Independent Laboratory Validation of EPL Bio Analytical Services Method 477G696C for the Determination of XDE-848 Benzyl Ester (SX-1552) and Five Metabolites (1552-Acid, 1552-OHBE, 1552-OHA, 1552-DBE and 1552-DA) in Sediment

INTRODUCTION

Scope

The objective of this study was to assess and to independently validate EPL Bio Analytical Services Method 477G696C, "Determination of XDE-848 Benzyl Ester (SX-1552) and Five Metabolites (1552-Acid, 1552-OHBE, 1552-OHA, 1552-DBE and 1552-DA) in Sediment" [1]. The independent laboratory validation demonstrated that the method can be considered applicable for use in the determination of residues of XDE-848 benzyl ester and five metabolites (1552-Acid, 1552-OHBE, 1552-OHA, 1552-DBE and 1552-DA) in sediment. The methodology was successfully independently validated over the concentration range of 0.003-0.03 µg/g for XDE-848 benzyl ester (SX-1552) and five metabolites (1552-Acid, 1552-OHBE, 1552-OHA, 1552-DBE and 1552-DA) with an independently validated limit of quantification of 0.003 µg/g for XDE-848 benzyl ester (SX-1552) and five metabolites (1552-Acid, 1552-OHBE, 1552-OHA, 1552-DBE and 1552-DA).

The chemical names, molecular structures, molecular formulae and molecular weights for the analytes are given in Table 1.

This study was conducted to fulfil data requirements outlined in EU Commission Regulation No 283/2013 setting out the data requirements, in accordance with Regulation (EC) No 1107/2009 [2] and EPA Guideline OCSPP 850.6100 [3] and Guidance Document SANCO/825/00 rev.8.1 and [4].

Method Principle

A 5 g subsample of sediment was extracted with 4 × 20 mL volumes of 90/10 acetonitrile/0.1N hydrochloric acid. The combined extracts were adjusted to a final volume of 100 mL. Samples were then diluted 1:5 and an internal standard solution was added. Diluted samples with internal standards were analysed by High Performance Liquid Chromatography-Tandem Mass Spectrometry (HPLC-MS/MS).

Test Substances/Reference Compounds/Analytical Standards

All analytical standards were provided by Dow AgroSciences LLC.

Abbreviated ID	Analytical Standard[a]	Percent Purity	Re-Certification Date	Lot Number
SX-1552	XDE-848 Benzyl Ester	99.2	11 Jul 2015	JY-001-174-22
1552-Acid or 1552-A	X11438848	99	25 Oct 2015	201102463-6A
1552-OHBE	X12300837	99	18 Oct 2015	BD-B130064-40-1
1552-OHA	X11966341	98	21 Oct 2015	JY-001-181-40
1552-DBE	X12131932	97	04 Oct 2016	DE3-137773-1
1552-DA	X12393505	98	04 Oct 2016	DE3-133876-86
IS-SX-1552[b]	X12293407	100	09 Oct 2015	XS9-120633-39
IS-1552-Acid[b]	X12293409	100	05 Oct 2016	XS9-120633-41
IS-1552-OHBE[b]	X12400867	99	05 Oct 2016	YC2-134955-54
IS-1552-OHA[b]	X12401027	99	21 Oct 2016	YC2-134955-53-1

[a]The molecular formulae and structures are given in Table 1. The certificates of analysis are given in Figure 1 to Figure 10.
[b]Internal standards were supplied as a mixed 1 µg/mL solution in methanol.

EXPERIMENTAL

Sample Origin and Storage

The independent validation was carried out using a characterised sediment sample, provided by the Sponsor (Sponsor Reference NC401). The sediment sample was characterised by Agvise, Northwood, ND, 58267, in a separate study and full characterisation details are given in Appendix 2. A unique sample number was assigned to the sample to track it during storage and analysis.

