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 Water Using LC/MS/MS" with Stability Data

DATA REQUIREMENTS

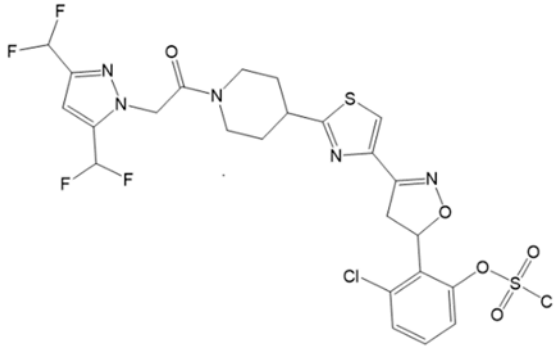
EPA OCSPP 850.6100
EU Guidance SANTE/2020/12830, Rev.1 (2021)
CA DPR SOP QAQC012.00

2.0 MATERIALS

2.1 Analytical Reference Standard(s)

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

BCS-CS55621

Synonym:	Fluoxapiprolin
Chemical Name:	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:	$C_{25}H_{24}ClF_4N_5O_5S_2$
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 ºC)

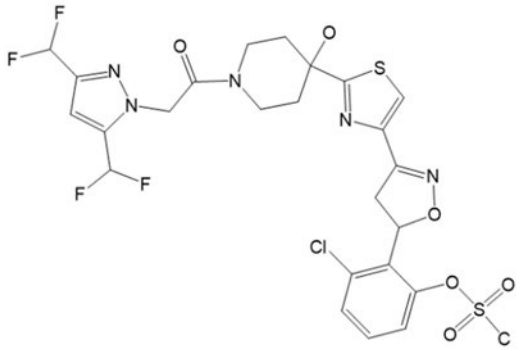
BCS-CY96288

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: $C_{25}H_{24}ClF_4N_5O_6S_2$

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 ºC)

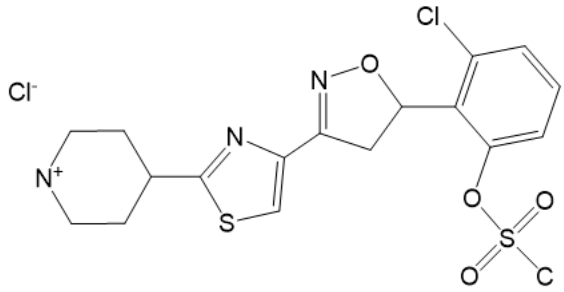
BCS-CU97237

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: $C_{18}H_{21}ClN_3O_4S_2Cl$

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 ºC)

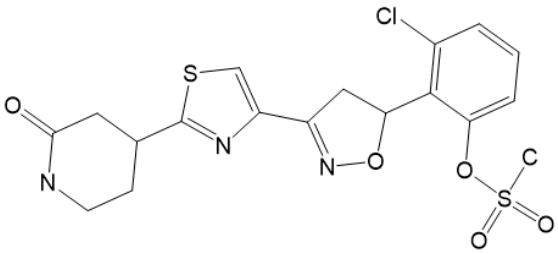
BCS-DA63612

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 (~ −20 ºC)

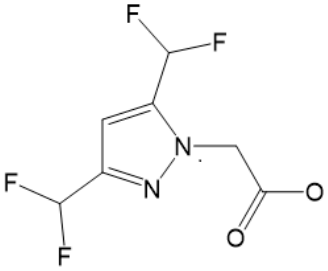
BCS-CC26101

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

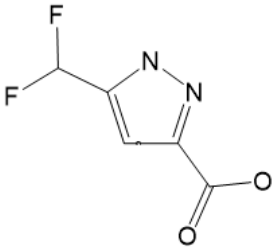
Purity: 98.5%

Expiration Date: 26-Aug-24

Storage: Freezer (~ −20 ºC)

BCS-CZ38260

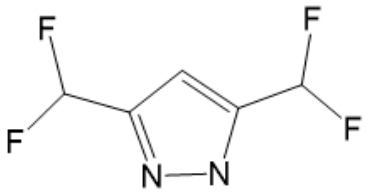
Chemical Name: 5-(difluoromethyl)-1H-pyrazole-3-carboxylic acid
Molecular Formula: $C_5H_4F_2N_2O_2$
Molecular Weight: 162.09 g/mol
Molecular Structure:



Receipt Date: 13 April 2021
Source: Bayer CropScience
Batch/Lot Number: K-2233 / 1228201705
Purity: 99.8%
Expiration Date: 02-Nov-25
Storage: Freezer (~ −20 ºC)

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 (~ −20 ºC)

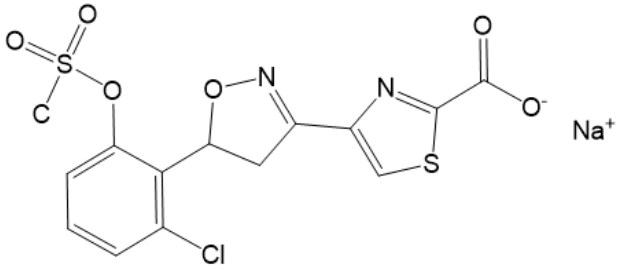
BCS-DH17585

Chemical Name: Sodium 4-[5-(2-chloro-6-methylsulfonyloxy-phenyl)-4,5-dihydroisoxasol-3-yl]thiazole-2-carboxylate

Molecular Formula: $C_{14}H_{10}ClN_2O_6S_2Na$

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

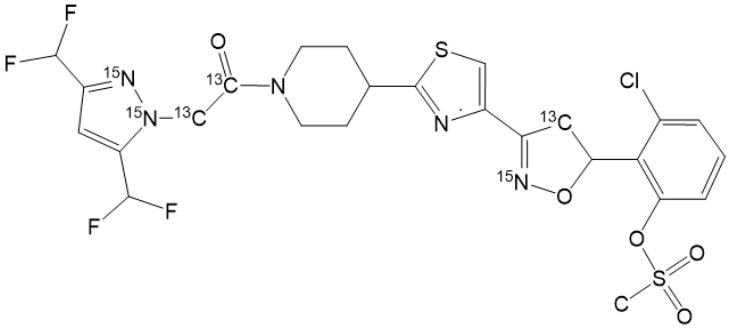
Storage: Freezer (~ −20 ºC)

BCS-CS55621-$^{13}C_3$, $^{15}N_3$

Molecular Formula: $^{13}C_3C_{22}H_{24}ClF_4{}^{15}N_3N_2O_5S_2$

Molecular Weight: 656.02 g/mol

Molecular Structure:



Receipt Date: 13 April 2021

Source: Bayer CropScience

Batch/Lot Number: K-2246

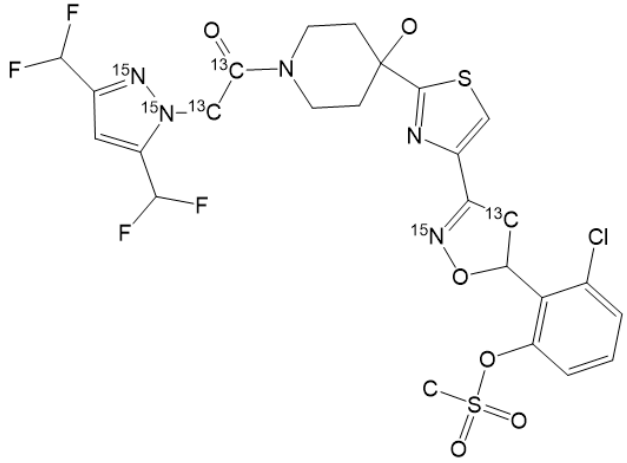
Purity: 100%

Expiration Date: 05-Apr-27

Storage: Freezer (~ −20 ºC)

BCS-CY96288-$^{13}C_3$, $^{15}N_3$

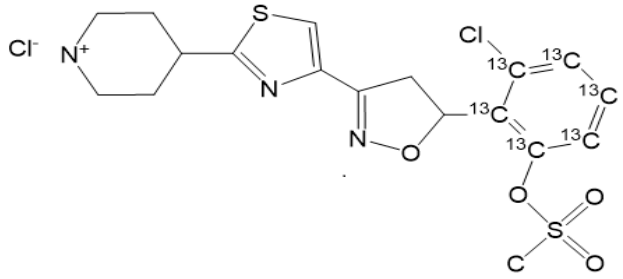
Molecular Formula: $^{13}C_3C_{22}H_{24}ClF_4{}^{15}N_3N_2O_6S_2$
Molecular Weight: 672.02 g/mol
Molecular Structure:



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

BCS-CU97237-$^{13}C_6$

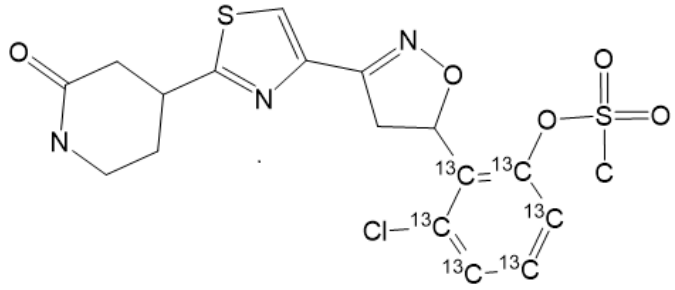
Molecular Formula: $^{13}C_6C_{12}H_{21}ClN_3O_4S_2 \cdot Cl$
Molecular Weight: 484.37 g/mol
Molecular Structure:



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

BCS-DA63612-$^{13}C_6$

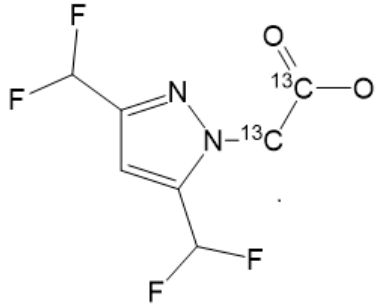
Molecular Formula: $^{13}C_6C_{12}H_{18}ClN_3O_5S_2$
Molecular Weight: 461.89 g/mol
Molecular Structure:



Receipt Date: 13 April 2021
Source: Bayer CropScience
Batch/Lot Number: K-2318
Purity: 99.3%
Expiration Date: 05-Dec-28
Storage: Freezer (~ −20 ºC)

BCS-CC26101-$^{13}C_2$

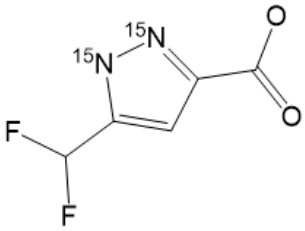
Molecular Formula: $^{13}C_2C_5H_6F_4N_2O_2$
Molecular Weight: 228.11 g/mol
Molecular Structure:



Receipt Date: 13 April 2021
Source: Bayer CropScience
Batch/Lot Number: K-2306
Purity: 100%
Expiration Date: 04-Sep-28
Storage: Freezer (~ −20 ºC)

BCS-CZ38260-$^{15}N_2$

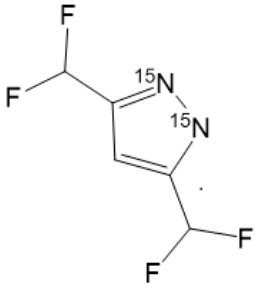
Molecular Formula: $C_5H_4F_2{}^{15}N_2O_2$
Molecular Weight: 164.08 g/mol
Molecular Structure:



Receipt Date: 13 April 2021
Source: Bayer CropScience
Batch/Lot Number: K-2316
Purity: 100%
Expiration Date: 05-Dec-28
Storage: Freezer (~ −20 ºC)

BCS-BP32808-$^{15}N_2$

Molecular Formula: $C_5H_4F_4{}^{15}N_2$
Molecular Weight: 170.08 g/mol
Molecular Structure:



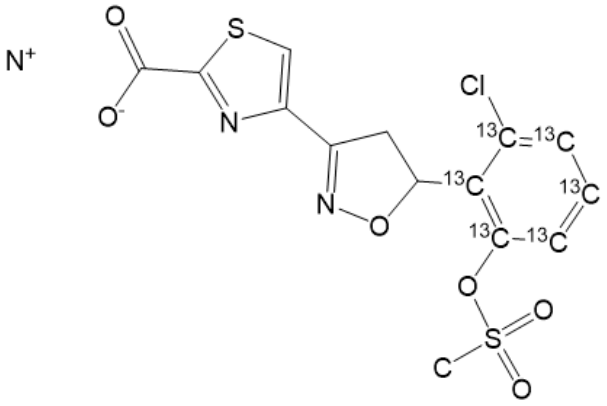
Receipt Date: 13 April 2021
Source: Bayer CropScience
Batch/Lot Number: K-2035 / 0907201802
Purity: 99.6%
Expiration Date: 16-Nov-24
Storage: Freezer (~ −20 ºC)

BCS-DH34157-$^{13}C_6$

Molecular Formula: $^{13}C_6C_8H_{10}ClN_2O_6S_2 \cdot H_4N$

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 ºC)

