STUDY TITLE

Method Validation of XDE-659 and its Metabolite in Water

DATA REQUIREMENTS

SANCO/825/00 rev. 8.1
SANCO/3029/99 rev. 4
PMRA Dir98-02
OCSPP Guideline 850.6100

AUTHOR(S)

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STUDY COMPLETED ON

15, January 2020

PERFORMING LABORATORY

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STUDY SPONSOR

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STUDY NUMBER

JRF America Study No: AU-2019-11
Sponsor Study ID.: 180503

PAGE COUNT

1 of 271

1.0 INTRODUCTION

1.1 Scope

The objective of this study was to develop and prepare an analytical method for the determination of XDE-659 and X12485649 in Water. This rapid analytical method is applicable for the quantitative determination of residues of XDE-659 and X12485649 in a representative Water (ground, surface and drinking).

The method is detailed in JRFA Analytical Method AU-297R0 "Analytical Method for the Determination of XDE-659 and X12485649 in Water." See Appendix VI. The method was validated over the concentration range of 0.10-1.0 ng/mL with a validated limit of quantitation of 0.10 ng/mL. The Limit of detection was set to 0.03 ng/mL. Common names, chemical names, and molecular formulas for the analyte are given in Table 1.

This study was conducted to fulfill data requirements that satisfy SANCO/825/00 rev 8.1, SANCO/3029/99 rev 4, PMRA Dir98-02, and USEPA OCSPP Guideline 850.6100, Environmental Chemistry Methods and Associated Independent Laboratory Validation (January 2012). This study was conducted following JRF America Standard Operating Procedures. There were no protocol amendments or deviations associated with this study.

1.2 Method Principle

Samples were fortified then vortexed. Samples were then analyzed through LC-MS/MS).

Test Substances/Reference Compounds/Analytical Standards

Test Substance[a]	TSN	Lot No	Percent Purity	Recertification Date
XDE-659	TSN310889	YN4-156527-070-B	99.1%	17-May-2020
X12485649	TSN313826	GZX-01-015-1	98.0%	21-May-2021

The test substances was received on October 17, 2019. The XDE-659 material was stored in refrigerated conditions, while the material X12485649 was store in ambient conditions. The certificates of analysis for the reference substance can be found in Appendix V. The above standard may be obtained from Dow AgroSciences LLC, 9330 Zionsville Road, Building 304, Indianapolis, IN 46268-1053.

Equipment, Glassware, and Materials

Laboratory Equipment:

Equipment	Size, Description	Manufacturer	Catalog No.
Balance	Analytical Balance	Mettler Toledeo	K51405

Flasks, Volumetric	Various sizes	Various	----
Pipettes	Various volumes	Eppendorf /Oxford	----
Vortex Mixer	BenchMark, vortex	BenchMark Scientific, Inc.	13112215
HPLC Vials	2 mL	Agilent	5182-0716
HPLC vial caps	2 mL	Agilent Technologies	5182-0717

UHPLC-UV/MS/MS:UHPLC-RES9			
Module Type	Manufacturer	Model No.	Serial/ID No.
Column Oven	Aglient 1290	G1316C	DEBAC08348
Auto sampler	Aglient 1290	G2446A	DEBAP05490
Solvent Delivery System	Aglient 1290	G4220A	DEBAA04368
Mass Spectrometer	AB Sciex 6500 QTrap TripQuad	6500	BL23451404
3 x 50mm , 1.8 µm	Zorbax	Eclipse Plus Phenyl Hexyl	USDER01319

Chemical	Grade	Manufacturer/Supplier	Lot No.
Formic Acid	LC/MS	Honeywell/Fluka Sigma-Aldrich St. Louis, MO	J1070
Water	LC/MS	Honeywell/Fluka Sigma-Aldrich St. Louis, MO Honeywell/Fluka Fisher Chemical	195465 196233 195466
Acetonitrile	LC/MS	Honeywell/Fluka Sigma-Aldrich St. Louis, MO OmniSolv/Millipore Billerica, MA Honeywell/Fluka Fisher Chemical	DW926-US 193278
Methanol	LC/MS	OmniSolv/Millipore Billerica, MA Honeywell/Fluka Fisher Chemical	59235 194362

Prepared Solutions:

Mobile Phase A: 0.1 % Formic Acid in Water

Measure 800 mL of LC/MS water into a 1000 mL volumetric flask and then add 1 mL of LC/MS grade formic acid. Following bring the solution to the mark by adding 200 mL of LC/MS water. Invert three times and sonicate briefly. Pour into 1L bottle using funnel if needed.

