STUDY TITLE

Independent Laboratory Validation for the Determination of Residues of Florpyraxifen Benzyl (XDE 848 BE), X11438848 and X11966341 in Compost by Liquid Chromatography with Tandem Mass Spectrometry

DATA REQUIREMENTS

SANCO/825/00, rev. 8.1

SANCO/3029/99, rev. 4

OCSPP 850.6100

STUDY COMPLETED ON

13 September 2018

INTRODUCTION

Scope

The objective of the study was to independently validate analytical method as given in the DAS study 171407 [1] for the determination of Florpyrauxifen benzyl (XDE-848 BE), X11438848 and X11966341 in compost in accordance to the guidance documents SANCO/825/00, rev. 8.1 [2] and SANCO/3029/99 rev. 4 of the European Commission [3], and OCSPP 850.6100 of the United States Environmental Protection Agency [4]. The limit of quantification was 0.00015 mg/kg for Florpyrauxifen benzyl (XDE-848 BE), 0.00045 mg/kg for X11438848 and 0.009 mg/kg for X11966341

Analytical Procedure

Compost samples were extracted by shaking with acetonitrile/0.1N hydrochloric acid (90:10, v/v), centrifuging, and decanting into a separate tube containing QuEChERS Citrat kit. An aliquot of 1N 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 evaporated to near dryness under a stream of nitrogen. 1N HCl was added and the samples were incubated for one hour at 80 °C. Ethyl acetate was added and the samples were transferred to a Supel QuE Z-Sep tube and centrifuged. After centrifuging, the samples were placed in a dry ice bath to flash freeze the aqueous layer and the organic layer was poured off into a glass tube. The samples were evaporated under a stream of nitrogen, reconstituted in methanol and 0.1% formic acid in water, and transferred into an autosampler vial for analysis.

The samples were analyzed for XDE-848 BE and its metabolites by liquid chromatography with positive ion electrospray ionization tandem mass spectrometry

Selectivity

Quantification was performed by use of LC-MS/MS detection. Two (2) mass transitions were evaluated in order to demonstrate that the method achieves a high level of selectivity. No significant interference above 30 % of LOQ was detected in any of the reagent blanks or the control sample extracts of, so that a high level of selectivity was demonstrated. Unambiguous identification is ensured by the observation of a precursor ion plus a structurally significant product ion observed at the same retention time [5].

Matrix Effects

Matrix effects on the detection of XDE-848 BE, X11438848 and X11966341 in extracts of compost were found to be insignificant ($\leq \pm$ 20 %). Additionally, an internal standard was used in the analytical method for each anayte to normalize the matrix effect. Therefore solvent calibration standards were used for all analytical sets.

Linearity

The linearity of the detector response was demonstrated by single determination of solvent calibration standards at eight (8) concentration levels ranging from 0.0114 ng/mL to 2.29 ng/mL for XDE-848 BE, from 0.0343 ng/mL to 6.86 ng/mL for X11438848 and from 0.686 to 137 ng/mL for X11966341 This range is equivalent to final concentration of calibration standard from 0.045 µg/kg to 9 µg/kg for XDE-848 BE, from 0.135 µg/kg to 27.0 µg/kg for X11438848 and 2.70 µg/kg to 540 µg/kg for X11966341, and thus covers the range from no more than 5 % of the LOQ and at least + 20 % of the highest analyte concentration level detected in a (diluted) sample extract.

The calibration curves obtained for both mass transitions were linear since correlation coefficients (R) were ≥ 0.995. Linear regression was performed with 1/x-weighting.

Quantification

Quantification was performed by using linear regression with additional correction for bracketing standards. Internal standards were used for quantification together with the calibration standards.

Accuracy and Precision

Accuracy was determined by fortification of control samples with known amounts of the test items and subsequent determination of the recoveries when applying the analytical method. Precision was determined by repeatability (relative standard deviation). The analytes were fortified jointly and quantified separately.

All mean recovery values at fortification levels of LOQ (0.00015 mg/kg for XDE-848 BE, 0.00045 mg/kg for X11438848 and 0.009 mg/kg for X11966341), 10x LOQ (0.0015 mg/kg for XDE-848 BE, 0.0045 mg/kg for X11438848 and 0.09 mg/kg for X11966341) and 50x LOQ (0.0075 mg/kg for XDE-848 BE, 0.0225 mg/kg for X11438848 and 0.45 mg/kg for X11966341) for two (2) mass transitions comply with the standard acceptance criteria of the guidance documents SANCO/825/00, rev. 8.1, SANCO/3029/99 rev. 4 and EPA OPPTS 860.6100.

