Method Validation Study for the Determination of Residues of XDE-848 Benzyl Ester and Three Metabolites (X11438848, X12300837 and X11966341) in Soil and Sediment by 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, and X11966341 in soil and sediment. The method was validated over the concentration range of 0.003 - 0.300 µg/g with a validated limit of quantitation of 0.003 µg/g. Common names, chemical names, and molecular formulas for the analytes are given in Table 1 and Figures 1 - 8.

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 5 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, and X11966341 are extracted from soil with 90/10 acetonitrile/0.1N hydrochloric acid and from sediment with 90/9.5/0.5 acetonitrile/water/formic acid. The extracts are decanted, collected in one vial and the volume is adjusted. An aliquot of the extract is evaporated to 200-300 µL in a Turbo-vap. After reconstitution, the sample is loaded onto a solid phase extraction (SPE) cartridge. Rinse and elution steps follow. After elution from the SPE cartridge with 50/50 acetonitrile/methanol, the concentrated eluate is evaporated to dryness on a Turbo-vap. The sample is 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, and X11966341 by two structurally characteristic MS/MS transitions for each analyte by LC with tandem mass spectrometry. Analytical method may be found in Appendix I.

Test Substances/Reference Compounds/Analytical Standards

Test Substance	TSN	Percent Purity	Recertification Date	Reference
X11438848	TSN301691	99%	25-Oct-2015	FAPC13-000580
X11966341	TSN305649	98%	18-Oct-2015	FAPC13-000339
X12300837	TSN305650	99%	18-Oct-2015	FAPC13-000340
XDE-848 Benzyl Ester	TSN301734	99.70%	20-Oct-2015	FAPC13-000456

Table 1. Identities and Structures of Analytes

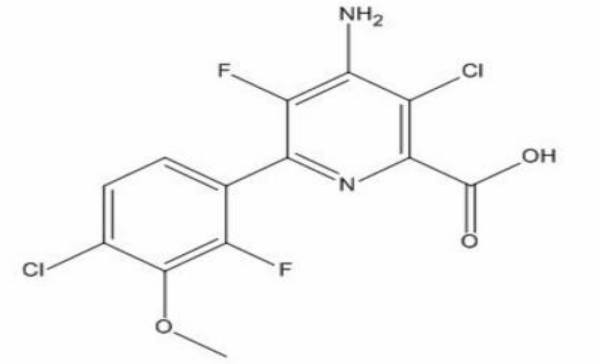
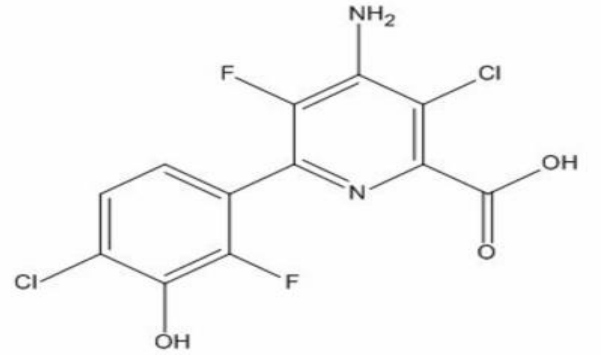
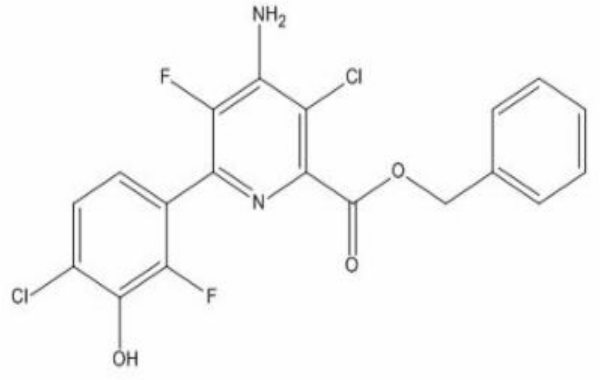
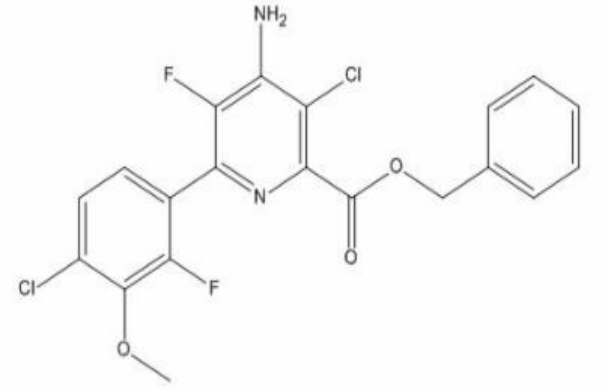
Common Name	Structural Formula
X11438848 Molecular Formula: $C_{13}H_8C_{l2}F_2N_2O_3$ CAS Number: 943832-81-3 Molecular Weight: 349.12	 4-amino-3-chloro-6-(4-chloro-2-fluoro-3- methoxyphenyl)-5-fluoro-2-pyridinecarboxylic acid
X11966341 Molecular Formula: $C_{12}H_6Cl_2F_2N_2O_3$ CAS Number: NA Molecular Weight: 335.09	 4-Amino-3-chloro-6-(4-chloro-2-fluoro-3-hydroxyphenyl)-5-fluoropyridine-2-carboxylic acid
X12300837 Molecular Formula: $C_{19}H_{12}Cl_2F_2N_2O_3$ CAS Number: NA Molecular Weight: 425.21	 benzyl 4-amino-3-chloro-6-(4-chloro-2-fluoro-3-hydroxyphenyl)-5-fluoropyridine-2-carboxylate

