MRID No.: 51185102

STUDY TITLE

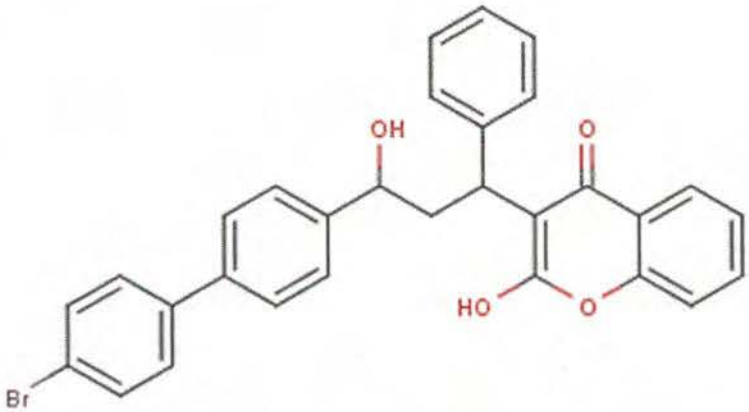
Validation of the Environmental Analytical Method 184A for Bromadiolone Residues in Soil

TEST GUIDELINES

CFR Title 40, Part 160
EPA OCSPP 850.6100 and 830.1800

INTRODUCTION

Bromadiolone has the following chemical structure:



Chemical Names:	3-[3-[4-(4-bromophenyl)phenyl]-3-hydroxy-1-phenylpropyl]-4-hydroxychromen-2-one; 3-[3-(4'-Bromo-4-biphenylyl)-3-hydroxy-1-phenylpropyl]-4-hydroxy-2H-chromen-2-one
CAS #:	28772-56-7
Formula:	$C_{30}H_{23}BrO_4$
Molecular Weight:	527.41 g/mole

The objective of the protocol was to validate the method described in draft NWRC method 184A for the determination of bromadiolone in soil. This study is being submitted to fulfill the EPA laboratory efficacy data requirements for OCSPP 850.6100 and 830.1800 as outlined in 40 CFR 160 for the validation of draft NWRC Method 184A, "Determination of Bromadiolone Residues in Soil".

EXECUTIVE SUMMARY

An analytical method has been developed and validated for the determination of bromadiolone residues in soil. Control soil was fortified at concentrations equivalent to bromadiolone residues of 0.01 mg/kg and 0.1 mg/kg. Seven replicate samples at both fortification levels were tested over two days to demonstrate method accuracy and precision.

was stored refrigerated at 4 °C (Room B215, Fridge #4) in the original container. Refer to Appendix II for COA.

Test System

Clean soil was obtained and characterized under GLP by Agvise Laboratories (Appendix I) and sent to NWRC Chemistry Laboratory Unit. Bromadiolone reference standard was obtained and used to prepare standards and fortify soil at 0.01 mg/kg and 0.10 mg/kg. A deuterium labeled surrogate analyte (d_5-bromadiolone) was obtained from CDN Isotopes Inc. (Appendix III). Draft Method 184A was validated using the clean soil fortified with bromadiolone and d_5-bromadiolone.

METHODS

The protocol for this study was prepared according to NWRC standards and procedures and approved on June 11, 2019. It was assigned NWRC Study Number QA-3078 (Appendix IV). The protocol includes a draft version of NWRC method 184A, "Determination of Bromadiolone Residues in Soil". The general method is summarized as follows:

> A 10 g soil sample is fortified with deuterium labeled surrogate analyte (d_5-bromadiolone) and extracted with acetonitrile and excess sodium chloride. The extract is clarified by centrifugation and a portion of the supernatant cleaned-up by solid-phase extraction using a reversed phase sorbent. The eluate is reconstituted in mobile phase and quantified by ultra-performance liquid chromatography (UPLC) coupled to a tandem mass spectrometer (MS/MS) with electrospray ionization (ESI) source.

Matrix Interference:

Seven control soil samples were fortified with only surrogate analyte (d_5-bromadiolone), and then extracted and analyzed.

Results:
The analyses were performed using LC-MS/MS instrumentation operated in multiple-reaction-monitoring (MRM) mode. No significant interferences were observed in the control soil samples (Figure 4). The results demonstrated the method to be highly selective for bromadiolone.