Limit of Quantification (LOQ) and Limit of Detection (LOD)

The LOQ is the lowest validated fortification level for each analyte and was thus successfully established at 0.00015 mg/kg for XDE-848 BE, 0.00045 mg/kg for X11438848 and 0.009 mg/kg for X11966341 in compost for the two (2) mass transitions.

The LOD was set at 0.000045 mg/kg for XDE-848 BE, 0.000135 mg/kg for X11438848 and 0.0027 mg/kg for X11966341, which is 30 % of the LOQ.

Stability of Stock (and Fortification) Solutions

Testing of stability of analyte in stock and fortification solution was not part of this study. The stability of the analyte in stock and fortification solutions has already been tested in DAS Study 140956 [ref].

XDE-848 BE, X11438848, and X11966341 were found to be stable in methanol for up to 158 days. Stock and fortification solutions were stored under refrigerated conditions when not in use

Stability of Calibration Solutions

XDE-848 BE, X11438848 and X11966341 were found to be stable for 7 days when prepared in a mixture of 250 µL of methanol and 600 µL 0.1 % formic acid, and stored at 1 °C to 10 °C in the dark.

Extract Stability

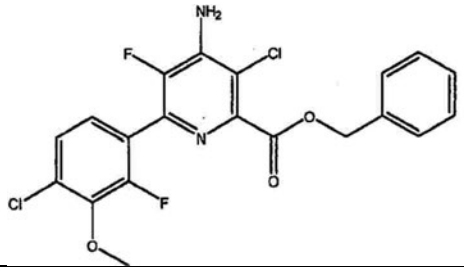
XDE-848 BE, X11438848 and X11966341 were found to be stable in final extracts of compost for at least 5 days when stored at 1 °C to 10 °C in the dark. This period covers the storage of the extracts used in the study.

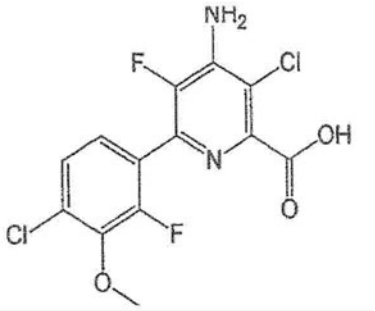
Conclusion

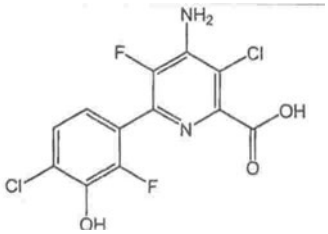
The method was found to be valid according to the guidance documents SANCO/825/00, rev. 8.1, SANCO/3029/99 rev. 4 and EPA OPPTS 860.6100 for the determination of Florpyrauxifen benzyl (XDE-848 BE), X11438848 and X11966341 in compost with the tested LOQ of 0.00015 mg/kg for XDE-848 BE, 0.00045 mg/kg for X11438848 and 0.009 mg/kg for X11966341

MATERIALS AND METHODS

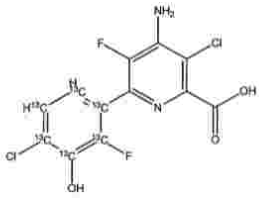
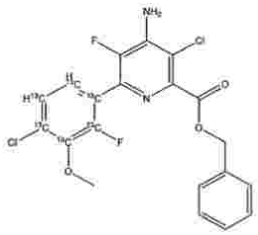
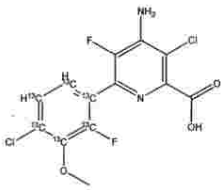
Test Items

Test Item 1			
Test item name	X11959130	Batch number / Test substance number	JY-001-174-22 / TSN305894
Other name(s)	XDE-848 BE florpyrauxifen-benzyl		
EAS Test item code	M-00002044	Appearance / colour	solid / white
Chemical name	benzyl 4-amino-3-chloro-6-(4-chloro-2-fluoro-3-methoxyphenyl)-5-fluoropyridine-2-carboxylate		
CAS number	not available	Purity analysed	99.2 % w/w
Chemical structure		Molecular weight	439.2 g/mol
Density	not applicable	Signal word(s)	warning
Issue date of certificate	28 Sep 2017	Expiry date	22 Oct 2021
		Storage conditions	ambient (5 °C - 30 °C), dark, dry

