Independent Laboratory Validation of Analytical Method Study Number 110716 for the Determination of XDE-729 Methyl Ester and its Metabolite Residues in Soil

ABSTRACT

This study was conducted to provide independent laboratory validation data for the determination of residues of XDE-729 methyl ester (X11393728), XDE-729 Acid (X11393729) and des-methyl-XDE-729 Acid (X11449757) in two types of soil, following the analytical method, Dow AgroSciences LLC, Study Number 110716, "Validation Study for the Determination of Residues of X11393728 (XDE-729 Methyl), X11393729 (XDE-729 Acid) and X11449757 (des-Methyl XDE-729 Acid) in Soil using High Performance Liquid Chromatography with Positive-Ion Electrospray Ionization Mass Spectrometry Detection"(reference 5). The validated limit of quantification was 0.05 µg/kg, for all the soil samples.

For the independent validation of the method, for both types of soil, after fortification with the analytes, where applicable, the following specimens were analysed by LC-MS/MS:

- 5 specimens fortified at the LOQ level of 0.05 µg/kg
- 5 specimens fortified at 10 LOQ level of 0.50 µg/kg
- 2 unfortified, untreated control specimens,
- 1 specimen fortified at the LOD level of 0.02 µg/kg
- 1 reagent blank (containing no matrix), taken through sample cleanup with the samples

All of the individual recovery values for XDE-729 methyl ester (X11393728), XDE-729 acid (X11393729) and des-methyl-XDE-729 acid (X11449757) in all of the soil samples were within the acceptance range of 70-120%. Average recoveries at each fortification level were within the acceptance range of 70-110%. The relative standard deviation (RSD) never exceeded ± 20% at any fortification level. There were no interferences present greater than 30% of the LOQ seen in the chromatograms of any of the blank and unfortified specimens for either the quantification or confirmatory transitions.

This independent laboratory validation was conducted to satisfy requirements of EU Regulation (EC) No 1107/2009, and the European Commission Guidance Document on

Residue Analytical Methods, SANCO/825/00 rev. 8.1 and SANCO/3029/99 rev. 4. The study was also conducted to satisfy the requirements of U.S. EPA Guideline OCSPP 850.6100 and PR Notice 2011-3.

INTRODUCTION

An analytical method was developed and validated for the determination of XDE-729 methyl ester (X11393728), XDE-729 acid (X11393729) and des-methyl-XDE-729 acid (X11449757) in two types of soil. The method is identified as Dow AgroSciences Study Number 110716, "Method Validation Study for the Determination of Residues of X11393728 (XDE-729 Methyl), X11393729 (XDE-729 Acid) and X11449757 (des-Methyl XDE-729 Acid) in Soil using High Performance Liquid Chromatography with Positive-Ion Electrospray Ionization Mass Spectrometry Detection" (reference 5). This method is referenced as AGR/MOA/XDE-2 at Eurofins Agroscience Services Chem for the independent validation study.
The method was found to be suitable for the determination of residues of XDE-729 methyl ester (X11393728), XDE-729 acid (X11393729) and des-methyl-XDE-729 acid (X11449757) in two types of soil over the concentration range of 0.01 to 6 µg/kg. The validated limit of quantification was confirmed to be 0.05 µg/kg for both soil types.

An independent laboratory validation of the analytical method was conducted on two types of soil to satisfy the requirements of the European Commission Guidance Document on Residue Analytical Methods, SANCO/825/00 rev. 8.1 (Reference 1) and SANCO/3029/99 rev. 4 (Reference 2). The study was also conducted to satisfy the requirements of U.S. EPA Guideline OCSPP 850.6100 (Reference 3) and PR Notice 2011-3 (Reference 4).

The independent laboratory, the Study Director, and the analyst chosen to conduct the ILV were unfamiliar with the method, both in its development and subsequent use in analyzing samples. The independent laboratory used all of its own equipment and supplies, so that there was no common link between Dow AgroSciences and the ILV analyst. Throughout the conduct of the study, any communications between Dow AgroSciences and the Study Director and/or the analyst were logged for inclusion in the report. No one from Dow AgroSciences was allowed to visit the independent laboratory during the ILV trial to

observe, offer help, or assist the chemists or technicians. These steps successfully maintained the integrity of the ILV study.

ANALYTICAL

Preparation and Storage of Samples

The independent laboratory validation was carried out on two soil specimens: sandy soil, and clay soil. The soils were purchased from Lufa Speyer.

Specimen	Lufa Sample Reference Number	EAS Sample Reference Number
Sandy Soil	2.1(Sp2.12911)	232
Clay Soil	6S (Sp6S2111)	234

Upon receipt, the soil specimens were stored frozen at -20±5 °C.

