

Method Validation Study for the Determination of Residues of XDE-848 Benzyl Ester and Five Metabolites (X11438848, X12300837, X11966341, X12131932, and X12393505) in Ground, Surface, and Drinking Water Using Liquid Chromatography with Tandem Mass Spectrometry

INTRODUCTION

Scope

This method is applicable for the quantitative determination of residues of XDE-848 benzyl ester, X11438848, X12300837, X11966341, X12131932, and X12393505 in ground, surface, and drinking water. The method was validated over the concentration range of 0.02 - 5.0 µg/L with a validated limit of quantitation of 0.02 µg/L for the XDE-848 benzyl ester and 0.05 µg/L for the five metabolites. Common names, chemical names, and molecular formulas for the analytes are given in Table 1.

This study was conducted to fulfill data requirements outlined in the EPA Residue Chemistry Test Guidelines, OCSPP 850.6100 (1). The validation also complies with the requirements of EU Council Regulation (EC) No. 1107/2009 with particular regard to Section 4 of SANCO/3029/99 rev.4 and Section 6 of SANCO/825/00 rev.8.1 as well as PMRA Residue Chemistry Guidelines as Regulatory Directive Dir98-02 (2-4). The validation was conducted following Dow AgroSciences SOP ECL-24 with exceptions noted in the protocol or by protocol amendment.

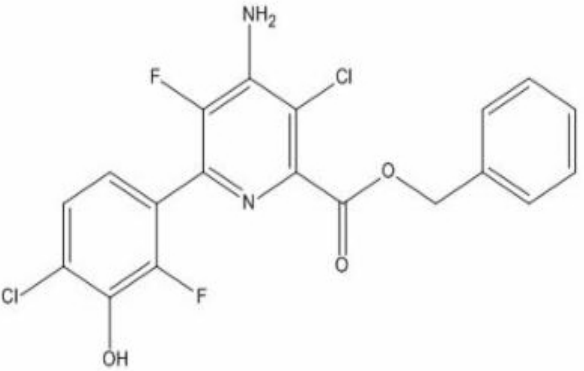
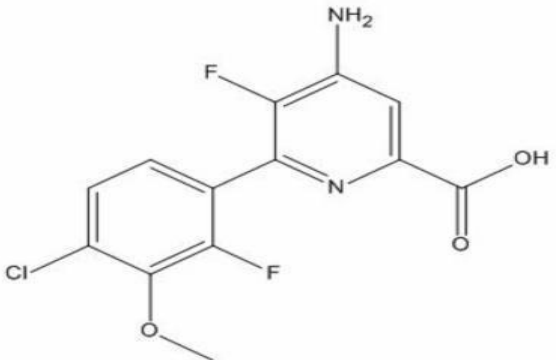
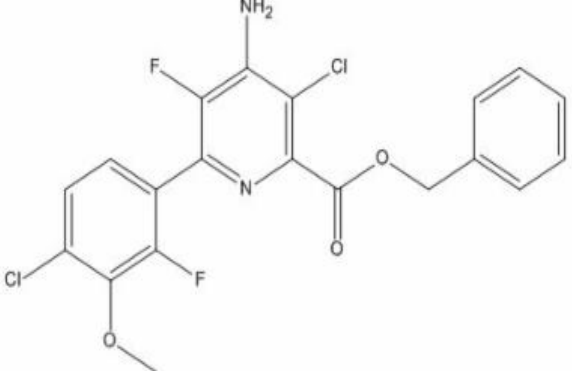
Method Principle

Residues of XDE-848 benzyl ester, X11438848, X12300837, X11966341, X12131932, and X12393505 are determined using a 10 mL sample of water which is acidified and loaded onto a solid phase extraction (SPE) cartridge. Following a rinse step, the analytes are eluted from the SPE cartridge with 50/50 acetonitrile/methanol followed by acetonitrile. A mixed internal standard solution is added and the samples are evaporated to dryness in a Turbo-Vap before being reconstituted with 25/25/50 acetonitrile/ methanol/ water containing 0.1% formic acid. The final sample is analyzed for XDE-848 benzyl ester, X11438848, X12300837, X11966341, X12131932, and X12393505 by two structurally characteristic MS/MS transitions for each analyte by tandem mass spectrometry.