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 ºC) 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 (mg)	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 (mg)	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 (mg)	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 ºC) when not in use. None of the standard solutions in this study were used beyond their 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 into a 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			

Parent Solution	Concentration of Parent Solution	Aliquot Volume	Diluent	Dilution Volume (mL)	Final Concentration of Mixed Native Standard Solution
Mixed Native Standard	1000 ng/mL	10 mL	1:1 ACN:Water	100	100 ng/mL
Mixed Native Standard	100 ng/mL	10 mL	1:1 ACN:Water	100	10.0 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	5.00 mL	1:1 ACN:Water	50	100 ng/mL
		2.00 mL		100	20.0 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 ºC) when not in use. None of the standard solutions in this study were used beyond their expiration dates.

Calibration standards were prepared using a positive-displacement pipette to measure and transfer an aliquot each of mixed native standard solution into a class A volumetric flask, then to measure and transfer 200 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 (µL)	Dilution Volume (mL)	Final Concentration of Calibration Standard Solution (ng/mL)
1000	500	50	10.0 / 4.00
	400	50	8.00 / 4.00
	200	50	4.00 / 4.00
100	500	50	1.00 / 4.00
	200	50	0.400 / 4.00
10.0	1000	50	0.200 / 4.00
	500	50	0.100 / 4.00

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 XP205DR	Mettler Toledo
Freezer (Walk-in)	Model FSA-2D-S1	Victory
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 9-13WR	Environmental Specialties, Inc.
	Model HR2-1S	Beverage-Air
	Model STM1R-IS	True
Refrigerator/Freezer Combo	LRF201WWW/0	Nor-lake 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
Water	Optima	Fisher
Methanol	Optima	Fisher

2.3.3 Preparation of Laboratory 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.

<u>0.5% Acetic Acid in Water (Mobile Phase)</u>

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

<u>0.5% Acetic Acid in Acetonitrile (Mobile Phase)</u>

Prepared by adding 5 mL of 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.

1:1 Acetonitrile:Water (v:v)

Prepared by mixing 1000 mL of water with 1000 mL of acetonitrile. 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 100 mL of acetonitrile with 900 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 Sample Origin, Characterization, and Storage

Duplicate ambient control drinking water samples were collected from the cafeteria at the Eurofins Testing Facility on October 06, 2021 and logged according to Eurofins SOPs. One of the samples was transferred to a refrigerator (approximately 2-8 °C) where it remained until sample preparation and/or analysis; the other was sent to AGVISE Laboratories located in Northwood, North Dakota for characterization. The results of the AGVISE Water Characterization Report are presented in Appendix 3 and summarized below.

Water Property	Result	Water Property	Result
pH	8.2	Total Dissolved Solids (ppm)	350
Potassium (ppm)	5.1	Turbidity (NTU)	0.16
Calcium (ppm)	64	Alkalinity (mg CaCO3/L)	293
Magnesium (ppm)	28	Carbonates (meq/L)	0.00
Sodium (ppm)	20	Bicarbonates (meq/L)	5.57
Hardness (mg equiv. CaCO3/L)	278	Sulfate-Sulfur (ppm)	6.4
Conductivity (mmhos/cm)	0.50	Nitrogen (Nitrate) (ppm)	<0.1
Sodium Adsorption Ratio (SAR)	0.53	Chloride (ppm)	6.1

3.0 METHODS

The analytical method independently validated and used for the evaluation of refrigerated storage stability in this study was Bayer CropScience method TY-004-W21-01, entitled "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 Water Using LC/MS/MS" (Appendix 1).

3.1 Sample Preparation and Analysis

3.1.1 Independent Laboratory Method Validation

Method validation consisted of one reagent blank, two unfortified control samples, five fortifications each at the proposed LOQ (0.500 ng/mL) and 10×LOQ (5.00 ng/mL), and one each of a solvent and matrix post-spike to evaluate matrix effects.

Prior to sample preparation, the bulk control drinking water sample was removed from refrigerated storage, allowed to come to room temperature, and shaken by hand to mix well. Clean autosampler vials were labelled with a unique Eurofins ID beginning with the five-digit Eurofins study number (90890) followed by an additional designation. Method validation samples (reagent blank, untreated controls, and fortifications) were given consecutive number designations. For example, the first sample (a reagent blank) was assigned the unique identification of 90890-001, the second sample (a control) 90890-002, and so on. Solvent and matrix post-spikes were labelled 90890-SPS-DW and 90890-MPS-DW, respectively.

