

### **3.5 Determination of SX-1552 and Five Metabolites in Water Samples by HPLC-MS/MS**

Water samples were analyzed for active ingredient and the five metabolites content using EPL BAS method 477G696A-1.

### 3.5.1 Reagents

Methanol (MeOH), HPLC Grade, EMD

Formic Acid, 88%, Fisher Scientific

Water, Deionized (DI), as delivered by a Barnstead NANOpure water system

0.1 N Hydrochloric Acid, Fisher Scientific

0.1% Formic Acid in DI Water: Prepared by adding 1.0 mL of formic acid to a 1 L volumetric flask containing approximately 500 mL of DI water and diluting to volume with DI water. Solution was mixed thoroughly and stored ambient.

0.1% Formic Acid in MeOH: Prepared by adding 1.0 mL of formic acid to a 1 L volumetric flask containing approximately 500 mL of MeOH. Brought to volume with MeOH. Solution was mixed thoroughly and stored ambient.

50:50:0.1 Methanol:DI water: Formic Acid: Prepared by mixing 500 mL of methanol and 500 mL of DI water in a 1L bottle. 1.0 mL of formic acid was pipetted into the solution. Solution was mixed thoroughly and stored ambient.

Sample Dilution Solution: Prepared by mixing 9.0 mL of Methanol, 1.0 mL of 10 ng/mL internal standard, and 10.0 mL of 0.1% Formic Acid in DI water into an appropriate vessel. Solution was mixed thoroughly and stored refrigerated.

### 3.5.2 Equipment and Instruments

Class A Volumetric glassware

Temperature controlled sonicating water bath

Amber scintillation vials

Transfer pipettes, Pasteur, glass

Culture tubes, glass, 15 mL with screw caps

Orbital Shaker

Bench-top tube vortexer

Analytical Columns: Kinetex PFP (100 x 2.10 mm, 1.7  $\mu$ m)

High-Performance Liquid Chromatograph (HPLC), Agilent 1290

HPLC autosampler glass vials with screw caps, Waters

Mass spectrometer detector, 6500 Q Trap, AB Sciex

Pipetters, fixed and adjustable volume

HPLC-MS/MS data system, Analyst version 2.1, AB Sciex

### 3.5.3 Water Travel Spike Preparation and Shipment

Water travel spikes were prepared at EPL BAS. Travel spikes were prepared and shipped to both study sites, received back at EPL BAS and tested prior to the discovery of the parent compound and benzyl ester compounds (OHBE and DBE) adsorbing to the glass vials. Per the request of the study director (protocol amendment 4), the water

travel spikes were re-prepared and shipped after the method modification was in place. Aliquots (5 mL) of control water were dispensed into 40 mL amber glass scintillation vials. Two sets of travel spikes were prepared (one for each site). Each set contained an unspiked control and triplicate sub-samples fortified with only one compound each for a total of 19 samples per set. A separate set of verification samples (one for each site) were prepared concurrently with the travel spike samples consisting of one unspiked control and a single sub-sample fortified with only one compound for a total of 7 samples per set. For each compound, 0.125 mL of a single component reference substance solution at a concentration of approximately 1,000 ng/mL was added directly to the water in the scintillation vial. This resulted in a travel spike concentration of 25 ppb (ng/mL). Travel spikes were shipped to the respective field sites frozen on dry ice. After the re-preparations, the initial set of travel spikes sent to the Florida site arrived thawed. Another set of travel spikes and verifications were prepared and shipped to the Florida site. The second set of travel spike verifications prepared for the Florida site will not be reported due to unacceptable recovery of the fortification samples ran with the set. Water travel spikes were received from the field with the North Carolina site event 19 and 20 (7 and 8 month) water dissipation samples and with the Florida site event 18-21 (6-9 month) water dissipation samples. For set TS005, travel spike samples from North Carolina, sample NC756 was broken in transit. Only two values will be used to report the average recovery of SX-1552 for the North Carolina travel spikes.

### 3.5.4 Sample Preparation: SX-1552 and Five Metabolites in Water

Water samples were transferred to refrigerated storage just prior to testing in order to thaw. Once thawed, the samples were thermally equilibrated to ambient temperature before processing in the laboratory. Water samples were inverted to mix thoroughly.

