INTRODUCTION

Dow AgroSciences LLC Analytical Method GRM 05.05, "Determination of Residues of XDE-742 and its Metabolites in Soil and Sediment by Liquid Chromatography with Tandem Mass Spectrometry Detection" (Appendix A), was developed and validated at Dow AgroSciences LLC. The method was found to be suitable for the determination of residues of XDE-742 and its metabolites in soil over the concentration range 1.0-100 ng/g with a validated lower limit of quantitation of 1.0 ng/g. An independent laboratory validation of method GRM 05.05 was conducted to satisfy the requirements of the European Council Directive 91/414/EEC, as amended by European Commission Directive 96/46/EC, and the European Commission Guidance Document on Residue Analytical Methods, SANCO/825/00 rev. 7 (1,2,3). The study was also conducted to satisfy the requirements of U.S. EPA Guideline OPPTS 860.1340(c) (6) and PR Notices 96-1 and 86-5 (4, 5, 6).

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 soil samples. The independent laboratory used all of its own equipment and supplies, so that there was no common link between Dow AgroSciences LLC and the Study Director or analyst. Throughout the conduct of the study, any communications between Dow AgroSciences LLC and the Study Director and/or the analyst were logged for inclusion in the report. No one from Dow AgroSciences LLC 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

Sample Preparation and Storage

Two European standard soils were obtained from LUFA Speyer[1] sieved (2 mm) and stored air dried. Soil characterization information is given in Appendix B. Prior to use in this study, water

[1] Landwirtschaftliche Untersuchungs- und Forschungsanstalt Speyer, D-67346 Speyer.

was added as to establish approximately 40 – 50 % of the maximum water holding capacity.

Preparation of Solutions and Standards

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

Test Items/ Analytical Standards	AGR/TSN No.	Percent Purity	Certification Date	Reference
XDE-742 (Lot No.: E0728-78A)	TSN102482	100	04-May-2005 Re-certification Date 02-May-2009	05-729-L
5-OH-XDE-742 (Lot No.: E1631-23)	TSN104232	100	08-Sep-2003 Re-certification Date 28-Jul-2005	FA&PC 033171
7-OH-XDE-742 (Lot No.: E1631-21)	TSN104231	99	08-Sep-2003 Re-certification Date 28-Jul-2005	FA&PC 033172
6-Cl-7-OH-XDE-742 (Lot No.: E1791-21)	TSN104660	96	22-Jul-2004 Re-certification Date 07-Jun-2006	FA&PC 043198

Internal Standards	TSN No.	Percent Purity	Certification Date	Reference
$^{15}N_3$-XDE-742 (Lot No.: F1064-006)	TSN104269	99	13-Nov-2003 Re-certification Date 29-Jul-2005	FA&PC 033211 R1
$^{15}N,D_3$-5-OH-XDE-742 (Lot No.: E1791-23)	TSN104659	99	30-Jun-2004 Re-certification Date 03-Jun-2006	FA&PC 043197
$^{15}N,D_3$-7-OH-XDE-742 (Lot No.: E1791-25)	TSN104661	95	30-Jun-2004 Re-certification Date 03-Jun-2006	FA&PC 043199
$^{15}N,D_3$-6-Cl-7-OH-XDE-742 (Lot No.: E1791-41)	TSN104670	99	21-Jul-2004 Re-certification Date 08-Jun-2006	FA&PC 043217

Standard solutions were prepared as described in Section 7 of method GRM 05.05.

Fortification of Recovery Samples

ILV trials for ten times LOQ and hundred times LOQ fortifications for each of the 2 soil types consisted of the following:

2 (two) unfortified control samples
5 (five) control samples fortified at 10 ng/g (ten times (10x) the LOQ of the method)
5 (five) control samples fortified at 100 ng/g (hundred times (100x) the LOQ of the method)

ILV trials for LOQ fortifications for each of the 2 soil types consisted of the following:

1 (one) unfortified control sample
5 (five) control samples fortified at 1.0 ng/g (the LOQ of the method)

Sample Extraction, Purification and Analysis

The ILV trial was conducted as described in Section 9 of method GRM 05.05 with some minor adaptations.