Method validation samples were processed as follows.

1. 1.00 mL of control water was transferred to labelled autosampler vials for all samples except the reagent blank.
2. 1.00 mL of Fisher water was transferred to the reagent blank autosampler vial.
3. Samples were fortified directly into the autosampler vials by adding 50.0 µL of a standard fortification solution to obtain the fortification levels shown below.

Sample Description	No. of Samples Prepared	Mixed Standard Fortification Solution		Fortification Level
		Volume	Concentration	
Reagent Blank	1	NA	NA	NA
Unfortified Control	2	NA	NA	NA
LOQ Fortification	5	50.0 µL	10.0 ng/mL	0.500 ng/mL
10×LOQ Fortification	5	50.0 µL	100 ng/mL	5.00 ng/mL
Solvent Post Spike	1	50.0 µL	100 ng/mL	5.00 ng/mL
Matrix Post Spike	1	50.0 µL	100 ng/mL	5.00 ng/mL

4. 0.25 mL of a 20 ng/mL mixed internal standard was added to each autosampler vial, including the reagent blank.
5. Autosampler vials were capped and vortexed to mix.
6. Equilibration vials were prepared by combining unknown volumes of multiple samples/standards.
7. Sample, standard, and equilibration vials were submitted for LC-MS/MS analysis.

The bulk control drinking water sample was returned to refrigerated storage after samples were prepared.

3.1.2 Refrigerated Storage Stability

With the exception of Day 0, storage stability sets consisted of one reagent blank, one unfortified control sample, three aged samples fortified at 10×LOQ, and three control samples fortified at 10×LOQ on the day of analysis. Day 0 analysis consisted of one reagent blank, one unfortified control sample, and three control samples fortified at 10×LOQ.

Prior to sample preparation, the bulk control drinking water sample was removed from refrigerated storage, allowed to come to room temperature, and shaken by hand to mix well. Clean autosampler vials were labelled with a unique Eurofins ID beginning with the five-digit Eurofins study number (90890) followed by the designation "FS" then by consecutive two-digit numbers according to the order in which the samples were prepared. For example, the first set of samples prepared was for the analysis of all analytes on Day 0. The first sample (a reagent blank) was assigned the unique identification of 90890-FS01, the second sample (a control) 90890-FS02, and so on until all of the stability samples being analyzed for all analytes were prepared. The Day 0 samples for the analysis of BCS-CZ38260 and BCS-BP32808 only were prepared, and started with the Eurofins ID 90890-FS30 and so on until all samples designated for stability analysis had been prepared.

Six sets of samples were prepared (i.e., four sets plus two additional contingency sets) for the analysis of BCS-CS55621 and its metabolites BCS-CY96288, BCS-CU97237, BCS-DA63612, BCS-CC26101, BCS-CZ38260, BCS-BP32808, and BCS-DH17585 on Day 0 and after refrigerated storage periods of 7, 15, and 30 days. An additional six sets of samples were prepared (i.e., four sets plus two additional contingency sets) for the analysis of BCS-CZ38260 and BCS-BP32808 on Day 0 and after refrigerated storage periods of 1, 3, 5, and 7 days. Each set of stored samples consisted of four unfortified controls and three controls fortified at 10×LOQ as shown below. Note that the same fortification solution was used for all stability samples.

Sample Description	No. of Samples Prepared	Mixed Standard Fortification Solution		Fortification Level
		Volume	Concentration	
Control	4	NA	NA	NA
Fortification	3	50.0 µL	100 ng/mL	5.00 ng/mL

All except Day 0 stability samples were placed in refrigerated storage until removed for analysis. The bulk control drinking water sample was also returned to refrigerated storage after samples were prepared.

The Day 0 sets were prepared and processed as follows.

1. 1.00 mL of control water was transferred to pre-labelled autosampler vials.
2. Appropriate samples (FF samples) were fortified at 5.00 ng/mL by adding 50.0 µL of a 100 ng/mL mixed standard solution directly into the autosampler vial containing the control water.
3. 1.00 mL of Fisher water was transferred to an autosampler vial pre-labeled for the Day 0 reagent blank.