Confirmatory Analysis:

The validation was performed with LC-MS/MS, a technique that is quantitative and highly specific. The selection of precursor ions for bromadiolone and d_5-bromadiolone under negative ionization conditions are presented in Figures 6 and 7. The molecular ion ($M\text{-}H^-$) chosen as the precursor for bromadiolone was *m/z* 525, and *m/z* 530 for d_5-bromadiolone. As shown in Figure 8, the most abundant product ion for bromadiolone (*m/z* 250) was chosen as the quantifier ion, and the second most abundant (*m/z* 181) as the qualifier ion. For d_5-bromadiolone, the *m/z* 255 product ion was chosen for quantitation (Figure 9).

Detection Limit (DL):

The Detection Limit (DL) was estimated from the baseline noise observed in the control soil samples and the peak height observed in soil fortified with 0.01 mg/kg bromadiolone. The estimated DL is defined as the concentration of bromadiolone required to generate a signal equal

to 3X the average baseline noise (measured peak-to-peak) observed in the control soil.

Result: The estimated detection limit for bromadiolone in soil was 0.000063 mg/kg.

Quantitation Limit (QL):

The Quantitation Limit (QL) was estimated from the baseline noise observed in the control soil samples and the peak heights observed in soil fortified with 0.01 mg/kg bromadiolone. The estimated QL is defined as the concentration of bromadiolone required to generate a signal equal to 10X the average baseline noise (measured peak-to-peak) observed in the control soil.

Result: The estimated quantitation limit for bromadiolone in soil was 0.000209 mg/kg. This is over 40X more sensitive than the required QL of 0.01 mg/kg. A representative ion chromatogram of soil fortified with 0.01 mg/kg bromadiolone is presented in Figure 5.

Bias and Repeatability:

Control soil samples were fortified with 0.01 mg/kg and 0.10 mg/kg bromadiolone. Seven replicates samples at both fortification levels were tested over two days.

System Suitability:

As required in Draft Method 184A, instrument repeatability was demonstrated by injecting Standard 6 (121 ng/mL bromadiolone, 131 ng/mL d_5-bromadiolone) five times before each sequence of samples. The percent relative standard deviation (% RSD) in response ratios for

each analysis was required to be less than or equal to 2.0 %. In addition, CH010.01 required the percent match of the quality control working standard be within 95.0 - 105 % of target for an analysis to be accepted.

Result: Requirements for instrument repeatability and quality control working standard percent match were achieved for each of the three analyses

Bromadiolone Residue Confirmation:

For bromadiolone residues to be confirmed, draft Method 184A required that acceptance criteria for bromadiolone retention time, bromadiolone qualifier transition percent match, and d_5-bromadiolone surrogate recovery be met for each sample.

APPENDIX V

Wildlife Services NWRC National Wildlife Research Center Analytical Method	United States Department of Agriculture Animal Plant Health Inspection Service Wildlife Services National Wildlife Research Center Chemistry Laboratory Unit	Number: 184A Supersedes: NA Date effective: 08-05-2019 Page: 1 of 18

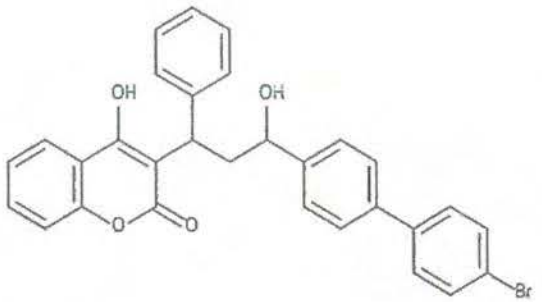
DETERMINATION OF BROMADIOLONE RESIDUES IN SOIL

I. CHEMICAL DATA (ACTIVE INGREDIENT)

Common Names: Bromadiolone

Chemical Names: 3-[3-[4-(4-bromophenyl)phenyl]-3-hydroxy-1-phenylpropyl]-4-hydroxychromen-2-one; 3-[3-(4'-Bromo-4-biphenylyl)-3-hydroxy-1-phenylpropyl]-4-hydroxy-2H-chromen-2-one

Structure:



Formula: $C_{30}H_{23}BrO_4$
CAS #: 28772-56-7
Molecular Weight: 527.41 g/mole
Melting Point: 200-210°C
Physical State: White to off-white powder
Solubility (mg/mL): 730 mg/mL in dimethylformamide; 25.0 in ethyl acetate; 22.3 in acetone; 10.1 in chloroform; 5.6 in methanol; 0.2 in hexane; 0.019 in water.