Table 1. Identities and Structures of Analytes

Common Name	Structural Formula
X11438848 Molecular Formula: C ₁₃ H ₈ C ₁₂ F ₂ N ₂ O ₃ CAS Number: 943832-81-3	
X11966341 Molecular Formula: C ₁₂ H ₆ C ₁₂ F ₂ N ₂ O ₃ CAS Number: NA	
X12131932 Molecular Formula: C ₂₀ H ₁₅ ClF ₂ N ₂ O ₃ CAS Number: NA	

Table 1. (con't) Identities and Structures of Analytes

Common Name	Structural Formula
<p>X12300837</p> <p>Molecular Formula: $C_{19}H_{12}Cl_2F_2N_2O_3$ CAS Number: NA</p>	
<p>X12393505</p> <p>Molecular Formula: $C_{13}H_9ClF_2N_2O_3$ CAS Number: NA</p>	
<p>XDE-848 Benzyl Ester</p> <p>Molecular Formula: $C_{20}H_{14}Cl_2F_2N_2O_3$ CAS Number: 1390661-72-9</p>	

Dow AgroSciences_ Analytical Method for Determination of Residues of XDE-848 Benzyl Ester and Five Metabolites (X11438848, X12300837, X11966341, X12131932 and X12393505) in Surface, Ground and Drinking Water with Tandem Mass Spectrometry

Scope

This method is applicable for the determination of residues of XDE-848 Benzyl Ester and five metabolites (X11438848, X12300837, X12131932 and X12393505) in water. This method is applicable over a concentration range of 0.02 – 5.0 ng/mL.

Principle

Using a 10 mL sample of water, residues of XDE -848 and five metabolites are acidified and loaded onto a solid phase extraction (SPE) cartridge using a glass pasteur pipette. Following a rinse step, the analytes are eluted from the SPE cartridge with 50/50 acetonitrile/methanol and acetonitrile. Internal standard is added. The eluate is evaporated to dryness on a Turbo-Vap and reconstituted with 25/25/50 acetonitrile/ methanol/ water containing 0.1% formic acid. The sample is analyzed for XDE-848BE and 5 metabolites by liquid chromatography with positive ion electrospray ionization tandem mass spectrometry (LC-MS/MS).

Safety Precautions

Each analyst must be acquainted with the potential hazards of the equipment, reagents, products, solvents, and procedures used in this method before commencing laboratory work. Sources of information include operation manuals, material safety data sheets, literature, and other related data. Safety information should be obtained from the supplier. Disposal of waste materials, reagents, reactants, and solvents must be in compliance applicable governmental requirements.

Methanol, acetonitrile and 2-propanol are flammable and should be used in well-ventilated areas away from ignition sources. Formic acid is corrosive and can cause severe burns. It is imperative that proper eye and personal protection equipment be worn when handling these reagents.

Laboratory Equipment

Balance, analytical, Model AE100, Mettler - Toledo
Balance, top loader, PG2002, Mettler – Toledo
Culture Tube, Disposable, 16 x 100mm, cat # 14-961-29, Fisher Scientific
Culture Tube, Disposable, screw top, 16 x 100mm, cat # 73770-16100, Kimble Chase
Pipette, positive-displacement, 1-10 μ L capacity, catalog number F148501G, Gilson
Pipette, positive-displacement, 10-100 μ L capacity, catalog number F148504G, Gilson
Pipette, positive-displacement, 20-50 μ L capacity, catalog number F148503G, Gilson
Pipette, positive-displacement, 50-250 μ L capacity, catalog number F148505G, Gilson
Pipette, positive-displacement, 100-1000 μ L capacity, catalog number F48506G, Gilson
Oasis HLB 3cc (60mg), SPE, part# WAT 094226, Waters
Repeater Plus Pipette, 1.0 μ L-10 mL capacity, catalog # 21-380-9, Eppendorf
Turbo-Vap, Zymark
Vacuum Manifold, IST VacMaster
Vial, glass, 45-mL, part# 60958A 11, Kimble Chase