Characterisation of Samples

The soil specimens were characterized by the supplier, details of the characterization results are as follows:

Specimen	Sandy soil 2.1	Clay soil 6S
Sampling date	19.07.11	25.05.11
Org. C in %	0.62 ± 0.07	1.64 ± 0.12
Nitrogen in %N	0.05 ± 0.01	0.20 ± 0.02
Particles <0.02 mm in %	7.5 ± 2.1	64.8 ± 3.6
pH-Value (0.01 M CaCl2)	5.1 ± 0.4	7.1 ± 0.1
Cation Exchange Capacity	3.8 ± 0.8	23.7 ± 7.0
Particle Size According to USDA (%)		
< 0.002 mm	2.7± 1.1	41.0 ± 1.9
0.002 – 0.05 mm	10.1± 1.6	36.8 ± 2.0
0.05 – 2.0 mm	87.3 ± 1.2	22.2 ± 1.8
Soil Type	Sand	Clay
Water Holding Capacity (g/100g)	31.2 ± 2.0	38.9 ± 4.6
Weight per Volume (g/1000 mL)	1462 ± 39	1372 ±60

Preparation of Solutions and Standards

Reagents used were of equivalent specifications as described in the analytical method.

The following analytical test substances/analytical standards were utilized during the independent laboratory method validation:

Test Substance/ Analytical Standard:	XDE-729 Methyl Ester (X11393728)
Supplier:	Sponsor
Reference Number:	TSN031117-0002
Batch/Lot no:	XW7-38246-49
Purity:	97.4%
Expiry date:	09 Nov 2013
Storage:	20±4°C

Test Substance/ Analytical Standard:	XDE-729 acid (X11393729)
Supplier:	Sponsor
Reference Number:	TSN030751-004
Batch/Lot no:	DC6-E2622-77
Purity:	99%
Expiry date:	2 Nov 2013
Storage:	20±4°C

Test Substance/ Analytical Standard:	Des-methyl-XDE-729 acid (X11449757)
Supplier:	Sponsor
Reference Number:	TSN031413-0003
Batch/Lot no:	YB1-100780-103
Purity:	99%
Expiry date:	25 Jan 2014
Storage:	between 0 and 9°C

The certificates of analysis were provided by Dow AgroScience LLC. These are located in appendix B.
Standard solutions, calibration standard solutions and fortification standards were prepared as described in the analytical method presented in appendix C. The test/reference items and specimens will be retained until expiry and then disposed of with the approval of the Study Director and the Study Monitor.

Fortification of Recovery Samples

The control specimens were fortified as described below with XDE-729 methyl ester (X11393728), XDE-729 acid (X11393729) and des-methyl-XDE-729 acid (X11449757):

Matrix	Untreated Control Specimen Replicates	Replicates at Fortification Level (LOD)*	Replicates at Fortification Level (LOQ)**	Replicates at Fortification Level
Clay Soil	2	1 at 0.02 µg/kg	5 at 0.05 µg/kg	5 at 0.5 µg/kg
Sandy Soil	2	1 at 0.02 µg/kg	5 at 0.05 µg/kg	5 at 0.5 µg/kg

*LOD – Limit of determination
**LOQ – Limit of Quantification

Each sample was fortified as per the table above. One sample was fortified to achieve a fortification level of 0.02 µg/kg (LOD), five samples were fortified to achieve the fortification level of 0.05 µg/kg (LOQ) and five samples were fortified to achieve the upper fortification level of 0.5 µg/kg for two types of soil. The fortification solution was injected directly into the matrix.

Sample Extraction, Purification and Analysis

Specimens were assayed according to the analytical method Dow AgroSciences Study Number 110716, "Method Validation Study for the Determination of Residues of X11393728 (XDE-729 Methyl), X11393729 (XDE-729 Acid) and X11449757 (des-Methyl XDE-729 Acid) in Soil using High Performance Liquid Chromatography with Positive-Ion Electrospray Ionization Mass Spectrometry Detection" (reference 5). The method was internally referenced at Eurofins Agroscience Services Chem under the number AGR/MOA/XDE-2.

Residues of XDE-729 methyl and its major soil metabolites were extracted from soil by accelerated solvent extraction (ASE) at 90 °C and 1500 psi for 5 minutes with methanol/water (50:50) solution containing 0.1% phosphoric acid. A 5-mL aliquot of the extraction solution was placed in a 50-mL vial and a stable isotope internal standard mixture was added. The sample was evaporated to approximately 1 mL, a small amount of acetonitrile was added and the sample was then filtered prior to LC-MSMS analysis. The sample was analyzed by liquid chromatography with positive-ion electrospray ionization tandem mass spectrometry (LC-MS/MS).