8/5/2019 Developed By Date	8-7-19 Approved By Date
8/5/2019 Validated By Date	8/6/19 QA/QC Specialist Date

II. MATRICES TESTED

Sandy loam consisting of 63% sand, 20% clay, 17% silt, 21% moisture, 3.5% organic matter and a pH of 6.6 (Agvise Laboratories, P/N MSL-PF).

III. GENERAL METHOD

A 10 g soil sample is fortified with deuterium labelled surrogate analyte (d_5-bromadiolone) and extracted with acetonitrile and excess sodium chloride. The extract is clarified by centrifugation and a portion of the supernatant cleaned-up by solid-phase extraction using a reversed phase sorbent. The eluate is reconstituted in mobile phase and quantified by ultra-performance liquid chromatography (UPLC) coupled to a tandem mass spectrometer (MS/MS) with electrospray ionization (ESI) source.

IV. REAGENTS

Name	CAS #
1. Bromadiolone reference standard	28772-56-7
2. d_5-bromadiolone (phenyl-d_5)	N/A
3. Acetonitrile (ACN), HPLC grade	75-05-8
4. Sodium chloride (NaCl), Certified ACS grade	7440-23-5
5. Formic acid, LCMS grade	64-18-6
6. Methanol (MeOH), HPLC grade	67-56-1
7. Ultrapure (18 MΩ) deionized water	7732-18-5
8. Acetone, HPLC grade	67-64-1
9. Ethyl acetate, HPLC grade	141-78-6

V. SPECIAL EQUIPMENT/SUPPLIES

1. Strata-X SPE cartridges, 60 mg/3 mL (Phenomenex, Inc., Madrid, CA, USA), or equivalent.
2. SPE manifold and vacuum source.
3. 0.45-µm glass fiber filters, 25-mm diameter.
4. Xbridge BEH C18, 2.5 µm, 2.1 x 50 mm, (Waters Corp., Milford, MA, USA), or equivalent.
5. Flatbed mechanical shaker, capable of ~300 cycles/min.
6. 50-mL conical-bottom polypropylene tubes.

7. Centrifuge capable of 3500 RCF with 50-mL conical bottom polypropylene tubes.
8. 15-mL conical-bottom polypropylene tubes.
9. Repeat pipettor with tips ranging from 0.5-mL to 50-mL.
10. 1000-µL air-displacement pipettor with 1-mL tips.
11. 8-mL amber glass vials with PTFE-lined caps

VI. SOLUTIONS

0.1% Formic acid: Combine 1.0 mL formic acid with 1000 mL of ultra-pure deionized water. Invert not less than 5X to mix. Transfer ~200 mL to a separate glass bottle for dilutions.

0.1% Formic acid in Acetonitrile: Combine 1.0 mL formic acid with 1000 mL acetonitrile. Invert not less than 5X to mix.

Wash solution: Combine 15 mL methanol with 85 mL ultra-pure deionized water and 0.100 mL formic acid in a glass bottle. Invert not less than 5X to mix.

VII. STANDARDS PREPARATION

Note: All Class A volumetric glassware and beakers used to prepare standard solutions should be pre-cleaned prior to use. Factory-new amber glass vials and caps do not need to be pre-cleaned. Pre-cleaning is accomplished by:

1. Rinse each piece of glassware three times with 2-3 mL of ethyl acetate, discarding each wash to hazardous waste.
2. Rinse each piece of glassware two times with 2-3 mL of ultra-pure deionized water.
3. Invert each piece of glassware in a metal rack and dry in an oven heated to 100-110°C for approximately two hours or until dry.
4. Allow the glassware to cool to room temperature prior to use.

