

3.4 Determination of SX-1552 and Five Metabolites in Sediment Samples by HPLC-MS/MS

Sediment samples were analyzed for active ingredient and the five metabolites content using EPL BAS method 477G696C.

3.4.1 Reagents

Methanol (MeOH), HPLC Grade, EMD
Formic Acid, 88%, Fisher Scientific and EMD
Acetonitrile (ACN), HPLC Grade, 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.

90:10 Acetonitrile:0.1N Hydrochloric Acid: Prepared by adding 400 mL of 0.1 N hydrochloric acid with 3600 mL of Acetonitrile in a 4 L bottle. 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.

3.4.2 Equipment and Instruments

Balance, analytical, capable of weighing to the nearest 0.1 mg
Balance, analytical, capable of weighing to the nearest 0.01 mg accuracy
Class A glass volumetric glassware
Transfer pipettes, Pasteur glass
50 mL centrifuge tube
Centrifuge
HPLC Column, Kinetex PFP (100 x 2.10 mm, 1.7 μ m), Phenomenex
HPLC System, 1290, Agilent
HPLC autosampler vials with screw caps, Waters
Mass spectrometer detector, 6500 Q Trap, AB Sciex
HPLC-MS/MS data system, Analyst version 2.1, AB Sciex
Pipettors, fixed and adjustable volume
Platform Shaker

Bench-top tube vortexer

3.4.3 Determination of Sediment Moisture Content

Sediment moisture content was not required for this study.

3.4.4 Sediment Travel Spike Verification, Preparation and Shipment

Sediment travel spikes were prepared at EPL BAS. Sub-samples (5.000-5.100 g) of bulk control sediment were weighed into 20 ml amber glass scintillation vials. Samples are transferred to 50 mL centrifuge tubes for extraction. Two sets of travel spikes were prepared (one for each site). Each set contained an un-spiked 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 un-spiked control and a single sub-sample fortified with only one compound for a total of 7 samples per set. For each compound, 0.100 mL of a single component reference substance solution at a concentration of approximately 1,000 ng/mL was added directly to the sediment in the scintillation vial. This resulted in a travel spike concentration of 20 ppb (ng/g). Travel spike verification samples were frozen and analyzed at EPL BAS without being shipped. Travel spikes were shipped to the respective field sites frozen on dry ice. Sediment travel spikes were received from the Florida site with the event 16 and 17 (4 and 5 month respectively) field dissipation samples and were analyzed with those event's samples. Sediment travel spikes were received from the North Carolina site with the event 16 samples (4 month) field dissipation samples and were analyzed with those event's samples.

3.4.5 Sediment Sample Extraction

A sub-sample (5.000-5.100 g) of sediment was weighed into a 50 mL centrifuge tube. Samples designated as laboratory fortified samples were weighed and spiked at this time. To the tube, 20 mL of extraction solvent (90:10 acetonitrile:0.1N HCl) was added. The sample was vortexed and then placed horizontally on a platform shaker and shaken at a setting of 180 rpm for about 30 minutes. The sample was centrifuged at 2,000 rpm for 5 minutes. The supernatant was decanted into a 100 mL volumetric flask. The extraction, shaking and centrifugation steps were repeated three more times, combining all extracts in the 100 mL volumetric flask. The flask was brought to volume with extraction solvent. A 200 μ L aliquot of the combined extract was transferred to an autosampler vial with 750 μ L of the 50:50:0.1 Methanol:DI water:Formic Acid. 50 μ L of the 10 ng/ml mixed internal standard was added to the autosampler vial. The sample was thoroughly mixed and sealed for analysis.

3.4.6 Laboratory Fortified Samples

Laboratory fortified control sediment samples were prepared and analyzed with each set of field sediment samples. Fortification levels utilized over the course of the study were

0.003 µg/g, the limit of quantitation (LOQ) and 0.03 µg/g (10x LOQ). After weighing the control sub-samples identified for fortification, an appropriate volume of a fortification standard solution was added directly to the sediment. The fortified samples were then carried through the method steps described in section 3.4.5 above.

3.4.7 HPLC/MS/MS Analysis of Sediment Extracts

Representative calibration curves and chromatograms for sediment analyses appear in Figures 1-96. 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.