Mobile Phase B: 0.1 % Formic Acid in 80: 20 ACN: MeOH

Measure 800 mL of LC/MS Acetonitrile and 200 mL LC/MS MeOH into a 1000 mL volumetric flask, removed 1mL and then added 1 mL of reagent grade formic acid. Invert three times. Pour into 1L bottle using funnel if needed.

Dilution Solvent: 100% Acetonitrile + 0.1% formic acid

Measure 1 L ACN and remove 1 mL and replace with 1 mL formic acid.

Water Matrices: 60/40 Matrix/ACN + 0.1% formic acid

Measure 600 mL matrix (ground, surface, or drinking) water, add 400 mL ACN. Shake and remove 1mL and replace with 1 mL formic acid.

2.0 EXPERIMENTAL

2.1 Sample Origin, Numbering, Preparation and Storage

Surface water was obtained from Site D- Emperor Lake, Chatsworth, Derbyshire United Kingdom. Complete source documentation is included in the study file. During the course of the study, the samples were stored in temperature-monitored refrigerator at approximately 4 °C, except when removed for analysis. Ground water was obtained from Bennett Residence 591 West Boot Road, West Chester, PA 19380. Complete source documentation is included in the study file. During the course of the study, the samples were stored in temperature-monitored freezers at approximately 4 °C, except when removed for analysis. Drinking water was obtained from JRF America, 2650 Eisenhower Ave. Audubon, PA 19403. Complete source documentation is included in the study file. Detailed characterization is shownin Table 90 and Appendix IV During the course of the study, the samples were stored in temperature-monitored refrigerators at approximately 4 °C, except when removed for analysis.

2.2 Instrumentation

XDE-659

HPLC: Agilent 1290 Infinity

Mobile Phase A:	0.1 % Formic Acid in Water
Mobile Phase B:	0.1 % Formic Acid in 80: 20 ACN: MeOH
Flow Rate:	500 µl/min
Column:	Zorbax Eclipse Plus Phenyl Hexyl 3 x 50 mm, 1.8 µm (sn: USDER01319)
Column Oven Temperature:	30 °C
Injection Volume:	10 µL
Run Time:	5 min
Detector:	Analyst™ software version 1.6.2
Retention Time:	~3.24 min
Pre-Equilibration before standards/samples analyzed:	~15-30 min
Pre-equilibration within each Injection:	40 s

X12485649

HPLC: Agilent 1290 Infinity

Mobile Phase A:	0.1 % Formic Acid in Water
Mobile Phase B:	0.1 % Formic Acid in 80: 20 ACN: MeOH
Flow Rate:	500 µl/min
Column:	Zorbax Eclipse Plus Phenyl Hexyl 3 x 50 mm, 1.8 µm (lot USDER01319
Column Oven Temperature:	30 °C
Injection Volume:	5 or 10 µL
Run Time:	5 min
Detector:	Analyst™ software version 1.6.2
Retention Time:	~2.9 min
Pre-Equilibration before standards/samples analyzed:	~30 min
Pre-equilibration within each Injection:	40 s

A gradient elution, using an increased percentage of organic solvent (80:20 ACN:MeOH + 0.1% formic acid) in the mobile phase, is used to resolve interferences and improve separation. See the specific gradient listed below:

Time (Min)	A% 0.1 % formic acid in Water	B% 0.1 % formic acid in 80: 20 ACN: MeOH	Flow (µL/min)
0.00	50	50	500
1.30	40	60	500
2.00	40	60	500
3.00	10	90	500
3.10	0	100	500
4.50	0	100	500
4.60	50	50	500
5.00	50	50	500

Note: Retention times may differ depending upon the flow rate, column, and gradient used.

Acquisition Ions and Compound Dependent Parameters:

Analyte	Mass Transition (*m/z*)	Dwell (msec)	DP (V)	CE (V)	CXP (V)
XDE-659 (Quantitation)	*512.960→231.100*	150.00	86.00	31.00	6.00
XDE-659 (Confirmatory)	*512.960→108.900*	150.00	86.00	73.00	18.00
X12485649 (Quantitation)	*470.813→230.900*	150.00	66.00	25.00	18.00
X12485649 (Confirmatory)	*470.813→109.000*	150.00	66.00	55.00	10.00

Typical MS/MS Conditions Used:

Sample Analysis	
Ionization Mode	ESI
Scan Type	MRM
Polarity	Positive
Curtain gas (N_2) (psi)	50
GS1 (psi)	80
GS2 (psi)	40
CAD gas (N_2)	Medium
Ion Spray (V)	5500
Temperature (ºC)	600

The above conditions may need to be optimized on each instrument.

