STUDY TITLE

Method Validation Study for the Determination of Residues of Florpyrauxifen-benzyl, X11438848 and X11966341 in Compost by Liquid Chromatography with Tandem Mass Spectrometry

DATA REQUIREMENTS

OCSPP 850.6100
EU Council Regulation (EC) 1107/2009
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STUDY COMPLETED ON

05-Sep-2018

Method Validation Study for the Determination of Residues of Florpyrauxifen-benzyl, X11438848 and X11966341 in Compost by Liquid Chromatography with Tandem Mass Spectrometry

INTRODUCTION

Scope

This method is applicable for the quantitative determination of residues of florpyrauxifen-benzyl (referred to as XDE-848 Benzyl Ester, or XDE-848 BE) and its metabolites (X11438848 and X11966341) in compost. The method was validated over the concentration range of 0.15 – 7.5 ng/g for XDE-848 BE, 0.45 – 22.5 ng/g for X11438848, and 9.0 – 450 ng/g for X11966341 with validated limits of quantitation of 0.15 ng/g, 0.45 ng/g, and 9.0 ng/g, respectively. Common names, chemical names, and molecular formulas for the analytes are given in Table 1.

Method Principle

Residues of XDE-848 BE and its metabolites (X11438848 and X11966341) were extracted from compost by shaking with acetonitrile/0.1N hydrochloric acid (90:10, v/v), centrifuging, and then decanting into a separate container containing rOQ QuEChERS (EN Method). An aliquot of 1 N HCl was added to the extract followed by centrifugation. After a portion of the organic layer was aliquoted, internal standard was added and the sample was taken to near dryness. Then, 1 N HCl was added and the sample was incubated for an hour at 80°C. Ethyl acetate was added and the sample was transferred to a Supel QuE Z-Sep tube. After centrifuging, the sample was placed in a dry ice bath to flash freeze the aqueous layer and the organic layer was poured off into a flexi-tier glass tube. The sample was dried down, reconstituted in methanol and 0.1% formic acid in water, and transferred to a 96-well plate. The samples were analyzed for XDE-848 BE and its metabolites by liquid chromatography with positive ion electrospray ionization tandem mass spectrometry.

Test Substances/Reference Compounds/Analytical Standards

Test Substance	TSN	Percent Purity	Recertification Date	Reference
XDE-848 BE	TSN305894	99.2%	22-OCT-2021	FAPC17-000463
X11438848	TSN304667	100%	26-OCT-2021	FAPC17-000479
X11966341	TSN306022	98%	05-OCT-2021	FAPC17-000538

In addition, the internal standards listed below were used.

Internal Standard	TSN	Percent Purity	Recertification Date	Reference
X12293407	TSN301884	100%	04-OCT-2020	FAPC15-000522
X12293409	TSN308600	99%	22-OCT-2018	FAPC16-000545
X12401027	TSN308642	98%	13-OCT-2021	FAPC16-000546

The Certificates of Analysis for the test substances can be found in Figures 1-3. The Certificates of Analysis for the internal standards can be found in Figures 4-6. The above standards may be obtained free of charge from Dow AgroSciences LLC, 9330 Zionsville Road, Building 304, Indianapolis, IN 46268-1054.

EXPERIMENTAL

Sample Origin, Numbering, Preparation, Storage, and Characterization

For this study, a pasture compost and a manure compost were obtained from Woods End Laboratories Inc. Fresh cut grass and alfalfa were mixed together and composted for 96 days to form the pasture compost. Cow manure and rye straw were mixed together and composted for 95 days to create the manure compost (*5*).

All samples were tracked upon receipt in the Dow AgroSciences LLC Regulatory Labs Information Management System (RLIMS) database. Unique sample numbers were assigned to the samples to track them during receipt, preparation, storage, and analysis. Complete source documentation was included in the study file.

During the course of the study, the samples were stored in temperature-monitored freezers at approximately -20 °C, except when removed for analysis.

Determination of Isotopic Crossover

In this assay, the 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 analytes and the internal standards is determined empirically by analyzing standard solutions of each compound separately at any analytically relevant concentration and if observed, should be addressed for accurate determination of analyte concentrations.