Test Item 2			
Test item name	X11438848	Batch number / Test substance number	XP3-124113-033 / TSN304667
Other name(s)	XDE-848 acid		
EAS Test item code	2013-005649	Appearance / colour	solid / white
Chemical name	4-amino-3-chloro-6-(4-chloro-2-fluoro-3-methoxyphenyl)-5-fluoropyridine-2-carboxylic acid		
CAS number	not available	Purity analysed	100 % w/w
Chemical structure		Molecular weight	349.1 g/mol
Density	not applicable	Signal word (s)	not available
Certificate of analysis	03 Oct 2017	Expiry date	26 Oct 2021
		Storage conditions	ambient (5 °C – 30 °C), dark, dry

Test Item 3			
Test item name	X11966341	Batch number	JY-001-181-40
		Test substance number	TSN306022
EAS Test item code	2013-005537	Appearance / colour	solid / white
Chemical name	4-amino-3-chloro-6-(4-chloro-2-fluoro-3-hydroxyphenyl)-5-fluoropyridine-2-carboxylic acid		
CAS number	943832-81-3	Purity analysed	98 % w/w
Chemical structure		Molecular weight	335.1 g/mol
Density	not applicable	Signal word(s)	not available
Issue date of certificate	13 Oct 2017	Expiry date	05 Oct 2021
		Storage conditions	ambient (≤ +30 °C), dark, dry

Internal Standard Solution for Test Items

Test item name	XDE-848 mixed stable internal standard in methanol	Batch number	201700716-39-ISWS
EAS Test item code	M-00018890	Appearance / colour	liquid / colourless
Reference compound 1	X12401027		
Chemical name	4-amino-3-chloro-6-(4-chloro-2-fluoro-3-hydroxy($^{13}C_6$)phenyl)-5-fluoropyridine-2-carboxylic acid	Test substance number	TSN308642
		Lot number	YH2-144026-074-S
CAS number	not available	Content nominal	0.1 µg/mL
Chemical structure		Molecular weight	341.1 g/mol
Reference compound 2	X12293407		
Chemical name	benzyl 4-amino-3-chloro-6-(4-chloro-2-fluoro-3-methoxy($^{13}C_6$)phenyl)-5-fluoropyridine-2-carboxylate	Test substance number	TSN301884
		Lot number	XS9-120633-39
CAS number	not available	Content nominal	0.01 µg/mL
Chemical structure		Molecular weight	445.2 g/mol
Reference compound 3	X12293409		
Chemical name	4-amino-3-chloro-6-(4-chloro-2-fluoro-3-methoxy($^{13}C_6$)phenyl)-5-fluoropyridine-2-carboxylic acid	Test substance number	TSN308600
		Lot number	YH2-144026-073-S
CAS number	not available	Content nominal	0.1 µg/mL
Chemical structure		Molecular weight	355.1 g/mol
Density	not available	Signal word(s)	not available
Issue date of certificate	not available	Expiry date	06 Jun 2019
		Storage conditions	cool (1 °C - 10 °C), dark, dry

Specifications essential for correct identification of the test items and for use under GLP are based on the information as provided by the study sponsor (e.g. certificate(s) of analysis). They have not been verified by the test facility and might have not been generated under GLP, except where this is explicitly claimed.

Additional specifications for test item characterisation originate from (non-GLP) sources other than the study sponsor / supplier.

Test System(s), Sample Origin, Preparation and Storage

Test System (Commodity)	Preparation	Origin
Compost	The sample material was thoroughly homogenised in a cutter with dry ice.	Untreated material was purchased from the local market

Weighed sub-samples were stored at typically ≤ -18 °C in the dark until fortification and extraction.

Method Summary

In brief, compost samples were extracted by shaking with acetonitrile/0.1N hydrochloric acid (90:10, v/v), centrifuging, and decanting into a separate container containing QuEChERS Citrat kit. An aliquot of 1N 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 evaporated to near dryness under a stream of nitrogen. 1N HCl was added and the samples were incubated for one hour at 80 °C. Ethyl acetate was added and the samples were transferred to a Supel QuE Z-Sep tube and centrifuged. After centrifuging, the samples were placed in a dry ice bath to flash freeze the aqueous layer and the organic layer was poured off into a glass tube. The samples were evaporated under a stream of nitrogen, reconstituted in methanol and 0.1% formic acid in water, and transferred into an autosampler vial for analysis.

The samples were analyzed for XDE-848 BE and its metabolites by liquid chromatography with positive ion electrospray ionization tandem mass spectrometry

No addition or modification to the original method other than optimisation of instrumental parameters and no communication with the method developers or others familiar with the method was necessary in order to carry out the analysis.

