

INTRODUCTION

Dow AgroSciences (DAS) residue analytical method 110354, "Determination of Recovery of Methoxyfenozide, the A-ring Phenol Metabolite and the B-ring Mono Acid Metabolite of Methoxyfenozide in Soil and Sediment" (1) (Appendix A), was developed and validated at Dow AgroSciences LLC. The method was found to be suitable for the determination of residues of methoxyfenozide and its major metabolites (A-ring phenol metabolite and B-ring mono acid metabolite) in soil over the concentration range of 0.01 to 0.1 mg/kg. The validated limit of quantification of the method was 0.01 mg/kg.

An independent laboratory validation following DAS Study Number 110354 was conducted on soil and sediment to satisfy the requirements of the European Council Directive 91/414/EEC (2), as amended by European Commission Directive 96/46/EC, and the European Commission Guidance Document on Residue Analytical Methods, SANCO/3029/99 rev. 4 (3), and SANCO/825/00 rev.8.1 (4). The study was also conducted to satisfy the requirements of U.S. EPA Guideline OPPTS 850.7100 (c)(2) (5), PR Notice 2011-3 (6), and PR Notice 96-1 (7).

Soil and sediment were characterized according to GLP to satisfy the requirements of SANCO/825/00 rev 8.1 and characterisation data is included in the Appendix B. Please note soil purchased from Lufa Spayer was characterized prior to and following the analysis, both characterisation certificates are included in the Appendix B.

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 analysing 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 analysts. Throughout the conduct of the study, communications between Dow AgroSciences and the Study Director and/or the analyst if any 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

Untreated sediment samples were taken from another EAS study. Untreated soil samples were obtained from Lufa Speyer, from meadow sites in Germany. Upon receipt at EAS, the soil and sediment samples were assigned EAS identification numbers. Characterization data for both soils is provided in Appendix B. Following arrival at EAS, the soils were placed in a freezer set to maintain a temperature of typically -18 C° where they were retained at all times unless removed for preparation or analysis. No preparation was deemed to be necessary for the soil purchased from Lufa Speyer. Sediment was prepared by hand, by removing of stones and any organic matter. Any further preparation for sediment deemed to be not necessary.

Preparation of Solutions and Standards

Reagents used were of equivalent specifications as described in DAS Study Number 110354.

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

Description	Analytical Test Substances/Reference Standards		
	Name / Abbreviation	Methoxyfenozide RH-2485 (RH-112485)	A-ring phenol metabolite of RH2485
Dow TSN #	TSN104129	TSN300811	TSN029592-0001
Dow Lot #	F50-C1572-46	SYN-FS08019-056	SYN-6925-76-02
EAS #	EAS00021	EAS00022	EAS00023
Purity (wt %)	99.9	99	99
Appearance	Solid	Solid	Solid
Storage Conditions	Ambient	Ambient	Refrigerator
Certification Date	Nov 19, 2010	June 29, 2011	Dec 17, 2010
Recertification Date	Oct 17, 2014	July. 28, 2013	July 11, 2012

These materials were received from the Sponsor on Jan 5, 2012 and further identified with EAS reference numbers. Documentation of the methods of synthesis and characterization are maintained by the Sponsor.

Stock standard solutions and calibration standard solutions were prepared as described in DAS Study Number 110354 with minor modifications. Full details of these materials are included in the raw data package for the study along with the preparation of all analytical and fortification standards prepared from the primary reference items. The test/reference items will be retained until expiry and then disposed of after completion and issuance of the final study report, upon which disposal will be authorized by the Study Monitor.

Fortification of Recovery Samples

One ILV trial following Study number 110354 was attempted with each trial consisting of the following treatments:

- 1 reagent blank (containing no matrix or analyte)
- 2 unfortified control samples for each matrix
- 5 control samples of each soil type were fortified with a mixed solution of the test substances at 0.01 mg/kg (the LOQ of the method).
- 5 control samples of each soil type were fortified with a mixed solution of the test substances at 0.1 mg/kg (10X the LOQ of the method).