2.3 Calculation of Standard Calibration Curve

Calculation of a standard curve begins with the injection of a series of calibration standards and acquisition of peak areas for the following analytes.

XDE-659	m/z Q1/Q3 *512.960/231.100* (quantitative)
	m/z Q1/Q3 *512.960/108.900* (confirmatory)
X12485649	m/z Q1/Q3 *470.813/230.900* (quantitative)
	m/z Q1/Q3 *470.813/109.000* (confirmatory)

For each analyte, the linearity of detector response was evaluated. A standard curve was generated by plotting the analyte concentration on the abscissa (x-axis) and the quantitation ratio on the ordinate (y-axis) in Analyst®. Using regression analysis, the equation was determined for the curve with respect to the abscissa. Refer to Figures 1 through Figures 3 for method validation and extract stability. Refer to Table 2 through Table 25 for method validation data and extract stability data.

2.4 Confirmation of Residue Identity

The method is selective for the determination of XDE-659 and X12485649, by virtue of the chromatographic separation and selective detection system used. To demonstrate further confirmation, an additional MS/MS ion transition was monitored for the analyte.

2.5 Statistical Treatment of Data

Statistical treatment of data included, but was not limited to, the calculation of regression equations, correlation coefficients (r) and coefficient of determination (r^2) for describing the linearity of calibration curves, and means, standard deviations, and relative standard deviations of the results for the fortified recovery samples.

3.1 Assay Time

A typical analytical set contains six calibration standards at different concentration levels. The samples for the method validation consisted of a reagent blank, two controls (a non-fortified sample), and five fortified controls for the LOQ and five for the 10x LOQ. The methodology is normally performed with a batch of 14 samples including LOD. On average, one chemist can complete the analysis of one batch of 14 samples including instrument analysis and data processing in a period of 8 working hours.

3.2 Specificity of Method and Confirmation of Residue Identity

The method is selective for the determination of XDE-659 and X12485649, by virtue of the chromatographic separation and MS/MS detection. Significant peak response was not observed in reagent blank and extracts of untreated blank control samples at the expected retention times of the analyte(s). Unambiguous identification was ensured by the observation of a precursor ion plus a structurally significant product ion observed at the same retention time.

Data obtained using the confirmatory MS/MS transition(s) met the acceptance criteria using the quantitative MS/MS transition(s), therefore, demonstrating that the analyte signal of the quantitative MS/MS transition was correct and not affected by any other compound.

3.3 Extraction Efficiency

In the current study, extraction efficiency was not evaluated experimentally because the radiolabeled samples with incurred residues were identical to those in metabolism studies. Extractability of the fortified analyte based on recovery was shown found to be consistent.

3.6 Example Calculations

$$Test\ Item\ Found\ \left(^{\mu g}/_{L}\right) = \frac{Calc\ Conc\ \left(^{\mu g}/_{L}\right) * Final\ Volume * Dilution\ Factor}{Sample\ Volume}$$

Sample: XDE-659 Quantitative Surface Water (204220) LOQ R1 Volume: 10 mL

Peak area in the quantitation transition was 119526.6345 counts

Calibration curve generated in the run was y = 1095088.207 * x + 7798.28517

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

$$x = \frac{119526.6345 - 7798.28517}{1095088.207}$$

$$x = 0.10\,{}^{ng}/_{mL}$$

Recovery Results

The recovery factor, expressed as a percentage (Recovery %), is calculated using the following equation:

$$\%\text{Recovery} = \frac{Measured\ concentration\ (ng/mL)}{Theoretical\ concentration\ (ng/mL)} * 100\%$$

Where the measured and control concentrations are taken from instrument outputs for the sample and UTC, respectively, and the theoretical concentration is the known amount of analyte added to the sample, if applicable.

Example:

XDE-659 Quantitative Surface Water (204220) LOQ R1 was analyzed.

$$\%\text{Recovery} = \frac{0.10\ ng/mL}{0.10\ ng/mL} * 100\% = 102\%$$

3.12 Allowable Instrumental Changes to Method

Changes to the analytical method may be made to the optimization parameters and instrumental conditions.

3.13 Critical Steps of the Method

Matrix matched standards were prepared using serial dilution of stock standards.

4.0 CONCLUSIONS

The analytical method for the determination of XDE-659 and X12485649 in water has been demonstrated to be satisfactory in terms of accuracy, precision, linearity, and specificity. The method was validated over the concentration range of 0.1 – 1.0 ng/mL with a validated limit of quantitation of 0.1 ng/mL.

