Independent Laboratory Validation of a Dow AgroSciences Method for the Determination of XDE-848 Benzyl Ester and Five Metabolites (X11438848, X12300837, X11966341, X12131932 and X12393505) in Water

INTRODUCTION

Scope

The objective of this study was to assess and to independently validate the method described in Dow AgroSciences Study 140952, "Method for the Analysis of XDE-848 Benzyl Ester and Five Metabolites (X11438848, X12300837, X11966341, X12131932 and X12393505) in Water" [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 five metabolites (X11438848, X12300837, X11966341, X12131932 and X12393505) in drinking water, ground water and surface water. The methodology was successfully independently validated over the concentration range of 0.02-2.0 ng/mL for XDE-848 benzyl ester and 0.05-5.0 ng/mL for its five metabolites with an independently validated limit of quantification of 0.02 ng/mL for XDE-848 benzyl ester and 0.05 ng/mL for its five 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 Guidance Documents EPA Guideline OCSPP 850.6100 [3] and SANCO/825/00 rev.8.1. [4].

Method Principle

Residues of XDE-848 benzyl ester and five metabolites (X11438848, X12300837, X11966341, X12131932 and X12393505) were extracted from water samples by adding formic acid and acetonitrile/methanol (50/50) before purification using Oasis HLB 3cc (60 mg) SPE cartridges. A solution of 0.01 µg/mL mixed internal standard was added and the samples were evaporated to dryness before reconstitution using acetonitrile/methanol/formic acid (50/50/0.1). The samples were then diluted with water containing 0.1% formic acid. The final sample was analysed for XDE-848 benzyl ester and five metabolites and their respective internal standards by liquid chromatography coupled with positive-ion electrospray 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
X12131932	TSN304497	97	04 Oct 2016	FAPC14-000785
X12393505	TSN304479	98	04 Oct 2016	FAPC14-000784
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 10.
[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 fully characterised drinking water, ground water and surface water samples, obtained from Battelle UK stocks of control samples. The water samples were characterised by Agvise, Northwood, ND, 58267, in a separate study and full characterisation details are given in Appendix 2. Unique sample numbers were assigned to the samples to track them 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 439/91 (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 351/270 (quantitative)
X11438848	*m/z* Q1/Q3 349/268 (confirmatory)
X12293409 IS	*m/z* Q1/Q3 355/274
X12300837	*m/z* Q1/Q3 427/91 (quantitative)
X12300837	*m/z* Q1/Q3 425/91 (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
X12131932	*m/z* Q1/Q3 405/65 (quantitative)
X12131932	*m/z* Q1/Q3 407/91 (confirmatory)
X12293407 IS	*m/z* Q1/Q3 447/91
X12393505	*m/z* Q1/Q3 315/234 (quantitative)
X12393505	*m/z* Q1/Q3 315/124 (confirmatory)
X12293409 IS	*m/z* Q1/Q3 355/274

Isotopically labeled internal standards were not available for X12131932 and X12393505.

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 11 through Figure 22 for example calibration plots and to Figure 23 for example calculations. Individual calibration results can be found in Table 2 through Table 13.

Confirmation of Residue Identity

The method is selective for the determination of XDE-848 benzyl ester and five 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 all representative sample matrices. 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 24 to Figure 33 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 five metabolites in drinking water, ground water and surface water. 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 five metabolites and analyzing the set following the procedures described in this report. Samples were fortified at the limit of detection (LOD) of 0.006 ng/mL for XDE-848 benzyl ester and 0.015 ng/mL for its five metabolites, the limit of quantitation (LOQ) of 0.02 ng/mL for XDE-848 benzyl ester and 0.05 ng/mL for its five metabolites, and at the higher fortification level of 2.0 ng/mL for XDE-848 benzyl ester and 5.0 ng/mL for its five 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.

Critical Steps

Plastic or Nalgene must not be used in any part of this procedure. The Solid Phase Extraction (SPE) elution step and the reconstituting step at the end of the method are critical steps. These critical steps are stated in the method.

Changes to the Method

The injection volume was increased from 15 µL to 30 µL due to poor sensitivity at the lower injection volume.