4. 0.25 mL of a 20 ng/mL mixed internal standard was added to each autosampler vial, including the reagent blank.
5. Autosampler vials were capped and vortexed to mix.
6. Equilibration vials were prepared by combining unknown volumes of multiple samples/standards.
7. Sample, standard, and equilibration vials were submitted for LC-MS/MS analysis.

Sample sets analyzed for each stability interval were prepared and processed as follows.

1. On Day 0, 1.00 mL of control water was transferred to pre-labelled autosampler vials.
2. Samples labelled as stability samples (AF) were fortified at 5.00 ng/mL by adding 50.0 µL of a 100 ng/mL mixed standard solution directly into the autosampler vial containing the control water.
3. The autosampler vials were capped and vortexed to mix.
4. The stability sample vials, as well as the unfortified control water vials that would be used for procedural recoveries for each time interval, were placed in refrigerated storage.
5. At the given storage interval, a set of stability vials were removed from refrigerated storage and allowed to come to ambient temperature.
6. 1.00 mL of Fisher water was transferred to an autosampler vial pre-labeled for the reagent blank.
7. Caps were carefully removed from the stability vials.
8. Three procedural recovery samples were prepared at 5.00 ng/mL by adding 50.0 µL of a 100 ng/mL mixed standard solution directly into pre-labeled autosampler vials containing control samples (FF samples).
9. 0.25 mL of a 20 ng/mL mixed internal standard was added to each autosampler vial, including the reagent blank.
10. Autosampler vials were capped and vortexed to mix.
11. Equilibration vials were prepared by combining unknown volumes of multiple samples/standards.
12. Sample, standard, and equilibration vials were submitted for LC-MS/MS analysis.

3.2 Instrumentation

All samples were analyzed by liquid chromatography employing tandem mass spectrometric detection (LC-MS/MS). At least two ion transitions were acquired for each analyte. Typical operating conditions are listed below.

3.2.1 LC Operating Conditions

System:	Applied Biosystems/Sciex System with Degasser, Solvent Valve Autosampler, Column Oven, and System Controller					
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					
Weak Needle Wash:	1:1:2 Acetonitrile:Methanol:Water					
Conditions:	Time	%A	%B	Valco Diverter Valve:	Time	Eluate Flow
	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			
	6.80	95	5			
	6.90	95	5			
	7.00	95	5			
Total Run Time:	7.00 minutes					
Approximate Retention Time(s):	Analyte	Minutes				
	BCS-CS55621	3.7				
	BCS-CY96288	3.5				
	BCS-CU97237	2.7				
	BCS-DA63612	3.1				
	BCS-CC26101	3.0				
	BCS-CZ38260	2.6				
	BCS-BP32808	3.1				
	BCS-DH17585	3.1				

3.2.2 MS/MS Operating Conditions

System:	Applied Biosystems/Sciex API 6500+ Q-Trap with Ion Chromatograph SelexION+
Data Acquisition Software:	Applied BioSystems/Sciex Analyst, Version 1.6.3
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 (quantitative)	20	41
	650.0 → 378.0 (confirmatory)	20	46
	656.0 → 616.2 (internal standard)	20	41
BCS-CY96288	666.0 → 626.1 (quantitative)	20	28
	666.0 → 191.0 (confirmatory)	20	46
	672.0 → 634.1 (internal standard)	20	28
BCS-CU97237	442.2 → 332.0 (quantitative)	20	38
	442.2 → 208.0 (confirmatory)	20	38
	448.0 → 338.0 (internal standard)	20	38
BCS-DA63612	456.0 → 345.2 (quantitative)	20	50
	456.0 → 222.1 (confirmatory)	20	37
	462.0 → 351.0 (internal standard)	20	50
BCS-CZ38260	163.1 → 125.0 (confirmatory)	20	28
	163.1 → 97.0 (confirmatory)	20	32
	165.0 → 127.0 (internal standard)	20	28
BCS-BP32808	169.0 → 101.0 (quantitative)	20	37
	169.0 → 149.0 (confirmatory)	20	27
	171.0 → 101.0 (internal standard)	20	37
BCS-DH17585	403.0 → 169.0 (quantitative)	20	27
	403.0 → 157.0 (confirmatory)	20	37
	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 (quantitative)	100	-20
	225.0 → 141.0 (confirmatory)	100	-25
	227.0 → 162.0 (internal standard)	100	-20
BCS-CZ38260	161.0 → 76.0 (quantitative)	100	-21
	163.0 → 77.0 (internal standard)	100	-21