5.0 ARCHIVING

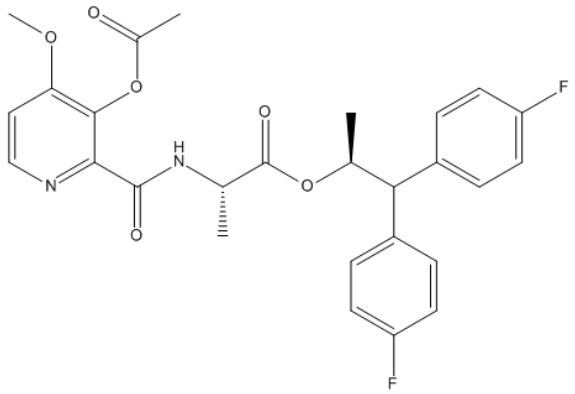
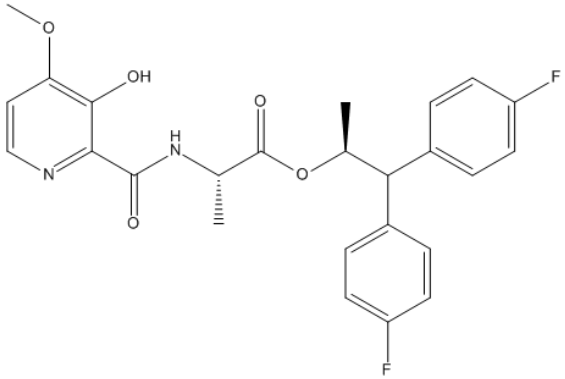
The protocol, raw data, and the original version of the final report will be filed in the Dow AgroSciences LLC archives at 9330 Zionsville Road in Indianapolis, Indiana 46268-1053. JRF America will keep copies of raw data and final report for two years. Sample extracts will be retained and disposed of following the completion of study and authorization of sponsor.

7.0 APPENDICES

7.1 Appendix I: Tables

Please note that some values in the tables in this section may differ from the original spreadsheets due to changes in significant figures.

Table 1 Identity and Structure of XDE-659 and X12485649

Identifying Information	Structure and CAS Name
Common Name of Compound: XDE-659 Molecular Formula: C27H26F2N2O6 Formula Weight: 512.51 Nominal Mass: 512 CAS Number: 1961312-55-9	(2S)-1-1-bis(4-fluorophenyl)propan-2-yl N-{[(3-acetyloxy)-4-methoxypyridin-2-yl]carbonyl}-L-alaninate 
Common Name of Compound: X12485649 Molecular Formula: $C_{25}H_{24}F_2N_2O_5$ Formula Weight: 470.47 Nominal Mass: 470 CAS Number: N/A	(2S)-1-1-bis(4-fluorophenyl)propan-2-yl N-[(3-hydroxy-4-methoxypyridin-2-yl)carbonyl]-L-alaninate 

Analytical Method – (continued)

1.0 INTRODUCTION

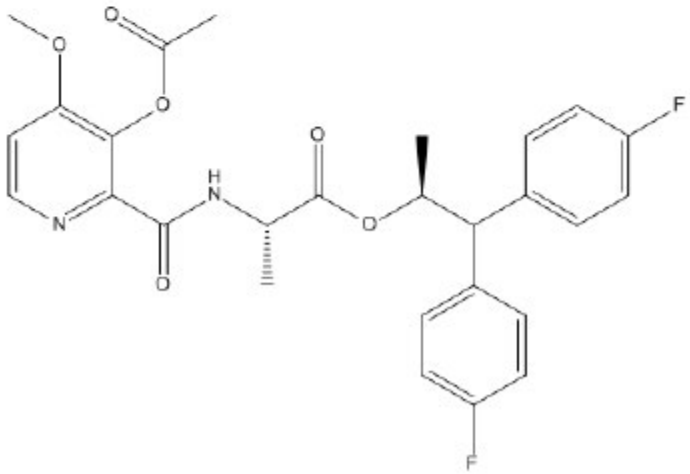
1.1. Scope and Chemical Structures

An analytical Method AU-297R0 was developed for analysis of XDE-659 and it's metabolite in drinking, surface and ground water. The method was developed using LC-MS/MS for detection. The limit of quantitation (LOQ) of the method has been established at 0.1 ng/mL and 10x LOQ at 1.0 ng/mL. This method satisfies SANCO/825/00 rev 8.1, SANCO/3029/99 rev 4, PMRA Dir98-02, and USEPA OCSPP Guideline 850.6100, Environmental Chemistry Methods and Associated Independent Laboratory Validation (January 2012).