Chromatographic System

Column, analytical, Kinetex 1.7 μ PFP 100A, 100 x 2.1mm, part# 00D-4476-AN,
Phenomenex
Guard column, SecurityGuard ULTRA cartridges, UHPLC PFP for 2.1mm ID columns,
part # AJO-8787, Phenomenex
Guard Column holder, SecurityGuard ULTRA cartridge holder, part# AJO-9000,
Phenomenex
Liquid chromatography, Model Agilent 1290, Agilent Technologies
Mass spectrometer, QTRAP 5500, AB SCIEX
Mass spectrometer data system, Analyst 1.6.2, AB SCIEX

Reagents

Acetonitrile, Chromasolv HPLC grade, catalog number 439134-4L, Sigma - Aldrich
Formic Acid, LC/MS grade, \geq 99.5% purity, catalog number A117-50, [Fisher Scientific](#).
Glycerol, Reagent ACS, 99.6%, Acros Organics
Methanol, Chromasolv HPLC grade, catalog number 34885-4L-R, [Sigma Aldrich](#)
2-Propanol, HPLC grade, catalog number A451-4L, [Fisher Scientific](#)
Water, Chromasolv HPLC grade, catalog number 270733-4L, [Sigma Aldrich](#)

Prepared Solutions

Water containing 0.1% Formic Acid (v/v)

Measure 4000 mL of HPLC grade water, using a graduated cylinder, and transfer into a 4 L bottle. Add 4.0 mL of formic acid into the 4L bottle and mix.

Methanol containing 0.1% Formic Acid (v/v)

Measure 4000 mL of methanol, using a graduated cylinder, and transfer into a 4 L bottle. Add 4.0 mL of formic acid into the 4L bottle and mix.

50/50/0.1, (v/v/v) Methanol/ Water/ Formic Acid

Transfer 50 mL of methanol and 50 mL of water, each measured by graduated cylinder, into a 4 ounce jar. Pipette 100- μ L of formic acid into the 4 ounce jar and mix. Allow solution to reach room temperature before use.

50/50,(v/v) Acetonitrile/ Methanol

Transfer 500-mL of acetonitrile and 500-mL of methanol, each measured by graduated cylinder, into a 1-L bottle. Mix. Allow solution to reach room temperature before use.

10/90, (w/v), Glycerol/Methanol

Weigh 10 grams of glycerol into a 4 ounce jar. Add 90-mL of methanol, measured using a graduated cylinder, and mix.

50/50/0.1%, (v/v/v)Acetonitrile/ Methanol/Formic Acid

Transfer 500-mL of acetonitrile and 500-mL of methanol, each measured by graduated cylinder, into a 1-L bottle. Add 1 mL of formic acid into the 1 L bottle. Mix. Allow solution to reach room temperature before use.

2/2/1, (v/v/v) Methanol/2-Propanol/Water

Measure 400-mL of methanol, 400-mL of 2-propanol and 200-mL of water, using a graduated cylinder, transfer into a 1-L bottle and mix.

Sample Dilution Solution (v/v)

Pipette 9.0 mL of 50/50/0.1 (v/v/v) acetonitrile/methanol/formic acid and 1.0 mL of 0.01 μ g/mL internal standard and 10.0 mL of water containing 0.1% formic acid into a small vial. Mix. Store in refrigerator.

25/25/50/0.1%, (v/v/v/v) Acetonitrile/ Methanol/Water/Formic Acid

Transfer 500-mL of the prepared 50/50/0.1 (v/v/v), acetonitrile/methanol/formic acid solution and 500-mL of water containing 0.1% formic acid, each measured by graduated cylinder, into a 1-L bottle. Mix. Allow solution to reach room temperature before use.