To determine the contribution of the unlabeled XDE-848 BE, X11438848, X11966341 to their internal standards X12293407, X12293409, and X12401027, respectively, a sample spiked with XDE-848 BE, X11438848, and X11966341 was prepared at the highest calibration standard solution without internal standard. No peak area was observed in any of the three internal standard transitions.

In a similar manner, to determine the contribution of the labeled internal standard to the unlabeled analytes, a sample was spiked with X12293407, X12293409, and X12401027 at the internal standard working concentration with no XDE-848 BE, X11438848, or X11966341. In addition, a sample was spiked with XDE-848, X11438848, and X11966341 at the lowest calibration standard concentration without internal standard. The peak area in the analyte transitions from the internal standard solution was less than the analyte peak areas in the lowest calibration standard.

During method development, the concentration range of the calibration curve and the concentration of the labeled internal standard were chosen to minimize the effect of the crossover contribution between the analyte and internal standard. As a result, no significant mass spectral isotopic crossover was observed. The results are summarized in Table 2.

Calculation of Standard Calibration Curve

Calculation of a standard curve begins with the injection of a series of calibration standards described in Appendix I and acquisition of peak areas for the following analytes.

XDE-848 BE	*m/z* Q1/Q3 439/91 (quantitative) *m/z* Q1/Q3 441/91 (confirmatory)
X11438848	*m/z* Q1/Q3 349/268 (quantitative) *m/z* Q1/Q3 349/253 (confirmatory)
X11966341	*m/z* Q1/Q3 335/254 (quantitative) *m/z* Q1/Q3 337/256 (confirmatory)

For each analyte, the linearity of detector response was evaluated using solvent standard solutions. In order to generate a standard curve, the analyte concentration was plotted on the abscissa (x-axis) and the respective quantitation ratio on the ordinate (y-axis) in Analyst 1.6.3. Using regression analysis, the equation for the curve was determined with respect to the abscissa. Refer to Figures 7-12 for example calibration plots and to Figure 13 for an example calculation.

Confirmation of Residue Identity

The method is specific for the determination of XDE-848 BE, X11438848, and X11966341 by virtue of the chromatographic separation and selective detection system used. To demonstrate further confirmation, an additional MS/MS ion transition was monitored for 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.

