparation

DFB Alcohol calibration standard solutions ranging from 2.50 to 50.0 µg/L were prepared by dilution of the above 1.00 µg/mL secondary fortification/calibration stock solution in dichloromethane (DCM) dilution solvent. The calibrations standards were prepared in 10-mL class A volumetric flasks using gas-tight syringes to measure fortification volumes. The final prepared calibrations standards were mixed well. The following shows the dilution scheme for a set of DFB Alcohol calibration standards:

Stock Concentration (µg /mL)	Aliquot (mL)	Final Volume (mL)	Calibration Standard Concentration (µg /L)
1.00	0.0250	10.0	2.50
1.00	0.0500	10.0	5.00
1.00	0.100	10.0	10.0
1.00	0.150	10.0	15.0
1.00	0.250	10.0	25.0
1.00	0.500	10.0	50.0

All stocks and standards were stored refrigerated at approximately 4°C when not in use.

Matrix Validation Samples– Surface and Ground Water

Separate analytical methods were developed and validated for each analyte. Untreated control surface and ground water matrix samples (10.0 mL) were fortified separately with each of the reference substances at two different concentrations for each of the two independent methods validated. For the Methiozolin method validations, five replicates were fortified at 0.0500 µg/L (LOQ) and five replicates were fortified at 0.500 µg/L (10X LOQ) in each of the matrices. For DFB Alcohol method validations,

five replicates were fortified at 5.00 µg/L (LOQ) and five replicates were fortified at 50.0 µg/L (10X LOQ) in each of the matrices. One reagent and two matrix blanks were also prepared for each method validation analysis to evaluate potential analytical interferences. All samples were processed and analyzed based on methodologies developed for use by EAG Laboratories-Easton and described below.

Analytical Method – Methiozolin

The analytical method applied to analysis of Methiozolin in surface and ground water is described below and presented schematically in Figure 1. The method consisted of an initial 1:1 dilution with acetonitrile solvent, succeeded by filtration and analysis by HPLC/MS/MS.

For surface and ground water method validation samples, approximately 5 mL of untreated control matrix was added for each sample to labeled 10-mL class A volumetric flasks. The validation samples were subsequently fortified using acetonitrile fortification stock solutions as shown below:

Fortification Stock Concentration (µg/mL)	Fortification Volume (mL)	Sample Volume (mL)	Sample Concentration (µg/L)
0.0100	0.0500	10.0	0.0500
0.100	0.0500	10.0	0.500

After fortification, the samples were adjusted to 10.0 mL final volume using appropriate matrix and mixed well by inversion. Aliquots of the samples were then diluted with an equal volume of acetonitrile in 15-mL glass culture tubes (i.e. 2.00 mL of aqueous sample + 2.00 mL of acetonitrile = 2X dilution factor). The dilutions were filtered using a disposable luer-lock syringe connected to a Whatman 0.2 µm Puradisk 25 TF (PTFE) syringe filter into 15-mL tubes. (Note: while not necessary during this study, further dilutions into the calibration range should be performed using acetonitrile: HPLC grade water, 1;1, v/v, dilution solvent). Aliquots of the final dilutions and calibration standards were transferred to auto-sampler vials and submitted for analysis by HPLC/MS/MS. Unfortified non-treated control samples for each matrix, and reagent blanks consisting of HPLC grade water, were also prepared using the same process and dilution scheme to evaluate potential analytical matrix interferences.

Concentrations of Methiozolin in processed samples were determined using an Agilent Technologies 1200 Infinity Series High Performance Liquid Chromatograph (HPLC) coupled with an Applied Biosystems/MDS Sciex API 5000 Mass Spectrometer (MS/MS) using a Turbo-Ion Spray source operated in

the positive ion, multiple reaction monitoring (MRM) mode. The HPLC was connected to the mass spectrometer (MS/MS) through a Valco valve that diverted only the eluate from 2.5 to 8.0 minutes post-injection to the HPLC/MS/MS . Chromatographic separations were achieved using a THERMO EC Betasil C18 analytical column (50 mm x 2.1 mm, 5 µm), preceded by a THERMO EC Javelin Betasil C18 guard column (10 mm x 2.1 mm) utilizing a gradient elution profile. Quantitation was performed using the response of the primary ion transitions for Methiozolin. Confirmation analysis was performed using the response of the secondary confirmation ion transition. The ion transitions monitored are summarized below:

Analyte	Primary (Quantitation)	Secondary (Confirmation)
Methiozolin	338→127 amu	338→211 amu

The High Performance Liquid Chromatograph/ Mass Spectrometer (HPLC/MS/MS) operating parameters are summarized in Table 1.