Table 1. Identities and Structures of Analytes

Common Name	Structural Formula
XDE-848 Benzyl Ester Molecular Formula: $C_{20}H_{14}Cl_2F_2N_2O_3$ CAS Number: 1390661-72-9 Molecular Weight: 439.24	 2-pyridinecarboxylic acid, 4-amino-3-chloro-6-(4-chloro-2-fluoro-3-methoxyphenyl)-5-fluoro-, phenylmethyl ester

Analytical Method for the Analysis of XDE-848 Benzyl Ester and Three Metabolites (X11438848, X12300837 and X11966341) in Soil and Sediment

Scope

This method is applicable for the determination of residues of XDE-848 Benzyl Ester and three metabolites (X11438848, X12300837 and X11966341) in soil and sediment. This method is applicable over a concentration range of 0.003 – 0.3 µg/g.

Principle

Residues of XDE -848 and three metabolites are extracted from soil with 90/10 acetonitrile/0.1N hydrochloric acid and from sediment with 90/9.5/0.5 acetonitrile/water/formic acid. The extracts are decanted, collected in one vial and the volume is adjusted to 70 mL. An aliquot of the extract is evaporated to 200-300 µL on a Turbo-vap. After reconstitution, the sample is loaded onto a solid phase extraction (SPE) cartridge. A rinse and elution steps follow. After elution from the SPE cartridge with 50/50 acetonitrile/methanol, the concentrated eluate is evaporated to dryness on a Turbo-vap. The sample is reconstituted with 25/25/50 acetonitrile/ methanol/ water containing 0.1% formic acid. The sample is analyzed for XDE-848BE and 3 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.

