Independent Laboratory Validation of a Dow AgroSciences Method for the Determination of XDE-848 Benzyl Ester and Three Metabolites (X11438848, X12300837 and X11966341) in Soil

INTRODUCTION

Scope

The objective of this study was to assess and to independently validate the method described in Dow AgroSciences Study 140956, "Analytical Method for the Analysis of XDE-848 Benzyl Ester and Three Metabolites (X11438848, X12300837 and X11966341) in Soil and Sediment" [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 three metabolites (X11438848, X12300837 and X11966341) in soil. The methodology was successfully independently validated over the concentration range of 0.003-0.3 mg/kg for XDE-848 benzyl ester and three metabolites with an independently validated limit of quantification of 0.003 mg/kg for XDE-848 benzyl ester and three metabolites.

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 Commission Regulation (EU) No 283/2013 setting out the data requirements, in accordance with Regulation (EC) No 1107/2009 [2] and EPA Guideline OCSPP 850.6100 [3] and Guidance Document SANCO/825/00 rev.8.1 [4].

Method Principle

Residues of XDE-848 benzyl ester and three metabolites were extracted from soil with acetonitrile/0.1N hydrochloric acid (90/10). The extracts were decanted, collected in one container and the volume was adjusted to 70 mL. An aliquot of the extract was evaporated to 200-300 µL on a Turbo-vap. After reconstitution, the sample was loaded onto a solid phase extraction (SPE) cartridge. After elution from the SPE cartridge with acetonitrile/methanol (50/50), the concentrated eluate was evaporated to dryness on a Turbo-vap. The sample was reconstituted with acetonitrile/methanol/ water containing 0.1% formic acid (25/25/50). The sample was analyzed for XDE-848 benzyl ester and three metabolites by liquid chromatography with positive ion electrospray ionization tandem mass spectrometry (LC-MS/MS).

Test Substances/Reference Compounds/Analytical Standards

Analytical Standard[a]	TSN	Percent Purity	Re-Certification Date	Reference
XDE-848 Benzyl Ester	TSN305894	99.2	11 Jul 2015	FAPC13-000386
X11438848	TSN301691	99	25 Oct 2015	FAPC13-000580
X12300837	TSN305650	99	18 Oct 2015	FAPC13-000340
X11966341	TSN306022	98	21 Oct 2015	FAPC13-000454
X12293407[b]	TSN301884	100	09 Oct 2015	FAPC14-000027
X12293409[b]	TSN301886	100	05 Oct 2016	FAPC15-000284
X12400867[b]	TSN305028	99	05 Oct 2016	FAPC15-000283
X12401027[b]	TSN305041	99	21 Oct 2016	FAPC15-000139

[a]The molecular formulae and structures are given in Table 1. The certificates of analysis are given in Figure 1 to Figure 8.
[b]Provided as a 1.0 µg/mL mixed solution in methanol by the sponsor.

EXPERIMENTAL

Full details of the instrumental conditions used during this independent validation are given in Appendix 1.

Sample Origin and Storage

The independent validation was carried out using a characterised soil sample, obtained from Battelle UK stocks of control samples. A sandy loam was chosen to represent the most difficult soil type to analyse. The soil sample was characterised by Agvise, Northwood, ND, 58267, in a separate study and full characterisation details are given in Appendix 2. A unique sample number was assigned to the sample to track it during receipt, 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:

XDE-848 Benzyl Ester	*m/z* Q1/Q3 441/65 (quantitative)
XDE-848 Benzyl Ester	*m/z* Q1/Q3 441/91 (confirmatory)
X12293407 IS	*m/z* Q1/Q3 447/91

X11438848	*m/z* Q1/Q3 349/268 (quantitative)
X11438848	*m/z* Q1/Q3 349/225 (confirmatory)
X12293409 IS	*m/z* Q1/Q3 357/276
X12300837	*m/z* Q1/Q3 425/91 (quantitative)
X12300837	*m/z* Q1/Q3 427/91 (confirmatory)
X12400867 IS	*m/z* Q1/Q3 433/91
X11966341	*m/z* Q1/Q3 335/254 (quantitative)
X11966341	*m/z* Q1/Q3 337/256 (confirmatory)
X12401027 IS	*m/z* Q1/Q3 341/260

In order to generate a standard curve, plot the analyte concentration/internal standard concentration 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, determine the equation for the curve with respect to the abscissa. Refer to Figure 9 through Figure 16 for example calibration plots and to Figure 17 for example calculations. Individual calibration results can be found in Table 2 through Table 9.