Table 1. Identity and Structures of Analytes and Internal Standards

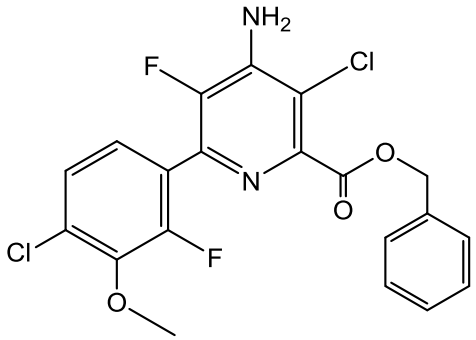
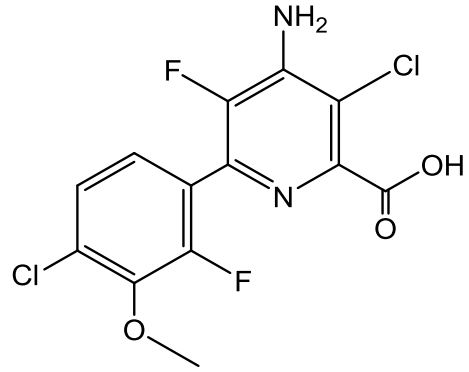
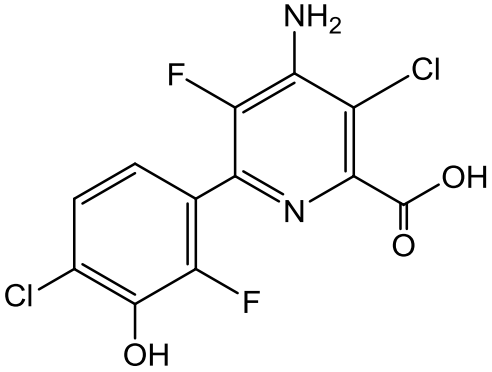
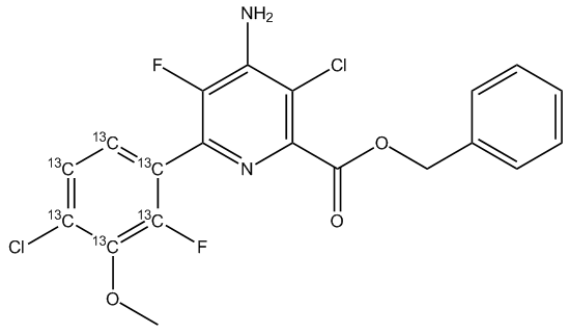
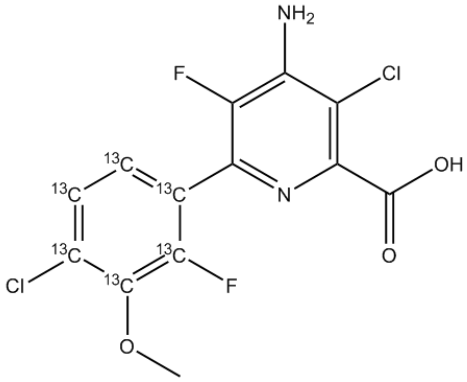
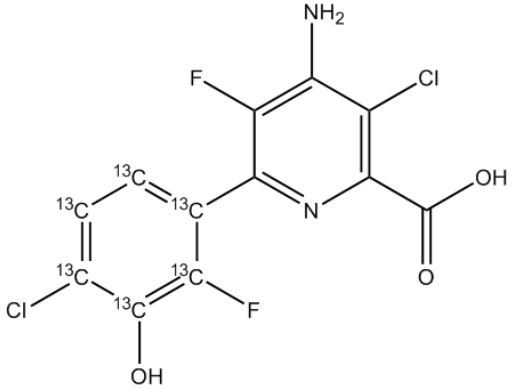
Identifying Information	Structure and IUPAC Name
Common Name of Compound: florpyrauxifen-benzyl (XDE-848 BE) Molecular Formula: $C_{20}H_{14}Cl_2F_2N_2O_3$ Formula Weight: 439.24 Nominal Mass: 439	 benzyl 4-amino-3-chloro-6-(4-chloro-2-fluoro-3-methoxyphenyl)-5-fluoropyridine-2-carboxylate
Common Name of Compound: X11438848 Molecular Formula: $C_{13}H_8Cl_2F_2N_2O_3$ Formula Weight: 349.11 Nominal Mass: 349	 4-amino-3-chloro-6-(4-chloro-2-fluoro-3-methoxyphenyl)-5-fluoropyridine-2-carboxylic acid
Common Name of Compound: X11966341 Molecular Formula: $C_{12}H_6Cl_2F_2N_2O_3$ Formula Weight: 335.09 Nominal Mass: 335	 4-Amino-3-chloro-6-(4-chloro-2-fluoro-3-hydroxyphenyl)-5-fluoropyridine-2-carboxylic acid

Table 1 (Cont.). Identity and Structures of Analytes and Internal Standards

Identifying Information	Structure and IUPAC Name
Common Name of Compound: X12293407 Molecular Formula: $C_{14}{}^{13}C_6H_{14}Cl_2F_2N_2O_3$ Formula Weight: 445.20 Nominal Mass: 445	 benzyl 4-amino-3-chloro-6-(4-chloro-2-fluoro-3-methoxy(13C6)phenyl)-5-fluoropyridine-2-carboxylate
Common Name of Compound: X12293409 Molecular Formula: $C_7{}^{13}C_6H_8Cl_2F_2N_2O_3$ Formula Weight: 355.07 Nominal Mass: 355	 4-amino-3-chloro-6-(4-chloro-2-fluoro-3-methoxy(13C6)phenyl)-5-fluoropyridine-2-carboxylic acid
Common Name of Compound: X12401027 Molecular Formula: $C_6{}^{13}C_6H_6Cl_2F_2N_2O_3$ Formula Weight: 341.05 Nominal Mass: 341	 4-Amino-3-chloro-6-(4-chloro-2-fluoro-3-hydroxy(13C6)phenyl)-5-fluoropyridine-2-carboxylic acid

APPENDIX I Analytical Method

DETERMINATION OF RESIDUES OF XDE-848 BE, X11438848, AND X11966341 IN COMPOST AND COMPOST MATERIALS USING LIQUID CHROMATOGRAPHY WITH TANDEM MASS SPECTROMETRY

Scope

This method is applicable for the determination of residues of XDE-848 benzyl ester (referred to as XDE-848 BE) and its metabolites (X11438848 and X11966341) in compost materials. This method is applicable over a concentration range of 0.15–7.5 ng/g for XDE-848 BE, 0.45 - 2.25 ng/g for X11438848, and 9.0–450 ng/g for X11966341.