Table 1 Identity and Structure of XDE-848 Benzyl Ester and Five Metabolites (X11438848, X12300837, X11966341, X12131932 and X12393505) and Internal Standards

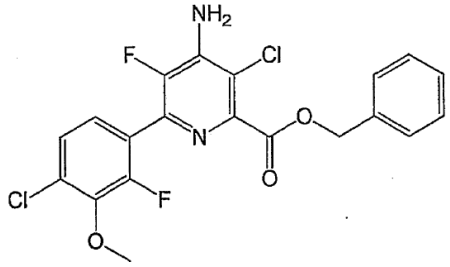
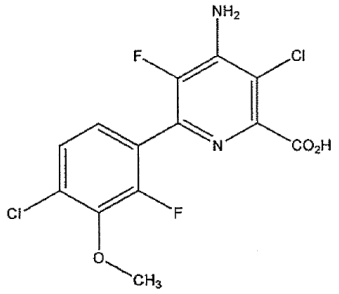
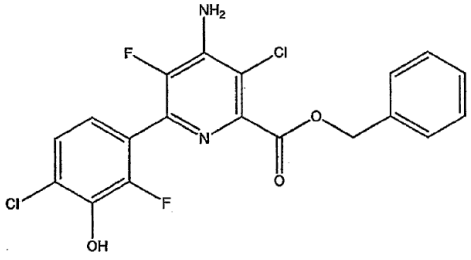
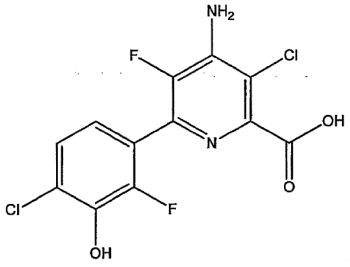
Common Name	Structural Formula and Chemical Name
XDE-848 Benzyl Ester Molecular Formula: $C_{20}H_{14}Cl_2F_2N_2O_3$ Molecular Weight: 439.24 CAS Number: 1390661-72-9	 Benzyl 4-amino-3-chloro-6-(4-chloro-2-fluoro-3-methoxyphenyl)-5-fluoropyridine-2-carboxylate
X11438848 Molecular Formula: $C_{13}H_8Cl_2F_2N_2O_3$ 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 Five Metabolites (X11438848, X12300837, X11966341, X12131932 and X12393505) and Internal Standards

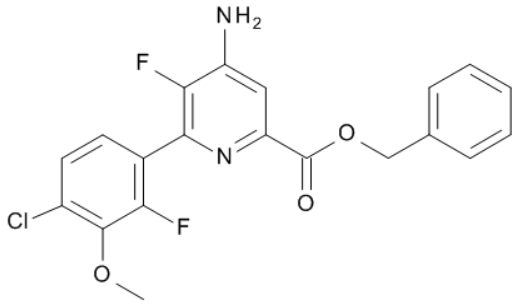
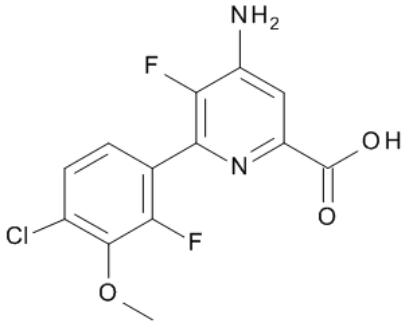
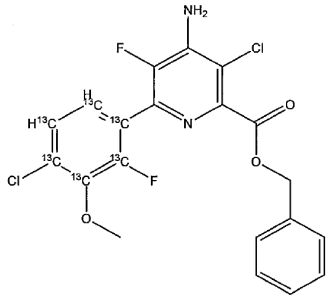
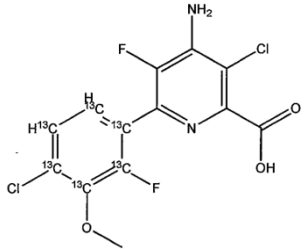
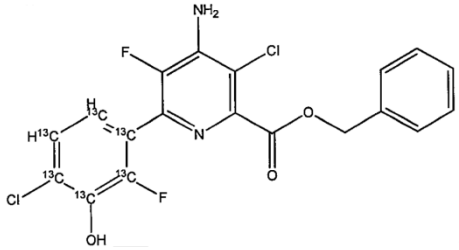
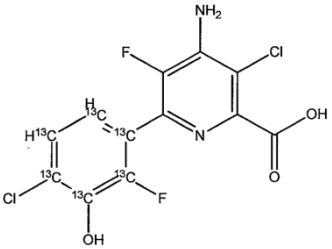
X12131932 Molecular Formula: $C_{20}H_{15}ClF_2N_2O_3$ Molecular Weight: 404.79	 Benzyl 4-amino-6-(4-chloro-2-fluoro-3-methoxyphenyl)-5-fluoropyridine-2-carboxylate
X12393505 Molecular Formula: $C_{13}H_9ClF_2N_2O_3$ Molecular Weight: 314.67	 4-amino-6-(4-chloro-2-fluoro-3-methoxyphenyl)-5-fluoropyridine-2-carboxylic acid