Acetonitrile, isopropanol and methanol are flammable and should be used in well-ventilated areas away from ignition sources. Formic acid and hydrochloric acid are 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

Bottle, 125 mL nalgene, part # 0331379C, Fisher Scientific

Bottle-Top Dispenser, 25 mL, catalog # 13-688-277, Fisher Scientific

Centrifuge Tube, 50 mL, catalog # 06-443-20, Fisher Scientific

Culture Tube, Disposable, 16 x 100mm, catalog #14-961-29, Fisher Scientific

Culture Tube, Disposable, screw top, 16 x 100mm, catalog # 73770-16100, Kimble Chase

Oasis HLB 3cc (60mg), SPE, part# WAT 094226, Waters

Pipette, positive-displacement, 1-10 µL capacity, catalog number F148501G, Gilson

Pipette, positive-displacement, 10-100 µL capacity, catalog number F148504G, Gilson

Pipette, positive-displacement, 20-50 µL capacity, catalog number F148503G, Gilson

Pipette, positive-displacement, 50-250 µL capacity, catalog number F148505G, Gilson

Pipette, positive-displacement, 100-1000 µL capacity, catalog number F48506G, Gilson

Repeater Plus Pipette, 1.0µL-10 mL capacity, catalog # 21-380-9, Eppendorf

Turbo-vap, Zymark

Vacuum Manifold, IST VacMaster

Chromatographic System

Column, analytical, Kinetex 1.7µ 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, Applied Biosystems

Mass spectrometer, QTRAP 5500, Applied Biosystems

Mass spectrometer data system, Analyst 1.6.2, Applied Biosystems

Reagents

Acetonitrile, HPLC grade, Sigma - Aldrich

Formic Acid, Optima, Fisher Scientific

Glycerol, Reagent ACS, 99.6%, Acros Organics

0.1N Hydrochloric Acid, Fisher Scientific

1 N Hydrochloric Acid, Fisher Scientific

Isopropanol, Fisher Scientific

Methanol, HPLC grade, Sigma - Aldrich

2-propanol, Sigma - Aldrich

Water, HPLC grade, 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. Pipette 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. Pipette 4.0 mL of formic acid into the 4L bottle and mix.

90/10, Acetonitrile/0.1 N Hydrochloric Acid (v/v)
Measure 3600 mL of acetonitrile, using a graduated cylinder, and transfer into a 4 L bottle. Add 400 mL of 0.1N Hydrochloric acid into the 4 L bottle and mix.

90/9.5/0.5, Acetonitrile/Water/Formic Acid (v/v/v)
Measure 3600 mL of acetonitrile, 380 mL of water and 20 mL of formic acid, using a graduated cylinder, and transfer into a 4L bottle and mix. Allow solution to reach room temperature before use.

50/50/0.1, Methanol/ Water/ Formic Acid (v/v)
Transfer 50 mL of methanol and 50 mL of water, each measured by graduated cylinder, into a 4 ounce jar. Pipette 100-µL of formic acid into the 4 ounce jar and mix. Allow solution to reach room temperature before use.

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

2/2/1, Methanol/Isopropanol/Water (v/v)
Measure 400-mL of methanol, 400-mL of isopropanol and 200-mL of water, using a graduated cylinder, and transfer into a 1-L bottle and mix.

10/90, Methanol/Water (v/v)
Measure 20 mL of methanol and 180 mL of water using a graduated cylinder, transfer each into an 8 ounce (250 mL) jar, and mix.

Preparation of Fortification Solutions of XDE-848 BE and Three Metabolites

1. Weigh out 0.0250 g of XDE-848 benzyl ester analytical standard and quantitatively transfer the analyte into a 25-mL volumetric flask with methanol. Dilute to volume with methanol to obtain a 1000-µg/mL stock solution of XDE-848 benzyl ester.

2. Weigh out 0.0250 g of X11438848, (XDE-848 acid) analytical standard and quantitatively transfer the analyte into a 25-mL volumetric flask with methanol. Dilute to volume with methanol to obtain a 1000-µg/mL stock solution of X11438848.

3. Weigh out 0.0250 g of X12300837, (XDE-848 BH) analytical standard and quantitatively transfer the analyte into a 25-mL volumetric flask with methanol. Dilute to volume with methanol to obtain a 1000-µg/mL stock solution of X12300837.