Concentrated d_5-Bromadiolone Stock: Accurately weigh approximately 1 mg of d_5-bromadiolone and quantitatively transfer to a 5-mL Class A volumetric flask. Dissolve in approximately 4 mL ethyl acetate, sonicate 1 minute (or until all solids are dissolved), and then bring to volume with ethyl acetate. Mix thoroughly and transfer to an amber 8-mL glass vial with PTFE-lined cap. The concentration of d_5-bromadiolone will be approximately 200 µg/mL. Store at 4°C.

Concentrated Bromadiolone Stock: Accurately weigh approximately 10 mg of bromadiolone reference standard and quantitatively transfer to a 10-mL Class A volumetric flask. Dissolve in

approximately 8 mL ethyl acetate, sonicate 1 minute (or until all solids are dissolved), and then bring to volume with ethyl acetate. Mix thoroughly and transfer ~ 8 mL to an amber glass vial with PTFE-lined cap. Dispose of excess solution to hazardous waste. The concentration of bromadiolone will be approximately 1000 µg/mL. Store at 4°C.

40X Surrogate Stock: Accurately transfer 0.200 mL of Concentrated d_5-Bromadiolone Stock to an amber 8-mL glass vial. Remove ethyl acetate with a gentle flow of nitrogen gas in a 50-60°C evaporator (1-2 minutes). Add 8.00 mL ACN, cap securely with a PTFE-lined cap, sonicate 5 minutes, and vortex mix 5-6 s. The concentration of d_5-bromadiolone will be approximately 5000 ng/mL (equivalent to 40 times the final analytical concentration). Store at 4°C.

40X-7 Bromadiolone Stock: Accurately transfer 0.250 mL of Concentrated Bromadiolone Stock to a 25-mL Class-A volumetric flask. Remove ethyl acetate with a gentle flow of nitrogen gas in a 50-60°C evaporator (1-2 minutes). Add ~20 mL ACN, cap securely, and sonicate 5 minutes. Allow the flask to equilibrate at room temperature for at least 5 minutes before diluting to volume with ACN. Mix thoroughly and transfer ~ 8 mL to one or more amber glass vials with PTFE-lined caps. Dispose of excess solution to hazardous waste. The concentration of bromadiolone will be approximately 10 µg/mL, or 10,000 ng/mL. Store at 4°C.

40X Bromadiolone Stocks in ACN:

Prepare six additional 40X bromadiolone stocks by combining the volumes described below in 8-mL amber glass vials with PTFE-lined caps. Store at 4°C.

ID		Approximate Concentration (ng/mL)
40X-7	See above for preparation	10,000
40X-6	Combine 2.500 mL of 40X-7 with 2.500 mL ACN	5000
40X-5	Combine 1.000 mL of 40X-7 with 4.000 mL ACN	2000
40X-4	Combine 0.500 mL of 40X-7 with 4.500 mL ACN	1000
40X-3	Combine 0.250 mL of 40X-7 with 4.750 mL ACN	500
40X-2	Combine 1.000 mL of 40X-4 with 4.000 mL ACN	200
40X-1	Combine 0.500 mL of 40X-4 with 4.500 mL ACN	100

1.25X Bromadiolone and d_5-Bromadiolone Stocks in ACN:

Prepare eight 1.25X stocks by combining the volumes described below in 8-mL amber glass vials with PTFE-lined caps. Store at 4°C.

ID		Approximate Bromadiolone (ng/mL)	Approximate d_5-Bromadiolone (ng/mL)
1.25X-7	0.100 mL 40X-7 + 0.100 mL 40X Surr. + 3.00 mL ACN	313	156
1.25X-6	0.100 mL 40X-6 + 0.100 mL 40X Surr. + 3.00 mL ACN	156	156
1.25X-5	0.100 mL 40X-5 + 0.100 mL 40X Surr. + 3.00 mL ACN	62.5	156
1.25X-4	0.100 mL 40X-4 + 0.100 mL 40X Surr. + 3.00 mL ACN	31.3	156
1.25X-3	0.100 mL 40X-3 + 0.100 mL 40X Surr. + 3.00 mL ACN	15.6	156
1.25X-2	0.100 mL 40X-2 + 0.100 mL 40X Surr. + 3.00 mL ACN	6.25	156
1.25X-1	0.100 mL 40X-1 + 0.100 mL 40X Surr. + 3.00 mL ACN	3.13	156
1.25X-0	0.100 mL 40X Surr. + 3.10 mL ACN	0	156

Standard Curve in 80% (ACN)/20% (0.1% formic acid):

Prepare the standard curve in amber 2-mL autosampler vials by combining the 0.200 mL of 0.1% formic acid with 0.800 mL of each 1.25X Stock. Store at 4°C.