APPENDIX A

Analytical Method

Reagents and Materials

Information pertaining to the identity and source of reagents used is summarised in Table 1. Alternatively, equivalent reagents and materials may be used, unless specifically stated otherwise.

Table 1: Identification of Reagents and Materials

- Acetonitrile HPLC grade
  (Prolabo, Art. No. 83639.320, Prolabo Art. No. 20060.320)
- Formic acid p.a.
  (Prolabo, Art. No. 20318.320)
- Hydrochlorid acid 37 % p.a.
  (Merck. Art. No. 1.00317)
- Methanol LCMS grade
  (Fluka, Art. No. 34966-2.5L)
- Water HPLC grade
  (Merck, Art. No. 1.15333.2500)
- Demineralized water
  (prepared at laboratory)
- Ethyl acetate
  (Prolabo, Art. No. 83660.320; Fluka, Art. No. 31063-2.5L)
- Supel QuE Z-Sep 15 mL Tubes
  (Supelco, Art. No. 55491-U)

Instrumentation and Apparatus

Information pertaining to the identity of instruments and apparatus used is summarised in Table II. Alternatively, equivalent instrumentation and apparatus may be used, unless specifically stated otherwise.

Table 2: Identification of Instrumentation and Apparatus

- Adjustable pipettes
  (Eppendorf: Research 10-100 µL, Research 100-1000 µL, Multipipette Plus 50-10000 µL; Brand: Transferpipette S 500-5000 µL, Transferpipette S 1000-10000 µL)
- Common laboratory glassware
- Balances
  (Sartorius CPA224S, Kern PFB 1200-2, Kern PCB 1000-2)
- Sample Concentrator
  (Biotage TurboVap LV)
- Horizontal flatbed shaker
  (Bühler SM 25 A, Bühler SM 30 A)
- Water bath
  (GFL Water bath Typ 1086)
- Laboratory centrifuge
  (Hettich Rotina 380)
- Vortex mixer
  (Scientific Industries Genie 2)
- Sample Cutter
  (Stephan U1400236)
- HPLC- MS/MS
  (Applied Biosystems API6500 with Agilent Infinity II HPLC)

Reagent Solutions and Mobile Phases

Mobile Phase A: Water + 0.1 % formic acid

Add 2.5 mL of formic acid into a 2.5 L HPLC grade water bottle. Cap the bottle and invert to mix well.

Mobile Phase B: Acetonitrile/Methanol (80:20, v/v) + 0.1 % formic acid

Add 1.6 L of acetonitrile and 0.4 L of methanol into a 2 L bottle. Add 2 mL of formic acid into the bottle. Cap the bottle and invert to mix well.

Acetonitrile/0.1 N Hydrochloric Acid (90:10, v/v) (Extraction Solvent)

Measure 900 mL of acetonitrile, using a graduated cylinder, and transfer into a 1 L bottle. Add 100 mL of 0.1N Hydrochloric acid into the 1 L bottle and mix.

Glycerol/Methanol (10:90, w/v), (Keepers Solution)

Weigh 10 g of glycerol into a 100 mL bottle. Add 90 mL of methanol, measured using a graduated cylinder, and mix.

Preparation of Standard Solutions

Stock solutions of the analytes were prepared by dissolving a weight of the test items with the aid of an ultrasonic bath. Each stock solution is allocated a reference number.

The stock solutions were further diluted for use as fortification solutions in the procedural recovery process and as (intermediate) standard solutions for subsequent use as solvent calibration solutions calibration solutions.

Matrix-matched calibration solutions for the assessment of the matrix effects were prepared using final sample extracts of control (untreated) samples of a respective matrix which are then fortified with (intermediate) solvent standard solutions.

All solutions were stored 1 °C to 10 °C (target) in a glass vial in the dark.

A summary of the typical dilutions to be carried out is presented in the following tables.