4. Weigh out 0.0250 g of X11966341, (XDE-848 HA) analytical standard and quantitatively transfer the analyte into a 25-mL volumetric flask with methanol. Dilute to volume with methanol to obtain a 1000-µg/mL stock solution of X11966341.

5. Pipette 10 mL of each of the four 1000-µg/mL solution of analytes prepared in steps 1 - 4 into the same 100-mL volumetric flask. Dilute to volume with methanol to obtain a 100-µg/mL mixed spiking solution containing the four analytes.

6. Pipette 10 mL of the 100-µg/mL mixed spiking solution prepared above into a 100-mL volumetric flask. Dilute to volume with methanol to obtain a 10-µg/mL mixed spiking solution containing the four analytes.

7. Pipette 10 mL of the 10-µg/mL mixed spiking solution prepared above into a 100 mL volumetric flask. Dilute to volume with methanol to obtain a 1.0-µg/mL mixed spiking solution containing the four analytes.

8. Pipette 1.0 mL of the 1.0-µg/mL mixed spiking solution prepared above into a 20 mL volumetric flask. Dilute to volume with methanol to obtain a 0.05-µg/mL mixed spiking solution containing the four analytes.

9. Pipette 300µL of the 1.0-µg/mL mixed spiking solution prepared in step 7 into a 20mL volumetric flask. Dilute to volume with methanol to obtain a 0.015-µg/mL mixed spiking solution containing the four analytes.

10. Pipette 1.0-mL of the 1.0-µg/mL mixed spiking solution prepared in step 7 into a 100mL volumetric flask. Dilute to volume with methanol to obtain a 0.01-µg/mL mixed spiking solution containing the four analytes.

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

Preparation of the Internal Standard Solutions of XDE-848 BE and Three Metabolites

1. Weigh out 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 1000-µg/mL stock solution of X12401027.

2. Weigh out 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-µg/mL stock solution of X12293409.

3. Weigh out 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-µg/mL stock solution of X12400867.

4. Weigh out 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-µg/mL stock solution of X12293407.

5. Pipette 10 mL of each 100-µg/mL solution of 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-µg/mL mixed internal standard solution.

6. Pipette 10 mL of the 10-µg/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-µg/mL mixed internal standard solution.

7. Pipette 1.0 mL of the 1.0-µg/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-µg/mL mixed internal standard solution.

Preparation of the Isotopic Crossover Solutions

1. Prepare a 20 ng/mL calibrator with no internal standard. Pipette 0.040 mL of the 1.0 µg/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 µg/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.

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

Preparation of Calibration Standards

Prepare calibration standards by using the spiking solutions described above as shown in the following table. Use the 0.01µg/mL mixed internal standard to prepare the calibration standard solutions. Dilute the calibrators to volume with 50/50/0.1, (v/v/v) methanol/water/formic acid solution. Store all calibration solutions in the refrigerator.

Original Spiking Solution Concentration	Aliquot of Spiking Solution	Aliquot of Internal Standard	Volume of Final Calibration Solution	Final Calibration Solution Concentration
µg/mL	mL	mL	mL	ng/mL
1.00	0.40	1.0	20	20.0
1.00	0.20	1.0	20	10.0
1.00	0.10	1.0	20	5.0
0.05	1.00	1.0	20	2.5
0.05	0.40	1.0	20	1.0
0.05	0.20	1.0	20	0.5
0.015	0.20	1.0	20	0.15
0.01	0.10	1.0	20	0.05

Instrumental Conditions

Typical LC-MS/MS Operating Conditions for XDE-848 Benzyl Ester and Three Metabolites Determination in Soil and Sediment

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:	10 µL
Autosampler Wash	30 seconds of methanol/isopropanol/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

Gradient:

Time, min	Solvent A, %	Solvent B, %
-3.0	50.0	50.0
0.0	50.0	50.0
8.0	0.0	100.0
10.0	0.0	100.0