Calibration curves were generated from analyses of Methiozolin standard solutions analyzed concurrently with each series of method validation samples. Each analytical sequence consisted of injection of the calibration standard solution series, followed by sample injections interspersed with standard solution injections and concluding with the same complete series of calibration standard solutions. A calibration standard solution was injected during each analytical sequence following no more than five sample injections.

Analytical Method – DFB Alcohol

The analytical method applied to analysis of DFB Alcohol in surface and ground water is described below and presented schematically in Figure 2. The method consisted of a single DCM liquid-liquid partition, followed by centrifugation, further dilution, if necessary, and analysis by GC/MSD.

For surface and ground water method validation samples, 10.0 mL aliquots of matrix were measured into labeled 50-mL plastic graduated centrifuge tubes and corrected for fortification co-solvent volumes. The validation samples were subsequently fortified using fortification stock solutions as shown below:

Fortification Stock Concentration (µg/mL)	Fortification Volume (mL)	Sample Volume (mL)	Sample Concentration (µg/L)
1.00	0.0500	10.0	5.00
10.0	0.0500	10.0	50.0

Following fortification, 5.00 mL volumes of DCM extraction solvent were added to each sample tube. The sample/solvent mixture was allowed to vent first by swirling gently by hand, followed by capping and a brief shaking. Pressure was carefully released by slowly breaking the seal of the cap and tube. The tubes were recapped tightly and the samples were extracted by shaking on a SPEX GenoGrinder sample processor for approximately 2 minutes at a setting of 1250 RPM. Following partitioning, the samples were centrifuged at ~ 4000 RPM for approximately five minutes. Using a 9 inch glass disposable pipet, aliquots of the lower DCM solvent layers were removed from each sample. Note: air was gently expelled while inserting through the upper aqueous layer to ensure DCM only aliquot was removed during this process. The content of each pipet was visually inspected to ensure the absence of water and the outside of each pipet surface was also wiped using a lab tissue to remove any additional moisture. The DCM aliquots were added directly to auto-sampler vials. If necessary, aliquots of the final solvent extracts were diluted further into the calibration range using DCM solvent. The final diluted extracts were transferred to auto-sampler vials and submitted for analysis by GC/MSD. Unfortified non-treated control samples for each matrix, and reagent blanks consisting of HPLC grade water, were also prepared using the same process and dilution scheme to evaluate potential analytical matrix interferences.

Concentrations of DFB Alcohol in processed samples were determined using an Agilent Technologies 6890N Gas Chromatograph (GC) coupled with an Agilent Technologies 5975 inert Mass Selective Detector (MSD) operated in the electron impact (EI) mode. Chromatographic separations were achieved using an Agilent DB-624 column (30 m x 0.25 mm, 1.4 µm film thickness). Quantitation was performed using the response of the primary ion transitions for Methiozolin. Confirmation analysis was performed using the response of the secondary confirmation ion transition. The ions monitored are summarized below:

Analyte	Primary Ion (Quantitation)	Secondary Ion (Confirmation)
DFB Alcohol	144 amu	123 amu

Other fragment ions (127 and 95 m/z) were observed during preliminary instrumental scans as part of GC/MSD method setup; however these ions lacked sufficient sensitivity and/or selectivity to be of any beneficial use, justifying the selection of the above ions for monitoring in this methodology.

The Gas Chromatograph/ Mass Selective Detector (GC/MSD) operating parameters are summarized in Table 2.

Calibration curves were generated from analyses of Methiozolin standard solutions analyzed concurrently with each series of method validation samples. Each analytical sequence consisted of injection of the calibration standard solution series, followed by sample injections interspersed with standard solution injections and concluding with the same complete series of calibration standard solutions. A calibration standard solution was injected during each analytical sequence following no more than five sample injections.