Analytical Instrumentation and Equipment

The ILV trial was conducted as described in Section 8 of method GRM 05.05, with minor adaptations as given below:

Liquid Chromatography Operating Conditions

For quantitation and confirmation of results only one liquid chromatography method was used. For confirmatory purposes, a second MRM ion transition for each compound was monitored and evaluated.

Instrumentation:	CTC Analytics HTC PAL Agilent Model 1100 binary pump Agilent Model 1100 degasser Applied Biosystems API 3000 LC/MS/MS System Applied Biosystems Analyst 1.3.1 data system
Column:	Phenomenex Aqua C_{18},

	50 x 2.0 mm i.d., 5 micron particle size Securityguard: Hypersil Gold C_{18}, 10 x 3 mm, 5 µm particle size
Column Temperature:	35 °C
Injection Volume:	50 µL or 75 µL (for LOQ determination), respectively
Run Time:	7.5 minutes
Mobile Phase:	A – methanol with 2 mM ammonium acetate B – water with 2 mM ammonium acetate
Flow Rate:	500 µL/min

Gradient:

Time, min	A, %	B, %
0.0	10	90
3.0	100	0
4.5	100	0
4.6	10	90
7.5	10	90

Mass Spectrometry Operating Conditions

API 3000:	
Interface:	TurboIonSpray
Polarity:	Positive
Scan Type:	MRM
Resolution:	Q1 – unit, Q3 – unit
Nebulizer Gas (NEB):	15
Curtain Gas (CUR):	12
IonSpray Voltage (IS):	5000 V
Temperature (TEM):	450 °C
Collision Gas (CAD):	4
Period Duration:	7.5 minutes
MR Pause:	5 ms
Spray Position:	5 mm left of Orifice

Compound	Ion, m/z		Time, ms	CE, V
XDE-742	435.1	194.7	75 ms or	37
XDE-742 (confirmation)	435.1	81.9	100 ms (for	87
$^{15}N_3$-XDE-742 (internal standard)	438.1	198.2	LOQ determination),	37
5-OH-XDE-742	421.0	181.1	respectively	34
5-OH-XDE-742 (confirmation)	421.0	148.1		39
$^{15}N,D_3$-5-OH-XDE-742 (internal standard)	425.0	185.1		34
7-OH-XDE-742	421.0	181.1		34
7-OH-XDE-742 (confirmation)[2]	421.0	138.1		47

[2] For confirmation of 7-OH-XDE-742 the ion transition m/z Q1/Q3 421/138 was used due to higher sensitivity instead of the 421/148 ion transition as described in the original method.

$^{15}N,D_3$-7-OH-XDE-742 (internal standard)	425.0	185.1	34
6-Cl-7-OH-XDE-742	455.0	214.9	38
6-Cl-7-OH-XDE-742 (confirmation)	455.0	148.1	39
$^{15}N,D_3$-6-Cl-7-OH-XDE-742 (internal standard)	459.0	219.2	38

Equipment:

Analytical Balance –Sartorius RC 210 D

Top Loading Balance – Sartorius LP 620 S

Centrifuge, Hettich Rotixa 720 and Biofuge primo, Heraeus

Vacuum manifold, 96 well, catalog number AHO 7284, Strata, Phenomenex

Ultrasonic bath, Elma - Transsonic 700

Horizontal shaker, IKA 250 B

Vortex mixer, Assistant Reamix

Evaporator, Rotavapor R-200 V800, Büchi and Pierce Reacti-Vap™

Syringe filters 0.45 µm, Macherey-Nagel

Filter Aid, Empore

Spin-X Centrifuge Tube Filters, 0.22 µm, Costar

Acetonitrile, water, methanol, all Promochem, HPLC grade or Pesticide Grade

Bi-distilled water (Ampuwa) Fresenius

Hydrochloric acid, 32 %, Merck

Ammonium acetate, ≥ 98 %, Merck

SPE 96-well plate, STRATA X 33-µm, 30-mg packing, Phenomenex no. 8E-S100-TGB

Microsoft Excel 2000 operating in Windows 2000, Microsoft Corporation

Calculations

The calculation was performed as described in Section 10 of method GRM 05.05. Calibration standards (0.15, 0.25, 0.5, 2.5, 5.0, 10, 25, 50, and 75 ng/mL, corresponding to 0.0003, 0.0005, 0.001, 0.005, 0.01, 0.02, 0.05, 0.10, 0.15 mg/kg) were analyzed with the sample set. Linear regression equations were generated for XDE-742 and its metabolites calibration standards with

1/x weighting using the concentration of the analyte standard / concentration of the internal standard versus the analyte peak area / internal standard peak area. Concentrations of XDE-742 and its metabolites in the final extracts (resulting in ng/mL residue results) were determined by substituting the peak area ratios into the linear regression equation as shown below:

$Y = aX + b$

Y: Ratio: (Analyte peak area / IS peak area)

X: Ratio: (Analyte concentration (c_{End})/ IS concentration)

The IS concentration was always equivalent to 5 ng/mL, in calibration solutions as well as in final extracts (for LOQ determination the internal standard concentration in final extracts was 15 ng/mL).

Thus:

$c_{End} = ((Y - b) / a) \times$ IS concentration

$c_{End} =$ (((Analyte peak area / IS peak area) – b) / a) x IS concentration

The analyte concentration is thus obtained as residue R (in mg/kg) by the following calculation:

$R = c_{End} \times (V_{Ex} \times V_{End} / V_{R1} \times W) / 1000$ ng/µg

$= c_{End} \times$ Multiplier M

where:

c_{End}: = Concentration of final extracts in ng/mL

V_{Ex}: = Extraction volume: 40 mL

V_{End}: = Volume of final extracts: 0.75 mL (for LOQ determination: 0.50 mL)

V_{R1}: = Aliquot taken for clean-up: 3.0 mL (for LOQ determination: 6.0 mL)

W: = Specimen weight: 5 g

1000: = Factor used for adjustment of units

Recoveries (Rec.) were calculated for the fortified specimens as follows:

$Rec. = (R / R_{fortified}) \times 100$ %

Example for XDE-742:

The calculation is exemplified with a Soil 2.2 specimen (PTRL-ID P848-57):

5.0 g of soil 2.2 were fortified at 0.01 mg/kg (10 ng/g, ten times of LOQ) by dosing 0.05 mL of the 1.0 µg/mL fortification solution.

After extraction, 50 ng of the internal standards were added to the raw extract (40 mL). A 3.0 mL aliquot of the raw extract was evaporated to dryness and reconstituted in 0.1 N HCL, purified by

SPE, the eluate was evaporated to dryness and reconstituted in a final volume of 0.75 mL (corresponding to 0.375 g of sample weight).

The final extract was examined by LC/MS/MS in run file P848-050 (Figure 17 and Figure 18), resulting in a XDE-742 area of 49400 counts for the primary 435 m/z → 195 m/z MRM ion transition. The internal standard peak area in the 438 m/z → 198 m/z MRM ion transition was 53700 counts.

The Analyst software used for the 435 m/z → 195 m/z MRM ion transition the calibration function

Y = 0.909 x X + 0.00995 (Figure 1 top)

which was established by injecting calibration solutions interspersed with final extracts, whereby:

Y: Ratio: (Analyte peak area / IS peak area)

X: Ratio: (Analyte concentration / IS concentration)

with IS concentration always 5 ng/mL (for LOQ determination the internal standard concentration in final extracts was 15 ng/mL).