Sample Extraction, Purification and Analysis

Untreated and fortified soil and sediment were assayed according to the analytical method described in DAS Study Number 110354 with negligible variations due to slightly different laboratory equipment and practices.

In brief, residues of methoxyfenozide and its major metabolites were extracted from soil and sediment by vortex mixing and shaking with 90% methanol/10% 1.0N hydrochloric acid, after which the samples were centrifuged. The extraction was repeated and both extracts were combined and the volume adjusted to 40.0 mL using 90% methanol/10% 1.0N hydrochloric acid extraction solution. A 1.0-mL aliquot of the extract was taken, diluted with 2.0 mL of HPLC grade water and vortex mixed. The samples were then purified using SPE, 1.0 mL of the sample solution was loaded onto a preconditioned SPE cartridge and the extract was pulled through the plate at approximately 1 mL/min, discarding the eluate. The SPE cartridge was washed with 1 mL of 60% water/40%

methanol/ 0.1% formic acid solution, discarding the eluate. The cartridge was dried under full vacuum, and methoxyfenozide and its metabolites were eluted from the SPE cartridge at approximately 1 mL/min with two 750- μ L aliquots of acetonitrile. The acetonitrile was evaporated to dryness at approximately 40°C using nitrogen. The sample was reconstituted in 1.0 mL of 70% water/30% acetonitrile solution containing 0.1% formic acid and vortex mixed prior to analysis by LC-MS/MS.

Analytical Instrumentation and Equipment

The instrumental conditions used during the ILV trial were as described in DAS Study Number 110354, with minor adaptations as given on the following pages:

Typical LC-MS/MS Operating Conditions

Instrumentation: Applied Biosystems API 4000 MS System
Applied Biosystems Analyst 1.4.2 data system

Column: Ascentis Express C18
50 x 2.1 mm i. d., 2.7 μ m

Column Temperature: 40 °C

Injection Volume: 15 μ L

Injection Wash

Wash solvent 1 5/95 Methanol/Water

Wash solvent 2 Methanol

Wash solvent 3 50/50 Methanol/Water

Run Time: approximately 12 minutes

Mobile Phase: A – water containing 0.1% formic acid
B – acetonitrile

Gradient:	Time (min)	Flow Rate (mL/min)	Solvent A (percent)	Solvent B (percent)
	0:01	0.40	70	30
	1:00	0.40	70	30
	8:00	0.40	10	90
	9:00	0.40	70	30
	12:00	0.40	70	30

Flow Diverter

Flow to Waste 0.0 min → 2.0 min

Flow to Source 2.0 min → 6.0 min

Flow to Waste 6.0 min → end of run

Typical Mass Spectrometry Operating Conditions

Instrumentation:	Applied Biosystems API 4000 MS System Applied Biosystems Analyst 1.4.2 data system
Ionization Mode:	Turbo spray
Polarity:	positive
Scan Type:	MRM
Resolution:	Q1 – unit, Q3 – unit
Curtain Gas (CUR)	40 psi
Collision Gas (CAD):	8.0 psi
Ion Source Gas 1 (GS1)	50 psi
Ion Source Gas 2 (GS2)	50 psi
Temperature (TEM):	500 °C
Ion Spray Voltage (IS):	5500 volts
Acquisition Time Delay:	0.00 minutes
Period Duration:	12 minutes
Dwell Time:	100 msec

Analytes	Precursor Ion, Q1	Product Ion, Q3	Declustering Potential, v	Entrance Potential, v	Collision Energy, v	Cell Exit Potential, v
Methoxyfenozide						
quantification	369.2	313.3	54	8	11	10
confirmation	369.2	149.1	54	8	23	10
A-ring phenol metabolite						
quantification	355.1	299.2	54	8	11	10
confirmation	355.1	135.0	54	8	23	10
B-ring mono acid metabolite						
quantification	399.3	343.1	75	8	11	10
confirmation	399.3	149.2	75	8	23	10

Adaptations to the Analytical Method

Additional adaptations to the analytical method described in DAS Study Number 110354 are listed below:

- a) Soils samples purchased from Lufa Sprayer were already homogenized and required no further preparation. Sediment was prepared by hand by removing stones and organic matter instead of using dry ice and hammer mill.