Confirmation of Residue Identity

The method is selective for the determination of XDE-848 benzyl ester and three metabolites by virtue of the chromatographic separation and MS/MS detection. When detection is by tandem mass spectrometry, confirmation of the presence of the analyte requires the observation of a precursor ion plus a structurally significant product ion observed at the same retention time [5]. Confirmation demonstrates the selectivity of the primary method for the representative sample matrix. 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 18 to Figure 25 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 and three metabolites

in soil. 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 and three metabolites and analyzing the set following the procedures described in this report. Samples were fortified at the limit of detection (LOD) of 0.0009 mg/kg, the limit of quantitation (LOQ) of 0.003 mg/kg, and at the higher fortification level of 0.3 mg/kg for XDE-848 benzyl ester and three metabolites (100 x LOQ). Samples fortified at the LOD were analyzed 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 matrices and a reagent blank were also included in each set.

Changes to the Method

The injection volume was increased from 10 µL to 30 µL due to poor sensitivity at the lower injection volume.

Table 1 Identity and Structure of XDE-848 Benzyl Ester and Three Metabolites (X11438848, X12300837 and X11966341) and Internal Standards

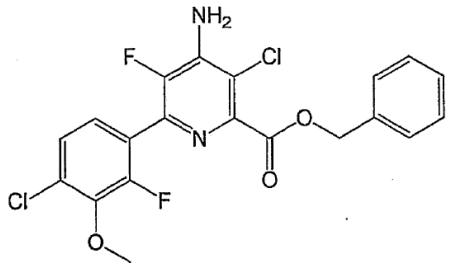
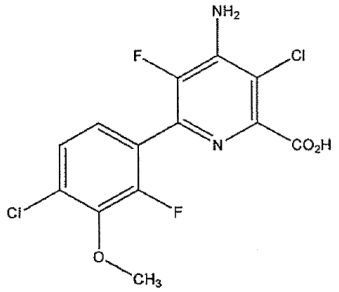
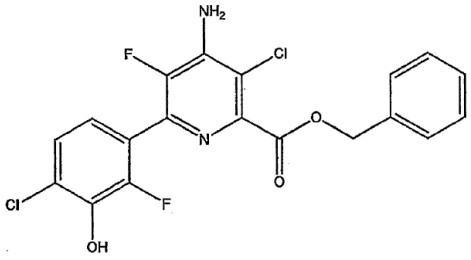
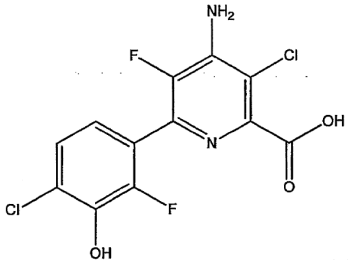
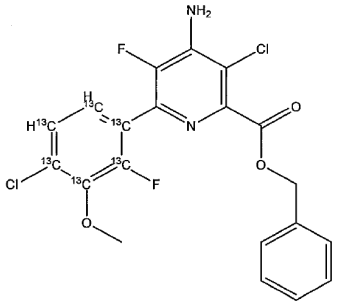
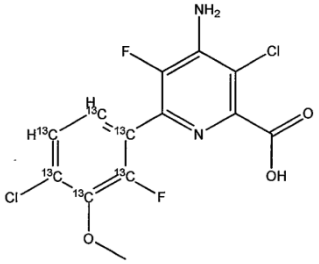
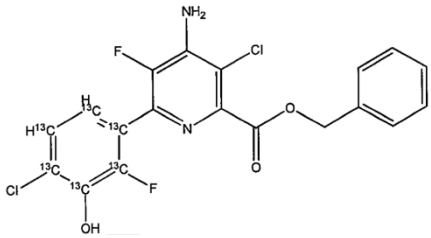
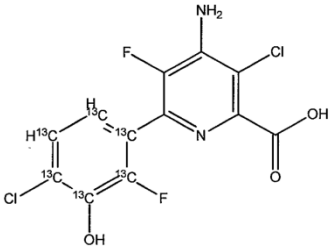
Common Name	Structural Formula and Chemical Name
XDE-848 Benzyl Ester Molecular Formula: $C_{20}H_{14}Cl_2F_2N_2O_3$ Molecular Weight: 439.24 CAS Number: 1390661-72-9	 Benzyl 4-amino-3-chloro-6-(4-chloro-2-fluoro-3-methoxyphenyl)-5-fluoropyridine-2-carboxylate
X11438848 Molecular Formula: $C_{13}H_8Cl_2F_2N_2O_3$ Molecular Weight: 349.12 CAS Number: 943832-81-3	 4-amino-3-chloro-6-(4-chloro-2-fluoro-3-methoxyphenyl)-5-fluoropyridine-2-carboxylic acid
X12300837 Molecular Formula: $C_{19}H_{12}Cl_2F_2N_2O_3$ Molecular Weight: 425.21	 Benzyl 4-amino-3-chloro-6-(4-chloro-2-fluoro-3-hydroxyphenyl)-5-fluoropyridine-2-carboxylate
X11966341 Molecular Formula: $C_{12}H_6Cl_2F_2N_2O_3$ Molecular Weight: 335.09	 4-amino-3-chloro-6-(4-chloro-2-fluoro-3-hydroxyphenyl)-5-fluoropyridine-2-carboxylic acid