The chemical structures of XDE-659 is summarized as follows:

Common Name:	XDE-659
Chemical Name (IUPAC):	(2S)-1-1-bis(4-fluorophenyl)propan-2-yl N-{[(3-acetyloxy)-4-methoxypyridin-2-yl]carbonyl}-L-alaninate
CAS Registry No.:	1961312-55-9
Molecular Formula:	$C_{27}H_{26}F_2N_2O_6$
Molecular Weight:	512.51 $g \cdot mol^{-1}$
Batch No.:	YN4-156527-070-B
Reassay Date:	May 17, 2020
Purity:	99.1%
Storage Condition:	Refrigerated
Source:	Dow Agrosciences LLC

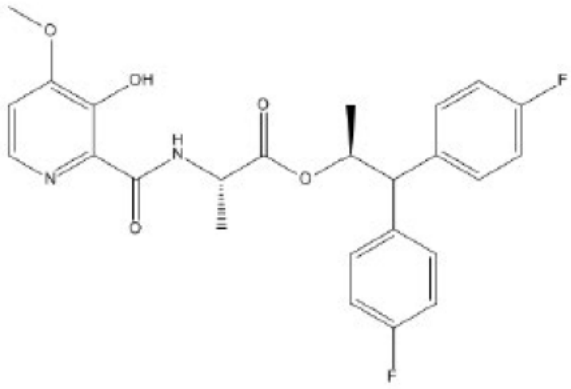
Structure:



Analytical Method – (continued)

The chemical structures of X12485649 is summarized as follows:

Common Name:	X12485649
Chemical Name (IUPAC):	(2S)-1-1-bis(4-fluorophenyl)propan-2-yl N-[(3-hydroxy-4-methoxypyridin-2-yl)carbonyl]-L-alaninate
CAS Registry No.:	N/A
Molecular Formula:	$C_{25}H_{24}F_2N_2O_5$
Molecular Weight:	470.47 $g \cdot mol^{-1}$
Batch No.:	GZX-01-015-1
Reassay Date:	May 21, 2021
Purity:	98%
Storage Condition:	Ambient
Source:	Dow Agrosciences LLC



Structure:

1.2. **Method Summary**

Samples were fortified then breifly shaken. Samples were then analyzed by LC/MS/MS.

Analytical Method – (continued)

2.0 MATERIALS AND APPARATUS

2.1. Apparatus

The recommended equipment and apparatus are listed in Appendix I.

Analytical Method – (continued)

2.2. **Reagents**

All solvents and other reagents are to be of high purity, e.g., glass distilled/HPLC grade solvents and analytical grade reagents. Water must be deionized prior to use or purchased HPLC grade water utilized. Particular care must be taken to avoid contamination of the reagents used. Reagents of comparable purity may be substituted as long as acceptable performance is demonstrated. A list of reagents used in this method along with details of preparation of solutions is included in Appendix II.

2.3. **Preparation of Analytical Standard Solutions**

It is recommended that the following precautions should be taken when weighing the analytical materials.

1. Ensure good ventilation.
2. Wear gloves, protective eyewear and lab coat.
3. Prevent inhalation and contact with mouth.
4. Wash any contaminated areas.

2.3.1. Stock Solutions

XDE-659 stock standard solution is prepared in LC/MS ACN +0.1% formic acid. A stock standard concentration of 994 µg/mL was used. The following is an example for preparing 10 mL of a 994 µg/mL stock standard.

1. A mass of 0.010034 g of reference standard is weighed (adjusted for purity of 99.1%) and transferred to a 10 mL class A volumetric flask.
2. Fill the volumetric flask halfway with LC/MS ACN + 0.1% formic acid and agitate gently (sonicate if necessary) until standard is completely dissolved.
3. Dilute to volume with LC/MS ACN + 0.1% formic acid and mix by inverting several times.
4 . Calculate the exact concentration using the exact weight and purity, for example:

$$\left(\frac{0.010034\ \text{g}*0.991}{10\ \text{mL}}\right)*\left(\frac{10^6\mu\text{g}}{\text{g}}\right) = 994\ \mu\text{g/mL}$$

X12485649 stock standard solution is prepared in LC/MS ACN +0.1% formic acid. A stock standard concentration of 1070 µg/L was used. The following is an example for preparing 10 mL of a 1070 µg/L stock standard.