ID		Bromadiolone (ng/mL)	d_5-Bromadiolone (ng/mL)
Standard 7	0.800 mL 1.25X-7 + 0.200 mL 0.1% formic acid	250	125
Standard 6	0.800 mL 1.25X-6 + 0.200 mL 0.1% formic acid	125	125
Standard 5	0.800 mL 1.25X-5 + 0.200 mL 0.1% formic acid	50	125
Standard 4	0.800 mL 1.25X-4 + 0.200 mL 0.1% formic acid	25	125
Standard 3	0.800 mL 1.25X-3 + 0.200 mL 0.1% formic acid	12.5	125
Standard 2	0.800 mL 1.25X-2 + 0.200 mL 0.1% formic acid	5.0	125
Standard 1	0.800 mL 1.25X-1 + 0.200 mL 0.1% formic acid	2.5	125
Standard 0	0.800 mL 1.25X-0 + 0.200 mL 0.1% formic acid	0	125
Blank	0.800 mL ACN + 0.200 mL 0.1% formic acid	0	0

VIII. SAMPLE PREPARATION

Sample Extraction:

1. For each sample and QC sample:
   1.1. Pre-weigh 1.9-2.1 g of NaCl into disposable plastic tubes or weigh boats. Set aside for Step 4.
   1.2. Remove plunger from a disposable 10-mL syringe and set aside. Attached a 25-mm diameter/0.45-µm glass fiber filter to the syringe. Set aside for Step 7.
2. Accurately weigh 9.6 – 10.4 g of soil into a 50-mL conical-bottom polypropylene tube. Record mass to ±0.0001 g.
3. Add 0.150 mL 40X Surrogate Stock (d_5-bromadiolone).
4. Add 12.0 mL ACN, followed by 2 g NaCl. Cap securely and vortex mix 4-5s.
5. Shake 15 minutes on a flatbed shaker at approximately 300 CPM.
6. Centrifuge 2 minutes at 3500 RCF to clarify the supernatant.
7. Hold the filter syringe (prepared in Section 1.2) over a 15-mL polypropylene tube and decant the supernatant into the syringe barrel. Reinstall the syringe plunger and filter the remaining solution into the 15-mL tube.
8. In a separate 15-mL polypropylene tube, combine 5.0 mL filtered extract with 2.5 mL 0.1% formic acid.
9. SPE: Strata-X: 60 mg/3 mL:
   9.1. Condition: 1 mL ACN, followed by 1 mL 0.1% formic acid. Do not dry sorbent.
   9.2. Load: 3.0 mL of diluted extract from Step 8 into SPE barrel. Do not dry sorbent.
   9.3. Wash: 1 mL Wash Solution. Dry sorbent.
   9.4. Elute: 3 mL acetone into a 15-mL polypropylene tube. Dry sorbent.
10. Evaporate eluate to dryness with a gentle flow of nitrogen gas in a 50-60°C nitrogen evaporator.
11. Add 0.800 mL ACN and vortex mix 4-5 s.
12. Add 0.200 mL 0.1% formic acid, vortex mix 4-5 s, and transfer to an amber autosampler vial for analysis.

IX. ANALYSIS PROCEDURE

Repeatedly inject 10 µL of Standard 6 to determine suitability for analysis. Inject 10 µL of each standard and sample and record the peak area response ratio (bromadiolone/d_5-bromadiolone).

X. SYSTEM SUITABILITY

System suitability is demonstrated when the percent relative standard deviation (% RSD) of the peak area response ratio (bromadiolone/d_5-bromadiolone) is ≤ 2.0 % for five consecutive injections of Standard 6.