Preparation of Fortification Solutions of XDE-848 and Five Metabolites

1. Weigh 0.0250 g of XDE-848 benzyl ester analytical standard and quantitatively transfer into a 25-mL volumetric flask with methanol. Dilute to volume with methanol to obtain a 1000- $\mu\text{g}/\text{mL}$ stock solution of XDE-848 benzyl ester.
2. Weigh 0.0250 g of X11438848, (XDE-848 acid) analytical standard and quantitatively transfer into a 25-mL volumetric flask with methanol. Dilute to volume with methanol to obtain a 1000- $\mu\text{g}/\text{mL}$ stock solution of X11438848.
3. Weigh 0.0250 g of X12300837, (XDE-848 BH) analytical standard and quantitatively transfer into a 25-mL volumetric flask with methanol. Dilute to volume with methanol to obtain a 1000- $\mu\text{g}/\text{mL}$ stock solution of X12300837.
4. Weigh 0.0250 g of X11966341, (XDE-848 HA) analytical standard and quantitatively transfer into a 25-mL volumetric flask with methanol. Dilute to volume with methanol to obtain a 1000- $\mu\text{g}/\text{mL}$ stock solution of X11966341.
5. Weigh 0.0250 g of X12131932, (dechlorinated XDE-848 BE) analytical standard and quantitatively transfer into a 25-mL volumetric flask with methanol. Dilute to volume with methanol to obtain a 1000- $\mu\text{g}/\text{mL}$ stock solution of X12131932.
6. Weigh 0.0250 g of X12393505, (dechlorinated XDE-848 acid) analytical standard and quantitatively transfer into a 25-mL volumetric flask with methanol. Dilute to volume with methanol to obtain a 1000- $\mu\text{g}/\text{mL}$ stock solution of X12393505.
7. Pipette 4.0 mL of the 1000 $\mu\text{g}/\text{mL}$ solution of XDE-848 benzyl ester (BE) prepared in step 1 and 10 mL of each of the 1000- $\mu\text{g}/\text{mL}$ solutions of five analytes prepared in steps 2 - 6 into the same 100-mL volumetric flask. Dilute to volume with methanol to obtain a mixed spiking solution containing 40- $\mu\text{g}/\text{mL}$ of XDE-848 BE and 100 $\mu\text{g}/\text{mL}$ of the five metabolites. **(Solution A)**
8. Pipette 10 mL of the mixed spiking solution prepared in step 7 (Solution A) into a 100-mL volumetric flask. Dilute to volume with methanol to obtain a mixed spiking solution containing 4.0 $\mu\text{g}/\text{mL}$ of XDE-848 BE and 10 $\mu\text{g}/\text{mL}$ of the five metabolites. **(Solution B)**
9. Pipette 10 mL of the mixed spiking solution prepared in step 8 (Solution B) into a 100 mL volumetric flask. Dilute to volume with methanol to obtain a mixed spiking solution containing 0.4 $\mu\text{g}/\text{mL}$ of XDE-848 BE and 1.0 $\mu\text{g}/\text{mL}$ of the five metabolites. **(Solution C)**

Note: All of the stock and spiking solutions should be stored in refrigerator

10. Pipette 10 mL of the mixed spiking solution prepared in step 9 (Solution C) into a 100 mL volumetric flask. Dilute to volume with methanol to obtain a mixed spiking solution containing 0.04- $\mu\text{g}/\text{mL}$ of XDE-848 BE and 0.1 $\mu\text{g}/\text{mL}$ of the five metabolites. **(Solution D)**
11. Pipette 100 μL of the mixed spiking solution prepared in step 9(Solution C) into a 10 mL volumetric flask. Dilute to volume with methanol to obtain a mixed spiking solution containing 0.004- $\mu\text{g}/\text{mL}$ of XDE-848 BE and 0.01 $\mu\text{g}/\text{mL}$ of the five metabolites. **(Solution E)**

Note: Solution E should be prepared fresh every 14 days.