Table 1 Cont. Identity and Structure of XDE-848 Benzyl Ester and Three metabolites (X11438848, X12300837 and X11966341) and Internal Standards

Common Name	Structural Formula and Chemical Name
X12293407 (XDE-848 Benzyl Ester IS) Molecular Formula: $C_{14}{}^{13}C_6H_{14}Cl_2F_2N_2O_3$ Molecular Weight: 445.20	 benzyl 4-amino-3-chloro-6-(4-chloro-2-fluoro-3-methoxy(13C6)phenyl)-5-fluoropyridine-2-carboxylate
X12293409 (X11438848 IS) Molecular Formula: $C_7{}^{13}C_6H_8Cl_2F_2N_2O_3$ Molecular Weight: 355.07	 4-amino-3-chloro-6-(4-chloro-2-fluoro-3-methoxy(13C6)phenyl)-5-fluoropyridine-2-carboxylic acid
X12400867 (X12300837 IS) Molecular Formula: $C_{13}{}^{13}C_6H_{12}Cl_2F_2N_2O_3$ Molecular Weight: 431.17	 benzyl 4-amino-3-chloro-6-(4-chloro-2-fluoro-3-hydroxy(13C6)phenyl)-5-fluoropyridine-2-carboxylate
X12401027 (X11966341 IS) Molecular Formula: $C_6{}^{13}C_6H_6Cl_2F_2N_2O_3$ Molecular Weight: 341.05	 4-amino-3-chloro-6-(4-chloro-2-fluoro-3-hydroxy(13C6)phenyl)-5-fluoropyridine-2-carboxylic acid

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The following provides an explanation of the actual materials and instrumentation used for this independent laboratory validation.

Laboratory Equipment

Balance, MSU225S Cubis 220-5, Sartorius Ltd

Balance, LP 4200, Sartorius Ltd

Centrifuge Varifuge 3.0R, Heraeus Instruments Ltd

Multipette Eppendorf Xstream®, Fisher Scientific

Pipettes, Gilson 'Microman', Anachem Ltd

Pipettes, Rainin Pos-D, Anachem Ltd

SPE manifold, Biotage AB

Turbo-Vap® II sample concentrator, Biotage

Vortex mixer, Fisher Scientific

Glassware and Materials

Centrifuge tubes, 50 mL plastic, VWR International

Glass Pasteur pipettes, Fisher Scientific

Glass tubes, disposable borosilicate, 16 x 100 mm, Corning

Plastic bottle, 125 mL plastic, VWR International

SPE cartridges, Oasis HLB 3cc, 3 mL, 60 mg, WAT 094226, Soils

Volumetric flasks, VWR

Chromatographic Systems

Autosampler, CTC Analytics HTS-xt PAL, Eksigent

Column, Kinetex PFP, 100 × 2.1 mm, 1.7 µm, 00D-4476-AN, Phenomenex

Guard column, SecurityGuard ULTRA cartridges, UHPLC PFP for 2.1mm ID columns, AJO-8787, Phenomenex