XI. TYPICAL LCMS CONDITIONS

Configure the LCMS with the following conditions. An Agilent 1290 UPLC with 6470 Triple Quadrupole MS/MS and Agilent Jet Stream ESI source were used to validate the method. Adjust acquisition parameters as necessary when using LCMS instruments from different manufacturers.

UPLC	Agilent 1290 Series Liquid Chromatograph
Column	Waters Xbridge BEH C18, 2.5 µm, 2.1 X 50 mm (or equivalent)
Column temperature	45 °C
Mobile phase A	0.1% formic acid in water
Mobile phase B	0.1% formic acid in acetonitrile
Flow rate	0.800 mL/min *
Injection volume	10 µL
Run time	3.00 min

Time (min)	% A	% B
0.00	60 %	40 %
0.50	60 %	40 %
2.00	0 %	100 %
2.50	0 %	100 %
2.51	60 %	40 %
3.00	60%	40 %

* Heat column to 45 °C prior to initiating column flow to prevent overpressure (600 bar)

Detector	Agilent 6470 Triple Quadrupole Mass Spectrometer (MS/MS)		
Ion source	AJS ESI	Sheath gas	250 °C
Gas temp	300 °C	Sheath gas	7 L/min
Gas flow	5 L/min	Capillary	-4000 V
Nebulizer	45 psi	Nozzle	0 V

Time Segment	Start (min)	End (min)	Type	Diverter	Delta EMV	Polarity	Data Stored
1	0.0	1.0	MS2 scan	To waste	0 V	Negative	No
2	1.0	2.0	MRM	To MS	-200 V	Negative	Yes
3	2.0	3.0	MS2 scan	To waste	0 V	Positive	No

MRM Transitions:

Analyte	Precursor Ion (m/z)	Product Ion (m/z) *	Fragmentor (V)	Dwell (ms)	Collision Energy (V)	Cell Accelerator (V)
Bromadiolone	525.1	**250.0**	190	30	40	7
	525.1	180.9	190	30	40	7
d_5-Bromadiolone	529.9	**254.9**	212	30	40	7

* Quantifier transitions are **bolded**.

Operating conditions may be adjusted to obtain optimum response and reproducibility. The retention time for both bromadiolone and d_5-bromadiolone are approximately 1.5 minutes

XII. DATA ANALYSIS AND CALCULATIONS

Record the peak area response ratio ($\frac{bromadiolone}{d_5 bromadiolone}$) for all injections. Using the data analysis software, generate a calibration curve using a quadratic regression equation, weighted 1/x and ignoring the origin, of relative peak area responses versus relative concentrations of the standards.

Calculate the bromadiolone residue "R" in units of µg bromadiolone per gram of soil using the following equation:

$$R = \frac{(X)(6)(V)}{(Wt)(1000\frac{ng}{\mu g})}$$

Where "X" is the observed analytical concentration of bromadiolone in units of ng/mL determined from the calibration curve, "6" is the dilution factor ($\frac{12\ mL\ x\ 7.5\ mL}{5\ mL\ x\ 3\ mL} = 6$), "V" is the final sample volume (1.0 mL), and "Wt" is the soil weight in grams.

The presence of bromadiolone is confirmed if the following three criteria are met:

(A) The chromatographic retention time of bromadiolone in the sample is within ±2% of the average bromadiolone retention time observed for the calibration standards.

(B) The recovery of d_5-bromadiolone surrogate analyte is ≥ 20% when compared to the average d_5-bromadiolone peak area response observed in the calibration standards.

(C) The peak area response ratio of the qualifier transition (525.1 → 180.9) divided by the quantifier transition (525.1 → 250.0) is ±20% of the average ratio determined for the calibration standards.

EXAMPLE CALCULATIONS

A soil sample weighing 9.8325 g produced a bromadiolone peak at 1.490 minutes with a response of 3577 units for the quantifier transition (525.1 → 250.0 m/z) and 909.6 units for the qualifier transition (525.1 → 180.9 m/z). The d_5-bromadiolone peak in the sample eluted at 1.502 minutes with a peak area response of 15559 units for the 529.9 → 254.9 m/z transition. The analytical concentration of d_5-bromadiolone in the sample (and all standards) was 116 ng/mL.