Table 1 Cont. Identity and Structure of XDE-848 Benzyl Ester and Five metabolites (X11438848, X12300837, X11966341, X12131932 and X12393505) and Internal Standards

Common Name	Structural Formula and Chemical Name
X12293407 (XDE-848 Benzyl Ester and X12131932 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 and X12393505 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

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

Glass Pasteur pipettes, Fisher Scientific

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

Glass vials, Fisher Scientific

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

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

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, X11966341, X12131932 and X12393505 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. 0.4 mL of the 1000 µg/mL XDE-848 BE stock solution and 1.0 mL of each of the 1000 µg/mL X11438848, X12300837, X11966341, X12131932 and X12393505 stock solutions were pipetted into a 10 mL volumetric flask and diluted to volume with methanol to obtain a mixed spiking solution containing 40 µg/mL of XDE-848 BE and 100 µg/mL of the five metabolites.
2. 1.0 mL of the 40/100 µg/mL mixed solution was pipetted into a 10 mL volumetric flask and diluted to volume with methanol to obtain a 4.0/10 µg/mL mixed solution.
3. 1.0 mL of the 4.0/10 µg/mL mixed solution was pipetted into a 10 mL volumetric flask and diluted to volume with methanol to obtain a 0.40/1.0 µg/mL mixed solution.
4. 1.0 mL of the 0.40/1.0 µg/mL mixed solution was pipetted into a 10 mL volumetric flask and diluted to volume with methanol to obtain a 0.040/0.10 µg/mL mixed solution.
5. 0.10 mL of the 0.40/1.0 µg/mL mixed solution was pipetted into a 10 mL volumetric flask and diluted to volume with methanol to obtain a 0.0040/0.010 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 50/50/0.1 acetonitrile/methanol/formic acid 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 BE, X11438848, X12300837, X11966341, X12131932 and X12393505 fortification solutions with a methanol/water (50/50) containing 0.1% formic acid solution to obtain calibration solutions over the concentration range of 0.03-4.0 ng/mL for XDE-848 BE and 0.075-10 ng/mL as described in the following table:

Concentration of Stock Solution (µg/mL) XDE-848 BE / 5 metabolites	Aliquot (mL)	Volume of 0.01 µg/mL ISTD (mL)	Final Volume (mL)	Calibration Solution Final Conc. (ng/mL)	Equivalent Sample Conc. (ng/mL)[a]
0.40 / 1.0	0.2	1.0	20	4.0 / 10	0.80 / 2.0
0.40 / 1.0	0.1	1.0	20	2.0 / 5.0	0.40 / 1.0
0.040 / 0.10	0.5	1.0	20	1.0 / 2.5	0.20 / 0.50
0.040 / 0.10	0.2	1.0	20	0.40 / 1.0	0.080 / 0.20
0.040 / 0.10	0.1	1.0	20	0.20 / 0.50	0.040 / 0.10
0.0040 / 0.010	0.5	1.0	20	0.10 / 0.25	0.020 / 0.050
0.0040 / 0.010	0.15	1.0	20	0.030 / 0.075	0.0060 / 0.0150

[a] The equivalent sample concentrations are based on taking an initial sample volume of 10 mL and a final volume of 2 mL.

Analytical Procedure

Note: - <u>Plastic or Nalgene was not used in any part of this procedure.</u>

1. For recovery samples:
   a. 10 mL of sample was measured into a glass vial.

b. For recovery samples, the appropriate volume of the spiking solution was added (a reagent blank contains no sample matrix). Refer to the following table for example fortification levels.
c. The samples were vortexed for 5 seconds.