Table 3: Preparation of a Stock Solution of XDE-848 BE in Methanol

Purity of test item* (%)	**Weighed amount of test item** (mg)	**Amount of analyte corrected for purity** (mg)	**Final volume** (mL)	**Equivalent concentration** (µg/mL)	**Reference of standard solution produced**
99.2	10.1	10.02	10.0	1002	S1002

* to be taken from the Certificate of Analysis

Table 4: Preparation of a Stock Solution of X11438848 in Methanol

Purity of test item* (%)	Weighed amount of test item (mg)	Amount of analyte corrected for purity (mg)	Final volume (mL)	Equivalent concentration (µg/mL)	Reference of standard solution produced
100	10.3	10.30	10.0	1030	S1030b

* to be taken from the Certificate of Analysis

Table 5: Preparation of a Stock Solution of X11966341 in Methanol

Purity of test item* (%)	Weighed amount of test item (mg)	Amount of analyte corrected for purity (mg)	Final volume (mL)	Equivalent concentration (µg/mL)	Reference of standard solution produced
98.0	10.5	10.29	10.0	1029	S1029a

* to be taken from the Certificate of Analysis

Table 6: Preparation of Mixed Fortification Solutions of XDE-848 BE, X11438848 and X11966341 in Methanol

Reference of standard solution used	Concentration (µg/mL)	Volume taken (µL)	Final volume (mL)	Equivalent concentration (µg/mL)	Reference of standard solution produced
S1002	1002	9.98	100	100	Spike Solution 1
S1030b	1030	29.1		300	
S1029a	1029	583		6000	
Spike Solution 1	100	1	10	10	Spike Solution 2
	300			30	
	6000			600	
Spike Solution 2	10	1	10	1	Spike Solution 3
	30			3	
	600			60	

The Internal Standard Working Solution was provided by the sponsor. The provided solution was already dissolved in methanol and diluted to the proper concentration. No further aliquotation/dilution was needed.

Table 7: Preparation of Mixed Solvent Calibration Stock Solutions of XDE-848 BE, X11438848 and X11966341 in Methanol

Reference of standard solution used	Concentration (ng/mL)*	Volume taken (µL)	Methanol volume (mL)	Equivalent concentration (ng/mL)*	Reference of standard solution produced
Spike 3	1/3/60	60	1.44	0.04/0.12/2.4	A
Spike 3	1/3/60	180	1.32	0.12/0.35/7.2	B
Spike 2	10/30/600	36	1.464	0.24/0.72/14.4	C
Spike 2	10/30/600	75	1.425	0.5/1.5/30	D
Spike 2	10/30/600	150	1.35	1/3/60	E
Spike 1	100/300/6000	30	1.47	2/6/120	F
Spike 1	100/300/6000	60	1.44	4/12/240	G
Spike 1	100/300/6000	120	1.38	8/24/480	H

* XDE-848 BE/X11438848/X11966341

Table 8: Preparation of Mixed Solvent Calibration Solutions of XDE-848 BE, X11438848 and X11966341 in Methanol/0.1 % formic acid in water mixture

Calibration standards were prepared fresh for each validation set by pipetting 250 µL of the Calibration Stock Solutions into separate autosampler vials that each contained 600 µL of 0.1% formic acid in water and 25 µL of Internal Standard Working Solution.

Reference of standard solution used	Concentration (ng/mL)*	Equivalent concentration (ng/mL)*	Equivalent concentration (ng/g)*	Reference of standard solution produced
A	0.04/0.12/2.4	2.29/6.86/137	0.045/0.135/2.7	Std A
B	0.12/0.35/7.2	1.14/3.43/68.6	0.135/0.405/8.1	Std B
C	0.24/0.72/14.4	0.571/1.71/34.3	0.27/0.81/16.2	Std C
D	0.5/1.5/30	0.286/0.857/17.1	0.5625/1.69/33.75	Std D
E	1/3/60	0.143/0.429/8.57	1.125/3.375/67.5	Std E
F	2/6/120	0.0686/0.206/4.11	2.25/6.75/135	Std F
G	4/12/240	0.0343/0.1/2.06	4.5/13.5/270	Std G
H	8/24/480	0.0114/0.0343/0.686	9/27/540	Std H

* XDE-848 BE/X11438848/X11966341

Equivalent concentration (ng/g) is based on extracting 1 g of sample with 18 mL of acetonitrile, taking a 4.0 mL aliquot and carrying out the remainder of the method until reconstituting with a final volume of 300 µL.

Laboratory Specimen Preparation

Specimen preparation as done in this validation study is described in section 3.2 of this report.

Specimen preparation should be in compliance with the test facilities/sites standard operation procedures (SOPs). In case of any conflict between the requirements of SOPs and study plan, the study plan would take priority.

Sample Weight(s) and Fortifications

Control (untreated) specimens of compost were fortified prior to extraction with the fortification solutions as described below.

