

Independent Laboratory Validation of EPL Bio Analytical Services Method 477G696A-1 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 Water

## INTRODUCTION

### Scope

The objective of this study was to assess and to independently validate EPL Bio Analytical Services Method 477G696A-1, “Determination of XDE-848 Benzyl Ester (SX-1552) and Five Metabolites (1552-Acid, 1552-OHBE, 1552-OHA, 1552-DBE and 1552-DA) in Water” [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 drinking water, ground water and surface water. The methodology was successfully independently validated over the concentration range of 0.02-0.2 ng/mL for XDE-848 benzyl ester (SX-1552) and 0.05-0.5 ng/mL for five metabolites (1552-Acid, 1552-OHBE, 1552-OHA, 1552-DBE and 1552-DA) with an independently validated limit of quantification of 0.02 ng/mL for XDE-848 benzyl ester (SX-1552) and 0.05 ng/mL for 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 OCSP 850.6100 [3] and Guidance Document SANCO/825/00 rev.8.1 [4].

### Method Principle

Water samples were analysed for residues of XDE-848 benzyl ester (SX-1552) and five metabolites (1552-Acid, 1552-OHBE, 1552-OHA, 1552-DBE and 1552-DA) by adding aliquots of formic acid, methanol and internal standard. The final sample was analysed for XDE-848 benzyl ester (SX-1552) and five metabolites (1552-Acid, 1552-OHBE, 1552-OHA, 1552-DBE and 1552-DA) 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 <sup>a</sup>	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 <sup>b</sup>	X12293407	100	09 Oct 2015	XS9-120633-39
IS-1552-Acid <sup>b</sup>	X12293409	100	05 Oct 2016	XS9-120633-41
IS-1552-OHBE <sup>b</sup>	X12400867	99	05 Oct 2016	YC2-134955-54
IS-1552-OHA <sup>b</sup>	X12401027	99	21 Oct 2016	YC2-134955-53-1

<sup>a</sup>The molecular formulae and structures are given in Table 1. The certificates of analysis are given in Figure 1 to Figure 10.

<sup>b</sup>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 fully characterised drinking water, ground water and surface water samples, obtained from Battelle UK stocks of control samples. The water samples were characterised by Agvise, Northwood, ND, 58267, in a separate study and full characterisation details are given in Appendix 3. Unique sample numbers were assigned to the samples to track them during storage, and analysis.

### 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	<i>m/z</i> Q1/Q3 441/65 (quantitative)
SX-1552	<i>m/z</i> Q1/Q3 441/91 (confirmatory)
SX-1552 IS	<i>m/z</i> Q1/Q3 447/91
1552-A	<i>m/z</i> Q1/Q3 349/268 (quantitative)
1552-A	<i>m/z</i> Q1/Q3 349/225 (confirmatory)
1552-A IS	<i>m/z</i> Q1/Q3 357/276
1552-OHBE	<i>m/z</i> Q1/Q3 425/91 (quantitative)
1552-OHBE	<i>m/z</i> Q1/Q3 427/91 (confirmatory)
1552-OHBE IS	<i>m/z</i> Q1/Q3 433/91
1552-OHA	<i>m/z</i> Q1/Q3 335/254 (quantitative)
1552-OHA	<i>m/z</i> Q1/Q3 337/256 (confirmatory)
1552-OHA IS	<i>m/z</i> Q1/Q3 341/260
1552-DBE	<i>m/z</i> Q1/Q3 405/65 (quantitative)
1552-DBE	<i>m/z</i> Q1/Q3 407/91 (confirmatory)
SX-1552 IS	<i>m/z</i> Q1/Q3 447/91
1552-DA	<i>m/z</i> Q1/Q3 315/234 (quantitative)
1552-DA	<i>m/z</i> Q1/Q3 315/124 (confirmatory)
1552-OHA IS	<i>m/z</i> 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 46 for example calibration plots and to Figure 47 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 48 to Figure 53 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 drinking water, ground water and surface water. 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 0.006 ng/mL for XDE-848 benzyl ester and 0.015 ng/mL for five metabolites (1552-Acid, 1552-OHBE, 1552-OHA, 1552-DBE and 1552-DA), the limit of quantitation (LOQ) of 0.02 ng/mL for XDE-848 benzyl ester and 0.05 ng/mL for five metabolites (1552-Acid, 1552-OHBE, 1552-OHA, 1552-DBE and 1552-DA), and at the higher fortification level of 0.2 ng/mL for XDE-848 benzyl ester and 0.5 ng/mL for five metabolites (1552-Acid, 1552-OHBE, 1552-OHA, 1552-DBE and 1552-DA) (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  $\mu\text{L}$  to 40  $\mu\text{L}$  due to poor sensitivity at the lower injection volume. This did not negatively impact the results, as satisfactory chromatography and recovery data were achieved.