Method Limits of Quantitation (LOQ)

The method limit of quantitation (LOQ) for the Methiozolin surface and ground water method was set at 0.0500 µg/L; the lowest level fortified and analyzed during each validation set. The 0.0100 µg/L low-level calibration standard was equivalent to an in sample value of 20% of the LOQ for Methiozolin method analyses. The method limit of quantitation (LOQ) for the DFB Alcohol surface and ground water method was set at 5.00 µg/L; the lowest level fortified and analyzed during each validation set. The 2.50 µg/L low-level calibration standard was equivalent to an in sample value of 50% of the LOQ for the DFB Alcohol method analyses. Therefore, any detected residues less than low-level calibration standard response for each analyte can be further defined as being <20% and <50% of the established LOQs for the Methiozolin and DFB Alcohol methods, respectively, during any residue study sample analyses. Reagent blank and matrix blank samples were evaluated to confirm any potential interference to be < 30% of the fortified LOQ for both quantitation methods.

Limits of Detection (LOD)

The theoretical in sample LOD for the Methiozolin analytical method was 0.0200 µg/L; calculated as the product of the lowest calibration standard analyzed (0.0100 µg/L) and the dilution factor of the blank and LOQ samples (2.0). The theoretical in sample LOD for DFB Alcohol analytical method was 1.25 µg/L; calculated as the product of the lowest calibration standard analyzed (2.50 µg/L) and the dilution factor of the blank and LOQ samples (0.50).

The actual calculated instrumental LODs for Methiozolin in surface and ground water matrices were determined to be 0.00514 µg/L and 0.00600 µg/L, respectively. These LOD values were calculated as the product of the one-tailed t-statistic at the 99% confidence level and the standard deviation of the five replicate LOQ matrix fortifications for each matrix as follows:

$$LOD = t^{99} \times SD$$

where t^{99} equals 3.747 for 5 replicate samples and SD is the standard deviation of the same five LOQ replicate samples for each matrix.

Surface water LOD = 3.747 x 0.0013725 = 0.00514 µg/L

Ground water LOD = 3.747 x 0.0016002 = 0.00600 µg/L

The actual calculated instrumental LODs for DFB Alcohol in surface and ground water matrices were determined to be 0.211 µg/L and 0.333 µg/L, respectively. These LOD values were calculated as the product of the one-tailed t-statistic at the 99% confidence level and the standard deviation of the five replicate LOQ matrix fortifications for each matrix as follows:

$$LOD = t^{99} \times SD$$

where t^{99} equals 3.747 for 5 replicate samples and SD is the standard deviation of the same five LOQ replicate samples for each matrix.

Surface water LOD = 3.747 x 0.0562162 = 0.211 µg/L

Ground water LOD = 3.747 x 0.0888186 = 0.333 µg/L

CALCULATIONS

Calibration Standard Curves and Regression Analyses (Methiozolin Method)

For Methiozolin analyses of surface and ground water samples, a linear regression analysis was applied to the chromatographic peak area responses for the calibration standard solutions versus their respective nominal concentrations. Standard curves were generated by plotting the regression functions consisting of the analyte concentration (µg/L) on the abscissa and the respective peak area responses on the ordinate. Each generated calibration curve was weighted 1/x with respect to concentration and expressed as a quadratic function as follows:

$$y = Ax^2 + Bx + C$$

where y = instrumental peak area response of concentration x of Methiozolin

A = quadratic coefficient

B = linear coefficient (Slope)

C =constant coefficient (Y-intercept)

Calculations of concentrations for injected calibration standards (µg/L) and residue samples (µg/L) were performed using Analyst Version 1.6 Applied Biosystems/MDS Sciex software. Values of dilution factors, nominal concentrations, and sample identifiers were entered into the software manually.

Calibration Standard Curves and Regression Analyses (DFB Alcohol Method)

For DFB Alcohol analyses in surface and ground waters, a linear regression was applied to the chromatographic peak area responses for the calibration standard solutions versus their respective nominal concentrations. Standard curves were generated by plotting the regression functions consisting of the analyte concentration (µg/L) on the abscissa and the respective peak area responses on the ordinate. Each generated calibration curve was expressed as a linear function as follows:

$$y = mx+b$$

where y = instrumental peak area response of concentration x of DFB Alcohol

m = slope

x = concentration

b = y-intercept

Calculations of concentrations for injected calibration standards (µg/L) and surface and ground water samples (µg/L) were performed using Agilent Technologies Chem Station software and Excel 2010. Values of dilution factors, nominal concentrations and sample identifiers were entered into the software manually.