Principle

Residues of XDE-848 BE and its metabolites (X11438848 and X11966341) are extracted from compost by shaking with acetonitrile/0.1N hydrochloric acid (90/10, v/v), centrifuging, and then decanting into a separate container containing rOQ QuEChERS (EN Method). An aliquot of 1N HCl is added to the extract followed by centrifugation. After a portion of the organic layer is aliquoted, internal standard is added and the sample is taken to near dryness. Then, 1N HCl is added and the sample is incubated for an hour at 80°C. Ethyl acetate is added and the sample is transferred to a Supel QuE Z-Sep tube. After centrifuging, the sample is placed in a dry ice bath to flash freeze the aqueous layer and the organic layer is poured off into a flexi-tier glass tube. The sample is dried down, reconstituted in methanol and 0.1% formic acid in water, and transferred to a 96-well plate. The samples are analyzed for XDE-848 BE and its metabolites by liquid chromatography with positive ion electrospray ionization tandem mass spectrometry.

Laboratory Equipment

2-dram vials, Kimble Chase, catalog number 60940A8, Fisher Scientific.

96-well plate – 1 mL, Advantage Clear Vu polypropylene round well, catalog number 17P557, Analytical Sales and Services.

Advantage Clear-Vu 1 mL Polypropylene Plate, Round Top, Round Wells 1-mL 96-well plates, catalog number 17P557, Analytical Sales & Services.

Analytical Balance

Bottle-Top Dispenser

Clear Flexi-Tier 2mL 96-well plate with cover, catalog number 96FP20-C, Analytical Sales & Services.

Conventional Nitrogen dry-down station, such as Biotage Turbo-Vap

Conventional 96-well plate Nitrogen dry-down station, such as Jones Chromatography SPE Dual Dry

PTFE/Silicone Cap mats for glass tubes, catalog number 96FSC2, Analytical Sales & Services.

Oven

roQ QuEChERS Extraction Kit, EN Method, 4.0 g MgSO4, 1.0 g NaCl, 1.0 g SCTD, 0.5 g SCDS, catalog number KS0-8909, Phenomenex.

Supel QuE Z-Sep 15 mL Tubes, catalog number 55491-U, Supelco.

Chromatographic and Spectrometric Systems

Column, analytical, Kinetex 1.7 um, C18 100 A, 2.1 x 150 mm, part# 00D-4475-AN, Phenomenex.

Liquid chromatograph, Agilent 1290, Agilent Technologies.

Mass spectrometer, QTRAP 5500, Sciex.

Mass spectrometer data system, Analyst 1.6, Sciex.

Reagents

2-Propanol, HPLC grade, catalog number A451-4, Fisher Scientific.

Acetonitrile, HPLC grade, catalog number A998-4, Fisher Scientific.

Dry Ice

Ethyl Acetate, HPLC grade, catalog number E195-1, Fisher Scientific.

Formic Acid, 99% purity, catalog number 27048-0010, ACROS Organics.

Glycerol, certified ACS grade, catalog number G33-1, Fisher Scientific.

Hydrochloric Acid, 0.1 N, catalog number SA54-1, Fisher Scientific.

Hydrochloric Acid, 1 N, certified, catalog number SA48-500, Fisher Scientific.

Isopropanol, Optima, ACS grade, catalog number A464-4, Fisher Scientific.

Methanol, HPLC grade, catalog number A452-4, Fisher Scientific.

Water, HPLC grade, catalog number W5-4, Fisher Scientific.

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.

Prepared Solutions

Water containing 0.1% Formic Acid (v/v) (Mobile Phase A)
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.

Acetonitrile: Methanol (80/20, v/v) containing 0.1% Formic Acid (v/v) (Mobile Phase B)
Measure 3200 mL of acetonitrile, using a graduated cylinder, and transfer into a 4 L bottle. Measure 800 mL of methanol, using a graduated cylinder, and transfer into the same 4 L bottle. Pipette 4.0 mL of formic acid into the 4L bottle and mix.