Information provided by NDR Research, Plainfield, IN 46168: The characterisation report from Agvise lists 3 different sample numbers (NC403-405) for sediment. Those samples were taken from the same pond, at 3 separate locations that were marked for stations for residue sampling. The bulk sediment received for this study was a cumulative sediment collected from various spots within the pond, so essentially a mix of the 3 Agvise characterisation samples. The pond (number 18) is located on the SePRO Research and Development Campus, near the town of Whitakers (Nash County), NC.

Calculation of Standard Calibration Curve

Calculation of a standard curve begins with the injection of a series of calibration standards described in Appendix 1 and acquisition of peak areas for the following analytes:

SX-1552	*m/z* Q1/Q3 441/65 (quantitative)
SX-1552	*m/z* Q1/Q3 441/91 (confirmatory)
SX-1552 IS	*m/z* Q1/Q3 447/91
1552-A	*m/z* Q1/Q3 349/268 (quantitative)
1552-A	*m/z* Q1/Q3 349/225 (confirmatory)
1552-A IS	*m/z* Q1/Q3 357/276
1552-OHBE	*m/z* Q1/Q3 425/91 (quantitative)
1552-OHBE	*m/z* Q1/Q3 427/91 (confirmatory)
1552-OHBE IS	*m/z* Q1/Q3 433/91
1552-OHA	*m/z* Q1/Q3 335/254 (quantitative)
1552-OHA	*m/z* Q1/Q3 337/256 (confirmatory)
1552-OHA IS	*m/z* Q1/Q3 341/260
1552-DBE	*m/z* Q1/Q3 405/65 (quantitative)
1552-DBE	*m/z* Q1/Q3 407/91 (confirmatory)
SX-1552 IS	*m/z* Q1/Q3 447/91
1552-DA	*m/z* Q1/Q3 315/234 (quantitative)
1552-DA	*m/z* Q1/Q3 315/124 (confirmatory)
1552-OHA IS	*m/z* Q1/Q3 341/260

Isotopically labeled internal standards were not available for 1552-DBE and 1552-DA.

In order to generate a standard curve, the analyte concentration/internal standard concentration was plotted on the abscissa (x-axis) and the respective analyte peak area/internal standard peak area on the ordinate (y-axis) in Analyst. Using linear regression analysis with 1/x weighting, the equation for the curve with respect to the abscissa was determined. Refer to Figure 11 through Figure 22 for example calibration plots and Figure 23 for example calculations. Individual calibration results can be found in Table 2 through Table 13.

Confirmation of Residue Identity

The method is selective for the determination of XDE-848 benzyl ester (SX-1552) and five metabolites by virtue of the chromatographic separation and MS/MS detection. Confirmation demonstrates the selectivity of the primary method for all representative sample matrices. It has to be confirmed that the primary method detects the correct analyte (analyte identity) and that the

analyte signal of the primary method is quantitatively correct and not affected by any other compounds. Full scan mass spectra and product ion spectra are provided in Figure 24 to Figure 29 to justify the selection of ions used for determination of each analyte.

Statistical Treatment of Data

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

Recovery Levels and Precision

The independent laboratory validation study was conducted to determine the recovery levels and the precision of the method for the determination of XDE-848 benzyl ester (SX-1552) and five metabolites in sediment. The performance of the analytical method was determined with each set of samples by fortifying aliquots of appropriate control matrix with XDE-848 benzyl ester (SX-1552) and five metabolites and analysing the set following the procedures described in this report. Samples were fortified at the limit of detection (LOD) of 0.0009 µg/g for XDE-848 benzyl ester and five metabolites, the limit of quantitation (LOQ) of 0.003 µg/g for XDE-848 benzyl ester and five metabolites, and at the higher fortification level of 0.03 µg/g for XDE-848 benzyl ester and five metabolites (10 x LOQ). Samples fortified at the LOD were analysed only to demonstrate that observable peaks at the LOD level could be distinguished from untreated control samples; the results were not included for average percent recovery calculations. Two unfortified control samples and a reagent blank were also included in each set.