Including the intercept b with 0.00995, the linear calibration function becomes:

c_{End} = ((Y –b) / a) x IS concentration

= (((Analyte peak area / IS peak area) – 0.00995) / 0.909) x 5.0 ng/mL

= (((49400 counts / 53700 counts) – 0.00995) / 0.909) x 5.0 ng/mL

= 5.01 ng/mL

The analyte concentration is thus obtained as residue R (in mg/kg) by the following calculation:

R = c_{End} x (V_{Ex} x V_{End} / V_{RI} x W) / 1000 ng/µg

= 5.01 ng/mL x (40 mL x 0.75 mL / 3.0 mL x 5 g) / 1000 ng/µg

= 5.01 ng/mL x 0.002 mL/g x µg/ng

= 0.010 µg/g

Recoveries (Rec.) were calculated for the fortified specimens as follows:

Rec. = (R / $R_{fortified}$) x 100 %

= 0.010 µg/g / 0.010µg/g) x 100 %

= 100 %

The XDE-742 and its metabolites values were calculated using a computerized spreadsheet that used more decimal places than those displayed. As a result, the calculated values shown in this example may vary slightly if the values are recalculated using the displayed parameters.

Statistical Treatment of Data

The mean recoveries for the fortified samples were calculated using the "AVERAGE (MITTELWERT)" 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 recovery for a sample was calculated using the "STDEV (STABW)" 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, 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.

Problems Encountered and Changes or Modifications Made

No problems were encountered with the methodology, and only minor modifications were found to be necessary (see below).

Critical Steps

The clean-up procedure with the Strata X 96-well plates was observed as the only critical step. For some soils (in this case soil 2.2, a loamy sandy soil) it seems to be necessary to centrifuge (about 5 min at 10000 rpm) the reconstituted HCl extracts before applying them on the 96-well SPE plate. Otherwise the SPE material may get clogged. Additionally, it is advisable to use filter aid at the clean-up step for some soil extracts (about 0.5 g filter aid was added by a spatula in each well of the Strata X 96-well plate before conditioning the Strata X SPE).
In the 2nd set for specimens fortified at 1.0 ng/g (the LOQ of the method) a higher aliquot of the raw extract (6 mL instead of 3 mL) was taken and the final volume was adjusted to 0.5 mL

(instead of 0.75 mL). Thus a better determination of all analytes with good reproducibility could be achieved at the LC-MS/MS.

Sample Analysis Time Requirements

One set of 12 samples required approximately eight person-hours or approximately one calendar day to complete.

Communications

No contacts between the Study Director at the independent laboratory and the method developers, or others familiar with the method were necessary.

CONCLUSION

Dow AgroSciences LLC soil residue analytical method GRM 05.05 has been successfully validated by an independent laboratory analyst who had no previous experience with the soil residue analytical methodology. The LOQ of the method was confirmed as 1.0 ng/g for XDE-742 and its metabolites in soil.

ARCHIVING

At the conclusion of the study, the raw data, the original study plan, amendments, deviations, and the original version of the final report will be archived at Datacare Business Systems Limited, 3012 Heyford Park, Heyford Park, Upper Heyford, Oxon, OX25 5HF, United Kingdom.

REFERENCES

1. *European Council Directive, 91/414/EEC*, Official Journal of the European Communities L 230, HMSO Publications, London, UK, 1991.

2. *European Commission Directive, 96/46/EC*, Official Journal of the European Communities L 214, HMSO Publications, London, UK, 1996.

3. *European Commission Guidance Document on Residue Analytical Methods, SANCO/825/00 rev. 7,* Directorate General for Health and Consumer Protection, 2000.

4. *Residue Chemistry Test Guidelines, OPPTS 860.1340, Residue Analytical Method,* U.S. Environmental Protection Agency. U.S. Government Printing Office: Washington, DC, 1996; EPA 712-C-96-174.

5. *Pesticide Regulation Notice 96-1,* U.S. Environmental Protection Agency, Office of Pesticide Programs: Washington, DC, 1996.

6. *Pesticide Regulation Notice 86-5,* U.S. Environmental Protection Agency, Office of Pesticide Programs: Washington, DC, 1986.