

INDEPENDENT LABORATORY VALIDATION REPORT

STUDY TITLE

Independent Laboratory Validation of "An Analytical Method for the Determination of Residues of BCS-CS55621, and Its Metabolites BCS-CY96288, BCS-CU97237, BCS-DA63612, BCS-CC26101, BCS-CZ38260, BCS-BP32808, and BCS-DH17585 in Soil and Sediment Using LC/MS/MS"

DATA REQUIREMENTS

EPA OCSPP 850.6100

2.0 MATERIALS

2.1 Analytical Reference Standard(s)

Analytical reference standards were provided by the Sponsor. Upon receipt, reference standards were logged according to Eurofins SOPs and placed in frozen storage (approximately -10 to -25 °C) according to their respective Certificates of Analysis.

BCS-CS55621

Synonym: Fluoxapiprolin

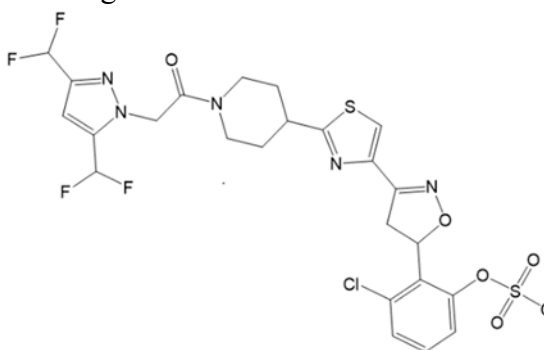
Chemical Name (IUPAC): 2-{3-[2-(1-{[3,5-bis(difluoromethyl)-1H-pyrazol-1-yl]acetyl} piperidin-4-yl)-1,3-thiazol-4-yl]-4,5-dihydro-1,2-oxazol-5-yl}-3-chlorophenylmethanesulfonate

CAS Number: 1360819-11-9

Molecular Formula: C₂₅H₂₄ClF₄N₅O₅S₂

Molecular Weight: 650.06 g/mol

Molecular Structure:



Receipt Date: 13 April 2021

Source: Bayer CropScience

Batch/Lot Number: K-2231

Purity: 98.2%

Expiration Date: 02-Jan-23

Storage: Freezer (~ -20 °C)

BCS-CY96288

Chemical Name:

[2-[3-[2-[1-[2-[3,5-bis(difluoromethyl)pyrazol-1-yl] acetyl]-4- hydroxyl-4-piperidyl]-4-yl)-4,5-dihydroisoxazol-5-yl]-3-chlorophenyl] methanesulfonate

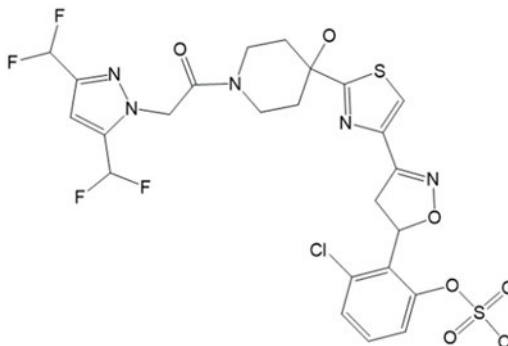
Molecular Formula:

C₂₅H₂₄ClF₄N₅O₆S₂

Molecular Weight:

666.06 g/mol

Molecular Structure:



Receipt Date:

13 April 2021

Source:

Bayer CropScience

Batch/Lot Number:

K-2232 / 1228201704

Purity:

97.4%

Expiration Date:

11-Nov-25

Storage:

Freezer (~ -20 °C)

BCS-CU97237

Chemical Name:

4-[4-(5-{2-chloro-6-[(methylsulfonyl)oxy]phenyl}-4,5-dihydro-1,2-oxazol-3-yl)-1,3-thiazol-2-yl]piperidinium chloride

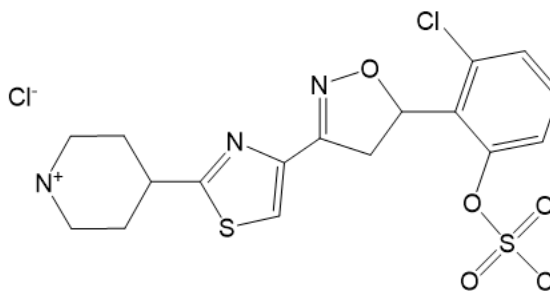
Molecular Formula:

C₁₈H₂₁ClN₃O₄S₂Cl

Molecular Weight:

478.41 g/mol

Molecular Structure:



Receipt Date:

13 April 2021

Source:

Bayer CropScience

Batch/Lot Number:

K-2261

Purity:

98.3%

Expiration Date:

17-Aug-25

Storage:

Freezer (~ -20 °C)

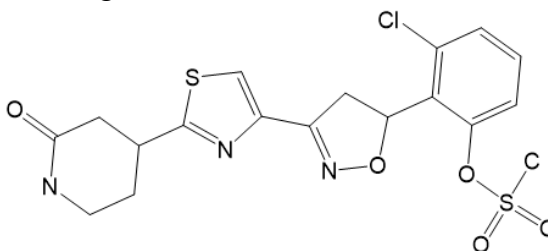
BCS-DA63612

Chemical Name: 3-chloro-2-{3-[2-(2-oxopiperidin-4-yl)-1,3-thiazol-4-yl]-4,5-dihydro-1,2-oxazol-5-yl}phenyl methanesulfonate

Molecular Formula: $C_{18}H_{18}ClN_3O_5S_2$

Molecular Weight: 455.94 g/mol

Molecular Structure:



Receipt Date: 13 April 2021

Source: Bayer CropScience

Batch/Lot Number: K-2279 / 0520201904

Purity: 92.7%

Expiration Date: 08-Apr-22

Storage: Freezer ($\sim -20^\circ\text{C}$)

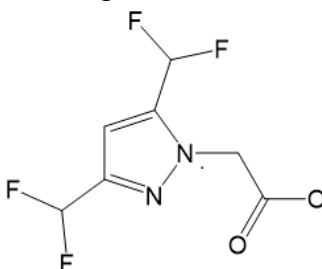
BCS-CC26101

Chemical Name: 2-[3,5-bis(difluoromethyl)pyrazol-1-yl]acetic acid

Molecular Formula: $C_7H_6F_4N_2O_2$

Molecular Weight: 226.13 g/mol

Molecular Structure:



Receipt Date: 13 April 2021

Source: Bayer CropScience

Batch/Lot Number: K-2234

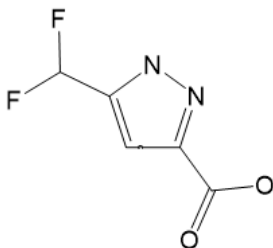
Purity: 98.5%

Expiration Date: 26-Aug-24

Storage: Freezer ($\sim -20^\circ\text{C}$)

BCS-CZ38260

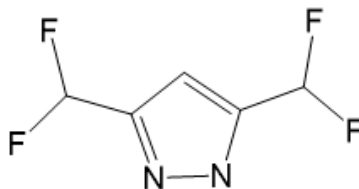
Chemical Name: 5-(difluoromethyl)-1H-pyrazole-3-carboxylic acid
Molecular Formula: $C_5H_4F_2N_2O_2$
Molecular Weight: 162.09 g/mol
Molecular Structure:



Receipt Date: 13 April 2021
Source: Bayer CropScience
Batch/Lot Number: K-2233 / 1228201705
Purity: 99.8%
Expiration Date: 02-Nov-25
Storage: Freezer ($\sim -20\text{ }^{\circ}\text{C}$)

BCS-BP32808

Chemical Name: 3,5-bis(difluoromethyl)-1H-pyrazole
Molecular Formula: $C_5H_4F_4N_2$
Molecular Weight: 168.09 g/mol
Molecular Structure:



Receipt Date: 13 April 2021
Source: Bayer CropScience
Batch/Lot Number: K-2301 / 0205202101
Purity: 95.3%
Expiration Date: 12-Mar-23
Storage: Freezer ($\sim -20\text{ }^{\circ}\text{C}$)

BCS-DH17585

Chemical Name:

Sodium 4-[5-(2-chloro-6-methylsulfonyloxy-phenyl)-4,5-dihydroisoxazol-3-yl]thiazole-2-carboxylate

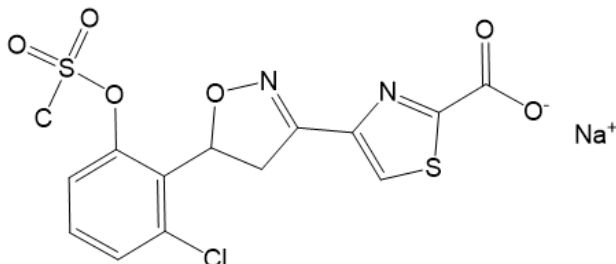
Molecular Formula:

$$\text{C}_{14}\text{H}_{10}\text{ClN}_2\text{O}_6\text{S}_2\text{Na}$$

Molecular Weight:

424.81 g/mol

Molecular Structure:



Receipt Date:

13 April 2021

Source:

Bayer CropScience

Batch/Lot Number:

K-2323

Purity:

90.8%

Expiration Date:

09-Nov-23

Storage:

Freezer (~ -20 °C)

BCS-CS55621-¹³C₃, ¹⁵N₃

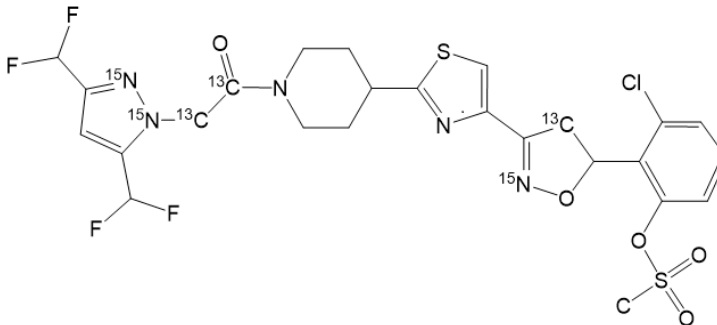
Molecular Formula:

$$^{13}\text{C}_3\text{C}_{22}\text{H}_{24}\text{ClF}_4^{15}\text{N}_3\text{N}_2\text{O}_5\text{S}_2$$

Molecular Weight:

656.02 g/mol

Molecular Structure:



Receipt Date:

13 April 2021

Source:

Bayer CropScience

Batch/Lot Number:

K-2246

Purity:

100%

Expiration Date:

05-Apr-27

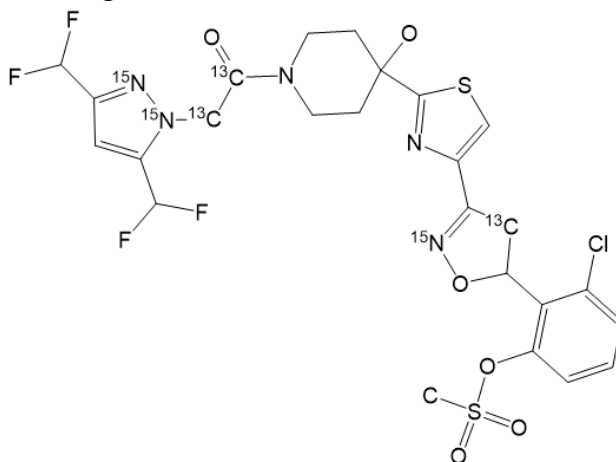
Storage:

Freezer (~ -20 °C)

BCS-CY96288-¹³C₃, ¹⁵N₃Molecular Formula: ¹³C₃C₂₂H₂₄ClF₄¹⁵N₃N₂O₆S₂

Molecular Weight: 672.02 g/mol

Molecular Structure:

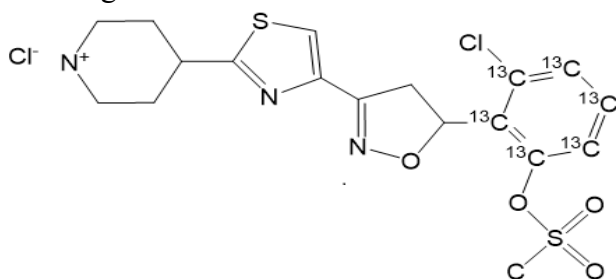


Receipt Date: 13 April 2021
Source: Bayer CropScience
Batch/Lot Number: K-2315
Purity: 98.9%
Expiration Date: 05-Dec-28
Storage: Freezer (~ -20 °C)

BCS-CU97237-¹³C₆Molecular Formula: ¹³C₆C₁₂H₂₁ClN₃O₄S₂·Cl

Molecular Weight: 484.37 g/mol

Molecular Structure:



Receipt Date: 13 April 2021
Source: Bayer CropScience
Batch/Lot Number: K-2317
Purity: 99.2%
Expiration Date: 05-Dec-28
Storage: Freezer (~ -20 °C)

BCS-DA63612-¹³C₆

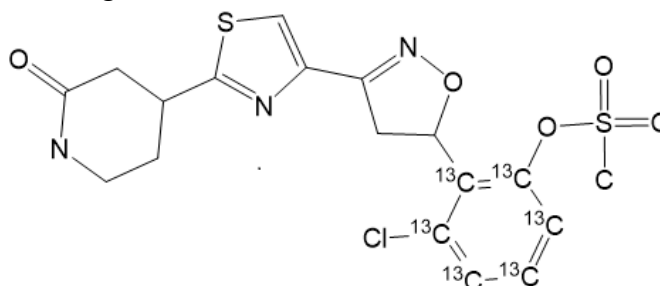
Molecular Formula:

¹³C₆C₁₂H₁₈ClN₃O₅S₂

Molecular Weight:

461.89 g/mol

Molecular Structure:



Receipt Date:

13 April 2021

Source:

Bayer CropScience

Batch/Lot Number:

K-2318

Purity:

99.3%

Expiration Date:

05-Dec-28

Storage:

Freezer (~ -20 °C)

BCS-CC26101-¹³C₂

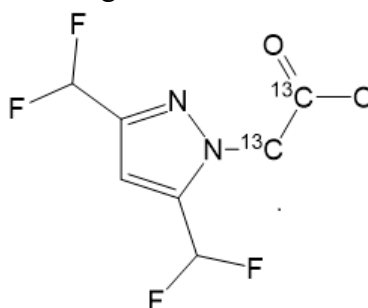
Molecular Formula:

¹³C₂C₅H₆F₄N₂O₂

Molecular Weight:

228.11 g/mol

Molecular Structure:



Receipt Date:

13 April 2021

Source:

Bayer CropScience

Batch/Lot Number:

K-2306

Purity:

100%

Expiration Date:

04-Sep-28

Storage:

Freezer (~ -20 °C)

BCS-CZ38260-¹⁵N₂

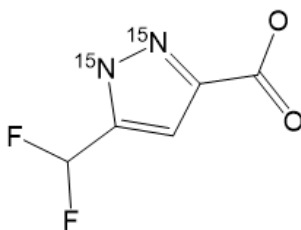
Molecular Formula:

 $C_5H_4F_2^{15}N_2O_2$

Molecular Weight:

164.08 g/mol

Molecular Structure:



Receipt Date:

13 April 2021

Source:

Bayer CropScience

Batch/Lot Number:

K-2316

Purity:

100%

Expiration Date:

05-Dec-28

Storage:

Freezer (~ -20 °C)

BCS-BP32808-¹⁵N₂

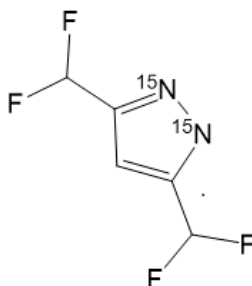
Molecular Formula:

 $C_5H_4F_4^{15}N_2$

Molecular Weight:

170.08 g/mol

Molecular Structure:



Receipt Date:

13 April 2021

Source:

Bayer CropScience

Batch/Lot Number:

K-2035 / 0907201802

Purity:

99.6%

Expiration Date:

16-Nov-24

Storage:

Freezer (~ -20 °C)