A seven-level standard curve was used to quantify bromadiolone in the sample. For the standards, the average bromadiolone retention time was 1.488 minutes, average bromadiolone qualifier ratio ($\frac{525.1 \rightarrow 180.9\ m/z}{525.1 \rightarrow 250.0\ m/z}$) was 0.2564, and average d_5-bromadiolone response for the 529.9 → 254.9 m/z transition was 22358 area units.

The seven-level standard curve was fit to a quadratic regression equation, weighted 1/x, and ignoring the origin. The general formula for a quadratic equation is:

$$y = ax^2 + bx + c$$

The coefficients determined by the data analysis software were: a = -0.017141, b = 1.5760, and c = -0.001253. The quadratic regression equation is therefore expressed as:

$$y = -0.017141x^2 + 1.5760x - 0.001253$$

Where "y" is the peak area response ratio ($\frac{bromadiolone\ peak\ area}{d_5 bromadiolone\ peak\ area}$) and "$x$" is the concentration ratio ($\frac{bromadiolone\ Conc.\ (\frac{ng}{mL})}{d_5 bromadiolone\ Conc.(\frac{ng}{mL})}$). The quadratic formula is used to determine the relative concentration (x):

$$x = \frac{-b \pm \sqrt{b^2 - 4a(c-y)}}{2a}$$

$$x = \frac{-1.5760 \pm \sqrt{1.5760^2 - (4)(-0.017141)(-0.001253 - \frac{3577}{15559})}}{(2)(-0.017141)}$$

$$x = 0.14690$$

The relative concentration (x) is then multiplied by the surrogate analyte concentration to calculate the analytical concentration of bromadiolone:

$$(0.14690)\left(116\frac{ng}{mL}\ d_5 bromadiolone\right) = 17.04\frac{ng}{mL} bromadiolone$$

The concentration of bromadiolone in soil is calculated by accounting for the dilution factor (6), final sample volume (1.0 mL), sample weight (10.1634 g), and converting ng to µg.

$$\frac{(17.04\frac{ng}{mL})(6)(1.0\ mL)}{(9.8325\ g)(1000\frac{ng}{\mu g})} = 0.0104\ \frac{\mu g}{g}\ bromadiolone$$

The presence of bromadiolone is confirmed because:

(A) Plus or minus 2% of the average retention time of the standards (1.488 minutes) equates to an acceptance window of 1.458 to 1.518 min. The sample retention time of 1.490 is within range.

(B) The d_5-bromadiolone surrogate analyte recovery is calculated by dividing the surrogate analyte response in the sample by the average surrogate analyte response of the standards:

$$\left(\frac{15559}{22358}\right)(100\ \%) = 69.6\ \%$$

The sample surrogate recovery of 69.6 % is greater than or equal to 20 %. Surrogate analyte recovery is accepted.

(C) The qualifier transition ratio observed in the sample is divide by the qualifier transition ratio of the calibration standards.

$$\frac{(909.6/3577)}{0.2564} \times 100\% = 99.2\%$$

The qualifier ratio percent match of 99.2 % is within the 80% - 120 % acceptance range.

XIII. METHOD VALIDATION

Note: All Class A volumetric glassware and beakers used to prepare standard solutions should be pre-cleaned prior to use. Factory-new amber glass vials and caps do not need to be pre-cleaned. Pre-cleaning is accomplished by:

1. Rinse each piece of glassware three times with 2-3 mL of ethyl acetate, discarding each wash to hazardous waste.
2. Rinse each piece of glassware two times with 2-3 mL of ultra-pure deionized water.
3. Invert each piece of glassware in a metal rack and dry in an oven heated to 100-110°C for approximately two hours or until dry.
4. Allow the glassware to cool to room temperature prior to use.

