INDEPENDENT LABORATORY VALIDATION REPORT

Study Title

Independent Laboratory Validation of "Determination of Bromadiolone Residues in Soil"

DATA REQUIREMENTS
Residue Chemistry Test Guidelines

U.S. EPA OCSPP 850.6100

SPONSOR

Liphatech, Inc.
3600 West Elm Street
Milwaukee, Wisconsin 53209
U.S.A.

STUDY COMPLETION DATE

11 June 2020

PERFORMING LABORATORY

Eurofins EAG Agroscience, LLC
7200 East ABC Lane
Columbia, Missouri 65202
U.S.A.

TABLE OF COMMONLY USED ABBREVIATIONS

ºC	degrees centigrade (Celsius)
aq.	Aqueous
CAS	Chemical Abstracts Service
CE	Collision Energy
CFR	Code of Federal Regulations
EPA	Environmental Protection Agency
FIFRA	Federal Insecticide, Fungicide, and Rodenticide Act
Fort.	Fortification
g	gram(s)
ID	Identification
LC-MS/MS	Liquid Chromatography w/ tandem Mass Spectrometry
LOD	Limit of Detection
LOQ	Limit of Quantitation
L	liter
min	minute
mg	milligram
mL	milliliter
mm	millimeter
mol	mole
MRM	multiple reaction monitoring
msec	millisecond
µg	microgram
µL	microliter
µm	micrometer
m/z	mass/charge ratio
n, No.	Number
NA	Not Applicable
ng	nanograms
OCSPP	Office of Chemical Safety and Pollution Prevention
ppm	parts per million (e.g. mg/kg)
Rec.	Recovery
RPM	Revolutions per Minute
RSD	Relative Standard Deviation
Samp	Sample
SOP	Standard Operating Procedure
Std. Dev.	Standard Deviation
UPLC	Ultra Performance Liquid Chromatography
Vol/v	Volume

2.0 INTRODUCTION

The purpose of this study was to demonstrate the method (*1*) accuracy, precision, ruggedness, linearity, specificity, matrix effects, and limits of detection and quantitation as well as demonstrate its suitability for the determination of bromadiolone in soil following the method (*1*) as written.

This study was designed to satisfy guideline requirements described in U.S. EPA OCSPP 850.6100 (*2*). This study was conducted in compliance with EPA FIFRA Good Laboratory Practice Standards, 40 CFR Part 160 (*3*).

The method was suitable for the extraction of bromadiolone in soil samples fortified at the required LOQ (0.0100 ppm) and at 10×LOQ (0.100 ppm). The homogenized sample was extracted with acetonitrile and sodium chloride, shaken, centrifuged, and filtered. Extracts underwent solid phase extraction (SPE) clean-up and were eluted from the SPE with acetone. Samples were then evaporated to dryness, reconstituted with acetonitrile and 0.1% formic acid (aq), and submitted for LC-MS/MS analysis. The procedure performed by Eurofins EAG is provided in Section 3.10, and the full method is presented in APPENDIX I.

3.0 EXPERIMENTAL DETAILS

3.1 Test Substance

The test substance for this study was bromadiolone.

3.2 Analytical Reference Substance

The test substance mentioned in the previous section also served as an analytical reference substance. Information regarding the reference substance is summarized below.

Bromadiolone

Common Name:	Bromadiolone
Chemical Name:	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 No.:	28772-56-7
Molecular Weight:	527.41 g/mol
Molecular Formula:	$C_{30}H_{23}BrO_4$
Source:	Liphatech
Lot Number:	229001
Purity:	99.0%
Do Not Use Beyond Date:	13 March 2021
Storage:	Room Temperature

3.3 Internal Standard

Information regarding the internal standard is summarized below.

Bromadiolone Internal Standard

Common Name:	Bromadiolone-d_5 (phenyl-d_5)
Chemical Name:	3-[3-(4'-Bromo-1,1'-biphenyl-4-yl)-3-hydroxy-1-phenylpropyl-4-hydroxycoumarin
CAS No.:	Not applicable
Molecular Weight:	532.44 g/mol
Molecular Formula:	$C_{30}H_{18}D_5BrO_4$
Source:	C/D/N Isotopes Inc.
Lot Number:	AB-126
Purity:	95%
Expiration Date:	07 February 2022
Storage:	Room Temperature

The test substance and internal standard were supplied by the Sponsor. Information pertaining to the characterization and stability of the test substance is archived by the Sponsor. Certificates of Analysis are included in APPENDIX II.