Changes to the Method

A minor modification was made to the method in order for it to be independently validated. The injection volume was increased from 15 µL to 40 µL due to poor sensitivity at the lower injection volume. This did not negatively impact the results, as satisfactory chromatography and recovery data were achieved.

To ensure the stability of standards throughout the study, standards in methanol were stored in a freezer not in a refrigerator as stated in the method.

Table 1 Identity and Structure of XDE-848 Benzyl Ester and Five metabolites (1552 Acid, 1552-OHBE, 1552-OHA, 1552-DBE and 1552-DA) and Internal Standards

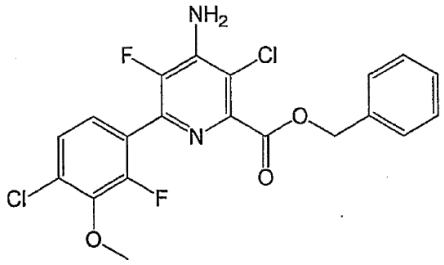
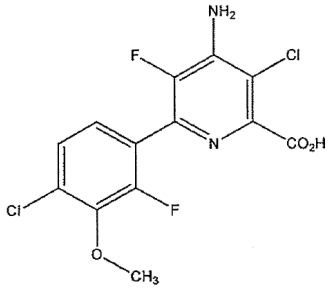
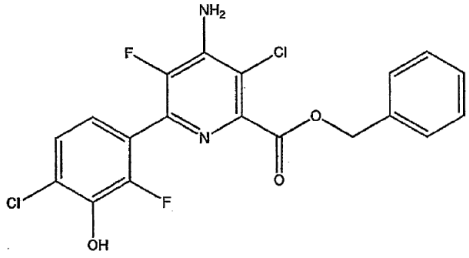
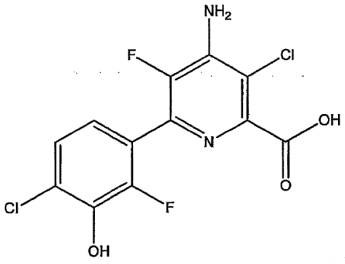
Name	Structural Formula and Chemical Name
SX-1552 XDE-848 Benzyl Ester (X11959130) Molecular Formula: $C_{20}H_{14}Cl_2F_2N_2O_3$ Molecular Weight: 439.24 CAS number: 1390661-72-9	 Benzyl 4-amino-3-chloro-6-(4-chloro-2-fluoro-3-methoxyphenyl)-5-fluoropyridine-2-carboxylate
1552-Acid Molecular Formula: $C_{13}H_8Cl_2F_2N_2O_3$ Molecular Weight: 349.12 CAS number: 943832-81-3	 4-amino-3-chloro-6-(4-chloro-2-fluoro-3-methoxyphenyl)-5-fluoropyridine-2-carboxylic acid
1552-OHBE Molecular Formula: $C_{19}H_{12}Cl_2F_2N_2O_3$ Molecular Weight: 425.21	 Benzyl 4-amino-3-chloro-6-(4-chloro-2-fluoro-3-hydroxyphenyl)-5-fluoropyridine-2-carboxylate
1552-OHA Molecular Formula: $C_{12}H_6Cl_2F_2N_2O_3$ Molecular Weight: 335.09	 4-amino-3-chloro-6-(4-chloro-2-fluoro-3-hydroxyphenyl)-5-fluoropyridine-2-carboxylic acid

Table 1 Cont. Identity and Structure of XDE-848 Benzyl Ester and Five metabolites (1552 Acid, 1552-OHBE, 1552-OHA, 1552-DBE and 1552-DA) and Internal Standards