Determination of Methiozolin Concentrations in Surface and Ground Water Samples

Concentrations of Methiozolin in method validation samples were determined by substituting peak area responses of the samples into the applicable rearranged regression equation above, corrected for dilution factor, as follows:

$$\text{Measured Concentration} = \frac{-B + \sqrt{B^2 - [4 \times A \times (C - \text{Peak Area})]}}{2 \times A} \times \text{Dilution Factor}$$

The above measured concentration was compared to the nominal concentration to calculate a percent of nominal concentration as follows:

$$\text{Percent of Nominal Concentration} = \frac{\text{Measured Concentration}}{\text{Nominal Concentration}} \times 100$$

Determination of DFB Alcohol Concentrations in Surface and Ground Water Samples

Concentrations of DFB Alcohol in the method validation samples for this study were determined by substituting peak area responses of the samples into the applicable rearranged regression equation above, corrected for dilution factor, as follows:

Measured Concentration = Peak Area - (y-Intercept)/Slope x Dilution Factor

The above measured concentration was compared to the nominal concentration to calculate a percent of nominal concentration as follows:

$$\text{Percent of Nominal Concentration} = \frac{\text{Measured Concentration}}{\text{Nominal Concentration}} \times 100$$

Representative Calculation of Methiozolin Concentration in Surface Water Validation Sample

A representative calculation is presented consisting of quantitation of Methiozolin concentration in surface water method validation sample 716C-106-SW-VMAS-1 using data and the results from the quadratic weighted (1/x) regression analysis listed below.

Peak area = 20628
Constant Coefficient (C) = -100.718
Linear Coefficient (B) = 880840
Quadratic Coefficient (A) = -229535
r = 0.9993653 equivalent to r^2, the coefficient of determination, = 0.998731
Dilution Factor ($V_{final}/M_{initial}$) = 2.00

The concentration of Methiozolin in the sample extract solution at instrument was determined by substituting the resulting analyte peak area of each sample into the rearranged regression equation as shown below.

$$\text{Measured Concentration At Instrument } (\mu g/L) = \frac{-B + \sqrt{B^2 - [4 \times A \times (C - \text{Peak Area})]}}{2 \times A}$$

$$= \frac{-880840 + \sqrt{(880840)^2 - [(4 \times (-229535) \times (-100.718 - 20628]}}{2 \times (-229535)}$$

$$= 0.023679$$

The calculated residue concentration of Methiozolin in the method validation samples were determined as the product of the Methiozolin concentration at instrument (µg/L) and the sample processing dilution factor as shown below:

Methiozolin
Concentration
in Residue Sample (µg/L) = Methiozolin at Instrument Conc. x Dilution Factor

= 0.023679 x 2.00

= 0.047357

$$\text{Percent of Nominal Concentration} = \frac{0.047357}{0.0500} \times 100$$

= 94.7%

Note: Calculation of concentrations for injected calibration standards were performed using Analyst Version 1.6 Applied Biosystems/MDS Sciex software algorithms following entry of the dilution factors, nominal concentrations, and sample identifiers. Values calculated using rounded numbers, either as presented above or in the tables, may differ slightly. These sample calculations were also used to quantitate the Methiozolin concentration using confirmatory method, and also in ground water method validation samples as well.

Representative Calculation of DFB Alcohol Concentration in Surface Water Validation Sample

A representative calculation is presented consisting of quantitation of DFB Alcohol concentration in surface water method validation sample 716C-106-SFW-VMAS-11, nominal concentration of 5.00 µg/L, using data and the results from the regression analysis listed below.