Fortify 10 mL of water with the six analytes:			
Description	Spiking Volume (µL)	Spiking Solution (µg/mL)	Fortification Level (ng/mL)
-	-	XDE-848 BE / 5 metabolites	
Control	-	-	-
LOD	15	0.0040/0.010	0.0060/0.015
LOQ	50	0.0040/0.010	0.020/0.050
100 x LOQ	50	0.40/1.0	2.0/5.0

2. 100 µL of formic acid was added to each sample.
3. The samples were vortexed for 5 seconds.
4. 500 µL of 50/50 acetonitrile/methanol solution was added to each sample.
5. The samples were vortexed for 5 seconds.

SPE Portion **Plastic or Nalgene was not used in any part of this procedure.**

6. A manifold was set up. Waters Oasis HLB 3cc (60 mg) cartridges were used. Part number WAT 094226.
7. SPE Conditioning:
   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.
8. The sample was loaded onto SPE – the vial and Pasteur pipette was saved for steps 9 and 11.
   a. Glass Pasteur pipettes were used.
   b. Samples were pulled through the SPE cartridge slowly at approximately 0.5 mL/minute.
   c. A 5 second vacuum was applied at the end of elution.
   d. The eluate was discarded.
9. The sides of vial were rinsed with 1.0 mL of HPLC grade water.
   a. The rinse was loaded onto the SPE cartridge.
   b. The rinse was pulled through the SPE cartridge at approximately 1 mL/minute.
   c. A 5 second vacuum was applied at the end of elution.
   d. The eluate was discarded.

10. The SPE cartridge was dried for 5 minutes under full vacuum.
11. Analytes were eluted 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 – The sides of sample vial were rinsed with the 1.5 mL of 50/50 acetonitrile/methanol solution prior to loading onto the SPE cartridge.
    b. Elution 3 – The sides of the sample vial were rinsed with 1.5 mL of acetonitrile prior to loading onto the SPE.
    c. Elution 4 – The analytes were eluted with 1.5 mL of 50/50 acetonitrile/methanol solution. The elution was loaded onto SPE cartridge only. No vial rinse was required.
    d. **Gravity** was used to pull slowly through the SPE.
    e. A 5 second vacuum was applied at the end of each elution.
12. 50 µL of keeper was added, (10/90 (w/v) glycerol/methanol).
13. 100 µL of 0.01 µg/mL mixed XDE-848 internal standard was added.
14. The samples were vortexed gently.
15. The samples were evaporated to dryness on a Turbo-vap set at 40°C with 7 psi nitrogen. (Approx. 30-35 min.)
16. Reconstitution:
    a. 1000 µL of 50/50/0.1 acetonitrile/methanol/formic acid solution was added. **(NOTE: This removes the analytes from the sides of the glass tube and is a very important step.)**
    b. The samples were vortexed for 5 seconds.
    c. 1000 µL of water containing 0.1% formic acid was added.
    d. The samples were vortexed for 5 seconds.
17. 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.

Instrumental Conditions

Instrumentation

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

Typical Liquid Chromatography Operating Conditions

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
7.00	0	100
9.00	0	100
9.10	50	50
12.00	50	50

Approximate Retention Time: XDE-848 Benzyl Ester 6.0 mins
X11438848 3.4 mins
X12300837 5.3 mins
X11966341 2.0 mins
X12131932 5.8 mins
X12393505 2.5 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):	High
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	438.9/91.0	100	60	69	8
	440.9/91.0	100	60	69	10
X11438848	350.9/270.0	100	60	41	24
	349.0/267.9	100	60	49	13
X12300837	427.0/90.9	100	60	41	8
	425.0/90.9	100	60	40	8
X11966341	334.9/254.0	100	60	47	18
	336.9/256.0	100	60	47	24
X12131932	405.1/64.9	100	60	109	10
	407.1/91.0	100	60	39	10
X12393505	315.0/234.0	100	60	41	18
	315.0/124.1	100	60	101	10
X12293407 (XDE-848 BE IS)	446.9/91.0	100	60	69	10
X12293409 (X11438848 IS)	355.0/274.0	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

Note: Two of the analytes have no internal standards. The following were used for the analysis of each.
X12131932 - use X12293407 internal standard
X12393505 - use X12293409 internal standard