BCS-DH34157-¹³C₆

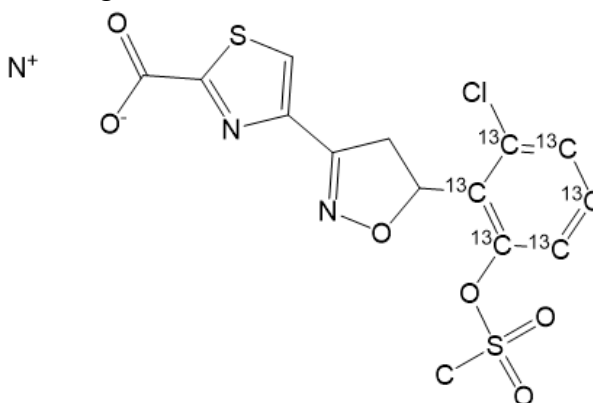
Molecular Formula:

¹³C₆C₈H₁₀ClN₂O₆S₂·H₄N

Molecular Weight:

425.82 g/mol

Molecular Structure:



Receipt Date:

13 April 2021

Source:

Bayer CropScience

Batch/Lot Number:

K-2332

Purity:

100%

Expiration Date:

12-Sep-29

Storage:

Freezer (~ -20 °C)

2.2 Preparation of Standard Solutions**2.2.1 Stock Standard Solutions**

All of the stock standard solutions prepared in this section were given an expiration date of approximately six months from the date of preparation and stored in amber glass bottles in the dark under freezer conditions (approximately -20 °C) when not in use. None of the standard solutions in this study were used beyond their expiration dates.

Stock standard solutions were prepared by quantitatively transferring a targeted amount (2.00 g, corrected for purity) of an analytical standard into a class A volumetric flask and diluting with either acetonitrile (ACN) or 80:20 ACN:water. The targeted concentration of each stock solution was 100 µg/mL; however, in some instances, the amount of standard available at the Eurofins testing facility was either less or slightly more than was the amount needed. In these instances, the total on-hand amount was used which resulted in final concentrations above or below the target concentration.

The stock standard solutions used in this study were prepared as follows.

Native Standard Solutions

Standard	Weight (g)	Diluent	Dilution Volume (mL)	Final Concentration (µg/mL)
BCS-CS55621	2.04	ACN	20	100
BCS-CY96288	2.06	ACN	20	100
BCS-CU97237	2.04	80:20 ACN:Water	20	100

Standard	Weight (g)	Diluent	Dilution Volume (mL)	Final Concentration (µg/mL)
BCS-DA63612	1.62	80:20 ACN:Water	20	75.1
BCS-CC26101	2.04	ACN	20	100
BCS-CZ38260	2.01	ACN	20	100
BCS-BP32808	2.10	ACN	20	100
BCS-DH17585	2.21	80:20 ACN:Water	20	100

Internal Standard Solutions

Standard	Weight (g)	Diluent	Dilution Volume (mL)	Final Concentration (µg/mL)
BCS-CS55621- ¹³ C ₃ , ¹⁵ N ₃	2.52	ACN	25	101
BCS-CY96288- ¹³ C ₃ , ¹⁵ N ₃	2.50	ACN	25	98.9
BCS-CU97237- ¹³ C ₆	2.42	80:20 ACN:Water	25	96.0
BCS-DA63612- ¹³ C ₆	2.48	80:20 ACN:Water	25	98.5
BCS-CC26101- ¹³ C ₂	2.53	ACN	25	101
BCS-CZ38260- ¹⁵ N ₂	2.40	ACN	25	96.0
BCS-BP32808- ¹⁵ N ₂	2.48	ACN	25	98.8
BCS-DH34157- ¹³ C ₆	2.47	80:20 ACN:Water	25	98.8

2.2.2 Intermediate/Fortification Standard Solutions

All of the intermediate/fortification standard solutions prepared in this section were given an expiration date of approximately four months from the date of preparation and stored in amber glass bottles in the dark under refrigerator conditions (approximately 2-8 °C) when not in use. None of the standard solutions in this study was used beyond assigned expiration dates.

Intermediate and fortification mixed-standard solutions were prepared for native and internal standards using class A volumetric pipettes and/or positive displacement pipettes to measure and transfer appropriate volumes of standard solution to class A volumetric flask before diluting to the final concentration as shown below.

Native Standard Solutions

Parent Solution	Concentration of Parent Solution	Aliquot Volume	Diluent	Dilution Volume (mL)	Final Concentration of Mixed Native Standard Solution
BCS-CS55621	100 µg/mL	1000 µL	80:20 ACN:Water	100	1000 ng/mL
BCS-CY96288	100 µg/mL	1000 µL			
BCS-CU97237	100 µg/mL	1000 µL			
BCS-DA63612	75.1 µg/mL	1332 µL			
BCS-CC26101	100 µg/mL	1000 µL			
BCS-CZ38260	100 µg/mL	1000 µL			
BCS-BP32808	100 µg/mL	1000 µL			
BCS-DH17585	100 µg/mL	1000 µL			
Mixed Native Standard	1000 ng/mL	12.5 mL		250	50.0 ng/mL

Internal Standard Solutions

Parent Solution	Concentration of Parent Solution	Aliquot Volume	Diluent	Dilution Volume (mL)	Final Concentration of Mixed Internal Standard Solution
BCS-CS55621- ¹³ C ₃ , ¹⁵ N ₃	101 µg/mL	990 µL	80:20 ACN:Water	100	1.00 µg/mL
BCS-CY96288- ¹³ C ₃ , ¹⁵ N ₃	98.9 µg/mL	1011 µL			
BCS-CU97237- ¹³ C ₆	96.0 µg/mL	1042 µL			
BCS-DA63612- ¹³ C ₆	98.5 µg/mL	1015 µL			
BCS-CC26101- ¹³ C ₂	101 µg/mL	990 µL			
BCS-CZ38260- ¹⁵ N ₂	96.0 µg/mL	1042 µL			
BCS-BP32808- ¹⁵ N ₂	98.8 µg/mL	1013 µL			
BCS-DH34157- ¹³ C ₆	98.8 µg/mL	1013 µL			
Mixed Internal Standard	1.00 µg/mL	10 mL			100 ng/mL

2.2.3 Calibration Standard Solutions

All of the standard solutions prepared in this section were given an expiration date of approximately three months from the date of preparation and stored in amber glass bottles in the dark under refrigerator conditions (approximately 2-8 °C) when not in use. None of the standard solutions in this study was used beyond their expiration dates.