Guard Column holder, SecurityGuard ULTRA cartridge holder, AJO-9000, Phenomenex

Liquid chromatograph, Agilent HPLC 1290, binary pump and column oven, Agilent Technologies UK

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

Mass spectrometer software, Analyst, version 1.6.2, AB Sciex

Reagents

Acetonitrile, HPLC grade, Fisher Scientific

Formic Acid (99-100%), VWR

Glycerol, Fisher Scientific

Hydrochloric Acid (32%), Fisher Scientific

Isopropanol, VWR

Methanol, HPLC grade, Fisher Scientific

Water, HPLC grade, Rathburn Chemicals Ltd

Preparation of Stock Solutions

25 mg (adjusted for purity) of XDE-848 benzyl ester (XDE-848 BE), X11438848, X12300837 and X11966341 were weighed into separate 25 mL volumetric flasks and diluted to volume with methanol to obtain a 1000 µg/mL stock solution of each analyte.

Preparation of Fortification Solutions

1. 1.0 mL of each of the 1000 µg/mL XDE-848 benzyl ester, X11438848, X12300837 and X11966341 stock solutions were pipetted into a 10 mL volumetric flask and diluted to volume with methanol to obtain a 100 µg/mL mixed solution.
2. 1.0 mL of the 100 µg/mL mixed XDE-848 benzyl ester, X11438848, X12300837 and X11966341 solution was pipetted into a 10 mL volumetric flask and diluted to volume with methanol to obtain a 10 µg/mL mixed solution.
3. 1.0 mL of the 10 µg/mL mixed XDE-848 benzyl ester, X11438848, X12300837 and X11966341 solution was pipetted into a 10 mL volumetric flask and diluted to volume with methanol to obtain a 1.0 µg/mL mixed solution.
4. 1.0 mL of the 1.0 µg/mL mixed XDE-848 benzyl ester, X11438848, X12300837 and X11966341 solution was pipetted into a 20 mL volumetric flask and diluted to volume with methanol to obtain a 0.05 µg/mL mixed solution.

5. 0.3 mL of the 1.0 µg/mL mixed XDE-848 benzyl ester, X11438848, X12300837 and X11966341 solution was pipetted into a 20 mL volumetric flask and diluted to volume with methanol to obtain a 0.015 µg/mL mixed solution.
6. 1.0 mL of the 1.0 µg/mL mixed XDE-848 benzyl ester, X11438848, X12300837 and X11966341 solution was pipetted into a 100 mL volumetric flask and diluted to volume with methanol to obtain a 0.01 µg/mL mixed solution.

Preparation of Internal Standard Working Solution and Sample Dilution Solution

1. 1.0 mL of the 1.0 µg/mL mixed X12293407, X12293409, X12400867 and X12401027 solution in methanol was pipetted into a 100 mL volumetric flask and diluted to volume with methanol to obtain a 0.01 µg/mL mixed internal standard solution.
2. 2.0 mL of the 0.01 µg/mL mixed internal standard solution was pipetted into a vial and 18 mL of methanol and 20 mL of water containing 0.1% formic acid were added and mixed to obtain a 0.5 ng/mL mixed internal standard solution used to dilute samples.