Note: All of the stock and spiking solutions should be stored in refrigerator

Preparation of Internal Standard Solutions of XDE-848 and Five Metabolites

1. Weigh 0.0050 g of X12401027 internal standard and quantitatively transfer the analyte into a 50-mL volumetric flask with methanol. Dilute to volume with methanol to obtain a 100- $\mu\text{g}/\text{mL}$ stock solution of X12401027.
2. Weigh 0.0050 g of X12293409 internal standard and quantitatively transfer the analyte into a 50-mL volumetric flask with methanol. Dilute to volume with methanol to obtain a 100- $\mu\text{g}/\text{mL}$ stock solution of X12293409.
3. Weigh 0.0050 g of X12400867 internal standard and quantitatively transfer the analyte into a 50-mL volumetric flask with methanol. Dilute to volume with methanol to obtain a 100- $\mu\text{g}/\text{mL}$ stock solution of X12400867.
4. Weigh 0.0050 g of X12293407 internal standard and quantitatively transfer the analyte into a 50-mL volumetric flask with methanol. Dilute to volume with methanol to obtain a 100- $\mu\text{g}/\text{mL}$ stock solution of X12293407.
5. Pipette 10 mL of each 100- $\mu\text{g}/\text{mL}$ solution of the four internal standard solutions prepared in steps 1 - 4 into the same 100-mL volumetric flask. Dilute to volume with methanol to obtain a 10- $\mu\text{g}/\text{mL}$ mixed internal standard solution.
6. Pipette 10 mL of the 10- $\mu\text{g}/\text{mL}$ mixed internal standard solution prepared in step 5 into a 100-mL volumetric flask. Dilute to volume with methanol to obtain a 1.0- $\mu\text{g}/\text{mL}$ mixed internal standard solution.
7. Pipette 1.0 mL of the 1.0- $\mu\text{g}/\text{mL}$ mixed internal standard solution prepared in step 6 into a 100 mL volumetric flask. Dilute to volume with methanol to obtain a 0.01- $\mu\text{g}/\text{mL}$ mixed internal standard solution.

Preparation of Isotopic Crossover Solutions

1. Prepare a 4.0 / 10 ng/mL calibrator with no internal standard. Pipette 0.2 mL of the 0.4 / 1.0 $\mu\text{g}/\text{mL}$ spiking solution into a 20 mL volumetric flask. Dilute to volume with 50/50/0.1, (v/v/v) methanol/water/formic acid solution.
2. Prepare a 0.5 ng/mL solution of internal standard. Pipette 1 mL of 0.01 $\mu\text{g}/\text{mL}$ mixed internal standard solution into a 20 mL volumetric flask. Add 9 mL of methanol and dilute to volume with water containing 0.1% formic acid.

Notes: All of the above internal standard and crossover solutions should be stored in refrigerator.

Preparation of Calibration Standards

Prepare calibration standards by using the spiking solutions described above as shown in the following table. Use the 0.01 μ g/mL mixed XDE-848 internal standard to prepare the following solutions. Use a methanol/water/formic acid (50/50/0.1, v/v/v) solution to dilute the standards to volume. Store all calibration solutions in the refrigerator.

Original Spiking Solution Concentration.		Aliquot of Spiking Solution	Aliquot of Internal Standard	Volume of Final Solution	Final Calibration Solution Concentration
XDE-848BE / 5 analytes μ g/mL	Solution ID	mL	mL	mL	XDE-848BE / 5 analytes ng/mL
0.4 / 1.0	C	0.20	1.0	20	4.0 / 10
0.4 / 1.0	C	0.10	1.0	20	2.0 / 5.0
0.04 / 0.1	D	0.50	1.0	20	1.0 / 2.5
0.04 / 0.1	D	0.20	1.0	20	0.4 / 1.0
0.04 / 0.1	D	0.10	1.0	20	0.2 / 0.5
0.004 / 0.01	E	0.50	1.0	20	0.1 / 0.25
0.004 / 0.01	E	0.15	1.0	20	0.03 / 0.075

EXPERIMENTAL

Instrumental Conditions

Typical LC-MS/MS Operating Conditions for XDE-848 BE and Metabolites Determination