1. A mass of 0.010874 g of reference standard is weighed (adjusted for purity of 98 %) and transferred to a 10 mL class A volumetric flask.
2. Fill the volumetric flask halfway with LC/MS ACN + 0.1% formic acid and agitate gently (sonicate if necessary) until standard is completely dissolved.

Analytical Method – (continued)

3. Dilute to volume with LC/MS ACN + 0.1% formic acid and mix by inverting several times.
4. Calculate the exact concentration using the exact weight and purity, for example:

$$\left(\frac{0.01034\ g*0.98}{10\ mL}\right)*\left(\frac{10^6 \mu g}{g}\right) = 1070\ \mu g/L$$

2.3.2. Preparation of Fortification Solutions

A mixed stock standard of XDE-659 and X12485649 was prepared at a concentration of 10 µg/mL.

Sample fortification solutions should be prepared by serial dilution of the stock standard in LC/MS ACN + 0.1% formic acid for both XDE-659 and X12485649. It is recommended that the following concentrations are prepared for fortification standards: 10 ng/mL, and 100 ng/mL.

2.3.3. Preparation of Calibration Standards for LC-MS/MS

Calibration solutions suitable for LC-MS/MS analysis should be prepared in matrix (Drinking water, ground water and surface water). At least five levels of external calibration standards should be prepared to develop calibration curves for calculation of sample residues. The following dilution schemes were used to prepare the LC-MS/MS calibration solutions are as follows:

Starting Concentration (µg/mL)	Volume Used (mL)	Final Volume (mL)	Final Concentration (µg/mL)
10	0.200	20	0.10
10	1.0	20	0.50
10	0.100	20	0.05
10	0.040	20	0.02
0.50	0.200	20	0.005
0.50	0.050	20	0.00125
0.50	0.020	20	0.0005
0.05	0.100	20	0.00025
0.05	0.040	20	0.0001
Starting Concentration (µg/mL)	Volume Used (mL)	Final Volume (mL)	Final Concentration (ng/mL)
10	0.200	20	0.1
10	0.100	20	0.05
10	0.040	20	0.02
0.50	0.200	20	0.005
0.50	0.050	20	0.00125
0.50	0.020	20	0.005
0.05	0.1	20	0.00025

Analytical Method – (continued)

0.05	0.040	20	0.0001
Starting Concentration (µg/mL)	Volume Used (mL)	Final Volume (mL)	Final Concentration (ng/mL)
0.10	0.020	1	2.0
0.05	0.020	1	1.0
0.02	0.020	1	0.40
0.005	0.020	1	0.10
0.00125	0.020	1	0.025
0.005	0.010	1	0.05
0.0005	0.020	1	0.01

2.3.4. Standard Solution Storage and Expiration

XDE-659 and X12485649 stock and fortification solutions should be stored in a refrigerator (~ 4 ° C) when not in use to prevent decomposition and/or concentration of the standard. Standard solutions should be allowed to equilibrate to room temperature prior to use.

An expiration date of 6 months is recommended for the XDE-659 and X12485649 stock standard solution, and 1 month for XDE-659 and X12485649 calibration standards and fortification solutions, based on company standard operating procedure and available data.

2.4. Safety Precautions and Hazards

All caution should be exercised when handling pure material or concentrated stock solutions. Avoid skin contact, swallowing and inhalation. See Safety Data Sheet (SDS) documentation accompanying standard shipment. All personnel should be familiar with all solvents and equipment precautions and hazards prior to use.

3.0 ANALYTICAL PROCEDURE

3.1. Sample Preparation

Samples should be prepared using an approved method for sample preparation for residue analysis.

1. Pipette 10 mL (for LOQ samples) and 10 mL (for 10x LOQ) of drinking, tap, or ground water into 20 mL glass vial.
2. Fortify samples with the proper amount (if necessary). To fortify the LOQ samples, 0.010 µL of a 100 ng/mL fortification solution was used (0.100 µL of a 10 ng/mL fortification solution was used for drinking water samples). To fortify the 10x LOQ samples, 0.100 µL of 100 ng/mL fortication solution.
3. Breifly shaken by hand for approximately 10 seconds to mix.

Analytical Method – (continued)

3.2. **Time Required for Analysis**

The methodology is normally performed with a batch of 14 samples. In average, one chemist can complete the analysis of one batch of 14 samples including instrument analysis and data processing in a period of eight (8) working hours.

3.3. **Modifications and Potential Problems**

Samples should be analyzed within a seven days after extraction. An expiration date of 6 months is recommended for the XDE-659 and X12485649 stock standard solution, and 1 month for XDE-659 and X12485649 calibration standards and fortification solutions.