Calibration standards were prepared using class A volumetric pipettes and/or positive-displacement pipettes to measure and transfer an aliquot each of mixed native standard solution into a Class A volumetric flask, then to measure and transfer 250 µL of the 1.00-µg/mL mixed internal standard solution into the same flask. Calibration standard solutions were diluted with 1:9 ACN:water to their final concentrations as shown below (native / internal standard).

Concentration of Parent Mixed Native Standard Solution (ng/mL)	Aliquot Volume (mL)	Dilution Volume (mL)	Final Concentration of Calibration Standard Solution (ng/mL)
1000	2.5	50	50.0 / 5.00
	2.0	50	40.0 / 5.00
	1.0	50	20.0 / 5.00
	0.5	50	10.0 / 5.00
50.0	4.0	50	4.00 / 5.00
	1.0	50	1.00 / 5.00
	0.50	50	0.500 / 5.00
	0.25	50	0.250 / 5.00

2.3 Equipment, Reagents, and Laboratory Solutions**2.3.1 Equipment**

Equipment used throughout the course of the study (including balances, pipettes, and various glassware) was the same or of similar technical quality as that specified in the analytical method. General laboratory supplies (such as graduated cylinders, volumetric flasks, bottles, vials, etc.) are not specified. Specific equipment used is presented in the following table.

Equipment Description	Product ID	Manufacturer
Analytical Balances	Model XPE205 Model ML3002E/03 Model MS12001L/03	Mettler Toledo
Centrifuge	AccuSpin Micro 17R	ThermoFisher Scientific
	Legend XTR	Sorvall
Freezer	Model 106.827598	Kenmore
	Model E39RF112LE-25522-1	Masterbilt
	Model T-49	True
	FSA-2D-S1	Victory
Hammermill	Various	Various
Microwave Extractor	Model MARS 6 230/60 (Model No. 910900)	CEM
Nitrogen Evaporator	Various	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 Positive displacement Model M25, 3-25 µL capacity	Gilson, Inc.
	Repeater Xstream, 10 µL-50 mL capacity	Eppendorf North America
Refrigerator	Model HR2-1S	Beverage-Air
	Model STM1R-1S	True
Refrigerator/Freezer Combo	LRF201WWW/0	Nor-lake Scientific
Vortex Mixer	M16715	ThermoFisher Scientific

2.3.2 Reagents

The reagents listed below were used during the course of this study and were of equivalent grade as those specified in the analytical method.

Reagent	Purity	Source
Acetic Acid	Glacial	Fisher
Acetonitrile	Optima	Fisher
Methanol	Optima	Fisher
Water	Optima	Fisher

2.3.3 Preparation of Reagent Solutions

The following reagent solutions were prepared for use in this study. Expirations of prepared reagent solutions were assigned per Eurofins SOP. Reagent solution stability was not determined. Volumes were adjusted as needed as long as ratios were maintained.

0.5% Acetic Acid in Water (Mobile Phase)

Prepared by adding 5 mL acetic acid to 1000 mL water. Assigned an expiration date of one month from the date of preparation and stored at room temperature.

0.5% Acetic Acid in Acetonitrile (Mobile Phase)

Prepared by adding 5 mL acetic acid to 1000 mL acetonitrile. Assigned an expiration date of one month from the date of preparation and stored at room temperature.

80:20 Acetonitrile:Water (v:v)

Prepared by mixing 800 mL of water with 3200 mL of acetonitrile. Assigned an expiration date of one year from the date of preparation and stored at room temperature.

4:1 Acetonitrile:Water (v:v)

Prepared by mixing 400 mL of acetonitrile with 100 mL of water. Assigned an expiration date of one year from the date of preparation and stored at room temperature.

1:9 Acetonitrile:Water (v:v)

Prepared by mixing 10 mL of acetonitrile with 90 mL of water. Assigned an expiration date of three months from the date of preparation and stored at room temperature.

1:1:2 Acetonitrile:Methanol:Water (v:v:v)

Prepared by mixing ~4000 mL methanol, ~4000 mL acetonitrile, and ~8000 mL water. Assigned an expiration date of one year from the date of preparation and stored at room temperature.

2.4 Test System

2.4.1 Sample Origin, Characterization, and Storage

The control soil sample used in this study was obtained from and characterized by AGVISE Laboratories located in Northwood, North Dakota.

Sand (%)	Silt (%)	Clay (%)	USDA Textural Class ^a	Bulk Density (g/cm ³)	Cation Exchange Capacity (meq/100 g)	Moisture Holding Capacity at 1/3 Bar (%)	Organic Matter ^b (%)	pH ^c
76	15	9	Sandy Loam	1.06	13.5	16.6	4.6	6.7

^a Hydrometer method

^b Walkley-Black method

^c pH in 1:1 soil:water ratio

The control soil sample was received ambient at Eurofins on June 01, 2021 via UPS. Upon receipt, the bulk sample was assigned a generic control identification number, logged in according to Eurofins SOPs, and transferred to a limited-access freezer (approximately -20 °C) where it remained until sample milling and/or analysis.