The analytes were fortified jointly

Table 9: Summary of Sample Weights and Fortifications

Fortified analytes	**Matrix**	**Sample weight (g)**	**Reference of mixed fortification solution used**	**Concentration of fortification solution (µg/mL)**	**Volume of fortification solution added (µL)**	**Fortification level (each) (mg/kg)**
XDE-848 BE, X11438848 and X11966341	Compost	1.00	Spike solution 3	1/3/60	50	0.000045 / 0.000135 / 0.0027
		1.00	Spike solution 3	1/3/60	150	0.00015 / 0.00045 / 0.009
		1.00	Spike solution 2	10/30/600	150	0.0015 / 0.0045 / 0.09
		1.00	Spike solution 1	100/300/6000	75	0.0075 / 0.0225 / 0.45

Recovery fortifications at the level of LOD (0.000045 / 0.000135 / 0.0027) were not used for quantification and will not be reported

Sample Work-Up 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.

For field samples:

4. Measure by weight, 1.0 ± 0.05 g of each sample into an empty 50 mL bottle.

For all samples:

5. Add 20 mL of extraction solution, 90/10 Acetonitrile/0.1N 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 Citrate Kit 01.
9. Vortex mix briefly.
10. Add 10 mL of 1N 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.
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 1N 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 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 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 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 an autosampler vial for analysis by LC-MS/MS.

Dilution of final extracts

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.

e.g.

To dilute a sample 5-fold, pipette 0.8 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.

Chromatographic and Mass Spectrometric Conditions

A summary of the chromatographic and mass spectrometric conditions used for quantification is included in the following tables:

Table 10: Summary of chromatographic and mass spectrometric conditions

Chromatographic conditions for XDE-848 BE, X11438848 and X11966341 in Compost				
HPLC system	Agilent 1290 Infinity II HPLC			
Pre-column	Not used			
Column	Phenomenex Kinetex 1.7 µm C18 100 A, 100 mm x 2.1 mm, 1.7 µm, (Part No. 00D-4475-AN)			
Column oven temperature	30 °C			
Injection volume	20 µL			
Mobile phases	Eluent A: Water containing 0.1 % (v/v) formic acid Eluent B: Acetonitrile/Methanol (80/20, v/v) containing 0.1 % (v/v) formic acid			
Gradient	Time [min]	% Eluent A	% Eluent B	Flow [µL/min]
	0.00	70	30	400
	0.50	70	30	400
	2.00	35	65	400
	6.00	30	70	400
	6.01	0	100	600
	7.00	0	100	600
	7.01	70	30	400
	8.00	70	30	400
Divert valve	Not used			
Retention time(s)	XDE-848 BE: approx. 5.4 min X11438848: approx. 3.0 min X11966341 : approx. 1.6 min			

Mass spectrometric conditions XDE-848 BE, X11438848 and X11966341 in Compost						
MS system	SCIEX QTRAP 6500 System, SCIEX (Linear ion trap quadrupole)					
Ionisation type	Electrospray ionisation (ESI, TurboIonSpray)					
Polarity	Positive ion mode					
Scan type	MS/MS, Multiple Reaction Monitoring (MRM)					
Capillary voltage (IS)	4000 V		Ionspray turbo heater (TEM)		600 °C	
Curtain gas (CUR)	Nitrogen set at 20 (arbitrary units)		Gas flow 1 (GS1)		Nitrogen set at 60 (arbitrary units)	
Collision gas (CAD)	Nitrogen set at 9 (arbitrary units)		Gas flow 2 (GS2)		Nitrogen set at 50 (arbitrary units)	
Analyte monitored	Mass transition monitored *(m/z)*	Declustering potential (DP) [V]	Entrance potential (EP) [V]	Collision energy (CE) [V]	Cell exit potential (CXP) [V]	Dwell time [ms]
XDE-848 BE	439 → 91[#]	41	10	57	10	50
	441 → 91	41	10	67	10	50
X11438848	349 → 268[#]	52	10	41	24	50
	349 → 253	52	10	55	22	50
X11966341	335 → 254[#]	91	10	45	18	50
	337 → 256	91	10	45	18	50
X12293407 (XDE-848 BE IS)	447 → 91	56	10	51	10	50
X12293409 (X11438848 IS)	357 → 276	51	10	41	24	50
X12401027 (X11966341 IS)	341 → 260	75	10	47	18	50

[#] used for quantification but both of the mass transitions listed can be used for quantification

Special Precautions

None

Calculation of Results

Quantification is performed using calibration plots with a minimum of five (5) different concentration levels covering the required calibration range.

Standard solutions representing a calibration curve are injected within the sample list. Additionally bracketing standards are analysed between the samples. Concentrations of the target analyte in the samples are corrected according to the response of the bracketing standards.