Full extraction details:

1. Forty 5-g portions of each soil sample were measured into a 50-mL centrifuge tube.

2. For preparing fortified samples, appropriate aliquots of the appropriate spiking solutions were added to untreated control soil to encompass the necessary concentration range:

Concentration of Fortified Sample (µg/kg)	Volume of Spiking Solution (µL)	Concentration of Spiking Solution (µg/mL)
0.02	50	0.002 µg/mL
0.05	125	0.002 µg/mL
0.5	125	0.02 µg/mL

3. Sample is supplemented with diatomaceous earth (DE) to obtain sample+DE mass to reach ~10mL marking on centrifuge tube.
4. Thoroughly mix sample and DE to obtain a homogeneous mixture
5. Prepare 10-mL ASE cells (see suggested cleaning procedures): Place 27 mm cellulose filters on bottom of 11-mL ASE cell.
6. Add samples to prepared 10-mL ASE cells, tighten ends of cells. Place in cell rack on ASE200.
7. Place vials in ASE200 vial rack.
8. 'Rinse' system
9. Load ASE method
    a. Preheat = 0 min.
    b. Heat = 5.0 min.
    c. Static = 5.0 min
    d. Flush[a] = 100%
    e. Purge = 60 sec.
    f. Cycles = 2
    g. Pressure = 1500 psi
    h. Temperature = 90°C
    i. Soln X[b] = 100%

    [a] Very important - Adjust flush to obtain as close to 25 mL of extraction as possible.

    [b] X correspond to the position reference (A, B, C or D) on system tray of extraction solvent MeOH:H_2O (50:50) + 0.1% H_3PO_4
10. Start ASE

11. Upon completion of ASE extraction, top off extract to 25 mL by placing sample vial between two 60-mL extraction vials containing 25.0 mL of extraction solution (check vials) and fill sample vial to level of check vials, cap and gently shake vial to mix.
12. Transfer a 5.0 mL aliquot into 50 mL polypropylene flask.
13. Add internal standard to each sample aliquot. Add 40 µL of 0.0125 µg/mL mixed (M+6) internal standard solution to result in the concentration of the IS to be approximately 0.50 ng/mL in the final volume.
14. Evaporate aliquots to 1 mL using a rotary evaporator at 40°C.
15. Add 100 µL acetonitrile to each sample.
16. Cap vials, briefly sonicate and vortex to mix.
17. Transfer a 500µL portion of the sample to Whatman syringeless filter vials, and filter sample.
18. Analyze the samples and calibration standards by LC-MS/MS with positive-ion electrospray tandem mass spectrometry.

Analytical Instrumentation and Equipment

- LC-MS/MS API 5500 (Sciex)
- Column oven CTO-20AC (Shimadzu)
- Automatic sampler SIL20AC (Shimadzu)
- Pump LC20AD (Shimadzu)
- Accelerated Solvent Extractor ASE200 (Dionex)
- Filter cellulose for ASE 200 (Thermo Scientific, ref 49458)
- Filter Whatman Mini-Uniprep syringless, PTFE membrane, 0.45 µm (Fisher Scientific, ref. 09-923-28)
- HPLC column Synergi Hydro-RP 80A, 4.6 x 75 mm, 4 µm (Phenomenex, ref. 00C-4375-E0)
- Polypropylene centrifugation tubes
- Precision balance (Mettler)
- PTFE syringe filters
- Rotary evaporator (Deltalabo)
- SecurityGuard Cartridge, AQ C18, 3 x 4 mm (Phenomenex, ref. AJ0-7511)
- Standard laboratory glassware (volumetric flasks, measuring cylinders)
- Ultrasonic bath (Bioblock)
- Various pipettes (Thermo Scientific)

- Vortex (VWR)

The instrumental conditions used during the ILV trial were as described in the analytical method, and are given below.

Typical HPLC Operating Conditions

Column:	Synergi Hydro-RP 80A, 4.6 x 75 mm, 4 µm
Column Temperature:	23°C
Automatic sampler temperature:	4°C
Injection Volume:	50 µL
Injection Wash	Isopropanol/Ultra-Pure Water (50/50, v/v)
Run Time:	13.0 minutes
Mobile Phase:	A – Ultrapure water + 0.1% formic acid B – Acetonitrile + 0.1% formic acid
Flow Rate:	1.0 mL/min.