2.4.2 Sample Milling

The control soil sample was ground using a Hammermill. Dry ice was passed through the Hammermill to cool it before sample processing. The control sample was then ground in the presence of enough dry ice to keep the sample frozen. After grinding, the sample was placed in pre-labeled containers and the dry ice was allowed to sublime in a freezer over multiple days.

11. Combined the extra control samples from Step 8 into a single tube and mixed. Prepared post spike samples in concentrations of 4.00 ng/mL (3.20 ppb equivalent) as follows:
 - a. Matrix post spike: Combined 0.080 mL of the 50.0 ng/mL fortification standard with 0.920 mL of the extra control final volume.
 - b. Solvent post spike: Combined 0.080 mL of the 50.0 ng/mL fortification standard with 0.920 mL of 1:9 ACN:H₂O.
 Capped and vortexed to mix.
12. Transferred each sample to an HPLC vial containing a 300-μL insert.
13. Prepared equilibration vial(s) by combining unknown volumes of multiple standards.
14. Submitted sample, standard, post spike, and equilibration vials for LC-MS/MS analysis.

3.2 Instrumentation

All samples were analyzed by liquid chromatography employing tandem mass spectrometric detection (LC-MS/MS). The operating conditions used are listed below.

3.2.1 LC Operating Conditions

System:	Shimadzu System with Autosampler, Column Heater, Controller, Degasser, and Pump				
Column:	Phenomenex Luna Omega C18, 100 mm × 2.1 mm, 1.6 μm				
Guard Column:	NA				
Column Temperature:	40 °C				
Autosampler Temperature:	10 °C				
Injection Volume:	20 μL				
Flow Rate:	0.3 mL/min				
Mobile Phase A:	0.5% Acetic Acid in Water				
Mobile Phase B:	0.5% Acetic Acid in Acetonitrile				
Strong Needle Wash:	NA				
Weak Needle Wash:	1:1:2 Acetonitrile:Methanol:Water				
Conditions:	<u>Time</u>	<u>%A</u>	<u>%B</u>	Valco Diverter Valve:	<u>Time</u> <u>Eluate Flow</u>
	0.00	95	5		0.0-1.0 Waste
	2.50	20	80		1.0-5.0 Mass Spec
	5.80	0	100		5.0-end Waste
	5.90	95	5		
	7.00	95	5		
Total Run Time:	7.00 minutes				
Approximate Retention Time(s):	<u>Analyte</u>	<u>Minutes</u>			
	BCS-CS55621	3.9			
	BCS-CY96288	3.8			
	BCS-CU97237	2.9			
	BCS-DA63612	3.3			
	BCS-CC26101	3.2			
	BCS-CZ38260	2.8			
	BCS-BP32808	3.3			
	BCS-DH17585	3.4			

3.2.2 MS/MS Operating Conditions

System:	Applied Biosystems/Sciex API 6500 Q-Trap
Data Acquisition Software:	Applied BioSystems/Sciex Analyst, Version 1.6.2
Interface:	TIS (turbo ion spray)
Scan Type:	MRM (multiple reaction monitoring)
Resolution:	Q1 – unit, Q3 – unit
Carrier Gas:	Nitrogen

Period 1 Experiment 1

Polarity:	Positive (+)	Declustering Potential (DP):	30
Ion Spray Voltage (IS):	5500	Collision Gas (CAD):	"Medium"
Probe Temperature (TEM):	600	Curtain Gas (CUR):	40
Ion Source Gas 1 (GS1):	55	Entrance Potential (EP):	10
Ion Source Gas 2 (GS2):	55	Cell Exit Potential (CXP):	10

Analyte	Transitions Monitored (Q1→Q3)	Dwell Time (msec)	Collision Energy (CE)
BCS-CS55621	650.0 → 610.2	20	41
	656.0 → 616.2 (internal standard)	20	41
BCS-CY96288	666.0 → 626.0	20	28
	672.0 → 634.0 (internal standard)	20	28
BCS-CU97237	442.0 → 332.0	20	38
	448.0 → 338.0 (internal standard)	20	38
BCS-DA63612	456.0 → 345.0	20	50
	462.0 → 351.0 (internal standard)	20	50
BCS-BP32808	169.0 → 101.0	20	37
	171.0 → 101.0 (internal standard)	20	37
BCS-DH17585	403.0 → 169.0	20	27
	409.0 → 169.0 (internal standard)	20	27

Period 1 Experiment 2

Polarity:	Negative (-)	Declustering Potential (DP):	-30
Ion Spray Voltage (IS):	-4500	Collision Gas (CAD):	"Medium"
Probe Temperature (TEM):	600	Curtain Gas (CUR):	40
Ion Source Gas 1 (GS1):	55	Entrance Potential (EP):	-10
Ion Source Gas 2 (GS2):	55	Cell Exit Potential (CXP):	-10

Analyte	Transitions Monitored (Q1→Q3)	Dwell Time (msec)	Collision Energy (CE)
BCS-CC26101	225.0 → 161.0	100	-20
	227.0 → 162.0 (internal standard)	100	-20
BCS-CZ38260	161.0 → 76.0	100	-21
	163.0 → 77.0 (internal standard)	100	-21