3.3 Calculations

3.3.1 Determination of Residue (ppb)

Calculations for instrumental analysis were conducted using validated software application AB SCIEX Analyst®, version 1.6.3, 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
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:

$$\%\ Recovery = \frac{ng/mL\ found}{ng/mL\ added} \times 100$$

3.3.3 Example Calculation

Analyte of Interest: BCS-CS55621
Sample Description: Control Drinking Water-1 + 0.500 ng/mL
Sample ID: 90890-008
Analytical Set: 001
where:

peak area	=	239102
internal standard peak area	=	2647653
peak area ratio (y)	=	0.0903070 (calculated below)
y-intercept (b)	=	0.004300466
slope (m)	=	0.2069477
dilution factor (DF)	=	1.25
ng/mL added	=	0.500

$$peak\ area\ ratio = \frac{239102}{2647653} = 0.0903070$$

$$ng/mL\ Found = \frac{0.0903070 - 0.004300466}{0.2069477} \times 1.25 = 0.5194942\ ng/mL$$

$$\%\ Recovery = \frac{0.5194942\ ng/mL\ found}{0.500\ ng/mL\ added} \times 100 = 104\ \%$$

4.1.2 Control Screen

The control sample selected for use in this study was analyzed per the method and was determined to be free of any interference in the area of analyte elution (corresponding to analyte residue levels <30% of the LOQ) of the expected retention time for each analyte.

4.1.3 Accuracy and Precision

The average recoveries of BCS-CS55621 and its metabolites in both quantitation and confirmation transitions were within the acceptable range of 60 to 120% with a relative standard deviation ≤20% at both the LOQ and 10×LOQ fortification levels.

4.1.4 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 5.00 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:

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

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 less than ±20% indicating that matrix effects were not a significant factor in analyte recovery. The results of the determination of matrix effects are reported in Table 4.

4.1.5 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. 0.100 to 10.0 ng/mL. 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 0.996 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.

4.1.6 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/mL (ppb) for BCS-CS55621 and its metabolites in drinking water.

The limit of detection (LOD) is defined as the lowest detectable concentration or amount of an analyte in a sample and is expressed as the lowest calibration standard in matrix, or approximately 30% of the LOQ (approximately 0.15 ng/mL; Reference 3). The lowest calibration standard used during method validation, and therefore the LOD, was 0.100 ng/mL.

The LOD was confirmed by multiplying the standard deviation of the analyte concentration at the LOQ by $t_{0.99}$, the appropriate one-tailed Student's t statistic. Results for the calculated limits of detection and quantitation are presented in Table 5.

4.1.7 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 at least two MS/MS transitions characteristic of each analyte and one MS/MS transition characteristic of each internal standard. Analysis of control samples resulted in no significant signals (≤ 30% loss of the LOQ) at the expected retention times of the analytes. This LC-MS/MS method is highly selective for both the quantitation and confirmation of BCS-CS55621 and its metabolites in drinking water. Mass spectra for all analytes are presented in Figure 1.

4.1.8 Time Required for Analysis

Method validation consisted of 14 calibration standards (at 7 different concentration levels) ranging from 0.100 to 10.0 ng/mL, a reagent blank, two unfortified control drinking water samples, five samples fortified at the LOQ (0.500 ng/mL), and five samples fortified at 10×LOQ (5.00 ng/mL). This set of 13 samples was taken through the analytical procedure in approximately 0.5 hour (excluding the time for the bulk control water to warm to ambient temperature). LC-MS/MS analyses were allowed to run unattended overnight and took approximately 3.5 hours. Data manipulation for the set took approximately one hour. The preparation of solutions and reagents took approximately 1.5 hours. Standard preparation took two analysts approximately 22 hours. Therefore, if standards, solutions, and reagents are prepared in advance, a set of 13 samples can be analysed within one business work day (8 hours).

4.1.9 Critical Steps

There were no critical steps encountered during this independent method validation.

4.1.10 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.

4.2 Refrigerated Storage Stability

The original storage stability setup consisted of the analysis for BCS-CS55621 and all of its metabolites at 0, 7, 15, and 30 days after refrigerated storage. Initial analysis showed poor stability for BCS-CZ38260 and BCS-BP32808. A new stability setup was added for the analysis of BCS-CZ38260 and BCS-BP32808 only at 0, 1, 3, 5, and 7 days after refrigerated storage to determine when the loss was occurring. Statistics for the procedural recovery results for the new stability analyses were calculated separately from the original.