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

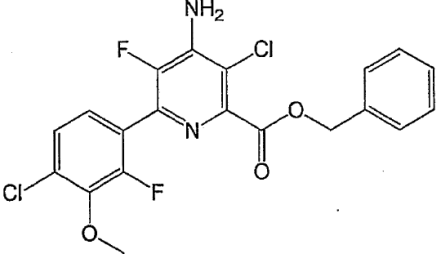
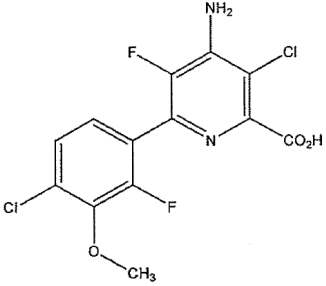
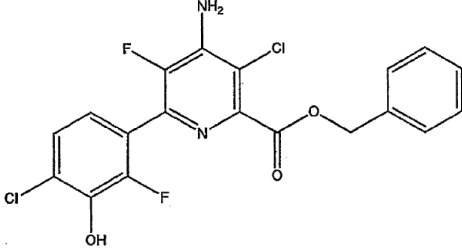
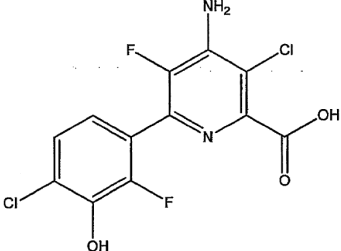
Name	Structural Formula and Chemical Name
<p>SX-1552 XDE-848 Benzyl Ester (X11959130)</p> <p>Molecular Formula: C<sub>20</sub>H<sub>14</sub>Cl<sub>2</sub>F<sub>2</sub>N<sub>2</sub>O<sub>3</sub></p> <p>Molecular Weight: 439.24</p> <p>CAS number: 1390661-72-9</p>	 <p>Benzyl 4-amino-3-chloro-6-(4-chloro-2-fluoro-3-methoxyphenyl)-5-fluoropyridine-2-carboxylate</p>
<p>1552-Acid</p> <p>Molecular Formula: C<sub>13</sub>H<sub>8</sub>Cl<sub>2</sub>F<sub>2</sub>N<sub>2</sub>O<sub>3</sub></p> <p>Molecular Weight: 349.12</p> <p>CAS number: 943832-81-3</p>	 <p>4-amino-3-chloro-6-(4-chloro-2-fluoro-3-methoxyphenyl)-5-fluoropyridine-2-carboxylic acid</p>
<p>1552-OHBE</p> <p>Molecular Formula: C<sub>19</sub>H<sub>12</sub>Cl<sub>2</sub>F<sub>2</sub>N<sub>2</sub>O<sub>3</sub></p> <p>Molecular Weight: 425.21</p>	 <p>Benzyl 4-amino-3-chloro-6-(4-chloro-2-fluoro-3-hydroxyphenyl)-5-fluoropyridine-2-carboxylate</p>
<p>1552-OHA</p> <p>Molecular Formula: C<sub>12</sub>H<sub>6</sub>Cl<sub>2</sub>F<sub>2</sub>N<sub>2</sub>O<sub>3</sub></p> <p>Molecular Weight: 335.09</p>	 <p>4-amino-3-chloro-6-(4-chloro-2-fluoro-3-hydroxyphenyl)-5-fluoropyridine-2-carboxylic acid</p>