3.2.3 Calibration/Sample Analysis

Calibration standard solutions were prepared in concentrations ranging from 0.250 to 50.0 ng/mL. Final calibration standard concentrations were converted from ng/mL to ppb (ng/g) equivalents by multiplying the concentration in ng/mL by the method factor, which was determined as follows:

$$\text{Method Factor} = \frac{\text{Extract Volume (mL)}}{\text{Sample Weight (g)}} \times \frac{\text{Final Volume (mL)}}{\text{Aliquot Volume (mL)}}$$

where:

$$\begin{aligned} \text{Extract Volume (mL)} &= 10 \\ \text{Sample Weight (g)} &= 5.00 \\ \text{Final Volume (mL)} &= 0.6 \\ \text{Aliquot Volume (mL)} &= 1.5 \end{aligned}$$

therefore:

$$\text{Method Factor} = \frac{10 \text{ mL}}{5.00 \text{ g}} \times \frac{0.6 \text{ mL}}{1.5 \text{ mL}} = 0.8$$

For example, a calibration standard of 50.0 ng/mL was converted to ppb equivalents as follows:

$$\text{ppb Equivalents} = 50.0 \text{ ng/mL} \times 0.8 = 40.0 \text{ ppb}$$

The following concentrations of calibration standard solutions were prepared in ng/mL, and their calculated ppb equivalents values entered into Analyst[®] for the determination of residues in ppb.

Calibration Standard Concentration								
ng/mL	0.250	0.500	1.00	4.00	10.0	20.0	40.0	50.0
ppb equivalents	0.200	0.400	0.800	3.20	8.00	16.0	32.0	40.0

An 8-point standard curve was constructed for each analytical set by injecting constant volumes of calibration standard solutions in concentrations listed above. Calibration standards were injected interspersed with test samples, with no more than three sample injections between standards. Constant volume injections were used for sample extracts as well. The performance of the instrument was evaluated during each injection set by monitoring the correlation coefficient of the calibration curve ($r \geq 0.995$).

3.3 Calculations

3.3.1 Determination of Residue (ppb)

Calculations for instrumental analysis were conducted using validated software application AB SCIEX Analyst[®], version 1.6.2, to create a standard curve based on linear regression. The regression function was used to calculate a best-fit line, with 1/x weighting, from a set of standard concentrations versus the analyte peak area ratio and to determine concentrations of the analyte found during sample analysis from the calculated best-fit line. The resulting equation used for linear regression was:

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

where:

x = analyte concentration in ppb equivalents
 y = peak area ratio (analyte peak area/internal standard peak area) for analyte
 b = y-intercept
 m = slope of the standard curve
 DF = dilution factor

Calculations were performed within Analyst[®] using a higher number of significant digits than is reported in the raw data; therefore, hand calculations based on the presented values may contain small rounding differences from the reported results.

3.3.2 Percent Recovery

Percent recoveries from fortified control samples were also calculated by Analyst[®] according to the following equation:

$$\% \text{ Recovery} = \frac{\text{ppb found}}{\text{ppb added}} \times 100$$

3.3.3 Example Calculation

Analyte of Interest: BCS-CS55621

Sample Description: Control Soil-1 + 5.00 ppb

Sample ID: 90854-009

Analytical Set: 001

where:

peak area = 813301
 internal standard peak area = 618042
 peak area ratio (y) = 1.3159327 (calculated below)
 y-intercept (b) = 0.02038201
 slope (m) = 0.2026793
 dilution factor (DF) = 1
 ppb added = 5.00

$$y = \frac{813301}{618042} = 1.3159327$$

$$x = \frac{1.3159327 - 0.02038201}{0.2026793} \times 1 = 6.3921205, \text{ Reported as } 6.39 \text{ ppb}$$

$$\% \text{ Recovery} = \frac{6.3921205 \text{ ppb found}}{5.00 \text{ ppb added}} \times 100 = 128 \%$$

3.4 Statistical Treatment of Data

Statistical evaluations including percent recoveries, mean percent recoveries, standard deviations, relative standard deviations, and confidence intervals were made using Microsoft Excel[®] 2016.

4.4 Matrix Effects

Matrix and solvent post-spike samples were prepared by fortifying both a control matrix sample and neat solvent sample with a mixed standard solution containing BCS-CS55621 and its metabolites at the concentration of 4.00 ng/mL. Matrix effects were evaluated by comparing the response of the analytes fortified in control matrix to the response of the analytes fortified in neat solvent according to the following equation:

$$\text{Matrix Effect [\%]} = 100 \times \left[\frac{\text{Peak Area of Spiked Matrix Sample}}{\text{Peak Area of Spiked Solvent Sample}} \right] - 100$$

A negative value for the matrix effects indicates matrix suppression, and a positive value for matrix effect indicates matrix enhancement. Matrix effects for all analytes at all monitored transitions were not less than $\pm 20\%$ indicating that matrix effects were a significant factor in analyte recovery. The results of the determination of matrix effects are reported in [Table 2](#). The method uses isotopically labelled internal standards to compensate for matrix effects. It has been demonstrated in this validation that the internal standard compensates for the matrix effects, based on acceptable recovery of fortification samples using non-matrix (solvent) calibration curve standards.

4.5 Standard Curve Linearity

Linearity of the detector response was evaluated for each transition using calibration standards prepared in solvent at concentrations ranging from approximately 30% of the LOQ to 20% above the highest anticipated residue level, i.e. 0.250 to 50.0 ng/mL (0.200 to 40.0 ppb equivalents). Calibration curves were generated by Analyst[®] using linear regression with 1/x weighting. For the least squares regression equations describing the detector response as a function of the standard calibration curve concentrations, the correlation coefficients (r) were greater than 0.998 for all of the calibration curve determinations during the method validation. The results indicate linearity of the detector response as a function of the standard concentration.