### 3.5.5 Water Sample Preparation

The reagent blank, unfortified control, fortified control, and water samples were extracted by adding the following to a 15 mL glass culture tube:

Reagent Blank	Unfortified Control	Fortified Control	Water Samples
<ul style="list-style-type: none"> <li>• 5 mL DI Water</li> <li>• 5 µL formic acid</li> <li>• 225 µL methanol</li> <li>• 25 µL of 100 ng/mL internal standard</li> </ul>	<ul style="list-style-type: none"> <li>• 5 mL reagent water</li> <li>• 5 µL formic acid</li> <li>• 225 µL methanol</li> <li>• 25 µL of 100 ng/mL internal standard</li> </ul>	<ul style="list-style-type: none"> <li>• 5 mL reagent water</li> <li>• 5 µL formic acid</li> <li>• 225 µL methanol</li> <li>• Specified amount of mixed analyte spike</li> <li>• 25 µL of 100 ng/mL internal standard</li> </ul>	<ul style="list-style-type: none"> <li>• 5 mL water sample</li> <li>• 5 µL formic acid</li> <li>• 225 µL methanol</li> <li>• 25 µL of 100 ng/mL internal standard</li> </ul> <p>Note: Water samples were vortexed to mix thoroughly.</p>

An aliquot of each sample was transferred using a glass Pasteur pipet into a glass HPLC autosampler vial.

### 3.5.6 Methanol Rinse Preparation

The remaining original water was transferred to a separate amber glass scintillation vial. After adding 5 mL of methanol to the original container, the sample was placed on an orbital shaker to shake horizontally for at least 12 hours (overnight). The samples were then placed in a sonicating water bath and sonicated at 40°C for 60 minutes. Following sonication, 5 mL of DI water was added to each methanol wash portion then mixed by gentle swirling. An aliquot (5 mL) of the sample was transferred to a 15 mL glass culture tube. Additionally, 5 µL of formic acid and 25 µL of the 100 ng/mL internal standard were added to the sample. The sample was vortexed to ensure thorough mixing (approximately 5 seconds). An aliquot of the sample was transferred to a glass autosampler vial using a glass Pasteur pipet. The sample was capped and analyzed or stored in a refrigerator until analysis. If analyte peak area exceeded the peak area of the highest standard by 5%, the sample was diluted using the sample dilution solution.

### 3.5.7 Laboratory Fortified Samples

Laboratory fortified control water samples were prepared and analyzed with each set of field water samples. Fortification levels utilized over the course of the study were at LOQ and 10x LOQ.

### 3.5.8 HPLC-MS/MS Parameters

Representative calibration curves and chromatograms for water analyses appear in Figures 97-216. Typical instrument parameters are presented below. Some parameters were modified to achieve adequate detector sensitivity or to alleviate matrix source suppression. The actual instrument parameters used for each set were documented in the raw data.

HPLC System: Agilent 1290 HPLC with 6500 Q Trap, AB Sciex Mass Spectrometer (MS) Detector  
HPLC Column: Kinetex PFP (100 x 2.10 mm, 1.7µm)  
Column  
Temperature: 35°C  
Mobile Phases: A = 0.1% Formic Acid in DI Water  
B = 0.1% Formic Acid in MeOH

Time (min)	% A	% B
0.00	90	10
7.00	0	100
8.50	0	100
8.60	90	10
11.00	90	10

Flow Rate: 0.3 mL/minute  
 Curtain Gas: 20 psi  
 Collision Gas: 12  
 IonSpray Voltage: 5500 V  
 Temperature: 650°C  
 Ion Source Gas 1: 30 psi  
 Ion Source Gas 2: 40 psi  
 Entrance Potential: 10 V  
 Injection Volume: 15 µL  
 MS Interface: Electrospray, Positive Ion  
 Data System: Analyst with companion software MultiQuant  
 Scan type: Multiple Reaction Monitoring (MRM)

MRM Program:

Compound	Ion (m/z)		Time (msec)	Declustering Potential (V)	Collision Energy (V)	Exit Potential (V)	Approximate Retention Time (min)
	Q1	Q3					
SX-1552	441.0	65.0 (Q)	75	66	119	10	6.85
	441.0	91.0 (C)	50	66	50	10	
1552-A	348.8	267.9 (Q)	75	46	41	14	5.50
	348.8	224.9 (C)	50	46	65	14	
1552-OHBE	424.9	91.0 (Q)	75	60	37	14	6.45
	426.8	91.0 (C)	50	60	43	12	
1552-OHA	334.8	253.9 (Q)	75	61	43	18	4.50
	336.8	255.9 (C)	50	61	43	18	
1552-DBE	404.9	65.0 (Q)	75	61	111	10	6.90
	406.9	91.0 (C)	50	61	53	10	
1552-DA	314.8	234.0 (Q)	75	51	39	12	4.90
	314.8	124.0 (C)	50	56	95	14	
IS-SX-1552	447.0	91.1	75	56	79	18	6.85
IS-1552-A	356.9	276.0	50	66	45	16	5.50
IS-1552-OHA	340.9	260.0	50	31	57	44	4.50
IS-1552-OHBE	432.9	91.0	75	79	68	12	6.45

(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 are not available for 1552-DBE and 1552-DA.

The HPLC-MS/MS system was calibrated by analysis of standard solutions ranging in concentration from approximately 0.005-50.0 ng/mL for each analyte. Calibration standards were prepared using 50:50:0.1 MeOH:DI water:Formic Acid as the solvent. A linear regression internal standard calibration curve was constructed using peak area (y-axis) and the standard solution concentration ratio (x-axis). Sample solution concentrations were calculated using the straight line equation from the calibration curve. A 1/x weighting function was used to construct the regression equation. The correlation coefficient (r) for each calibration curve was  $\geq 0.990$ . Peak areas, calibration curves and sample solution concentrations were calculated using the processing software (Multiquant).

## 4.0 Calculations

#### 4.4 Water Analysis

Calculation of the external standard regression equation was performed as described for sediment in section 4.3 above.

**Fortification Level (ng/mL) =**

$$\frac{\text{Spiking Soln. Conc. (ng/mL)} * \text{Spiking Soln. Volume (mL)}}{\text{Sample Volume (mL)}}$$

Example: 696-X015-S6 (NC747), Set W046 SX-1552-Acid  
Spiking Soln. Conc.: 10.00 ng/mL  
Spiking Soln. Volume: 0.250 mL  
Sample Volume: 5 mL

$$\text{Fortification Level} = 10.00 * 0.250 / 5 = 0.500 \text{ ng/mL}$$

**Fortification Recovery (%) =**

$$\frac{\text{Fortified Sample (ng/mL)} - \text{Unfortified Control Sample (ng/mL)}}{\text{Fortification Level (ng/mL)}} * 100$$

Example: 696-X015-S6 (NC747), Set W046, SX-1552-Acid  
Fortified Sample: 0.5067 ng/mL  
Unfortified Control Sample: 0.0000 ng/mL (696-X015-3)  
Fortification Level: 0.500 ng/mL

$$\text{Fortification Recovery} = \frac{0.5067 - 0.0000}{0.500} * 100 = 101.340 \%$$

**Sample Concentration (ng/mL) = Amount Found (ng/mL) \* Dilution Factor**

Example: 696-W233 (NC438), Set W046, SX-1552-OHBE  
Amount Found: 0.0065 ng/mL  
Dilution Factor: 1

$$\text{Sample Concentration} = 0.0065 * 1 = 0.0065 \text{ ng/mL}$$

***Corrected Sample Concentration (ng/mL)=***

$$\frac{\text{Sample Concentration (ng/mL)} - \text{Control Sample Concentration (ng/mL)}}{\text{Mean Set Fortification Recovery} / 100(\%)}$$

Sample Concentration: 0.0065 ng/mL

Control Sample Concentration: 0.000 ng/ml

Mean Set Fortification Recovery: 103.490 %

$$\text{Corrected Sample Concentration} = (0.0065 - 0.000) / (103.490 / 100) = 0.0063 \text{ ng/mL}$$

Note: Equation was used to calculate corrected sample concentration of sample analysis and methanol rinse analysis.