4.0 **FINAL DETERMINATION**

The method has been developed for use on a Sciex 6500 QTrap and Agilent 1290 Infinity system. The following instrumentation and conditions can be used as a general guidance. Other instrumentation, column and mobile phases can also be used, though optimization may be required to achieve the desired separation and sensitivity.

4.1. **Instrument Description**

HPLC System:	Agilent 1290 Infinity
Detector:	Analyst™ software version 1.6.2

4.2. **Chromatography Conditions for XDE-659 Analysis**

Mobile Phase A:	0.1% formic acid in LC-MS H_2O
Mobile Phase B:	0.1% formic acid in LC-MS grade 80: 20 ACN: MeOH + 0.1% formic acid
Flow Rate:	500 µL/min
Column:	Zorbax Eclipse Plus Phenyl Hexyl 3 x 50 mm, 1.8 µm (lot USDER01319
Column Oven Temp:	30°C
Injection Vol.:	5-10 µL
Run Time:	5 min
Detector:	Sciex 6500 QTrap
Retention Time:	~3.24 min

4.3. **Chromatography Conditions for X12485649 Analysis**

Mobile Phase A:	0.1% formic acid in LC-MS H_2O
Mobile Phase B:	0.1% formic acid in LC-MS grade 80: 20 ACN: MeOH + 0.1% formic acid
Flow Rate:	500 µL/min
Column:	Zorbax Eclipse Plus Phenyl Hexyl 3 x 50 mm, 1.8 µm (lot USDER01319)Column Oven Temp: 30°C

Analytical Method – (continued)

Injection Vol.: 5-10 µL
Run Time: 5 min
Detector: Sciex 6500 QTrap
Retention Time: ~2.90 min

Mobile Phase Composition (linear gradient changes):

A gradient elution, using an increased percentage of organic solvent (80: 20 ACN: MeOH +0.1% formic acid) in the mobile phase, is used to resolve interferences and improve separation. See the specific gradient listed below:

Time (Min)	A% 0.1 % formic acid in Water	B% 0.1 % formic acid in 80: 20 ACN: MeOH	Flow (µL/min)
0.00	50	50	500
1.30	40	60	500
2.00	40	60	500
3.00	10	90	500
3.10	0	100	500
4.50	0	100	500
4.60	50	50	500
5.00	50	50	500

Note: Retention times may differ depending upon the flow rate, column, and gradient used.

Acquisition Ions and Compound Dependent Parameters:

Analyte	Mass Transition (*m/z*)	Dwell (msec)	DP (V)	CE (V)	CXP (V)
XDE-659 (Quantitation)	*512.960→231.100*	150.00	86.00	31.00	6.00
XDE-659 (Confirmatory)	*512.960→108.900*	150.00	86.00	73.00	18.00
Analyte	Mass Transition (*m/z*)	Dwell (msec)	DP (V)	CE (V)	CXP (V)
X12485649 (Quantitation)	*470.813→230.900*	150.00	66.00	25.00	18.00
X12485649 (Confirmatory)	*470.813→109.000*	150.00	66.00	55.00	10.00

Typical MS/MS Voltage Conditions Used:

Ionization Mode	ESI
Scan Type	MRM
Polarity	Positive
Resolution Q1	unit
Resolution Q3	unit
Curtain gas (N_2, psi)	50
GS1 (psi)	80
GS2 (psi)	40

Analytical Method – (continued)

CAD gas (N_2)	Medium
Ion Spray (V)	5500
Temperature (°C)	600
EP (V)	10.00
ihe	ON

Initial and Final Q1 and Product Scans can be found in Appendix VI.

Note: The MS settings as provided above should be used as guidelines only. For optimal results, compound and source optimization should be performed by the analyst.

5.0 CALCULATION OF RESULTS

5.1. Multi Point Calibration Procedure

XDE-659 X12485649, may be calculated in ng/mL using a multi-point calibration procedure as follows.

1. Prepare standard solutions over a concentration range appropriate to the expected residues in the samples. An appropriate number of different concentrations within this range should be prepared (at least five).
2. Make an injection of each sample solution and measure the areas of the peaks corresponding to XDE-659 and X12485649. Calibration standard solutions should be interspersed throughout the analysis, after approximately four injections of sample solutions.
3. Calibration standards and samples were analyzed using HPLC/MS-MS. Calibration curves and residue values were calculated using Analyst 1.6.2 data handling software using linear regression (1/x weighting is recommended).