Peak Area = 58516
Slope = 6855.2730
y-Intercept = -8915.3753
r^2 = 0.9941
Dilution Factor ($V_{final}/M_{initial}$ x $V_{final}/V_{initial}$) = 0.500

The concentration of DFB Alcohol in the sample extract solution at instrument was determined by substituting the resulting analyte peak area of each sample into the rearranged regression equation as shown below.

$$\text{DFB Alcohol Concentration at instrument (µg/L)} = \frac{\text{Peak Area - (y-Intercept)}}{\text{Slope}}$$

$$\text{DFB Alcohol Concentration at instrument (µg/L)} = \frac{58516 + 8915.3753}{6855.2730}$$

DFB Alcohol Concentration at instrument (µg/L) = 9.8364

The calculated concentration of DFB Alcohol in the surface water sample was determined as the product of the DFB Alcohol concentration at instrument (µg/L) and the sample processing dilution factor as shown below:

DFB Alcohol Concentration in Sample (µg/L) = DFB Alcohol at Instrument Conc. x Dilution Factor
= 9.8364 µg/L x 0.500
= 4.9182

$$\text{Percent of Nominal Concentration} = \frac{4.9182}{5.00} \times 100$$

$$= 98.4\%$$

Note: Calculations of concentrations for injected calibration standards were performed using Excel 2010 algorithms following entry of the dilution factors, nominal concentrations, and sample identifiers. Values calculated using rounded numbers, either as presented above or in the tables, may differ slightly. These sample calculations were also used to quantitate the DFB Alcohol concentration using confirmatory method, and also in ground water method validation samples as well.

Table 1

Typical High Performance Liquid Chromatography/ Mass Spectrometer (HPLC/MS/MS)
Operational Parameters for the Analysis of Methiozolin

Instrument:	Agilent Technologies 1200 Infinity Series High Performance Liquid Chromatograph (HPLC) coupled with an Applied Biosystems/MDS Sciex API 5000 Mass Spectrometer (MS/MS) with QJet Ion guide operated in the positive ion multiple reaction monitoring (MRM) mode.
Analytical Column:	THERMO EC Betasil C-18 (50 x 2.1 mm, 5 µm particle size)
Guard Column:	THERMO EC Javelin Betasil C-18 (10 x 2.1 mm)
Column Oven Temperature:	40°C
Mobile Phases:	A – HPLC Grade Water:0.1% formic acid B – Acetonitrile:0.1% formic acid

Gradient Elution Profile :

Time	Flow Rate (µL/min.)	Percent A	Percent B
0.00	250	50.0	50.0
1.00	250	50.0	50.0
4.00	250	5.00	95.0
5.00	250	5.00	95.0
5.10	250	50.0	50.0
8.00	250	50.0	50.0

Injection Volume:	25.0 µL
Ion Source:	Turbo-V Ion Spray, positive mode

Parameter Table:

CUR:	25.0	IS:	5500.00
GS1:	35.0	DP:	40
GS2 :	45.0	EP:	10.00
CAD:	6.00	CE:	47,25
TEM:	400.00	CXP:	16,22

Monitored Transition(s):	338 → 127 m/z – Quantitation (dwell time 500 msec) 338 → 211 m/z – Confirmation (dwell time 500 msec)
Approximate Retention Time:	~ 4.8 minutes

Table 2

Typical Gas Chromatography/ Mass Spectrometer (GC-MSD)
Operational Parameters for the Analysis of DFB Alcohol

Instrument:	Agilent Technologies Model 6890N Gas Chromatograph coupled with a Model 5975 Inert Mass Selective Detector
Analytical Column:	Agilent DB-624 , Part No. 122-1334UI (30 m x 0.25 mm, 1.4-µm film thickness)
Carrier Gas:	Helium, @ 15 psi (1.6 mL/minute)
Injector:	Temperature: 120°C Volume: 1.00 µL, splitless
Oven Temperature Profile:	Initial temperature: 60°C Initial hold time: 1.00 minute Solvent Delay: 4.00 minutes

Ramp Rate (°C/minute)	Final Temperature (°C)	Final Time (minutes)
20.0	250	0.00

Detector Temperature:	300°C
Ions Monitored	SIM Mode: 144 m/z (Quantitation) 123 m/z (Confirmation) Dwell 100 msec
Peak Retention Time:	~7.5 minutes

METHOD OUTLINE FOR THE DIRECT INJECTION ANALYSIS OF METHIOZOLIN IN SURFACE AND GROUND WATER

PROCEDURE:

1. Prepare calibration standards in acetonitrile: HPLC Grade Water (1:1, v/v) using volumetric flasks and gas-tight syringes from methiozolin stock solutions, **STORE REFRIGERATED**.