3.4 Test System

The test system evaluated in this study was soil. This matrix was chosen as representative of the matrix for which the method was designed.

The control soil sample was obtained from, homogenized by (using a 2 mm sieve), and characterized by AGVISE Laboratories located in Northwood, North Dakota. The details of the characterization results are as follows:

Sample ID	Sand (%)	Silt (%)	Clay (%)	USDA Textural Class	% Moisture as Received	Organic Matter[a]	pH[b]
MSL-PF	59	20	21	Sandy Clay Loam	15.2	3.8	6.6

[a] Walkley-Black method
[b] pH in 1:1 soil:water ratio

The AGVISE Soil Characterization Report is presented in APPENDIX III.

3.5 Sample Receipt and Storage

The control soil sample was received ambient on February 21, 2019 at the Eurofins laboratory located at Discovery Ridge in Columbia, Missouri, stored overnight, and couriered to the Eurofins EAG Agroscience, LLC location the next day. The sample was received ambient and unopened at Eurofins EAG on February 22, 2019 where it was assigned a control identification number and logged in according to Eurofins SOPs. Except for the periods when the sample was first acquired and when it was removed for analysis, the sample was stored under frozen conditions (approximately -20 °C).

3.6 Sample Identification

All samples were assigned a unique sample identification number beginning with the five-digit Eurofins study number (88377) followed by consecutive number designations that were assigned as samples were prepared. For example, the first sample (a reagent blank) was assigned the unique identification of 88377-001, the second sample (a control) 88377-002, and so on.

3.7 Equipment

Equipment used throughout the course of the study (including balances, pipettors and various glassware) was the same or of similar technical quality as that specified in the analytical method. Equipment which varied from that listed in the method is presented in the following table.

Equipment Description	Product ID	Manufacturer
Analytical Balances	Model XPE205 Model ML3002E/03	Mettler Instrument Corp
Balance Weights	Model 7228-2W	Troemner
Centrifuge	Sorvall Legend XTR	ThermoFisher Scientific
Freezer (Walk-in)	Model E39RF112LE-25522-1	Masterbilt
Nitrogen Evaporator	N-EVAP Model 112	Organomation, Inc.
Pipettes	Positive displacement Model M1000, 100-1000 µL capacity Positive displacement Model M250, 50-250 µL capacity Positive displacement Model M100, 10-100 µL capacity	Gilson, Inc.
	Repeater Xstream, 10 µL-50 mL capacity	Eppendorf North America
Refrigerators	Model ESI 13-9CR	Environmental Specialties, Inc.
	Model 2R	Continental
	Model STM1R-IS	True
Sonicator	Bransonic 2210	Branson
Syringe Filters	0.45 µm	Whatman

3.8 Solvents, Chemicals, and Reagents

Solvents, chemicals and reagents used were of equivalent grade as those specified in the analytical method.

Solvent, Chemical, or Reagent	Grade or Purity	Supplier
Acetone	Optima	ThermoFisher Scientific
Acetonitrile	Optima	
Ethyl Acetate	Optima	
Formic Acid	Optima-LC/MS, 99.6%	
	99.4%	Sigma-Aldrich

Solvent, Chemical, or Reagent	Grade or Purity	Supplier
IPA*	HPLC, A.C.S.	ThermoFisher Scientific
Methanol	Optima, A.C.S.	
Sodium Chloride	Certified A.C.S., 99.8%	
Water	Optima-LC-MS and Optima	

* This solvent was not part of the method and was used as a needle wash.

3.9 Prepared Solutions

The preparation of stock, fortification, and calibration solutions is described in APPENDIX IV. Expirations of prepared reagent solutions were assigned per Eurofins SOP. Reagent solution stability was not determined. Volumes were adjusted accordingly for different quantities.