Instrumentation:	Agilent 1290 Infinity LC System AB SCIEX API 5500 LC-MS/MS System AB SCIEX Analyst 1.6.2 data system															
Column:	Phenomenex Kinetex 1.7u, PFP 100A 100 x 2.1 mm, 1.7 µm (Part #: 00D-4476-AN)															
Guard Column:	Phenomenex SecurityGuard ULTRA UHPLC PFP for 2.1 ID columns, part# AJO-8787															
Column Temperature:	ambient (approximately 20 °C)															
Sample Temperature	10°C															
Injection Volume:	15 µL															
Autosampler Wash	30 seconds of methanol/2-propanol/water (2/2/1) at the flush port															
Run Time:	approximately 10 minutes															
Mobile Phase:	A – water containing 0.1% formic acid B –methanol containing 0.1% formic acid															
Flow Rate:	300 µL/min															
Equilibration Time	3 minutes (-3 on table below)															
Gradient:	<table><thead><tr><th>Time, min</th><th>Solvent A, %</th><th>Solvent B, %</th></tr></thead><tbody><tr><td>-3.0</td><td>50.0</td><td>50.0</td></tr><tr><td>0.0</td><td>50.0</td><td>50.0</td></tr><tr><td>7.0</td><td>0.0</td><td>100.0</td></tr><tr><td>9.0</td><td>0.0</td><td>100.0</td></tr></tbody></table>	Time, min	Solvent A, %	Solvent B, %	-3.0	50.0	50.0	0.0	50.0	50.0	7.0	0.0	100.0	9.0	0.0	100.0
Time, min	Solvent A, %	Solvent B, %														
-3.0	50.0	50.0														
0.0	50.0	50.0														
7.0	0.0	100.0														
9.0	0.0	100.0														
Flow Diverter	1) 0.0 → 0.5 min – flow to waste 2) 0.5 → 9.0 min – flow to source 3) 9.0 → end of run – flow to waste															
Ionization Mode:	Electrospray															
Polarity:	Positive															
Scan Type:	MRM															
Resolution:	Q1 – unit, Q3 – unit															
Collision Gas (CAD):	Medium															
Curtain Gas (CUR):	20															
Ion Source Gas 1 (GS1):	50 psi															
Ion Source Gas 2 (GS2):	50 psi															
Temperature (TEM):	500 °C															
Entrance Potential:	10 volts															
IonSpray Voltage (IS):	5500 volts															
Acquisition Duration:	9 minutes															
Dwell Time:	100 ms															

Typical Mass Spectrometry Operating Conditions for XDE-848 BE and Metabolites Determination

Analytes:	Precursor Ion Q1 (m/z)	Product Ion Q3 (m/z)	Declustering Potential (v)	Collision Energy (v)	Cell Exit Potential (v)
XDE-848 BE (439/91)	438.9	91.0	71	69	8
XDE-848 BE (441/91)	440.9	91.0	56	69	10
X11438848 (351/270)	350.9	270.0	56	41	24
X11438848 (349/268)	349.0	267.9	120	49	13
X12300837 (427/91)	427.0	90.9	60	41	8
X12300837 (425/91)	425.0	90.9	60	41	8
X11966341 (335/254)	334.9	254.0	91	47	18
X11966341 (337/256)	336.9	256.0	71	47	24
X12131932 (405/65)	405.1	64.9	46	109	10
X12131932 (407/91)	407.1	91.0	46	39	10
X12393505 (315/234)	315.0	234.0	61	41	18
X12393505 (315/124)	315.0	124.1	61	101	10
X12293407	446.9	91.0	56	69	10
X12293409	355.0	274.0	66	43	22
X12400867	432.9	91.0	87	63	14
X12401027	340.9	260.0	91	47	18

Note: Two of the analytes have no internal standards. Use the following for the analysis of each.

X12131932 Dechlorinated XDE-848BE Use X12293407 internal standard
 X12393505 Dechlorinated XDE-848 acid Use X12293409 internal standard

The instrumental conditions may be modified to obtain optimal chromatographic separation and sensitivity.

Collection of Field Samples of XDE-848 and Five Metabolites

It is critical that water samples do not come in contact with plastic or Nalgene during any part of this procedure.

Preferred Sampling Method

1. Samples should be collected in triplicate for best results.
2. Weigh a 45mL glass vial with PTFE lined cap for each sample to be collected.
3. Sample 10 mL the water source by dipping the collection vial into the water source.
NOTE: Pipettes should not be used due to the “sticky” nature of the analytes.
4. Add diluted formic acid (1/50, formic/HPLC grade water) to adjust pH to 4
5. Samples should be stored in refrigerator (1 -2 days only) or freezer (longer storage)
6. Before analysis, sample vial is weighed to determine volume.
7. Sample is shaken well prior to analysis.

Former Sampling Method

1. Samples are collected in a larger glass container with PTFE lined cap.
2. Sample vials are not pre-weighed prior to collection of sample.
3. Water sample size may vary from 10 - 200 mL
4. Diluted formic acid (1 in 50, formic to HPLC grade water) is added to adjust pH to 4
5. Samples should be stored in refrigerator (1 -2 days only) or freezer (longer storage)
6. Prior to sampling:
 - a. 50/50 Methanol/Acetonitrile solution is added in quantity less than 5% of total volume
 - b. Solution is mixed well
 - c. A 10 mL aliquot is removed using a glass pipette.