Gradient:

Time, min	A, %	B, %
0.0	90	10
7.0	0	100
9.0	0	100
9.15	90	10
13.0	90	10

Flow Diverter Program:

1) 0.0→1.0 min: flow to waste
2) 1.0→10.0 min: flow to source
3) 10.0→end of run: flow to waste

Retention times:

Approx. 6.0 min for XDE-729 methyl ester
Approx. 3.0 min for XDE-729 acid
Approx. 2.5 min for des-methyl-XDE-729 acid

Typical Mass Spectrometry Operating Conditions

Instrumentation:	MDS SCIEX API 5500 LC-MS/MS System MDS SCIEX API 5500 QTrap LC-MS/MS System MDS SCIEX Analyst 1.5.1 data system
Polarity:	Positive
Interface	Electrospray
Scan Type:	MRM
Resolution:	Q1 – unit, Q3 – unit
Curtain Gas (CUR):	40 psi
Collision Gas	4 or Medium
Temperature (TEM):	550°C
Ion Source Gas 1	50 psi
Ion Source Gas 2	50 psi

Analyte	Parent ion (*m/z*)	Daughter ion (*m/z*)	DP (V)	EP (V)	CXP (V)	CE (eV)	Dwell (ms)
XDE-729 methyl ester	344.78	250 (quantification)	51	10	32	43	150
		285 (confirmatory)	51	10	38	31	150
XDE-729 acid	330.78	250 (quantification)	76	10	32	41	100
		284.9 (confirmatory)	76	10	40	29	100
des-methyl-XDE-729 acid	316.73	236.1(quantification)	86	10	32	45	125
	318.98	237.98 (confirmatory)	71	10	32	45	125

DP: Declustering Potential, CE: Collision Energy, CXP Cell Exit Potential, EP: Entrance Potential

Internal standards

Analyte	Parent ion (*m/z*)	Daughter ion (*m/z*)	DP (V)	EP (V)	CXP (V)	CE (eV)	Dwell (ms)
XDE-729 methyl ester M+6	350.79	256.1	86	10	34	43	150
XDE-729 acid M+6	336.86	256.1	81	10	34	41	100
X11449757 M+6	322.81	242	56	10	32	45	125

DP: Declustering Potential, CE: Collision Energy, CXP Cell Exit Potential, EP: Entrance Potential

Calculation of Results

For each analytical batch, a range of 8 calibration standards was injected over the range 0.01 ng/mL to 6.0 ng/mL. A calibration curve was prepared for each analyte by plotting the quantification peak area ratio obtained versus the analyte concentration ratio.

Example: XDE-729 methyl ester recovery at 0.05 µg/kg

A linear calibration curve was calculated using the method of least squares (1/x weighting):

$$Y = A \times C + B$$

Y = detector response (as peak area ratio) for XDE-729 methyl ester = 0.094

A = slope of the linear least squares fit of the calibration curve = 0.873

C = Quantitation ratio determined from standard curve $C = \frac{C_a}{IS}$

C_a = Analyte concentration

IS = internal standard concentration = 0.5 ng/mL

B = Y-intercept of the linear least squares fit of the calibration curve = 0.00415

The concentration determined from standard curve is $C_a = \frac{(Y - B)}{A} \times IS$ = 0.052 ng/mL

The residue of XDE-729 methyl ester in each test specimen is calculated as follows:

$$\text{Residue (µg/kg)} = EF \times \text{extract concentration (ng/mL)}$$

Where:

$$EF = \frac{V_1 \times V_f}{M \times V_2} \times n$$

V1 = Initial extraction volume (25 mL)

V2 = Volume of aliquot (5 mL)

Vf = Final volume (1 mL)

M = Sample weight (5 g)

n = final dilution

Extract concentration = 0.052 ng/mL

Residue (µg/kg) = 0.052 µg/kg

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

$$\text{Recovery } (\%) = \frac{A}{S} \times 100$$

Where:

A = concentration of XDE-729 methyl ester found in spiked sample = 0.052 µg/kg.

S = concentration of XDE-729 methyl ester added in spiked sample = 0.05 µg/kg.

Recovery = 103% (calculation performed on unrounded values)

Statistical Treatment of Data

The mean recoveries for the fortified samples were calculated using the "AVERAGE" function of the Microsoft Excel spreadsheet computer program, which divides the sum of the selected cells by the number of determinations. The standard deviation of the recoveries for each fortification level for each matrix type was calculated using the "STDEV" function of the same spreadsheet program, which sums the squares of the individual deviations from the mean, divides by the number of degrees of freedom (n-1), and extracts the square root of the quotient. Percent relative standard deviation, % RSD, was calculated by dividing the standard deviation by the mean, and then multiplying by 100.

Confirmation of Residue Identity

The LC-MS/MS method is highly selective for the determination of residues of XDE-729 methyl ester (X11393728) and its metabolites XDE-729 acid (X11393729), and des-methyl-XDE-729 acid (X11449757) in two types of soil by virtue of the chromatographic separation and selective detection system used. To demonstrate further confirmation, a second structurally characteristic MS/MS ion transition was monitored for each analyte. Calculations of %Recovery and %RSD were additionally carried out on the confirmatory ions data as well (Tables 1 and 2).