0.1% Formic Acid (aq) (Mobile Phase A and Extraction Diluent)

Using a 1000-mL graduated cylinder, added 1000 mL of water to a 1000-mL carboy. Using a pipet, added 1 mL of formic acid to same carboy and mixed. An expiration of at least one month from the date of preparation was assigned when stored at room temperature.

0.1% Formic Acid in Acetonitrile (Mobile Phase B)

Using a 5-mL serological pipet, added 4.0 mL into a previously unopened 4-L bottle of acetonitrile. An expiration of no more than one month from the date of preparation was assigned when stored at room temperature

0.1% Formic Acid in 15:85 Methanol:Water

Using a 100-mL graduated cylinder, added 15 mL of methanol to a 250-mL carboy. Using the same graduated cylinder, added 85 mL of water to the same carboy. Using a pipet, added 100 µL of formic acid to the same carboy and mixed. An expiration of no more than three months from the date of preparation was assigned when stored at room temperature

2:2:1 IPA:Methanol:Water (v:v:v; Strong Needle Wash)

Transferred 2000 mL of water to a 20-L carboy using a 2-L graduated cylinder. Added a 4-L bottle each of IPA and methanol to the same carboy and mixed. An expiration of no more than one year from the date of preparation was assigned when stored at room temperature.

1:1:2 Methanol:Acetonitrile:Water (v:v:v; Weak Needle Wash)

Added 8000 mL of water and 4000 mL each of acetonitrile and methanol to a 20-L carboy and mixed. An expiration of no more than one year from the date of preparation was assigned when stored at room temperature.

3.10 Sample Analysis

The analytical method independently validated in this study was entitled "Determination of Bromadiolone Residues in Soil." Below is an outline of the work performed by Eurofins EAG for the extraction and analysis of bromadiolone from soil. The complete text of the method and deviations (mentioned below) is included in APPENDIX I.

For the purposes of method validation, one reagent blank, two untreated control samples, and five fortifications each at the proposed LOQ (0.0100 µg/g) and 10×LOQ (0.100 µg/g) were prepared for soil. Recovery samples were prepared by adding appropriate aliquots of standard fortification solution to obtain concentrations of 0.0100 and 0.100 µg/g (ppm) bromadiolone as detailed in the table below.

Sample Description	Fortification Volume	Fortification Solution	Fortification Level
Control	NA	NA	NA
LOQ	100 µL	1000 ng/mL	0.0100 µg/g
10×LOQ	100 µL	10.0 µg/mL	0.100 µg/g
Reagent Blank	NA	NA	NA

Soil samples were processed as follows.

1. Weighed 10.00 g (± 0.4 g) of sample into a 50-mL BD Falcon conical bottom polypropylene tube with a screw cap. (Note: Reagent blank received no matrix.) Additionally, for each sample, pre-weighed 1.9-2.1 g of sodium chloride into disposable plastic tubes or weigh boats. Recorded exact weight and set aside for Step 3.
2. Fortified samples as shown above and added 150 µL of the internal standard at 5000 ng/mL to every sample.
3. Added 12.0 mL of acetonitrile followed by the ~2 g of sodium chloride weighed in Step 1 to each sample. A method deviation was prepared to document that the samples were not vortexed for 4-5 seconds after these additions as instructed in the method. See Section 4.5 for more information.
4. Shook samples on an Eberbach platform shaker for ~30 minutes on the high setting (~300 rpm). A method deviation was prepared to document that samples were shaken for 30 minutes instead of 15 minutes as described in the method. See Section 4.5 for more information.
5. Centrifuged samples at 3870 rpm (equivalent to 3500 × *g*) for ~2 minutes. Decanted the supernatant into a disposable 10-mL syringe with a 25 mm, 0.45 µm glass fiber filter attached. Filtered the extract into separate 15-mL BD Falcon conical bottom polypropylene centrifuge tubes.
6. In a separate 15-mL BD Falcon polypropylene conical bottom centrifuge tube, combined 5.0 mL of the filtered extract with 2.5 mL of 0.1% formic acid (aq). Stored remaining extracts as retains.