   The standards were fit to the linear equation $y = mx + b$

   Where: x is the concentration of sample in final extract
   m is the calibration line slope
   b is the calibration line intercept
   y is the peak area
4. The following equation can be rearranged and used to calculate residues as follows:

$$\text{XDE} - 659 \text{ or X12485649 Found } \left({}^{\text{ng}}/_{\text{mL}}\right) = \frac{\text{Peak Area} - \text{Curve Intercept}}{\text{Curve Slope}}$$

5.2. Example Calculation

1. Drinking Water sample 204960 LOQ R3 was analyzed.

   Sample volume 10 mL

   Peak area in the quantitation transition was 120472.0925 counts

Analytical Method – (continued)

Calibration curve generated in the run was y = 1083961.260 * x + 14929.2233

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

$$x = \frac{120472.0925 - 14929.2233}{1083961.260}$$

$$x = 0.10\ ^{ng}/_{mL}$$

2. Recovery Results

The recovery factor, expressed as a percentage (Recovery %), is calculated using the following equation:

$$\%\text{Recovery} = \frac{Measured\ concentration\ (ng/mL)}{Theoretical\ concentration\ (ng/mL)} * 100\%$$

Where the measured and control concentrations are taken from instrument outputs for the sample and UTC, respectively, and the theoretical concentration is the known amount of analyte added to the sample, if applicable.

Example:

Drinking Water sample 204960 LOQ R3 was analyzed.

$$\%\text{Recovery} = \frac{0.10\ ng/mL}{0.10\ ng/mL} * 100\% = 97\%$$

6.0 Untreated Control and Recovery Samples

If untreated control samples are available, untreated control samples should be analyzed for each set of samples analyzed to verify that samples are free from analyte contamination. A minimum of two controls should be analyzed with each batch of samples.

7.0 Specificity

7.1. Labware Interference

All reusable glassware is suggested to be detergent washed in hot water and then rinsed with deionized water and acetone prior to use.

7.2. Reagent and Solvent Interference

None.

Analytical Method – (continued)

8.6. **Stability of Analytes in Sample Extracts and in Final Solutions**

Based on sample stability data run one week after original preparation presented in the table below, samples should be analyzed within one week of receipt or preparation. XDE-659 and X12485649 stocks were stable when analyzed two weeks after preperation.

9.0 **LIMITATIONS**

The method has been tested on ground, surface and drinking water. It can reasonably be assumed that the method can be applied to other types of water. Test experiments for the recovery, matrix effects, interferences and sensitivity, etc. are strongly suggested prior to sample analysis.

10.0 **CONCLUSIONS**

This procedure has been demonstrated to be a reliable and accurate procedure for the determination of residues of XDE-659 and X12485649 in ground, surface and drinking water. The limit of quantitation (LOQ) of the method is 0.1 ng/mL for analysis of XDE-659 and X12485649. This method satisfies US EPA guidelines OCSPP (formerly OPPTS) 860.1340, OCSPP 850.6100, SANCO/3029/99 rev. 4, SANCO/825/00 rev. 8.1, and DIR98-02.

Analytical Method – (continued)

12.0 APPENDICES

12.1. Appendix I: Apparatus with Recommended Suppliers

A. HPLC/MS-MS System

1. Agillent 1290 Infinity HPLC System, S/N: DEBAF03419
2. Sciex 6500 QTrap MS with Analyst™ software version 1.6.2. S/N: BL23451401

B. Column: Zorbax Eclipse Plus Phenyl Hexyl 3 x 50 mm, 1.8 µm (lot USDER01319

C. Eppendorf adjustable pipettes, assorted sizes

D. Analytical Balance, for standard prep

E. Vortex, Benchmark Scientific

F. Glassware

Class A volumetric flasks, assorted sizes

Beakers, various sizes

Analytical Method – (continued)

12.2. **Appendix II: Reagents**

A. Solvents and Reagents.

1. LC/MS grade solvents or better should be utilized. Other brands and grades of solvents may be substituted as long as they do not produce interferences with the chromatography.

   a. Acetonitrile, Honeywell, Lot: DW926-US/ OmniSolv, Lot: 193278

   b. Methanol, Omni Solv, Lot: 59235, 194362
   c. Water, Omni Solv, Lot: 195465, 196233, 195466

2. Working Solutions

   a. Mobile phase A: 0.1% formic acid in water: 1.0 mL of formic acid to 1.0 L with water

   b. Mobile phase B: 0.1% formic acid in 80: 20 ACN: MeOH : dilute 1.0 mL of formic acid to 1.0 L with 80: 20 ACN: MeOH.