Analysis Procedure

Note: - Do not use plastic or Nalgene in any part of this procedure.

Note: Water volume for the sample analysis may be adjusted as long as all proportions remain the same.

For procedural recovery samples:

1. For control samples, transfer 10 mL of each type of control water into a 45 mL glass vial.
2. For recovery samples:
 - a. Transfer 10 mL of each type of control water into separate 45 mL glass vials.
 - b. Add the appropriate volume of the spiking solution to obtain fortified samples at LOD, LOQ, 10× LOQ and 100× LOQ (0.006, 0.02, 0.2 and 2.0 ng/mL for XDE-848 BE and 0.015, 0.05, 0.5 and 5.0ng/mL of the five metabolites respectively
 - c. Vortex for 5 seconds.

To fortify 10 mL of water with the six analytes:			
Description	Spiking Volume (µL)	Spiking Solution ID	Fortification Level (ng/mL)
			XDE-848BE / 5 analytes
Control	-	-	-
LOD	15	E	0.006 / 0.015
LOQ	50	E	0.02 / 0.05
10 x LOQ	50	D	0.2 / 0.5
100 x LOQ	50	C	2.0 / 5.0
1000 x LOQ	500	C	20 / 50

Spiking Solutions

Solution E – See step 11 of spiking solution preparation. It contains 0.004 µg/mL of XDE-848BE and 0.01 µg/mL of the 5 metabolites. Solution E should be prepared fresh every 14 days.

Solution D – See step 10 of spiking solution preparation. It contains 0.04 µg/mL of XDE-848BE and 0.1 µg/mL of the 5 metabolites

Solution C – See step 9 of spiking solution preparation. It contains 0.4 µg/mL of XDE-848BE and 1 µg/mL of the 5 metabolites

For field samples:

- Mix the sample thoroughly by shaking vigorously.
- Measure 10 mL of each water sample into a 45 mL glass vial by weight or by using a glass pipette.

Analysis Preparation

All samples (Control, recovery and field samples) are treated the same for the remaining part of the procedure.

3. Add 100 μ L of formic acid to each vial.
4. Vortex for 5 seconds.
5. Add 500 μ L of 50/50 acetonitrile/methanol solution to each sample. (Do not add to any samples using the former sampling procedure)
6. Vortex for 5 seconds.

SPE Portion

Do not use plastic or Nalgene in any part of this procedure

7. Set up manifold. Use Waters Oasis HLB 3cc (60 mg) cartridges. Part # WAT 094226.
8. Condition SPE
 - a. 3-mL of 50/50 acetonitrile/methanol followed by 3-mL of water containing 0.1% formic acid.
 - b. Pull a 5 second vacuum at the end of each elution.
 - c. Discard eluate.
9. Load sample onto SPE – save vial and pasteur pipette for steps 10 and 12.
 - a. Use glass pasteur pipettes
 - b. Pull samples through SPE cartridge slowly at approx 0.5 mL/minute.
 - c. Pull a 5 second vacuum at the end of elution.
 - d. Discard eluate.
10. Rinse sides of vial with 1.0 mL of HPLC grade water
 - a. Load onto SPE
 - b. Pull rinse through SPE cartridge at approximately 1mL/minute.
 - c. Pull a 5 second vacuum at the end of elution.
 - d. Discard eluate.
11. Dry for 5 minutes under full vacuum.
12. Elute analytes with 4 x 1.5-mL as follows: (NOTE: **Steps a. and b. help remove the analytes from the sides of the glass tube and are very important steps to achieve recoveries.**)