Solid Phase Extraction (SPE) Clean-Up

7. Conditioned SPE cartridges (Phenomenex Strata-X, 60 mg/3mL) with 1 mL of acetonitrile followed by 1 mL of 0.1% formic acid (aq). Did not allow cartridges to go dry. Did not collect eluates.
8. Loaded 3.0 mL of the diluted extract prepared in Step 6 into conditioned SPE cartridge. Allowed the load to go to waste. Did not allow the cartridge to go dry.
9. Washed the SPE cartridge with 1 mL of 15:85 (v:v) methanol:water in 0.1% formic acid. Did not collect eluate. Dried SPE cartridge using vacuum.
10. Placed 15-mL BD Falcon graduated conical bottom polypropylene tubes under each SPE cartridge. Eluted each cartridge with 3 mL of acetone. Dried SPE cartridge using vacuum.
11. Evaporated the samples to dryness under gentle stream of nitrogen at ~50-60 °C using an Organomation Inc. nitrogen evaporator.
12. Reconstituted samples with 0.80 mL of acetonitrile. Vortexed for 4-5 seconds.
13. Added 0.20 mL of 0.1% formic acid (aq) to each sample. Vortexed for 4-5 seconds.
14. Vialed a portion of each sample, appropriate calibration standards, and the QC standard into a 2-mL glass autosampler vial and submitted for analysis.

3.11 Instrumentation

All samples were analyzed by UPLC (Ultra-Performance Liquid Chromatography) tandem mass spectrometric (MS/MS) detection using the conditions listed below.

Operating Conditions

UPLC-MS/MS System:	Applied Biosystems/Sciex API 5500 Q-Trap with Waters Acquity Column Manager, Sample Manager, Sample Organizer, and Solvent Manager
Data Acquisition Software:	Applied BioSystems/Sciex Analyst, Version 1.6.2
Column:	Waters Xbridge C18, 50 mm × 2.1 mm, 2.5 µm particle size diameter
Column Temperature:	45 °C
Autosampler Temperature:	10 °C
Injection Volume:	1.0 µL
Flow Rate:	0.8 mL/min (No Split)
Mobile Phase A:	0.1% Formic Acid (aq)
Mobile Phase B:	0.1% Formic Acid in Acetonitrile

Conditions:	Time	%A	%B	Valco Diverter Valve:	Time	Eluate Flow
	Initial	60	40		0.0-0.5	Waste
	0.50	60	40		0.5-2.8	Mass Spec
	2.00	0	100		2.8-end	Waste
	2.50	0	100			
	2.51	60	40			
	3.00	60	40			
Total Run Time:	3.00 minutes					
Retention Time:	Bromadiolone - ~1.7 minutes					

Interface:	Turbo Spray		
Polarity:	Negative (-)		
Scan Type:	MRM (multiple reaction monitoring)		
Resolution:	Q1 – unit, Q3 – unit		
Ion Spray Voltage (IS):	-4500.00	Collision Gas (CAD):	"Medium"
Probe Temperature (TEM):	650.00	Curtain Gas (CUR):	40.00
Ion Source Gas 1 (GS1):	50.00	Entrance Potential (EP):	-10.00
Ion Source Gas 2 (GS2):	70.00	Cell Exit Potential (CXP):	-10.00

Analyte	**Ions Monitored (*m/z*)**	**Dwell Time (msec)**	**Collision Energy (CE)**	**Declustering Potential (DP)**
Bromadiolone	525.2 → 250.0 (primary)	40	-70	-120
	525.2 → 181.0 (alternate)	40	-60	-120
	527.1 → 250.1 (alternate)*	40	-50	-120
	527.1 → 181.0 (alternate)*	40	-47	-120
d5-Bromadiolone	530.2 → 255.0 (primary)	40	-50	-100
	530.2 → 181.0 (alternate)*	40	-47	-100
	532.2 → 255.0 (alternate)*	40	-51	-100
	532.2 → 181.0 (alternate)*	40	-48	-100

*Monitored but not reported

System Suitability

The 125 ng/mL (0.0750 µg/g equivalent) calibration standard was injected repeatedly until the percent relative standard deviation (%RSD) of the peak area response ratio is ≤ 2.0% for five consecutive injections. This was conducted prior to any sample analysis.