***Total Sample Concentration (ng/mL)=***

$$\text{Corrected Sample Concentration (ng/mL)} + \text{Corrected MeOH Rinse Sample Concentration (ng/mL)}$$

Corrected Sample Concentration: 0.0063 ng/mL

MeOH Rinse Concentration: 0.0040 ng/mL

$$\text{Total Sample Concentration} = 0.0063 + 0.0040 = 0.0103 \text{ ng/mL}$$



**Table 1. Reference Substances**

Description:	XDE-848 BE (Parent: SX-1552 or 1552-P)
Chemical Name:	Benzyl 4-amino-3-chloro-6-(4-chloro-2-fluoro-3-methoxyphenyl)-5-fluoropyridine-2-carboxylate
Lot Number:	201100802-69A
Purity (%):	99.7%
Assay Date:	9/16/2013
Expiration Date:	10/20/2015
Description:	X11438848 (metabolite and process impurity) (1552-A)
Chemical Name:	4-amino-3-chloro-6-(4-chloro-2-fluoro-3-methoxyphenyl)-5-fluoropyridine-2-carboxylic acid
Lot Number:	201102463-6A
Purity (%):	99%
Assay Date:	9/26/2013
Expiration Date:	10/25/2015
Description:	X12293407 (SX-1552 Internal Standard)
Chemical Name:	Benzyl 4-amino-3-chloro-6-(4-chloro-2-fluoro-3-methoxy(13C6)phenyl)-5-fluoropyridine-2-carboxylate
Lot Number:	XS9-120633-39
Purity (%):	100%
Assay Date:	1/22/2014
Expiration Date:	10/9/2015
Description:	X12293409 (1552-A Internal Standard)
Chemical Name:	4-Amino-3-chloro-6-[4-chloro-2-fluoro-3-methoxy(13C6)phenyl]-5-fluoropyridine-2-carboxylic acid
Lot Number:	XS9-120633-41
Purity (%):	100%
Assay Date:	3/28/2013
Expiration Date:	3/15/2015
Description:	X12393505 (1552-DA)
Chemical Name:	4-amino-6-(4-chloro-2-fluoro-3-methoxyphenyl)-5-fluoropyridine-2-carboxylic acid
Lot Number:	DE3-133876-86
Purity (%):	98%
Assay Date:	3/4/2013
Expiration Date:	1/11/2015
Description:	X12131932 (1552-DBE)
Chemical Name:	Benzyl 4-amino-6-(4-chloro-2-fluoro-3-methoxyphenyl)-5-fluoropyridine-2-carboxylate
Lot Number:	DE3-137773-1
Purity (%):	97%
Assay Date:	3/4/2013
Expiration Date:	1/11/2015

**Table 1. Reference Substances (Continued)**

Description:	X12400867 (1552-OHBE Internal Standard)
Chemical Name:	Benzyl 4-amino-3-chloro-6-[4-chloro-2-fluoro-3-hydroxy(13C6)phenyl]-5-fluoropyridine-2-carboxylate
Lot Number:	YC2-134955-54
Purity (%):	99%
Assay Date:	3/28/2013
Expiration Date:	3/15/2015
Description:	X12401027 (1552-OHA Internal Standard)
Chemical Name:	4-amino-3-chloro-6-[4-chloro-2-fluoro-3-hydroxy(13C6)phenyl]-5-fluoropyridine-2-carboxylic acid
Lot Number:	YC2-134955-53-1
Purity (%):	99%
Assay Date:	3/28/2013 3/11/2015 (after recertification)
Expiration Date:	3/15/2015 10/21/2016 (after recertification)
Description:	X11966341 (1552-OHA)
Chemical Name:	4-amino-3-chloro-6-(4-chloro-2-fluoro-3-hydroxyphenyl)-5-fluoropyridine-2-carboxylic acid
Lot Number:	BD-B130064-38-1
Purity (%):	98%
Assay Date:	7/29/2013
Expiration Date:	10/18/2015
Description:	X12300837 (1552-OHBE)
Chemical Name:	Benzyl 4-amino-3-chloro-6-(4-chloro-2-fluoro-3-hydroxyphenyl)-5-fluoropyridine-2-carboxylate
Lot Number:	BD-B130064-40-1
Purity (%):	99%
Assay Date:	7/29/2013
Expiration Date:	10/18/2015