- b) Different LC-MS/MS conditions were used:

Column: Ascentis Express C18 50 x 2.1 mm i.d 2.7 μ m particle size

Mobile phase A: 0.1% formic acid in water

Mobile phase B: Acetonitrile

Mobile phase gradient:

Time (min)	Flow Rate (mL/min)	Solvent A (percent)	Solvent B (percent)
0:01	0.40	70	30
1:00	0.40	70	30
8:00	0.40	10	90
9:00	0.40	70	30
12:00	0.40	70	30

- c) The elevated HPLC column temperature was at 40°C rather than ambient as listed in the method.
- d) Calibration standards were prepared in 70% water, 30% acetonitrile instead of 70% water 30% acetonitrile solutions containing 0.1% formic acid.
- e) Bracketing standard technique was used for the calculation of residues rather than interspersed standards.

All above analytical method adaptations were considered as minor changes and approved by the Study Director.

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 a fortification level of one 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, and extracts the square root of the quotient. Percent relative standard deviation

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9. DETERMINATION OF RECOVERY OF METHOXYFENOZIDE, THE A-RING PHENOL METABOLITE AND THE B-RING MONO ACID METABOLITE OF METHOXYFENOZIDE IN SOIL AND SEDIMENT

9.2. Sample Preparation

Method Departure No. 004

Prepare soil or sediment samples for analysis by freezing the sample with dry ice or liquid nitrogen and then grinding or chopping with a hammer mill equipped with a 3/16-inch screen size. Soil or sediment samples should be stored deep-frozen prior to analysis.

9.3. Sample Analysis

9.3.1. Weigh 5.0 ± 0.05 g of each soil or sediment sample into individual 45-mL vials equipped with PTFE-lined caps (or 50-mL polypropylene graduated centrifuge tubes equipped with caps). (Acceptable stopping point if sample is kept frozen.)

9.3.2. For recovery samples, add appropriate aliquots of the mixed spiking solutions to obtain concentrations ranging from 0.01-1.0 $\mu\text{g/g}$ for methoxyfenozide, the A-ring phenyl metabolite and the B-ring mono acid metabolite. (A reagent blank contains no sample matrix.) Refer to table below for example fortification levels to obtain this concentration range.

Sample Description	Spiking Volume μL	Spiking Solution $\mu\text{g/mL}$	Fortification Level $\mu\text{g/g}^a$
CONTROL	---	---	---
LOD	15	1.00	0.003
LOQ	50	1.00	0.010
MID (10 x LOQ)	50	10.0	0.100
HIGH (100 x LOQ)	50	100.0	1.00

^a Based on a 5.0-g initial soil or sediment sample.

9.3.3. Add 20 mL of the 90% methanol/10% 1.0N hydrochloric acid extraction solution to the sample vial.

9.3.4. Cap the sample vial with a PTFE-lined cap, pulse vortex mix for about 10 seconds, and shake the sample on a reciprocating shaker for at approximately 180 excursions/minute for a minimum of 30 minutes.

9.3.5. Centrifuge the sample for approximately 5 minutes at approximately 2000 rpm.

9.3.6. Transfer the extract to a 50-mL graduated mixing cylinder (or 50-mL polypropylene graduated centrifuge tube equipped with cap may be substituted).