Calibration/Sample Analysis

A 14-point (7 concentrations) standard curve was prepared by injecting constant volumes of calibration standards in concentrations ranging from 0.00150 to 0.150 µg/g (equivalent). Constant volume injections were used for sample extracts as well. A calibration curve standard was injected at least every five to six sample injections. With each analysis set, a bromadiolone quality control (QC) standard with a concentration of 125 ng/mL (0.0750 µg/g equivalent) was also injected as a check of the reproducibility of the reference material weigh outs. This QC standard was prepared from a separate stock weigh-out than the calibration curve.

3.12 Calculations

ppm Equivalence

Calibration standard solutions were prepared in concentrations of ng/mL which were converted to ppm equivalents prior to analysis in order to generate a calibration curve in ppm equivalents. The following equations were used to calculate ppm equivalents.

$$ppm\ equivalents = Final\ Concentration\ (ng/mL) \times Final\ Conversion\ Factor$$

$$Final\ Conversion\ Factor =$$

$$\frac{Extract\ Volume\ (mL)}{Aliquot\ Volume\ 1\ (mL)} \times \frac{Intermediate\ Volume\ (mL)}{Aliquot\ Volume\ 2\ (mL)} \times \frac{Final\ Volume\ (mL)}{Sample\ Weight\ (g)} \times \frac{1\ \mu g}{1000\ g}$$

where:

Extract Volume (mL)	=	12.0 mL
Aliquot Volume 1 (mL)	=	5.0 mL
Intermediate Volume (mL)	=	7.5 mL
Aliquot Volume 2 (mL)	=	3.0 mL
Final Volume (mL)	=	1.0 mL
Sample Weight (g)	=	10.00 g
1/1000 µg/g	=	unit conversion from ng/mL to µg/g (ppm)

For example, for a calibrations solution with a concentration of 250 ng/mL, ppm equivalents were determined as follows:

$$Conversion\ Factor = \frac{12.0\ mL}{5.0\ mL} \times \frac{7.5\ mL}{3.0\ mL} \times \frac{1.0\ mL}{10.00\ g} \times \frac{1\ \mu g}{1000\ g} = 0.0006\ \mu g/g\ (ppm)$$

$$ppm\ Equivalents = 250\ ng/mL \times 0.0006\ \mu g/g = 0.150\ \mu g/g\ (ppm)$$

Therefore, the following calibration standard values entered into Analyst® were:

Calibration Standard Concentration of Bromadiolone (ng/mL)	ppm Equivalence of Bromadiolone (µg/g)
250	0.150
125	0.0750
50.0	0.0300
25.0	0.0150
12.5	0.00750
5.00	0.00300
2.50	0.00150

Residue (ppm) and Percent Recovery

Calculations for instrumental analysis were conducted using validated software application Applied BioSystems/MDS Sciex Analyst®, version 1.6.2, to create a standard curve based on linear regression. The regression functions were used to calculate a best-fit line and to determine concentrations of the analyte found during sample analysis from the calculated best-fit line. A weighted linear curve (1/x) was used. The resulting equation defining the standard curve is shown below:

$$y = mx + b$$

where:

x = ppm found for peak of interest
y = peak area ratio (analyte peak area/internal standard peak area)
b = y-intercept
m = slope of the standard curve

The calculation performed by the instrument may be checked manually* by applying the following equation:

$$x = \frac{y - b}{m}$$

* Note that the curve constants (slope and y-intercept) were calculated by the computer system to a higher number of significant digits than is reported in the raw data; therefore, hand calculations based on these values may contain small rounding differences.