4.6 Limits of Quantitation and Detection

The limit of quantitation (LOQ) is defined as the lowest fortification level at which acceptable recovery data are obtained. This method has a stated LOQ of 0.5 ng/g (ppb) for BCS-CS55621 and its metabolites in soil.

The LOD was calculated by multiplying the standard deviation of the analyte concentration at the LOQ by $t_{0.99}$, the appropriate one-tailed Student's t statistic.

4.7 Specificity/Selectivity of the Method

Confirmation of the presence of the analytes in the recovery samples was indicated when the retention times of the analytes in the samples matched the retention times of the analytes in the calibration standards. Unambiguous identification was ensured by monitoring one MS/MS transition characteristic of each analyte and one MS/MS transition characteristic

of each internal standard. Analysis of control samples resulted in no significant signals at the expected retention times of the analytes. This LC-MS/MS method is highly selective for the quantitation of BCS-CS55621 and its metabolites in soil.

4.8 Time Required for Analysis

Method validation consisted of 8 calibration standards ranging from 0.250 to 50.0 ng/mL (0.200 to 40.0 ppb), a reagent blank, two unfortified control soil samples, five samples fortified at the LOQ (0.500 ng/g), and five samples fortified at 10×LOQ (5.00 ng/g). This set of 13 samples was taken through the analytical procedure in approximately 9 hours. LC-MS/MS analyses were allowed to run unattended overnight and took approximately 3 hours. Data manipulation for the set took approximately 45 minutes. The preparation of solutions and reagents took approximately 2.5 hours. Standard preparation took two analysts approximately 22 total hours, over the course of two work days. Therefore, if standards, solutions, and reagents are prepared in advance, a set of 13 samples can be analysed in just over one business work day (9 hours).

4.9 Critical Steps

The extraction procedures are critical.

4.10 Additional Method Comments/Changes

Table 8 of the method does not list an internal standard transition for BCS-CY96288 but lists two for BCS-CS55621. This appeared to be a typographical error, so the m/z 672/634.1 listed for the second BCS-CS55621 internal standard was used for the BCS-CY96288 internal standard. In Appendix 1: the molecular formula and IUPAC name for BCS-CY96288 appear to have typographical errors; the IUPAC names listed for BCS-DA63612 and BCS-CU97237 are the same; the molecular formula for BCS-CU97237- $^{13}\text{C}_6$ does not match the Certificate of Analysis. The expiration for standards is not listed. Based on the method, instrumentation uses a three pump system, which Eurofins does not have. Instrumental conditions for a more common, two pump system, should be added to the method. The method lists procedures when a multi-sample microwave extractor is used, which Eurofins does not have. Any changes to the procedures when a single-sample microwave extractor is used, should be added to the method. The injection sequence has multiple calibration curves injected. Alternate injection sequence parameters (allowance for interspersing calibration standards within the injection sequence) should be considered. The units for the standards in the representative linearity curves ($\mu\text{g/L}$) do not match the units of the calibration standards (ng/mL) in the method.

7.0 PROTOCOL/SOP/METHOD DEVIATIONS

Two protocol deviations were issued for this study. The first deviation recorded the lack of documentation showing that during the method preparation phase of the study, the Study Director and applicable analysts read the subject method completely and ensured necessary equipment and reagents were available. The second deviation documented that the lowest standard was 40% of the LOQ instead of 30%. These deviations had no impact on the study.

No method deviations occurred during the conduct of this study.

One Eurofins SOP deviation was generated during the course of the study and is documented in the raw data. It was not found to have had a significant impact on the integrity of the study.



6.2 Sample Extraction

Fortified recovery samples are prepared by spiking a known level of the appropriate standard(s) into a sample. A control sample should also be prepared, using the same samples as the fortified sample, to determine the residue levels, real or apparent, found in this sample and the % recovery calculated as described in [Section 8.1](#).

1. Weigh 5 ± 0.05 grams of the homogenized soil or sediment into a 4-oz bottle containing a magnetic bar.
Remark: Add the corresponding amount of spiking solution for the fortified recovery sample (e.g. 0.05 mL of a 50 ng/mL analyte mixture at LOQ level). Allow the sample to sit for 10 minutes.
2. Add 10 mL of 4:1 v/v acetonitrile:water.
3. Cap the jar, but do not fully close to avoid pressure build up.
4. Place samples into the microwave extractor. If you have more than 10 samples, adjust the power used in step 6.
5. Switch on the magnetic stirrer or confirm that samples will be stirred during extraction.
6. Extract for 15 minutes: From 0-3 minutes at 400 W (for 10 samples; ambient temperature to approx. 70°C) and from 3-15 minutes at 110 W (approx. 70°C).
7. Add 200 μ L of IS mixture solution with a concentration of 100 ng/mL.
8. Add 300 μ L acetic acid, mix well.
9. Let the sample sit ~5 min.
10. Transfer an aliquot of ~1.5 mL the supernatant of the sample into a microcentrifuge tube. Cap the microcentrifuge tube.
11. Centrifuge at ~13,000 rpm for 10 min.
12. Transfer the supernatant to a glass tube or 96-well plate. Evaporate to aqueous by using a TurboVap at 40°C and 25 psi for ~20 min.
13. Add 300 μ L 1:9 v/v acetonitrile:water. Mix well. Centrifuge as needed if the sample is cloudy.
14. Transfer the sample to the HPLC vial with insert or 96-well plate for LC/MS/MS analysis.