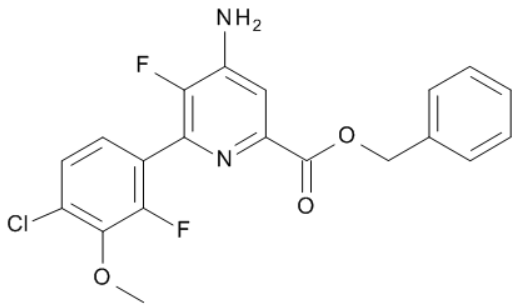
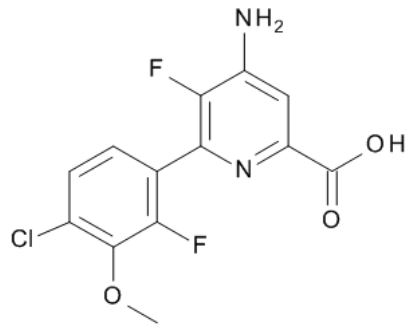
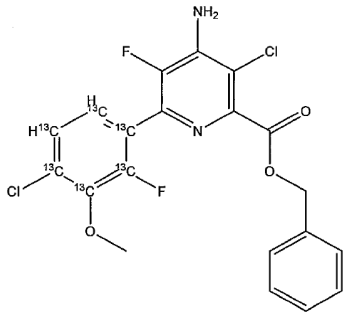
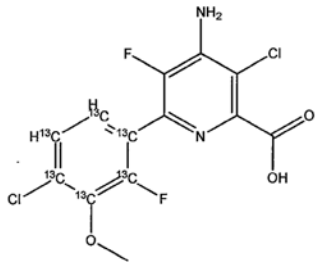
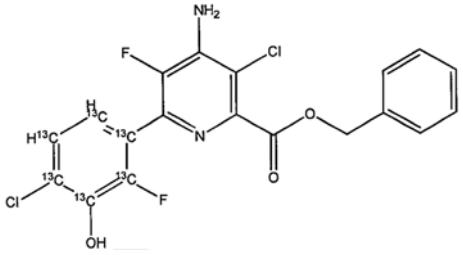
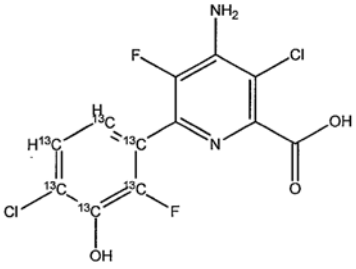
1552-DBE Molecular Formula: $C_{20}H_{15}ClF_2N_2O_3$ Molecular Weight: 404.79	 Benzyl 4-amino-6-(4-chloro-2-fluoro-3-methoxyphenyl)-5-fluoropyridine-2-carboxylate
1552-DA Molecular Formula: $C_{13}H_9ClF_2N_2O_3$ Molecular Weight: 314.67	 4-amino-6-(4-chloro-2-fluoro-3-methoxyphenyl)-5-fluoropyridine-2-carboxylic acid

Table 1 Cont. Identity and Structure of XDE-848 Benzyl Ester and Five metabolites (1552 Acid, 1552-OHBE, 1552-OHA, 1552-DBE and 1552-DA) and Internal Standards

Common Name	Structural Formula and Chemical Name
IS-SX-1552 XDE-848 Benzyl Ester IS Molecular Formula: $C_{14}{}^{13}C_6H_{14}Cl_2F_2N_2O_3$ Molecular Weight: 445.20	 Benzyl 4-amino-3-chloro-6-(4-chloro-2-fluoro-3-methoxy(13C6)phenyl)-5-fluoropyridine-2-carboxylate
IS-1552-A Molecular Formula: $C_7{}^{13}C_6H_8Cl_2F_2N_2O_3$ Molecular Weight: 355.07	 4-amino-3-chloro-6-(4-chloro-2-fluoro-3-methoxy(13C6)phenyl)-5-fluoropyridine-2-carboxylic acid
IS-1552-OHBE Molecular Formula: $C_{13}{}^{13}C_6H_{12}Cl_2F_2N_2O_3$ Molecular Weight: 431.17	 Benzyl 4-amino-3-chloro-6-(4-chloro-2-fluoro-3-hydroxy(13C6)phenyl)-5-fluoropyridine-2-carboxylate
IS-1552-OHA Molecular Formula: $C_6{}^{13}C_6H_6Cl_2F_2N_2O_3$ Molecular Weight: 341.05	 4-amino-3-chloro-6-(4-chloro-2-fluoro-3-hydroxy(13C6)phenyl)-5-fluoropyridine-2-carboxylic acid