Preparation of Calibration Standards

Calibration standards were prepared by dispensing 1.0 mL of the 0.01 µg/mL mixed internal standard solution into a 20 mL volumetric flask, before diluting the appropriate amount of mixed XDE-848 benzyl ester, X11438848, X12300837 and X11966341 fortification solutions with a methanol/water (50/50) containing 0.1% formic acid solution to obtain calibration solutions over the concentration range of 0.05-20.0 ng/mL as described in the following table:

Concentration of Stock Solution (µg/mL)	Aliquot (mL)	Volume of 0.01 µg/mL ISTD (mL)	Final Volume (mL)	Calibration Solution Final Conc. (ng/mL)	Equivalent Sample Conc. (mg/kg)[a]
1.0	0.4	1.0	20	20.0	0.280
1.0	0.2	1.0	20	10.0	0.140
1.0	0.1	1.0	20	5.0	0.070
0.05	1.0	1.0	20	2.5	0.035
0.05	0.4	1.0	20	1.0	0.014
0.05	0.2	1.0	20	0.5	0.0070
0.015	0.2	1.0	20	0.15	0.00021
0.01	0.1	1.0	20	0.05	0.00070

[a]The equivalent sample concentrations are based on taking an initial sample weight of 5 g, extracting with 70 mL and taking 2 mL through an SPE clean-up with a final volume of 2 mL.

Analytical Procedure

1. For the reagent blank, extraction solution in a 50-mL centrifuge tube containing no soil was used.
2. For control samples, 5.0 gram of soil was transferred into a 50-mL centrifuge tube.
3. For fortified samples, 5.0 gram of soil was transferred into separate 50-mL centrifuge tubes. The appropriate volume of the spiking solution was added to obtain fortified samples at LOD, LOQ and 100×LOQ (see table below for example fortifications).

To fortify 5 g of soil with the four analytes:			
Description	Spiking Volume (µL)	Spiking Solution (µg/mL)	Fortification Level (mg/kg)
Control	---	---	---
LOD	90	0.05	0.0009
LOQ	300	0.05	0.003
100× LOQ	150	10.0	0.3

4. Extraction procedure:
   a) 20 mL of extraction solution was added, 90/10, acetonitrile/0.1N hydrochloric acid, to each sample tube.
      i. The samples were vortexed to mix.
      ii. The samples were shaken for 30 minutes on a flatbed shaker set at 280 excursion/min.
      iii. The samples were centrifuge for 5 minutes at 2000 rpm.
      iv. The solution was decanted into a 125 mL plastic bottle.
   b) 15 mL of extraction solution was added and the above process was repeated.
   c) 15 mL of extraction solution was added and the above process was repeated.
   d) 15 mL of extraction solution was added and the above process was repeated.

5. The volume was adjusted to 70 mL with extraction solution.
   a) The solution level was compared against 2 bottles containing pre-measured 70 mL solution.
   b) The samples were shaken to mix.
6. A 2 mL aliquot of solution was pipetted into a 16 x 100mm culture tube.
7. 20 µL of 1N hydrochloric acid was added. The samples were vortexed for 5 seconds.

8. 50 µL of keeper solution, 10/90 (w/v), glycerol/methanol was added to each tube. The samples were vortexed for 5 seconds.
9. The samples were evaporated on a Turbo-vap set at 40°C and 7 psi nitrogen for approximately 15 minutes. There was between 200-300 µL of solution in the culture tube.
10. 500 µL of 50/50 acetonitrile/methanol was added to each sample. The samples were vortexed for 5-10 seconds.
11. 2 mL of water containing 0.1% formic acid was added. The samples were vortexed for 5-10 seconds.

SPE Portion

12. An SPE manifold was set up using Oasis HLB 3cc (60 mg) cartridges (WAT 094226).
13. The SPE cartridges were conditioned:
    a. 3 mL of 50/50 acetonitrile/methanol followed by 3 mL of water containing 0.1% formic acid.
    b. A 5 second vacuum was applied at the end of each elution.
    c. The eluate was discarded.
14. The samples were loaded onto the SPE cartridges – the tubes and Pasteur pipettes were saved for steps 15 and 17.
    a. Glass Pasteur pipettes were used.
    b. The samples were pulled through the SPE cartridges at approximately 0.5 mL/minute.
    c. A 5 second vacuum was applied at the end of the elution.
    d. The eluate was discarded.
15. The sides of the sample vials were rinsed with 1.0 mL of 10/90, methanol/water:
    a. This rinses were loaded onto the SPE cartridges.
    b. The samples were pulled through the SPE cartridges at approximately 0.5 mL/minute.
    c. A 5 second vacuum was applied at the end of elution.
    d. The eluate was discarded.
16. The SPE cartridges were dried for 5 minutes under full vacuum.
17. The analytes were eluted with 4 × 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 steps to achieve recoveries.)
    a. Elutions 1 and 2 – the sides of the sample vials were rinsed with the elution solution prior to loading onto the SPE cartridges.
    b. Elutions 3 and 4 – were loaded onto the SPE cartridges only. No rinsing of the vials was required.
    c. The elutions were performed slowly using gravity to pull through the cartridge.
    d. A 5 second vacuum was applied at the end of the elutions.
18. 100 µL of 0.01 µg/mL internal standard solution was added.
19. 50 µL of keeper solution, 10/90 (w/v), glycerol/methanol was added.
20. The samples were vortexed gently.
21. The samples were evaporated to dryness on a Turbo-vap set at 40°C with 7 psi nitrogen. (Approx. 30 - 40 minutes).