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

Glycerol/Methanol (10/90, w/v), (Keeper)
Weigh 10 g of glycerol into a 4 ounce jar. Add 90-mL of methanol, measured using a graduated cylinder, and mix.

Methanol/Isopropanol/Acetonitrile/Water (30/30/30/10, v/v/v/v) (Needle Wash)
Measure 1200-mL of methanol, 1200-mL of isopropanol, 1200-mL of acetonitrile, and 400-mL of water, using a graduated cylinder, and transfer into a 4-L bottle. Mix.

Preparation of Fortification Solutions of XDE-848 BE and Metabolites

1. Weigh 0.010 g of XDE-848 BE analytical standard and quantitatively transfer into a 10 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 0.010 g of X11438848 analytical standard and quantitatively transfer into a 10 mL volumetric flask with methanol. Dilute to volume with methanol to obtain a 1000 µg/mL stock solution of X11438848.

3. Weigh 0.010 g of X11966341 analytical standard and quantitatively transfer into a 10 mL volumetric flask with methanol. Dilute to volume with methanol to obtain a 1000 µg/mL stock solution of X11966341.

4. Pipette 0.01 mL of XDE-848 BE 1000 µg/mL solution, 0.03 mL of X11438848 1000 µg/mL solution, and 0.6 mL of X11966341 1000 µg/mL solution into the same 100 mL volumetric flask. Dilute to volume with methanol to obtain a 100 ng/mL XDE-848 BE, 300 ng/mL X11438848, and 6000 ng/ml X11966341 mixed spiking solution. This will be identified as Spike Solution 1.

5. Pipette 1.0 mL of Spike Solution 1 into a 10 mL volumetric flask. Dilute to volume with methanol to obtain a 10 ng/mL XDE-848 BE, 30 ng/mL X11438848, and 600 ng/ml X11966341 mixed spiking solution. This will be identified as Spike Solution 2.

6. Pipette 1.0 mL of Spike Solution 2 into a 10 mL volumetric flask. Dilute to volume with methanol to obtain a 1 ng/mL XDE-848 BE, 3 ng/mL X11438848, and 60 ng/ml X11966341 mixed spiking solution. This will be identified as Spike Solution 3.

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

Preparation of the Internal Standard Solutions

1. Weigh 0.010 g of X12293407 and quantitatively transfer into a 100 mL volumetric flask with methanol. Dilute to volume with methanol to obtain a 100 µg/mL stock solution of X12293407.

2. Pipette 1 mL of this 100 µg/mL X12293407 stock solution into a 10 mL volumetric flask and dilute to volume with methanol to obtain a 10 µg/mL stock solution of X12293407.

3. Weigh 0.010 g of X12293409 internal standard and quantitatively transfer into a 100 mL volumetric flask with methanol. Dilute to volume with methanol to obtain a 100 µg/mL stock solution of X12293409.

4. Weigh 0.010 g of X12401027 internal standard and quantitatively transfer into a 100 mL volumetric flask with methanol. Dilute to volume with methanol to obtain a 100 µg/mL stock solution of X12401027.

5. Pipette 0.100 mL of each of the 100 µg/mL of X12293409 and X12401027 internal standard stock solutions and 0.100 mL of the 10 µg/mL of X12293407 internal standard stock solution into a 100 mL volumetric flask and dilute to volume with methanol to obtain 0.1-µg/mL X12293409 and X12401027 and 0.01-µg/mL X12293407 mixed internal standards solutions labeled as the Internal Standard Working Solution.

Note: All of the above internal standard solutions should be stored refrigerated.

Preparation of Calibration Stock Solutions

Prepare calibration stock solutions using the spiking solutions as shown in the following table. Calibration stock solutions are prepared in glass autosampler vials with methanol for diluent. Solutions must be stored refrigerated.