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Laboratory Equipment

Balance, MSU225S Cubis 220-5, Sartorius Ltd
Balance, GX1000, A&D
Barnstead Smart2Pure 6UV Water Purification System, Thermo Scientific
Centrifuge Varifuge 3.0R, Heraeus Instruments Ltd
Pipettes, Gilson 'Microman', Anachem Ltd
Pipettes, Rainin Pos-D, Anachem Ltd
Vortex mixer, VWR International

Glassware and Materials

Centrifuge tubes, 50 mL plastic, VWR International
Volumetric flasks, Fisher Scientific

Chromatographic Systems

Autosampler, CTC Analytics HTS-xt PAL, Eksigent
Column, Kinetex PFP, 100 mm x 2.1 mm x 1.7 µm, Phenomenex
Liquid chromatograph, Agilent 1290, Binary Pump and Column Oven, Agilent Technologies UK
Mass spectrometer, QTRAP 6500, electrospray ionization with TurboIonSpray probe, AB Sciex
Software, Analyst version 1.6.2, AB Sciex

Reagents

Acetonitrile, HPLC Grade, Sigma Aldrich
Formic Acid (99-100%), Fisher Scientific
Hydrochloric Acid (32%), Fisher Scientific
Methanol, HPLC Grade, Sigma Aldrich
Propan-2-ol, HPLC Grade, Fisher Scientific

Prepared Solutions

0.1% Formic Acid in Ultrapure Water:
500 mL of ultrapure water was added to a 1 litre volumetric flask. 1 mL of formic acid was added. The solution was brought to volume with ultrapure water and mixed thoroughly.

0.1% Formic Acid in Methanol:
500 mL of methanol was added to a 1 litre volumetric flask. 1 mL of formic acid was added. The solution was brought to volume with methanol and mixed thoroughly.

Methanol/Water/Formic Acid 50/50/0.1 (v/v/v):
500 mL of methanol and 500 mL of ultrapure water were combined and 1 mL of formic acid was added into the mixture and mixed thoroughly.

0.1 N Hydrochloric Acid (v/v)
150 mL of ultrapure water was added to a 200 mL volumetric flask. 19.6 mL of hydrochloric acid (32%) was added. The solution was brought to volume with ultrapure water and mixed thoroughly.

90/10 Acetonitrile/0.1 N Hydrochloric Acid (v/v)
1350 mL of acetonitrile and 150 mL of 0.1 N hydrochloric acid were combined and mixed thoroughly.

50/50 Methanol/0.1% Formic Acid (v/v)
50 mL of methanol and 50 mL of 0.1% formic acid in ultrapure water were combined and mixed thoroughly.

Methanol/Isopropyl Alcohol/Ultrapure Water 40/40/20 (v/v/v):
400 mL of methanol, 400 mL of isopropyl alcohol and 200 mL of ultrapure water were combined and mixed thoroughly.

Methanol/Ultrapure Water 10/90 (v/v):

200 mL of methanol and 1800 mL of ultrapure water were combined and mixed thoroughly.

Preparation of Standard Solutions

All standard solutions prepared in methanol were stored in a freezer. All standard solutions prepared in methanol/water (50:50) containing 0.1% formic acid were stored in a fridge.

Preparation of Stock Solutions

25 mg (adjusted for purity) of each reference item was weighed into a separate 25 mL volumetric flask and diluted to volume with methanol to obtain 1000 µg/mL stock solutions of each analyte.