3.4.8 HPLC/MS/MS Parameters

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 Temp.: 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) Q1	Ion (m/z) Q3	Time (msec)	Exit Potential (V)	Collision Energy (V)	Declustering Potential (V)	Approximate Retention Time (min)
SX-1552	441	65 (Q)	75	10	119	66	6.85
	441	91 (C)	50	10	50	66	
1552-A	349	268 (Q)	75	14	41	46	5.50
	349	225 (C)	50	14	65	46	
1552-OHBE	425	91 (Q)	75	14	37	60	6.45
	427	91 (C)	50	12	43	60	
1552-OHA	335	254 (Q)	75	18	43	61	4.50
	337	256 (C)	50	18	43	61	
1552- DBE	405	65 (Q)	75	10	111	61	6.90
	407	91 (C)	50	10	53	61	
1552-DA	315	234 (Q)	75	12	39	51	4.90
	315	124 (C)	50	14	95	56	
IS-SX-1552	447	91	75	18	79	56	6.85
IS-1552-A	357	276	50	16	45	66	5.50
IS-1552-OHA	341	260	50	44	57	31	4.50
IS-1552-OBE	433	91	75	12	68	79	6.45

(Q) = Quantitation Ion

(C) = Confirmation Ion

The HPLC-MS/MS system was calibrated by analysis of external standard calibration solutions ranging in concentration from approximately 0.005-50 ng/mL for each analyte. Calibration standards were prepared using 50:50:0.1 methanol:DI water:formic acid as the solvent. A linear regression calibration curve was constructed using peak area (y-axis) and the standard solution concentration (x-axis). A weighting function (1/x) was used to construct the regression equation. The correlation coefficient (r) for each calibration curve was ≥ 0.990 . Sample solution concentrations were calculated using the straight line equation from the calibration curve. Data was acquired with Analyst software. The data processing was completed in MultiQuant, which is a companion software access via Analyst. The raw data for each analytical set was exported from MultiQuant as PDF report summaries.

4.3 Sediment Analysis

Sediment analysis was conducted using external standard calibration. Linear regression analysis of the standard peak areas (y-axis) and standard concentrations (x-axis) resulted in the straight line equation:

$$y = mx + b$$

Where y = Sample Peak Area
 m = Slope of Regression Line
 x = Amount Found (ng/mL)
 b = y-Intercept

A weighting function ($1/x$) was used when calculating the linear regression equation.

MultiQuant was used to calculate the regression equation for each set of sediment analyses and the Amount Found (ng/mL). Microsoft Excel 2007 was used for all other calculations.

Fortification Level ($\mu\text{g/g}$) =

$$\frac{\text{Spiking Soln. Concn. } (\mu\text{g/mL}) * \text{Spiking Soln. Volume (mL)}}{\text{Sample Weight (g)}}$$

Example: 696-X006-S19J (FL002), Set S003J, SX-1552-DA
Spiking Soln. Concn.: 0.098 $\mu\text{g/mL}$
Spiking Soln. Volume: 0.150 mL
Sample Weight: 5.001 g

$$\text{Fortification Level} = 0.098 * 0.150 / 5.001 = 0.00294 \mu\text{g/g}$$

Fortification Recovery (%) =

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

Example: 696-X006-S19J (FL002), Set S003J, SX-1552-DA
Fortified Sample: 0.00254 $\mu\text{g/g}$
Unfortified Control Sample: 0.00000 $\mu\text{g/g}$ (696-X006-5J)
Fortification Level: 0.00294 $\mu\text{g/g}$

$$\text{Fortification Recovery} = \frac{0.00254 - 0.00000}{0.00294} * 100 = 86.395\%$$

Sample Concentration ($\mu\text{g/g}$) =

$$\frac{\text{Amount Found (ng/mL)} * \text{Extract Volume (mL)} * \text{Dilution Factor}}{\text{Sample Weight (g)} * 1000 \text{ ng}/\mu\text{g}}$$

Example: 696-S080 (FL174), Set S005, SX-1552-OHA
Amount Found: 0.0032 ng/mL
Extract Volume: 100 mL
Dilution Factor: 5
Sample Weight: 5.078 g

$$\text{Sample Concentration} = (0.0032 * 100 * 5) / (5.078 * 1000) = 0.00032 \mu\text{g/g}$$

Sample Concentration was corrected for the mean fortification recovery of the sample set:

Corrected Sample Concentration ($\mu\text{g/g}$) =

$$\frac{\text{Sample Concentration } (\mu\text{g/g}) - \text{Control Sample Concentration } (\mu\text{g/g})}{\text{Mean Fortification Recovery } (\%) / 100}$$

Sample Concentration: 0.00032 µg/g
Control Sample Concentration: 0.00000 µg/g
Mean Fortification Recovery: 78.418 %
Corrected Sample Concentration = $(0.00032 - 0.00000) / (78.418 / 100) = 0.0004 \mu\text{g/g}$

Note: Samples appearing in Excel may appear differently due to rounding differences.

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