22. Reconstitution:
    a. 1 mL of 50/50 acetonitrile/methanol was added.
    (<u>NOTE:</u> This removes the analytes from the sides of the glass tube and is a very important step.)
    b. The sample was vortexed for 5-10 seconds.
    c. 1 mL of water containing 0.1% formic acid was added.
    d. The samples were vortexed for 5-10 seconds.
23. A portion of each sample was transferred to a HPLC vial. The 100 × LOQ samples were diluted by a factor of 10 with the 0.5 ng/mL mixed internal standard solution.
24. The calibration standards and samples were analyzed by LC-MS/MS with positive-ion electrospray tandem mass spectrometry, injecting the calibration standards interspersed with the samples throughout the run.

<u>Instrumental Conditions</u>

<u>Instrumentation</u>

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

<u>Typical Liquid Chromatography Operating Conditions</u>

Column:	Phenomenex Kinetex PFP, 100 mm x 2.1 mm x 1.7 µm
Column Temperature:	20ºC
Injection Volume:	30 µL
Flow Rate:	300 µL/min
Mobile Phase A:	HPLC grade water containing 0.1% formic acid
Mobile Phase B:	HPLC grade methanol containing 0.1% formic acid

Time – minutes	% Mobile Phase A	% Mobile Phase B
0.00	50	50
8.00	0	100
10.00	0	100
10.10	50	50
13.00	50	50

Approximate Retention Time: XDE-848 benzyl ester 6.4 mins
X11438848 3.4 mins
X12300837 5.6 mins
X11966341 2.1 mins

Solvent Wash 1:	Methanol/Isopropanol/Water 2/2/1 (v/v/v)	
Solvent Wash 2:	Methanol/Water (10/90 v/v)	
Injector Wash Program:	Post Clean with Solvent 1	× 2
	Post Clean with Solvent 2	× 2
	Valve Clean with Solvent 1	× 2
	Valve Clean with Solvent 2	× 2

Typical Mass Spectrometry Operating Conditions

Ion Source:	Turbo Spray IonDrive
Polarity:	Positive
Ion Spray Voltage (IS):	5500
Collision Gas (CAD):	Medium
Temperature (TEM):	500
Curtain Gas (CUR):	20
Ion Source Gas 1 (GS1):	50
Ion Source Gas 2 (GS2):	50
Entrance Potential (EP):	10

Analyte	Ion Mass Transitions (*m/z*)	Dwell Time (msec)	Declustering Potential (DP)	Collision Energy (CE)	Collision Cell Exit Potential (CXP)
XDE-848 BE	441.0/65.0	100	60	119	8
	441.0/91.0	100	60	69	10
X11438848	349.0/267.9	100	60	43	22
	349.0/225.1	100	60	69	16
X12300837	425.0/90.9	100	60	57	10
	427.0/90.9	100	60	63	14
X11966341	334.9/254.0	100	60	47	18
	336.9/256.0	100	60	47	24
X12293407 (XDE-848 BE IS)	446.9/91.0	100	60	69	10
X12293409 (X11438848 IS)	356.9/276.0	100	60	43	22
X12400867 (X12300837 IS)	432.9/91.0	100	60	63	14
X12401027 (X11966341 IS)	340.9/260.0	100	60	47	18