Preparation of Mixed Fortification and Calibration Intermediate Standard Solutions

Mixed fortification and calibration intermediate standard solutions were prepared by pipetting the appropriate amount of standard solutions with methanol in volumetric flasks as described in the following table:

Concentration of Solution	Aliquot (mL)	Final Volume (mL)	Solution Final Concentration
1000 µg/mL SX-1552	0.1	10	10 µg/mL
1000 µg/mL 1552-A	0.1		
1000 µg/mL 1552-OHBE	0.1		
1000 µg/mL 1552-OHA	0.1		
1000 µg/mL 1552-DBE	0.1		
1000 µg/mL 1552-DA	0.1		
10 µg/mL	1	10	1000 ng/mL
1000 ng/mL	1	10	100 ng/mL
100 ng/mL	1	10	10 ng/mL
10 ng/mL	1	10	1 ng/mL

A mixed solution for the preparation of matrix-matched standards was prepared by pipetting the appropriate amount of standard solution with methanol/water (50:50) containing 0.1% formic acid solution in a volumetric flask as described in the following table:

Concentration of Solution	Aliquot (mL)	Final Volume (mL)	Solution Final Concentration
10 ng/mL	1	10	1 ng/mL

Preparation of Internal Standard Solutions

1. 1 µg/mL XDE-848-BE mixed internal standard solution in methanol supplied by Dow AgroSciences.
2. 1 mL of the 1 µg/mL XDE-848-BE mixed internal standard solution was pipetted into a 10 mL volumetric flask and diluted to volume with methanol to obtain a 100 ng/mL solution.
3. 1 mL of the 100 ng/mL XDE-848-BE mixed internal standard solution was pipetted into a 10 mL volumetric flask and diluted to volume with methanol to obtain a 10 ng/mL solution.

Preparation of Mixed Calibration Standards

Mixed calibration standards were prepared by dispensing 0.5 mL of the 10 ng/mL mixed internal standard solution into a 10 mL volumetric flask, before diluting the appropriate amount of mixed standard solution with a methanol/water (50:50) containing 0.1% formic acid solution to obtain calibration solutions over the concentration range of 0.005-50 ng/mL as described in the following table:

Concentration of Solution (ng/mL)	Volume Taken (mL)	Volume 10 ng/mL ISTD (mL)	Final Volume (mL)	Calibration Solution Final Conc. (ng/mL)	Equivalent Sample Conc. (µg/g)[a]
1000	0.5	0.5	10	50	5
1000	0.1	0.5	10	10	1
100	0.1	0.5	10	1	0.1
100	0.05	0.5	10	0.5	0.05
10	0.15	0.5	10	0.15	0.015
10	0.05	0.5	10	0.05	0.005
1	0.15	0.5	10	0.015	0.0015
1	0.05	0.5	10	0.005	0.0005

[a] The equivalent sample concentrations are based on taking an initial sample weight of 5 g, extracting with 100 mL and diluting 1:5.

Preparation of Mixed Matrix-Matched Standards

Mixed 0.05 ng/mL matrix-matched calibration standards were prepared in triplicate by taking a 200 µL aliquot of a 100 mL control sample extract prior to the 1:5 dilution, adding 700 µL of methanol/water (50:50) containing 0.1% formic acid solution, 0.05 mL of the 10 ng/mL mixed internal standard solution, and 0.05 mL of the 1 ng/mL mixed standard solution methanol/water (50:50) containing 0.1% formic acid.

Analytical Procedure

A. **Sample Weighing and Fortification**

1. 5 g (5.0 - 5.1 g) of sediment was weighed into a plastic 50 mL centrifuge tube.
2. Laboratory fortifications for recovery determinations were prepared using control sediment subsamples.

   LOQ Fortification: 150 µL of the 100 ng/mL mixed standard solution was pipetted directly on to the sediment matrix.

   10x LOQ Fortification: 150 µL of the 1,000 ng/mL mixed standard solution was pipetted directly on to the sediment matrix.