Concentrated Bromadiolone QC Stock: Accurately weigh approximately 10 mg of bromadiolone reference standard and quantitatively transfer to a 10-mL Class A volumetric flask. Dissolve in approximately 8 mL ethyl acetate, sonicate 1 minute (or until all solids are dissolved), and then bring to volume with ethyl acetate. Mix thoroughly and transfer ~ 8 mL to an amber glass vial with PTFE-lined cap. Dispose of excess solution to hazardous waste. The concentration of bromadiolone will be approximately 1000 µg/mL. Store at 4°C.

QC High Stock: Accurately transfer 0.250 mL of Concentrated Bromadiolone QC Stock to a 25-mL Class-A volumetric flask. Remove ethyl acetate with a gentle flow of nitrogen gas in a 50-60°C evaporator (1-2 minutes). Add ~20 mL ACN, cap securely, and sonicate 5 minutes. Allow to the flask to equilibrate at room temperature for at least 5 minutes before diluting to volume with ACN. Mix thoroughly and transfer ~8 mL to one or more amber glass vials with PTFE-lined caps. Dispose of excess solution to hazardous waste. The concentration of bromadiolone will be approximately 10 µg/mL, or 10,000 ng/mL. Store at 4°C.

QC Mid Stock: Combine 2.50 mL of QC High Stock with 2.50 mL ACN in an amber 8-mL glass vial with PTFE-lined cap. Mix thoroughly and store at 4°C. The concentration of bromadiolone will be approximately 5000 ng/mL.

QC Low Stock: Combine 0.500 mL of QC High Stock with 4.50 mL ACN in an amber 8-mL glass vial with PTFE-lined cap. Mix thoroughly and store at 4°C. The concentration of bromadiolone will be approximately 1000 ng/mL.

QC 1.25X Stock in ACN: Combine 0.100 mL of QC Mid Stock with 0.100 mL of 40X Surrogate Stock and 3.00 mL ACN in an amber 8-mL glass vial with PTFE-lined cap. Mix thoroughly and store at 4°C. The concentration of both bromadiolone and the d_5-bromadiolone surrogate will be approximately 156 ng/mL.

Bromadiolone QC Standard: Combine 0.800 mL of the QC 1.25X Stock with 0.200 mL 0.1% formic acid in an amber 2-mL autosampler vial. The concentration of both bromadiolone and the d_5-bromadiolone surrogate will be approximately 125 ng/mL. Mix thoroughly and store at 4°C.

Fortification of Controls: Accurately weigh 9.6 – 10.4 g control soil into 50-mL conical-bottom polypropylene tubes. Record mass to ±0.0001 g. The table below indicates the volumes of QC bromadiolone stocks to be added to result in soil concentrations of approximately 0.01 and 0.1 µg/g:

Fortification Level	QC Low Stock	QC High Stock	Approximate Bromadiolone Concentration (µg/g)
Control	-	-	0.000
Low	0.100 mL	-	0.010
High	-	0.100 mL	0.10

Response Linearity:

Two bromadiolone (BRM) stocks were used to prepare two 7-level calibration standard curves ranging in concentration from 2.4 to 244 ng/mL. Each standard also contained 131 ng/mL of the surrogate analyte d_5-bromadiolone (d_5-BRM). The peak area responses for BRM and d_5-BRM were acquired from duplicate injections of each standard.

Matrix Interference:

Seven control soil samples were fortified with only surrogate analyte (d_5-bromadiolone), and then extracted and analyzed.

Results: No significant interferences were observed in the control soil samples.

Detection Limit (DL):

The Detection Limit (DL) was estimated from the baseline noise observed in the control soil samples and the peak heights observed in soil fortified with 0.01 µg/g bromadiolone. The estimated DL is defined as the concentration of bromadiolone required to generate a signal equal to 3X the average baseline noise (measured peak-to-peak) observed in the control soil.

Result: The estimated detection limit for bromadiolone in soil was 0.000063 µg/g.

Quantitation Limit (QL):

The Quantitation Limit (QL) was estimated from the baseline noise observed in the control soil samples and the peak heights observed in soil fortified with 0.01 µg/g bromadiolone. The estimated QL is defined as the concentration of bromadiolone required to generate a signal equal to 10X the average baseline noise (measured peak-to-peak) observed in the control soil.

Result: The estimated quantitation limit for bromadiolone in soil was 0.000209 µg/g.