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

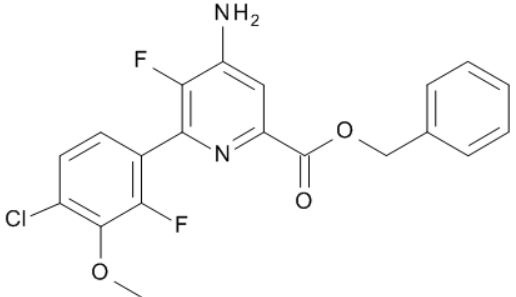
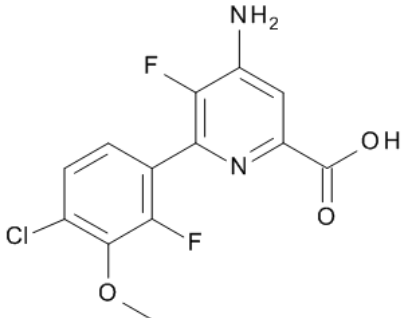
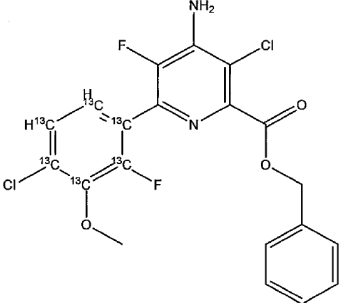
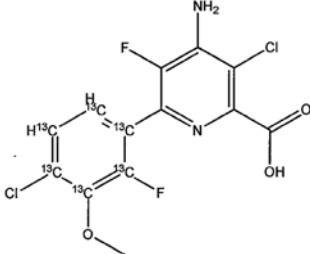
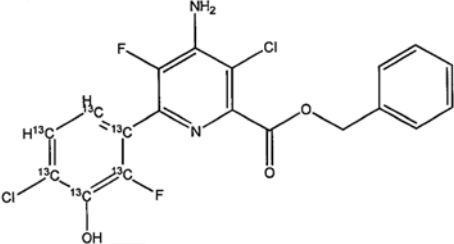
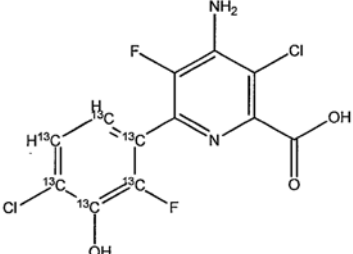
<p>1552-DBE</p> <p>Molecular Formula: <math>C_{20}H_{15}ClF_2N_2O_3</math></p> <p>Molecular Weight: 404.79</p>	 <p>Benzyl 4-amino-6-(4-chloro-2-fluoro-3-methoxyphenyl)-5-fluoropyridine-2-carboxylate</p>
<p>1552-DA</p> <p>Molecular Formula: <math>C_{13}H_9ClF_2N_2O_3</math></p> <p>Molecular Weight: 314.67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

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



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### Laboratory Equipment

Balance, MSU225S Cubis 220-5, Sartorius Ltd

Barnstead Smart2Pure 6UV Water Purification System, Thermo Scientific

Pipettes, Gilson 'Microman' and Pipetman, Anachem Ltd

Pipettes, Rainin Pos-D, Anachem Ltd

Vortex mixer, VWR International

### Glassware

Glass vials, Fisher Scientific

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  $\mu$ m, Phenomenex

Liquid chromatograph, Agilent 1290, Binary Pump and Column Oven, Agilent Technologies UK

Mass spectrometer, QTRAP 6500, electrospray ionization with TurboIonSpray probe, Applied BioSystems/AB Sciex

Software, Applied BioSystems/AB Sciex Analyst, version 1.6.2

### Reagents

Formic Acid (99-100%), 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 methanol and 500 mL of ultrapure water were combined and 1 mL of formic acid was added into the mixture 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 Calibration Intermediate Standard Solutions

Intermediate calibration standards 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
1 ng/mL	1	10	0.1 ng/mL

Preparation of Internal Standard of 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 1 mL of the 10 ng/mL mixed internal standard solution into a 20 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. (ng/mL) <sup>a</sup>
1000	1	1	20	50	50
1000	0.2	1	20	10	10
100	0.2	1	20	1	1
100	0.1	1	20	0.5	0.5
10	0.3	1	20	0.15	0.15
10	0.1	1	20	0.05	0.05
1	0.3	1	20	0.015	0.015
1	0.1	1	20	0.005	0.005

<sup>a</sup>The equivalent sample concentrations are based on taking an initial sample volume of 5 mL and having a final volume of 5 mL.