B. **Sample Extraction**

1. 20 mL of 90/10 acetonitrile/0.1 N HCl was added to the sample tube.
2. The sample was vortexed to mix.
3. The sample was shaken for 30 minutes on a flatbed shaker set at 180 rpm.
4. The sample was centrifuged at 2,000 rpm for 5 minutes.
5. The supernatant extract was decanted into a 100 mL volumetric flask.
6. Steps 1-5 were repeated three additional times, combining the extracts in the same 100 mL volumetric flask.
7. The 100 mL volumetric flask was brought to volume with 90/10 acetonitrile/0.1 N HCl and mixed. An aliquot was transferred to a glass vial or bottle and store refrigerated.

C. **Sample Extract Dilution**

1. 750 µL of 50/50 methanol/0.1% formic acid in ultrapure water was pipetted into an HPLC autosampler vial.
2. 200 µL of the sample extract was pipetted into the autosampler vial.
3. 50 µL of the 10 ng/mL mixed internal standard solution was pipetted into the autosampler vial. The vial was capped and mixed.

Instrumental Conditions

Instrumentation

Autosampler:	Eksigent CTC Analytics HTS-xt PAL
Liquid Chromatograph:	Agilent 1290 Binary Pump and Column Oven
Mass Spectrometer:	AB Sciex QTRAP 6500
Software:	AB Sciex Analyst, version 1.6.2

Typical Liquid Chromatography Operating Conditions

Column:	Phenomenex Kinetex PFP, 100 mm x 2.1 mm x 1.7 µm
Column Temperature:	35ºC
Injection Volume:	40 µL
Flow Rate:	300 µL/min
Autosampler Wash 1:	Methanol/Isopropyl Alcohol/Ultrapure Water (40/40/20)
Autosampler Wash 2:	Methanol/Water (10/90)
Mobile Phase A:	Ultrapure water containing 0.1% formic acid
Mobile Phase B:	Methanol containing 0.1% formic acid

Gradient:

Time – minutes	% Mobile Phase A	% Mobile Phase B
0.00	90	10
7.00	0	100
8.50	0	100
8.60	90	10
11.00	90	10

Typical Mass Spectrometry Operating Conditions

Ion Source:	Turbo Ion Spray
Polarity:	Positive
Ion Spray Voltage (IS):	5500
Collision Gas (CAD):	High
Curtain Gas (CUR):	20
Temperature (TEM):	650
Ion Source Gas 1 (GS1):	50
Ion Source Gas 2 (GS2):	60

Analyte	Ions (*m/z*)		Dwell Time (msec)	Declustering Potential DP (V)	Entrance Potential EP (V)	Collision Energy CE (V)	Collision Cell Exit Potential CXP (V)
	Q1 Mass (*m/z*)	Q3 Mass (*m/z*)					
SX-1552	441.1	65.1 (Q)	50	20	10	120	18
	441.1	91.0 (C)	40	20	10	60	18
1552-A	349.0	268.0 (Q)	50	45	10	42	25
	349.0	225.0 (C)	75	45	10	65	25
1552-OHBE	425.0	91.0 (Q)	40	50	8	60	15
	427.0	91.0 (C)	40	50	8	60	15
1552-OHA	334.9	254.0 (Q)	75	60	10	45	15
	336.9	256.0 (C)	150	60	10	45	15
1552-DBE	404.8	65.1 (Q)	40	50	10	110	20
	407.0	91.0 (C)	40	50	10	60	20
1552-DA	315.0	234.0 (Q)	40	55	10	39	15
	315.0	124.0 (C)	75	55	10	95	15
IS-SX-1552	447.0	91.0	25	20	10	60	18
IS-1552-A	357.0	276.0	25	45	10	42	25
IS-1552-OHA	341.0	260.0	25	60	10	45	15
IS-1552-OHBE	433.0	91.0	25	50	8	60	15

(Q) = Quantitation Ion, (C) = Confirmatory Ion

Note: IS-SX-1552 is used as the internal standard for 1552-DBE. IS-1552-OHA is used as an internal standard for 1552-DA. Isotopically labeled internal standards were not available for 1552-DBE and 1552-DA.