The linearity of the detection system is demonstrated by use of standard solutions covering a working range which is equivalent no more than 30 % of the LOQ and at least + 20 % of the highest analyte concentration level in a diluted sample extract.

A linear regression is performed with 1/x weighting. The correlation coefficient (R) must be greater or equal to 0.990 meaning that the coefficient of determination (R^2) must be greater or equal to 0.980.

A linear calibration function ($y = a + b \cdot x$) as determined by software Analyst 1.6.3, SCIEX is used to calculate the analyte concentration in final extracts as follows:

$C_A =$	$\frac{A_A - a}{b}$
C_A	Concentration of analyte in final extract (ng/mL) (x)
A_A	Peak area of analyte in the final solution (counts) (y) as obtained by integration with software Analyst 1.6.3, SCIEX
a	y -axis Intercept of the calibration curve (counts)
b	Slope of calibration curve (counts · mL/ng)

The residues are calculated by reference to the mean response of the appropriate bracketing matrix standards as follows:

$R =$	$\frac{C_1}{C_2} \times C_A \times \frac{V_{Ex} \times V_{End} \times DF}{W \times V_{Ali} \times CF}$
R	Analyte Residue (mg/kg)
C_1	Nominal concentration of bracketing matrix standards (ng/mL)
C_2	Average calculated concentration of matrix standards bracketing the samples, obtained from the matrix calibration function (ng/mL)
C_A	Analysed concentration of the sample, as calculated from the matrix calibration function (ng/mL)
V_{Ex}	Extraction volume (18 mL)
V_{End}	Final volume (300 µL)
V_{Ali}	Aliquot of the extract (4 mL)
W	Sample weight (1.00 g)
DF	Dilution factor
CF	Conversion from ng into µg (1000)

The recovery of a fortification experiment is calculated as follows:

Recovery (%) =	$\frac{R}{F}$ x 100
R	Analyte residue (mg/kg)
F	Nominal fortification level (mg/kg)

Example calculation

The equation used for the least squares fit is:

$$Y = \text{slope} \times X + \text{intercept}$$

Y = quantitation ratio = analyte peak area/internal standard peak area

$$X = \frac{Y - \text{intercept}}{\text{Slope}} = \text{ng/mL}$$

The standard (calibration) curve generated for each analytical set was used for the quantitation of XDE-848 BE and its metabolites in the samples from the set. For this study, the correlation coefficient (r) for each calibration curve was greater than 0.995.

For the determination of XDE-848 BE and metabolites in terms of ppb, the following equation is used:

$$\text{ppb } \left(\frac{\text{ng}}{\text{g}}\right) \text{Found} = \frac{\text{ng/mL found x Final volume (mL) x Extraction volume (mL) x Dilution Factor}}{\text{Sample weight (g)x Aliquot volume (mL)}}$$

Example: XDE-848 BE recovery of a Compost sample fortified at the 10xLOQ level (1.5 µg/kg)
(Sample ID 1022)

The concentration determined from the standard curve is (as per Analyst 1.6.3) with the correction for the mean response of bracketing injections = 1.18 ng/mL

The correction of the calculated concentration of XDE-848 BE trough bracketing in the final solution is calculated as follows:

$$\frac{\text{Nominal concentration of bracketing standards (ng/mL)} * \text{Analysed concentration of the sample (ng/mL)}}{\text{Average calculated concentration of standards bracketed between samples (ng/mL)}}$$

$$\text{XDE} - 848\ \text{BE corrected concentration} = \frac{0.143\ \text{ng/ml} * 1.21\ \text{ng/ml}}{0.1465\ \text{ng/ml}} = 1.18\ \text{ng/ml}$$

The residue of XDE-848 BE in the final solution is calculated as follows:

$$\text{XDE} - 848\ \text{BE} = \frac{1.18\ \frac{\text{ng}}{\text{mL}}\ \text{x}\ 0.3\ \text{mL x}\ 18\ \text{mL x}\ 1}{1\ \text{g x}\ 4\ \text{mL}} = 1.59\ \text{ng/g} = 1.59\ \mu\text{g/kg}$$

Procedural recovery data from fortified samples are calculated via the following equation:

$$\text{Percentage Recovery} = \frac{(\text{ng/g})\text{found}}{(\text{ng/g})\text{added}}\ \text{x}\ 100$$