#### Preparation of Mixed Matrix-Matched Calibration Standards

Mixed matrix-matched calibration standards were prepared by taking 5 mL aliquots of the water sample, adding 5 µL of formic acid, 0.025 mL of the 100 ng/mL mixed internal standard solution, the appropriate amount of mixed standard solution, and an appropriate amount of methanol so that the volume of standards in methanol and methanol together was 0.3 mL, 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 100 ng/mL ISTD (mL)	Volume Methanol (mL)	Volume Formic Acid (µL)	Final Volume Taken as (mL)	Calibration Solution Final Conc. (ng/mL)	Equivalent Sample Conc. (ng/mL) <sup>a</sup>
1000	0.25	0.025	0.025	5	5	50	50
1000	0.05	0.025	0.225	5	5	10	10
100	0.05	0.025	0.225	5	5	1	1
100	0.025	0.025	0.25	5	5	0.5	0.5
10	0.075	0.025	0.2	5	5	0.15	0.15
1	0.25	0.025	0.025	5	5	0.05	0.05
1	0.075	0.025	0.2	5	5	0.015	0.015
0.1	0.25	0.025	0.025	5	5	0.005	0.005

<sup>a</sup>The equivalent sample concentrations are based on taking an initial sample volume of 5 mL and having a final volume of 5 mL.

Preparation of Mixed Fortification Solutions

Mixed standards were prepared by pipetting the appropriate amount of mixed 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	50	2 µg/mL SX-1552 5 µg/mL Metabolites
1000 µg/mL 1552-A	0.25		
1000 µg/mL 1552-OHBE	0.25		
1000 µg/mL 1552-OHA	0.25		
1000 µg/mL 1552-DBE	0.25		
1000 µg/mL 1552-DA	0.25		
2 µg/mL SX-1552 5 µg/mL Metabolites	0.1	10	20 ng/mL SX-1552 50 ng/mL Metabolites
20 ng/mL SX-1552 50 ng/mL Metabolites	1	10	2 ng/mL SX-1552 5 ng/mL Metabolites
20 ng/mL SX-1552 50 ng/mL Metabolites	0.3	10	0.6 ng/mL SX-1552 1.5 ng/mL Metabolites

Analytical Procedure

1. Thawed water samples were mixed thoroughly by inverting several times.
2. 5 mL of ultrapure water was pipetted into a glass vial for the reagent blank.
3. 5 mL of the water samples were pipetted into glass vials for controls and fortified samples.
4. 5 µL of formic acid was added to each vial.
5. 225 µL of methanol was added to each vial.
6. 25 µL of the 100 ng/mL internal standard solution was added to each vial.

7. The samples were fortified with a mixed fortification standard as detailed below:

Sample Description	Fortification Volume (mL)	Fortification Solution	Fortification Level
LOD	0.05	0.6 ng/mL SX-1552	0.006 ng/mL SX-1552
		1.5 ng/mL Metabolites	0.015 ng/mL Metabolites
LOQ	0.05	2 ng/mL SX-1552	0.02 ng/mL SX-1552
		5 ng/mL Metabolites	0.05 ng/mL Metabolites
100 × LOQ	0.05	20 ng/mL SX-1552	0.2 ng/mL SX-1552
		50 ng/mL Metabolites	0.5 ng/mL Metabolites

8. The samples were vortexed thoroughly to mix.

9. Using a glass Pasteur pipette, an aliquot of the sample solution was transferred to a glass HPLC vial and capped. The vial samples were stored refrigerated.

### 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: 35°C  
 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	% Mobile
		Phase A	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)	50	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)	100	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.

## APPENDIX 2            COMMUNICATIONS

17 June 2015 – Communication by email – the Study Director informed the Study Monitor that the analysis of samples had failed due to high recoveries for 1552-DBE and that this was probably due to the different solvent ratios of samples and standards giving different responses on the mass spectrometer. 1552-DBE does not have its own internal standard and uses IS-SX-1552. They are affected differently by the change in solvent ratios. Standards are prepared in methanol/water/formic acid (50/50/0.1) and samples are prepared in water/methanol/formic acid (5/0.3/0.005). IS-SX-1552 is affected differently from 1552-DBE and therefore the peak area ratio for 1552-DBE is higher, leading to high recoveries.

17 June 2015 – Communication by email – Fred Claussen (EPL) asked the Study Director if they were using an AB Sciex 6500 and if not they may need to use matrix-matched standards.

17 June 2015 – Communication by email – the Study Director informed Fred Claussen (EPL) and the Study Monitor that the ILV performing laboratory was using an AB Sciex 6500 and also that changing the calibration standards for the ILV would be acceptable.