Flow Diverter

1) 0.0 → 0.5 min – flow to waste
2) 0.5 → 10.0 min – flow to source
3) 10.0 → end of run – flow to waste

Typical Mass Spectrometry Operating Conditions for XDE-848 Benzyl Ester and Three Metabolites Determination in Soil and Sediment

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:	10 minutes
Dwell Time:	100 ms

Analytes:	**Precursor Ion Q1 (*m/z*)**	**Product Ion Q3 (*m/z*)**	**Declustering Potential (v)**	**Collision Energy (v)**	**Cell Exit Potential (v)**
XDE-848 BE(441/65)	441.0	65.0	60	119	8
XDE-848 BE(441/91)	441.0	91.0	56	69	10
XDE-848 acid(349/268)	349.0	267.9	66	43	22
XDE-848 acid(349/225)	349.0	225.1	56	69	16
XDE-848 BH(425/91)	425.0	90.9	76	57	10
XDE-848 BH(427/91)	427.0	90.9	86	63	14
XDE-848 HA(335/254)	334.9	254.0	91	47	18
XDE-848 HA(337/256)	336.9	256.0	71	47	24
XDE-848 BE IS	446.9	91.0	56	69	10
XDE-848 acid IS	356.9	276.0	76	43	22
XDE-848 BH IS	432.9	91.0	87	63	14
XDE-848 HA IS	340.9	260.0	91	47	18

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

Analysis Procedure

For procedural recovery samples:
1. For reagent blank, use extraction solution in 50-mL centrifuge tube containing no soil or sediment.
2. For control samples, transfer 5.0 gram of each soil or sediment type into a 50-mL nalgene centrifuge tube.
3. For fortified samples, transfer 5.0 gram of each soil or sediment type into separate 50-mL nalgene centrifuge tubes. Add the appropriate volume of the spiking solution to obtain fortified samples at LOD, LOQ, 10× LOQ and 100× LOQ (0.9, 3, 30 and 300 ng/g, respectively). Shake gently by hand to mix into the soil. If soil is wet, do not shake. The soil will compact and be more difficult to extract. To mix the sediment samples, vortex for 5 – 10 seconds.

To fortify 5 g of soil/sediment with the four analytes:			
Description	Spiking Volumes (µL)	Spiking Solutions (µg/mL)	Fortification Level (µg/g)
Control	---	---	---
LOD	90	0.05	0.0009
LOQ	300	0.05	0.003
10× LOQ	150	1.0	0.03
100× LOQ	150	10.0	0.3

For field samples:

Measure, by weight, 5.0 $\pm$ 0.05 grams of each soil or sediment sample into a 50-mL nalgene centrifuge tube.

Extraction Portion

4. Extraction procedure
    a) For soil samples, add 20 mL of extraction solution, 90/10, Acetonitrile/0.1N Hydrochloric Acid, to each sample tube.
    b) For sediment samples, add 20 mL of extraction solution, 90/9.5/0.5, Acetonitrile/water/formic acid, to each sample tube.
        1. Vortex to mix.
        2. Shake for 30 minutes on a flatbed shaker set at 280 excursion/min.
        3. Centrifuge for 5 minutes at 2000 rpm.
        4. Decant solution into 125 mL nalgene bottle.
    c) Add 15 mL of extraction solution and repeat above process.
    d) Add 15 mL of extraction solution and repeat above process.
    e) Add 15 mL of extraction solution and repeat above process.
5. Adjust volume to 70 mL with extraction solution.
    a) Compare solution level against 2 bottles containing a pre-measured 70 mL solution.
    b) Shake to mix.