Calibration Stock Solution Name	Spiking Solution Used	Aliquot of Spiking Solution (µL)	Diluent Volume (mL)	Calibration Stock Solution Concentration (ng/mL)		
				XDE-848 BE	X11438848	X11966341
A	Spike 3	60	1.440	0.04	0.12	2.4
B	Spike 3	180	1.320	0.12	0.36	7.2
C	Spike 2	36	1.464	0.24	0.72	14.4
D	Spike 2	75	1.425	0.5	1.5	30
E	Spike 2	150	1.350	1	3	60
F	Spike 1	30	1.470	2	6	120
G	Spike 1	60	1.440	4	12	240
H	Spike 1	120	1.380	8	24	480

Preparation of Calibration Standards

Prepare calibration standards fresh for each validation set by pipetting 250 µL of the Calibration Stock Solutions into separate autosampler vials that each contain 600 µL of 0.1% formic acid in water and 25 µL of Internal Standard Working Solution. Vortex briefly to mix.

Calibration Standard Name	Calibration Stock Solution Name	Equivalent Final Concentration of Calibration Standard (ng/g)		
		XDE-848 BE	X11438848	X11966341
A	A	0.045	0.135	2.7
B	B	0.135	0.405	8.1
C	C	0.27	0.81	16.2
D	D	0.5625	1.69	33.75
E	E	1.125	3.375	67.5
F	F	2.25	6.75	135
G	G	4.5	13.5	270
H	H	9	27	540

Note: Equivalent Final Conc (ng/g) is based on extracting 1 g of sample with 18 mL of acetonitrile (taking into account that the extraction solution was 90/10 acetonitrile/0.1N HCl followed by a phase extraction), then taking a 4.0 mL aliquot and carrying out the remainder of the method until reconstituting with a final volume of 300 µL.

Preparation of Solvent Blanks

Prepare solvent blanks by pipetting 250 µL of methanol into an autosampler vial containing 600 µL of 0.1% formic acid in water. Vortex briefly to mix.

Instrumental Conditions

Instrumentation:	Agilent 1290 Infinity LC System AB SCIEX QTRAP 5500 LC/MS/MS System AB SCIEX Analyst 1.6.3 data system
Column:	Phenomenex Kinetex 1.7 µm, C18 100 Å 2.1 x 100 mm, 1.7 µm (Part #: 00D-4475-AN)
Column Temperature:	30 °C
Sample Temperature	10° C
Injection Volume:	20 µL (may be adjusted up to 60 µL as needed for sensitivity)
Autosampler Wash	30 seconds of Methanol/ Isopropanol/ Acetonitrile/ Water (30/30/30/10, v/v/v/v) at the flush port
Run Time:	8 minutes
Mobile Phase:	A – Water containing 0.1 % formic acid B –Acetonitrile/Methanol (80/20, v/v) containing 0.1% Formic Acid

Gradient:

Time, min	Solvent A, %	Solvent B, %	Flow, (µL/min)
0.0	70	30	400
0.5	70	30	400
2	35	65	400
6	30	70	400
6.01	0	100	600
7	0	100	600
7.01	70	30	400

Flow Diverter

1) 0.0 → 1.0 min – flow to waste
2) 1.0 → 6.0 min – flow to MS
3) 6.0 → end of run - flow to waste

Ionization Mode:	Electrospray
Polarity:	Positive
Scan Type:	MRM
Resolution:	Q1 – unit, Q3 – unit
Collision Gas (CAD):	High
Curtain Gas (CUR):	40
Ion Source Gas 1 (GS1):	60 psi
Ion Source Gas 2 (GS2):	50 psi
Temperature (TEM):	750 °C
Entrance Potential (V):	10
IonSpray Voltage (IS):	5500 volts
Acquisition Duration (min):	8
Dwell Time (ms):	50

MS condition:

	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)	439	91	41	51	10
XDE-848 BE (441/91)	441	91	41	51	10
X11438848 (349/268)	349	268	50	40	22
X11438848 (349/253)	349	253	50	55	22
X11966341 (335/254)	335	254	91	32	18
X11966341 (337/256)	337	256	91	47	18
X12293407 (XDE-848 BE IS)	447	91	41	51	10
X12293409 (X11438848 IS)	357	276	50	43	22
X12401027 (X11966341 IS)	341	260	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, add 20 mL of extraction solution into an empty 50-mL bottle.
2. For control samples transfer 1.0 g of compost material into an empty 50-mL bottle.
3. For fortified samples, transfer 1.0 g of compost material into an empty 50-mL bottle. Add the appropriate volume of the spiking solution to obtain fortified samples.