Percent recoveries were also calculated by Analyst® and may be checked manually by applying the following equation:

$$\%\ Recovery = \frac{ppm\ found\ in\ fortified\ sample}{ppm\ added} \times 100$$

For example, the amount of bromadiolone residue found in soil sample 88377-004 fortified with bromadiolone at 0.0100 ppm (Set 001) and the percent recovery were determined as follows:

Peak Area	=	20625
IS Peak Area	=	1882228
y-Intercept	=	0.00006621078
Slope	=	1.05247
ppm Added	=	0.0100

$$peak\ area\ ratio = \frac{20625}{1882228} = 0.0109580$$

$$ppm\ Found = \frac{0.0109580 - 0.00006621078}{1.05247} = 0.0103488, rounded\ to\ 0.0103\ ppm$$

$$\%\ Recovery = \frac{0.0103488\ ppm}{0.0100\ ppm} \times 100 = 103\%$$

APPENDIX I

Method and Method Deviations

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

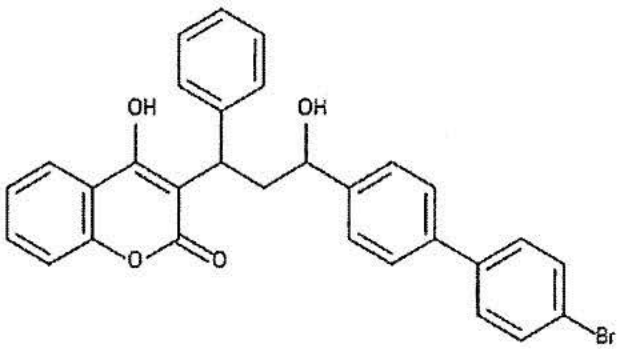
DETERMINATION OF BROMADIOLONE RESIDUES IN SOIL

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.

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

Wildlife Services NWRC National Wildlife Research Center	United States Department of Agriculture Animal Plant Health Inspection Service Wildlife Services National Wildlife Research Center Chemistry Laboratory Unit	Number:	Date effective:
		184A	08-05-2019
Analytical Method		Supersedes: NA	Page: 5 of 18

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).

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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)

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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:

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

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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.

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

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.

Bias and Repeatability:

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

XIV. STANDARD OPERATING PROCEDURES (SOPs)

The following SOP's were applicable at the time of method validation:

AD 011	Data Recording and Error Correction
CH 001	Receipt, Storage, Use, and Disposal of Chemicals
CH 002	Calculations, Significant Figures, and Rounding
CH 003	Analytical Chemistry Chain-of-Custody Procedures for samples, Raw Data, and QC matrices
CH 004	Chemical Reference Standards

CH 005	Laboratory Reagents
CH 006	Reference Standard Solutions
CH 007	Analytical Services Project Notebooks
CH 008	Pipettes, Repipettes, and Repeater Pipetters
CH 009	Analytical Method Format
CH 010	Quality Control Sample Preparations and Analysis
CH/CO 001	Chemical Accountability and Tracking System
CH/CO 007	Analytical Method Validation
HS 001	Chemical Spills, Exposures, and Emergencies
HS 002	Shipment of Dangerous Goods
HS 003	Respiratory Protection
HS 004	Personal Protective Equipment
HS 005	Control of Hazardous Energy
HS 006	Hazardous Waste, Collection, Storage, and Removal
HS 008	Hazard Communication
HS 009	Handling Gas Cylinders
IE 001	pH Meter and Electrodes
IE 002	Analytical Balances
IE 003	Class S Weight Set and QC Check Weights
IE 005	Laboratory Fume Hoods
IE 007	Analytical Columns
IE 008	Ultrasonic Cleaners
IE 009	Maintenance and Use of Centrifuges and Mechanical Shakers
IE 010	Thermometers
IE 011	Analytical Evaporators (N-Evaps)
IE 013	Security for Automated Data Collection Systems
IE 014	Analytical Instrument Documentation
IE 018	Refrigerators and Freezers
IE 026	Operation of HOBO Temperature or HOBO Temperature and Relative Humidity Data Logger

XV. REFERENCES

Analytical Chemistry Project Notebook: AC-161, pp. 195-200.

Analytical Chemistry Project Notebook: QC-34, pp. 99-102.

Analytical Services Project Number: Invoice 19-019 (QA-3078).

Preparation of Standard Solutions

All standard solutions prepared in this section were stored refrigerated at approximately 2 to 8 °C when not in use. Stock and intermediate/fortification solutions were assigned expiration dates of six months after the date of preparation. Because the solvent used to prepare the calibration solutions would expire earlier, calibration solutions were given the same expiration date as the solvent. None of the standard solutions were used beyond the expiration date.