- -

Estimated Time Required for Analysis

Recommended samples per set (incl. controls, recoveries and specimens)	12 samples
(Specimen preparation and) weighing of aliquots for analysis	2 hours
Extraction and clean-up, preparation of working solutions	4 hours
LC-MS/MS (GC-MS) instrument setup	1 hour
Analysis by LC-MS/MS (GC-MS) (incl. injection of calibration solutions)	3 hours (unattended instrument-hours)
Data evaluation	1 hour
Total (Specimen preparation to attainability of the results)	11 hours

Method Flow Chart

Sample weighing

1.00 ± 0.05 g of homogenised specimen of compost

⇩

Extraction

Add 20.0 mL of acetonitrile/0.1 N hydrochloric acid (90/10, v/v),
shake on a flatbed shaker for 60 minutes
centrifuge the samples for 5 minutes at 2000 rpm

⇩

Liquid-liquid partition

Pour off liquid extract into a 50 mL tube
Add citrate salt mixture,
vortex sample briefly
add 10 mL of 1N HCl and centrifuge for 5 min at about 2000 rpm
take 4 mL of the organic layer into a 45 mL vial containing keeper solution and internal standard

⇩

Clean-up

Evaporate the samples to dryness under stream of nitrogen
Add 1 mL of 1N HCl
Incubate at 80 °C for 60 minutes
Allow to cool to room temperature and add 1 mL of ethyl acetate
Pour contents into a 15 mL Supel QuE Z-Sep tube
Centrifuge tubes at 2500 rpm for 5 minutes
make a dry ice bath containing acetonitrile
freeze the water phase and pour the upper ethyl acetate layer into a 2 mL glass tube

⇩

Final fill-up

Dry tubes under a flow of nitrogen
reconstitute tubes with 125 µL of methanol followed by vortex-mixing
add 175 µL of 0.1% formic acid in water and vortex mix briefly
transfer the reconstituted extracts to an autosampler vial for analysis

Analyse final sample extracts by LC-MS/MS

Safety Information

Reagents	H- and P-Codes	H- and P-Phrases
Methanol	H225 H301+H311+H331 H370	• Highly flammable liquid and vapour. • Toxic if swallowed, in contact with skin or if inhaled. • Causes damage to organs
	P210	• Keep away from heat, hot surfaces, sparks, open flames and other ignition sources. No smoking.
	P280	• Wear protective gloves/protective clothing/eye protection/face protection.
	P302+P352+P312	• IF ON SKIN: Wash with plenty of soap and water, and call a POISON CENTER/doctor/… if you feel unwell.
	P304+P340+P311	• IF INHALED: Remove person to fresh air and keep comfortable for breathing and call a POISON CENTER/doctor/…
	P370+P378	• In case of fire: use extinguishing powder or dry sand.
	P403+P235	• Store in a well-ventilated place. Keep cool.
Acetonitrile	H225 H302, H312, H332 H319	• Highly flammable liquid and vapour. • Harmful if swallowed, in contact with skin, if inhaled. • Causes serious eye irritation.
	P210	• Keep away from heat, hot surfaces, sparks, open flames and other ignition sources. No smoking.
	P280	• Wear protective gloves/protective clothing/eye protection/face protection.
	P305+P351+P338	• IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing.
	P309+P311	• IF exposed or if you feel unwell: call a POISON CENTER or doctor/physician.
Formic acid	H314	• Causes severe skin burns and eye damage. • Wear protective gloves/protective clothing/eye protection/face protection.
	P280	
	P305+P351+P338	• IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing.
	P301+P330+P331	• IF SWALLOWED: Rinse mouth. Do NOT induce vomiting.
	P309+P311	• IF exposed or if you feel unwell: call a POISON CENTER or doctor/physician.

Ethylacetate	H225 H319 H336 EUH066 P210 P280 P305+P351+P338	• Highly flammable liquid and vapour. • Causes serious eye irritation. • May cause drowsiness or dizziness. • Repeated exposure may cause skin dryness or cracking. • Keep away from heat, hot surfaces, sparks, open flames and other ignition sources. No smoking. • Wear protective gloves/protective clothing/eye protection/face protection. • IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing.
Hydrochloric acid	H290 H314 H335 P280 P305+P351+P338 P301+P330+P331 P308+P310	• May be corrosive to metals. • Causes severe skin burns and eye damage. • May cause respiratory irritation. • Wear protective gloves/protective clothing/eye protection/face protection. • IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing. • IF SWALLOWED: Rinse mouth. Do NOT induce vomiting. • IF exposed or concerned: Immediately call a POISON CENTER/doctor/…