- a. Elutions 1 and 2 – Rinse the sides of sample vial with the 1.5 mL of 50/50 acetonitrile/methanol solution prior to loading onto the SPE cartridge.
 - b. Elution 3 – Rinse the sides of sample vial with 1.5 mL of acetonitrile prior to loading onto the SPE
 - c. Elution 4 – Elute with the 1.5 mL of 50/50 acetonitrile/methanol solution. Load elution onto SPE cartridge only. No vial rinse required.
 - d. **Use gravity** to pull slowly through the SPE.
 - e. Pull a 5 second vacuum at the end of each elution.
13. Add 50- μ L of keeper, (10/90, (w/v), glycerol/methanol).
 14. Add 100 μ L of 0.01 μ g/mL mixed XDE-848 internal standard.
 15. Vortex gently.
 16. Evaporate to dryness on a Turbo-Vap set at 40°C with 7 psi nitrogen. (Approx. 30-35 min.)
 17. Reconstitute
 - a. Add 1000- μ L of 50/50/0.1 acetonitrile/methanol/formic acid solution.
(NOTE: This removes the analytes from the sides of the glass tube and is a very important step.)
 - b. Vortex for 5 seconds.
 - c. Add 1000- μ L of water containing 0.1% formic acid.
 - d. Vortex for 5 seconds.
 18. Transfer portion to HPLC vials.
Dilute any samples above 4 ng/mL with dilution solution.
 19. Analyze the calibration standards and samples by LC-MS/MS with positive-ion electrospray tandem mass spectrometry, injecting the calibration standards interspersed with the samples throughout the run. Determine the suitability of the chromatographic system using the following performance criteria:
 - a. Standard curve linearity: Determine that the correlation coefficient equals or exceeds 0.995 for the least squares equation which describes the detector response as a function of standard curve concentration.
 - b. Peak resolution: Visually determine that sufficient resolution has been achieved for the analyte and internal standard relative to background interferences.
 - c. Appearance of chromatograms: Visually determine the chromatograms with respect to peak response, baseline noise, and background interference. Visually determine that a signal-to-noise ratio of 10:1 has been attained for analytes at the 0.02/0.05 ng/mL calibration standard.
 20. **XDE-848BE has carryover.** For samples above 5ng/mL or unknowns it will be necessary to have a blank (25/25/50/0.1 acetonitrile/methanol/water/formic acid) following each injection.
 21. Re-analyze the samples which contain concentrations of XDE-848 and 5 metabolites greater than 80% of the highest standard by diluting with dilution solution.

22. The gross analyte concentration should be at least 30% above the lowest calibration standard and at least 20% less than the highest calibration standard

Notes:

1. Water volume for the sample analysis may be adjusted as long as all proportions remain the same.
2. Samples that are not clear may require a filter aid for the SPE portion of the analysis. An example is: Filter Aid 400, 3M Empore, catalog # 143783, Fisher Scientific.
3. All stock, fortification and calibration solutions should be store in refrigerator.

Determination of Isotopic Crossover

In this assay, analytes and internal standards are quantified using MS/MS transitions characteristic of each compound. When using stable-isotope labeled internal standards, there is a possibility that isotopic contributions will occur between the transitions used for quantitation of the unlabeled and labeled compounds. This isotopic overlap between the analyte and the internal standard can be determined empirically by analyzing individual standard solutions of each compound and should be addressed for accurate determinations of concentrations.

To determine the isotopic crossover effect, inject the following samples:

1. 4.0/10.0 ng/mL calibrator with no internal standard
 - See preparation of isotopic crossover solutions
2. 4.0/10.0 ng/mL calibrator with internal standard
 - See preparation of calibration solutions
3. 0.5 ng/mL internal standard solution
 - See preparation of isotopic crossover solutions
4. 0.02/0.05 calibration solution with internal standard
 - See preparation of calibration solutions
 -

Be sure to have two sample blanks (50/50/0.1% methanol/water/formic acid) after each calibration injection. There may be carryover of analytes that may affect results.

For the internal standard at the working concentration with no analytes, the peak area of the analytes should be less than the peak area of the lowest standard peak area for each of the analyte transitions. For the highest calibration solution containing no internal standard, the peak area for the internal standard analytes should be less than 5% of the internal standard peak areas of the highest calibration solution containing the working concentration of the internal standards.

Supplemental Notes

1. Equipment, glassware, materials, reagents, and chemicals considered to be equivalent to those specified may be substituted with the understanding that their performance must be confirmed by appropriate tests. Common laboratory glassware and supplies are assumed to be readily available. Unless specified otherwise, class volumetric glassware is used to prepare analytical standards, fortification solutions, and calibration standards.
2. The instrumental conditions may be modified to obtain optimal chromatographic separation and sensitivity.
3. In the preparation of fortification, internal standard and calibration solutions, the quantity weighed may be adjust to meet needs. Also, other dilution schemes may be followed.