Stock Standard Solutions

Approximately 10 mg (corrected for purity) of bromadiolone analytical standard were accurately weighed, quantitatively transferred to a 10-mL volumetric flask, and brought to volume with ethyl acetate. The resulting concentration of the bromadiolone stock standard solution was 1000 µg/mL.

Approximately 1.0 mg (corrected for purity) of bromadiolone-d5 analytical standard were accurately weighed, quantitatively transferred to a 5-mL volumetric flask, and brought to volume with ethyl acetate. The resulting concentration of the bromadiolone-d5 internal standard stock solution was 200 µg/mL.

Fortification/Intermediate Standard Solutions

The following concentrations of fortification/intermediate standard solutions containing bromadiolone were prepared.

Final Concentration (µg/mL)	Concentration of Parent Solution (µg/mL)	Aliquot Volume (mL)	Dilution Volume[a] (mL)
10.0	1000	0.250[b]	25.0
5.00	10.0	2.500	5.00
2.00	10.0	1.000	5.00
1.00	10.0	0.500	5.00
0.500	10.0	0.250	5.00
0.200	1.00	1.000	5.00
0.100	1.00	0.500	5.00

[a] The solvent was acetonitrile.
[b] After transferring an aliquot of the parent stock solution to a 25-mL volumetric flask, the ethyl acetate was removed from the aliquot via a gentle flow of nitrogen. After blowing to dryness, approximately 20 mL of acetonitrile were added to the flask and sonicated for at least five minutes. The flask was allowed to equilibrate for at least another five minutes before bringing to volume.

The following concentration of standard solution containing bromadiolone-d5 internal standard was prepared.

Final Concentration (ng/mL)	Concentration of Parent Solution (µg/mL)	Aliquot Volume (mL)	Dilution Volume[a] (mL)
5000	200	0.200[b]	8.0

[a] The solvent was acetonitrile.
[b] After transferring an aliquot of the parent stock solution to a 4-dram amber bottle, the ethyl acetate was removed from the aliquot via a gentle flow of nitrogen. After blowing to dryness, 8.00 mL of acetonitrile were added and the bottle was closed with a PTFE-lined cap, sonicated for five minutes, and vortexed for five to six seconds to mix.

The following concentrations of standard solutions containing both bromadiolone and bromadiolone-d5 internal standard were prepared.

Final Concentration (ng/mL)	Concentration of Parent Solution (µg/mL)	Aliquot Volume (mL)	Dilution Volume[a] (mL)
313	10.0	0.100	3.200
156	5.00	0.100	3.200
62.5	2.00	0.100	3.200
31.3	1.00	0.100	3.200
15.6	0.500	0.100	3.200
6.25	0.200	0.100	3.200
3.13	0.100	0.100	3.200

[a] After transferring an aliquot of the parent solution to an 8-dram amber glass vial with a PTFE-lined cap, 0.100 mL of the 5000-ng/mL bromadioline-d5 internal standard solution were added to the vial followed by 3.00 mL of acetonitrile. The resulting final concentration of bromadiolone-d5 internal standard was 156 ng/mL.

Calibration Standard Solutions

The following concentrations of calibration standard solutions containing both bromadiolone and bromadiolone-d5 internal standard were prepared.

Final Concentration of Bromadiolone		Concentration of Parent Solution (ng/mL)	Aliquot Volume (mL)	Dilution Volume[b] (mL)
ng/mL	ppm Equivalent[a]			
250	0.150	313	0.800	1.000
125	0.0750	156	0.800	1.000
50.0	0.0300	62.5	0.800	1.000
25.0	0.0150	31.3	0.800	1.000
12.5	0.00750	15.6	0.800	1.000
5.00	0.00300	6.25	0.800	1.000
2.50	0.00150	3.13	0.800	1.000

[a] See Section 3.11 for calculations showing conversion from ng/mL to ppm equivalents.
[b] The solvent was 0.1% formic acid (aq). The resulting final concentration of bromadiolone-d5 internal standard was 125 ng/mL.