To fortify 1 g of compost (or compost materials) with the three analytes:					
Description	Spiking Volume (µL)	Spiking Solutions	XDE-848 BE Fortification Conc. (ng/g)	X11438848 Fortification Conc. (ng/g)	X11966341 Fortification Conc. (ng/g)
Control	---	---	---	---	---
LOD	50	Spike Solution 3	0.05	0.15	3
LOQ	150	Spike Solution 3	0.15	0.45	9
10X LOQ	150	Spike Solution 2	1.5	4.5	90
50X LOQ	75	Spike Solution 1	7.5	22.5	450

For field samples:

4. Measure by weight, 1.0 $\pm$ 0.05 g of each crop sample into an empty 50-mL bottle.

For all samples:

5. Add 20 mL of extraction solution, 90/10 Acetonitrile/0.1 N HCl, to each bottle.
6. Shake the sample for 60 min on a flatbed shaker set at approximately 180 excursions/minute.
7. Centrifuge the sample for 5 min at 2000 rpm.
8. Pour off liquid extract into a 50 mL tube containing a pouch of Phenomenex roQ QuEChERS (EN Method).
9. Vortex mix briefly.
10. Add 10 mL of 1 N HCl to each tube followed by brief vortex mix.
11. Centrifuge the sample for 5 min at 2000 rpm.
12. Pipette 4 mL of the upper organic layer into a 45-mL vial containing 100 uL of keeper solution and 25 uL of Internal Standard Working Solution.
    - Samples that have concentrations greater than or equal to 80% of the highest calibration standard (i.e. greater than or equal to 7.2 ng/g XDE-848 BE, 21.6 ng/g of X11438848, or 432 ng/g of X11966341) should be diluted 10-fold. To dilute a sample 10-fold, pipette 0.4 mL of the upper organic layer into a 45 mL vial containing 100 µL of keeper solution and 25 µL of Internal Standard Working Solution.
13. Take samples to near dryness on a Turbovap set at 40°C using a nitrogen flow of about 1.3 L/min (about 25-40 minutes).
14. Add 1 mL of 1 N HCl to each glass tube. Cap and vortex mix.

15. Incubate at 80°C for 60 minutes.
16. Allow to cool to room temperature.
17. Add 1 mL of ethyl acetate to each tube. Vortex mix tubes for 1 minute.
18. Pour contents into a 15-mL Supel QuE Z-Sep tube. Add another 1 mL of ethyl acetate to the 45 mL-vial and vortex-mix to rinse. Pour this additional 1 mL aliquot into the 15-mL Supel QuE Z-Sep tube.
19. Vortex mix the 15-mL Supel QuE Z-Sep tube briefly.
20. Centrifuge tubes at 2500 rpm for 5 minutes.
21. Make a dry ice bath that contains about 1-2 inches of dry ice in the bottom of a container, and then add acetonitrile so that it approximately reaches the 3-4 mL mark on the outside of the 15 mL Supel QuE Z-Sep sample tube. Place a single tube in the dry ice bath for 30 seconds at about a 45 degree angle to flash freeze the bottom aqueous layer of the extract.
22. Carefully pour the majority of the upper ethyl acetate layer from the 15 mL Supel QuE Z-Sep tube into a 2-mL glass flexi-tier glass tube, making sure not to transfer any of the aqueous layer that may not have fully frozen.
23. Place the Supel QuE Z-Sep 15 mL tube back in the dry ice bath for another 20-30 seconds at about a 45 degree angle, and repeat the pour off into the same glass tube to transfer any remaining ethyl acetate.
24. Place the glass tube in the appropriate location in the 96-well plastic holding rack.
25. Repeat steps 21-24 for each Supel QuE Z-Sep tube, performing the freezing and pour off one tube at a time.
26. Dry tubes using a 96-well nitrogen dry-down apparatus set at 45°C with a nitrogen flow of about 40 L/min (approximately 45-60 minutes).
27. Reconstitute tubes with 125 µL of methanol followed by vortex-mixing. Cover the plate with a cap mat and vortex-mix briefly.
28. Add 175 µL of 0.1% formic acid in water and vortex mix briefly.
29. Transfer the reconstituted extracts to a 1-mL 96-well plate for analysis by LC-MS/MS.