2. Prepare matrix fortification samples in appropriate aqueous control matrix using methiozolin stock solutions, volumetric flasks and gas-tight syringes. Prepare unfortified reagent and matrix blanks using HPLC grade water and appropriate matrix, respectively. Volumetrically dilute an aliquot of all aqueous samples with an equal volume of acetonitrile (i.e.; 2.00 mL H20 + 2mL of ACN = 4.00 mL final volume = 2X dilution factor).

3. Filter the above dilutions using a Whatman Puradisc 25 TF (PTFE) syringe filter connected to a 5-mL disposable B&D luer-lock syringe into 20-mL vials or equivalent.

4. If required, volumetrically dilute samples further using a solution of acetonitrile: HPLC Grade Water (1:1, v/v). using Class A volumetric flasks and gas-tight syringes, if necessary. Mix well.

5. Transfer aliquots of final sample dilutions and calibration standards to auto-sampler vials and submit for analysis by HPLC/MS/MS.

Figure 1. Analytical method outline for the analysis of Methiozolin in Surface and Ground Water.

METHOD OUTLINE FOR THE EXTRACTION AND ANALYSIS OF OF DFB ALCOHOL IN SURFACE AND GROUND WATER

PROCEDURE:

Prepare calibration standards in dichloromethane (DCM) using volumetric flasks and gas-tight syringes from DFB Alcohol stock solutions, STORE REFRIGERATED

1. Measure 10.0 mL of water sample into 50-mL disposable plastic centrifuge tubes.
2. Fortify the method validation samples as necessary **(LOQ/ 10X LOQ – 5.00/50.0)** using solvent stock solutions of DFB Alcohol. Reagent blank will consist of extraction solvent and any reagents carried though the methodology with no matrix or fortification of analytes. Matrix blank will be unfortified matrix carried through the methodology.
3. Volumetrically add 5.00 mL of DCM extraction solvent to each sample. Allow sample/solvent mixture to vent by first swirling gently by hand, followed by capping and a brief shaking. Carefully release any pressure by slowly breaking seal of cap and tube. Recap tightly for extraction below.
4. Extract samples by shaking for ~2 minutes on SPEX GenoGrinder sample processor at 1250 rpm setting
5. Centrifuge at ~ 4000 rpm for ~ 5 minutes.
6. Using a glass disposable pipet (9 "), carefully remove an aliquot of the lower DCM solvent layer from each sample. Note: gently expel air as inserting through aqueous phase to ensure DCM only aliquot removed. Visually inspect contents in pipet to ensure no water is present and also remove any moisture from the outside of the pipet surface using a lab tissue.
7. Transfer the solvent only layer directly into auto-sampler vials. Further dilute an aliquot of each final solvent extract into the calibration range using DCM, if necessary.
8. Aliquot final diluted extracts to auto-sampler vials for analysis by GC-MSD.

Figure 2. Analytical method outline for the extraction and analysis of DFB Alcohol in Surface and Ground Water.

EAG LABORATORIES

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AMENDMENT TO STUDY PROTOCOL

PROJECT NUMBER: 716C-106

AMENDMENT NUMBER: 1

EFFECTIVE DATE: May 02, 2017

AMENDMENT: Page 7; levels to be validated table; change levels to be validated for DFB Alcohol analyte to 5.00 (LOQ) and 50.0 (10X LOQ) µg/L.

REASON: A separate analytical method was required and developed for analysis of DFB Alcohol analyte. Due to its chemical properties (i.e. small molecular weight and volatility) its analysis was not adaptable to LC/MS/MS platform and typical sample concentration/evaporation procedures. As a direct consequence of this limitation, a GC-MSD analytical platform was adopted, which typically shows a decreased level of sensitivity in comparison to LC/MS/MS analysis. The original validation levels specified in the protocol and used for methiozolin validation (LC/MS/MS), were not achievable with DFB Alcohol (GC-MSD) and were adjusted as described above as a result of this difference in instrumental sensitivity and extraction/concentration limitations.

IMPACT: This amendment has no adverse effect on the integrity of the data generated during this study.

STUDY DIRECTOR — DATE: 05/02/2017

LABORATORY MANAGEMENT — DATE: 02 May 2017

QAU Review
Initials/Date: 5/2/17