6. Pipette a 2 mL aliquot of sample extract into a 16 x 100mm culture tube.
7. Add 20 µL of 1N Hydrochloric Acid. Vortex for 5 seconds.
8. Add 50 µL of keeper solution, 10/90 (w/v), glycerol/methanol to each tube. Vortex for 5 seconds.
9. Evaporate on a Turbo-vap set at 40°C and 7psi nitrogen for approximately 15 minutes. There should be between 200 – 300 µL of solution in the culture tube.
10. Add 500 µL of 50/50 acetonitrile/methanol to sample. Vortex for 5 – 10 seconds.
11. Add 2 mL of water containing 0.1% formic acid. Vortex for 5 – 10 seconds.

SPE Portion

12. Set up manifold. Use Oasis HLB 3cc (60 mg) cartridges. Part # WAT 094226.
13. 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.
14. Load sample onto SPE – save tube and Pasteur pipette for steps 15 and 17.
    a. Used glass Pasteur pipettes
    b. Pull samples through SPE cartridge at approximately 0.5 mL/minute.
    c. Pull a 5 second vacuum at the end of elution.
    d. Discard eluate.
15. Rinse sides of sample vial with 1.0 mL of 10/90, Methanol/ Water
    a. Load onto SPE
    b. Pull samples through SPE cartridge at approximately 0.5 mL/minute.
    c. Pull a 5 second vacuum at the end of elution.
    d. Discard eluate.
16. Dry for 5 minutes under full vacuum.
17. Elute analytes with 4 x 1.5-mL of 50/50 acetonitrile/methanol. (NOTE: Step a. helps remove the analytes from the sides of the glass tube and is a very important step to achieve recoveries.)
    a. Elutions 1 and 2 – Rinse the sides of the sample vial with the elution solution prior to loading onto the SPE cartridge.
    b. Elutions 3 and 4 – Load onto SPE cartridge only. No rinsing of vial required.
    c. Elute slowly using gravity to pull through the cartridge.
    d. Pulled a 5 second vacuum at the end of elution.
18. Add 100-µL of 0.01-µg/mL internal standard solution
19. Add 50-µL of keeper
20. Vortex gently
21. Evaporate to dryness on Turbo-vap set at 40°C with 7 psi nitrogen. (Approx. 30 - 40 min.)

22. Reconstitute
    a. Add 1 mL of 50/50 acetonitrile/methanol. **(NOTE: This removes the analytes from the sides of the glass tube and is a very important step.)**
    b. Vortex for 5 - 10 seconds.
    c. Add 1 mL of water containing 0.1% formic acid.
    d. Vortex for 5 – 10 seconds.
23. Transfer portion into HPLC vials.
24. 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 0.15 ng/mL calibration standard.
25. **XDE-848BE has carryover**. For samples above 5ng/mL or unknowns it will be necessary to inject a blank sample (25/25/50/0.1 acetonitrile/methanol/water/formic acid) following each injection.
26. Dilute any samples with concentrations greater than 80% of the highest standard with an appropriate amount of dilution solution. The dilution solution contains 0.9 mL of methanol + 0.1 mL of 0.01 µg/mL internal standard + 1.0 mL of water containing 0.1% formic acid.
27. The gross analyte concentration should be at least 30% above the lowest calibration standard and at least 20% less than the highest calibration standard

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:
4. 20 ng/mL calibrator with no internal standard
    - See preparation of isotopic crossover solutions
5. 20 ng/mL calibrator with internal standard
    - See preparation of calibration solutions

6. 0.5 ng/mL internal standard solution
    - See preparation of isotopic crossover solutions
7. 0.05 ng/mL calibration solution with internal standard.

Be sure to have two sample blanks (25/25/50 acetonitrile/methanol/water containing 0.1% formic acid) after each calibration injection. The carryover of XDE-848BE at the 20 ng/mL level will affect results.

For the internal standards only spiked sample, in order to be considered acceptable, the peak area in the analytes transition must be less than the analytes peak area in the lowest calibration standard.

For the high calibration standard prepared without internal standards, the peak area in the internal standard transition must be 5.0% or less than the internal standard peak area of the highest calibration standard spiked with internal standard.

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 A 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 adjusted to meet needs. Also, other dilution schemes may be followed.