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- 9.3.7. Add an additional 15 mL of the 90% methanol/10% 1.0N hydrochloric acid extraction solution to the original sample, pulse vortex mix for about 10 seconds, and shake for an additional 30 minutes (approximate) on a flat bed shaker at approximately 180 excursions/minute. (**Critical Step:** Ensure that the soil or sediment plug is broken up well by vortex mixing or manually shaking before placing the samples on the flat bed shaker.)
- 9.3.8. Centrifuge the sample for 5 minutes at 2000 rpm and combine the second extract with the first extract (Step 9.2.6) in the 50-mL graduated mixing cylinder.
- 9.3.9. Adjust the volume of the combined extract in the centrifuge tube to 40.0 mL using the 90% methanol/10% 1.0N hydrochloric acid extraction solution. Stopper the cylinder and mix thoroughly. (Acceptable stopping point if sample is kept refrigerated.)
- 9.3.10. Using a positive-displacement pipet, transfer 1.0 mL of the sample solution into a 8-mL (2-dram) vial.
- 9.3.11. Dilute the sample from Step 9.3.10 with 2.0 mL of HPLC grade water and vortex mix for approximately 5 seconds.
- 9.3.12. Purify samples using the following SPE procedure:
- Place a Phenomenex Strata-X 33 μ m polymeric sorbent reversed phase SPE cartridge (30-mg, 1-mL, catalog number 8B-S100-TAK) on a vacuum manifold box.
 - Condition the SPE cartridge with 1 mL of methanol followed by 1 mL of water, discarding the eluates. Apply full vacuum (approximately -15 to -25 inches of Hg) for about 10 seconds between solvent additions.
 - Transfer 1.0 mL of the sample solution from Step 9.3.11 to the SPE cartridge. Pull the sample through the plate at approximately 1 mL/min, discarding the eluate. Dry the plate under full vacuum for 10 seconds after sample has eluted.
 - Wash the SPE cartridge with 1 mL of a 60% water/40% methanol/0.1% formic acid solution, discarding the eluate. Dry the cartridge under full vacuum for 5 minutes.
 - Elute the methoxyfenozide from the SPE cartridge at approximately 1 mL/min with two 750- μ L aliquots of acetonitrile, collecting the eluate in a 12-mL (16 x 100 mm) culture tube.
- 9.3.13. Evaporate the acetonitrile to dryness at approximately 40 °C using nitrogen (approximately 10 psi) on a TurboVap evaporator.

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- 9.3.14. Reconstitute the samples in 1.0 mL of 70% water/30% acetonitrile solution containing 0.1% formic acid. Pulse vortex for about 10 seconds to mix well.
- 9.3.15. Transfer a portion of each sample that is ready for analysis to a 96-deep well plate.
- 9.3.16. Add approximately 1 mL of each of the calibration standards to the same plate and seal the plate. (Acceptable stopping point if sample is kept refrigerated.)
- 9.3.17. Analyze the calibration standards and samples by positive-ion ESI LC-MS/MS, injecting the calibration standards interspersed with the samples throughout the run.
- 9.3.18. Calculate the percent recovery found for each analyte.
- 9.3.19. Analyze the samples along with the calibration standards using the LC/MS/MS conditions listed. Determine the suitability of the chromatographic system using the following criteria:
- Standard curve linearity: Determine that the correlation coefficient equals or exceeds 0.995 for the least squares equation which describes the detector response as a function of standard curve concentration.
 - Appearance of chromatograms: Visually determine that the chromatograms resemble those shown in the final method with respect to peak response, baseline noise, and background interference. Visually determine that a minimum signal-to-noise ratio of 10:1 has been attained for the 0.5-ng/mL calibration standard (equivalent to 0.008 µg/g of methoxyfenozide, the A-ring phenyl metabolite and the B-ring mono acid metabolite in the soil or sediment sample).
- 9.3.20. Dilute any samples that have a concentration above the range of the calibration curve for re-analysis using the 70% water/30% acetonitrile solution containing 0.1% formic acid. Samples should be within the range of the curve and at least 20% below the highest standard.

Method Departure No. 000

8. INSTRUMENTAL CONDITIONS

8.1. Typical Liquid Chromatography Operating Conditions (Note 12.3.)

Instrumentation:	Symbiosis Pharma MDS SCIEX API 4000 LC/MS/MS System MDS SCIEX Analyst 1.4.2 data system
Column:	Gemini C18 110A, 2.00 x 50 mm, 5.0-µm (Security Guard column)

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Column Temperature: Ambient Method Departure No. 001

Injection Volume: 30 μ L

Injection Wash Program:

Autosampler loop and needle washed with:

- 1) 700 μ L of a 50% methanol/50% water solution
- 2) 2 x 700 μ L of acetonitrile
- 3) 2 x 700 μ L of water

Run Time: 12.00 minutes

Mobile Phase:

A –water containing 0.1% formic acid

B –acetonitrile containing 0.1% formic acid

Flow Rate:

0.40 mL/min, flow diverted to source after 1.0 min.
approx 250 μ L split to source

Gradient:

Time, min	A, %	B, %
0:00	70	30
1:00	70	30
8:00	10	90
9:00	70	30
12:00	70	30

Diverter Program:

Time, min	Position
0:00	A
1:00	B
8:00	A

8.2. Typical Mass Spectrometry Operating Conditions

Interface: ESI
Polarity: Positive
Scan Type: MRM (MRM)
Resolution: Q1 – unit, Q3 – unit
Curtain Gas (CUR): 30
Collision Gas (CAD): 6.0
Temperature (TEM): 350 $^{\circ}$ C
Ion Source Gas 1 (GS1): 50
Ion Source Gas 2 (GS2): 50

Period 1

Pre-acquisition Delay: 0.0 min

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- 7.2. Preparation of Methoxyfenozide, the A-ring Phenyl Metabolite and the B-ring Mono Acid Metabolite Mixed Fortification Solutions
- 7.2.1 Accurately pipet 10.0 mL of each of the 1000- $\mu\text{g}/\text{mL}$ standard solutions prepared in Step 7.1.1 through 7.1.3 above and quantitatively transfer each into the same 100-mL volumetric flask. Add approximately 60 mL of HPLC grade water and mix well. Equilibrate to room temperature and dilute to volume with HPLC grade water to obtain a mixed fortification solution containing 100.0 $\mu\text{g}/\text{mL}$ of each analyte in a 70% water/30% acetonitrile solution.
- 7.2.2 Pipet 10.0 mL of the 100- $\mu\text{g}/\text{mL}$ mixed standard solution prepared in Step 7.2.1 above into a 100-mL volumetric flask. Dilute to volume using a 70% water/30% acetonitrile solution containing 0.1% formic acid to obtain a 10.0- $\mu\text{g}/\text{mL}$ mixed fortification stock solution.
- 7.2.3 Pipet 10.0 mL of the 10- $\mu\text{g}/\text{mL}$ mixed standard solution prepared in Step 7.2.2 above into a 100-mL volumetric flask. Dilute to volume using a 70% water/30% acetonitrile solution containing 0.1% formic acid to obtain a 1.0- $\mu\text{g}/\text{mL}$ mixed fortification solution.
- 7.2.4 Pipet 10.0 mL of the 1.0- $\mu\text{g}/\text{mL}$ mixed standard solution prepared in Step 7.2.3 above into a 100-mL volumetric flask. Dilute to volume using a 70% water/30% acetonitrile solution containing 0.1% formic acid to obtain a 0.10- $\mu\text{g}/\text{mL}$ mixed fortification solution.
- 7.2.5 Pipet 10.0 mL of the 0.10- $\mu\text{g}/\text{mL}$ mixed standard solution prepared in Step 7.2.4 above into a 100-mL volumetric flask. Dilute to volume using a 70% water/30% acetonitrile solution containing 0.1% formic acid to obtain a 0.01- $\mu\text{g}/\text{mL}$ mixed fortification solution.

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Method Departure No. 002

7.3. Preparation of Methoxyfenozide, the A-ring Phenyl Metabolite and the B-ring Mono Acid Metabolite Mixed Calibration Standards

- 7.3.1. Prepare calibration standards by diluting the appropriate mixed calibration standard stock solutions (Steps 7.2.3-7.2.5) using a 70% water/30% acetonitrile solution containing 0.1% formic acid according to the following table:

Concentration of Fortification Solution	Aliquot of Fortification Solution	Final Soln. Volume	Calibration Soln. Final Conc.	Equivalent Sample Conc. ^a
µg/mL	mL	mL	ng/mL	µg/g
1.00	7.0	100	75.0	1.20
1.00	5.0	100	50.0	0.80
1.00	3.5	100	35.0	0.56
1.00	2.0	100	20.0	0.32
1.00	1.0	100	10.0	0.16
0.10	5.0	100	5.00	0.080
0.10	1.0	100	1.00	0.016
0.01	5.0	100	0.50	0.008
0.01	1.5	100	0.15	0.0024
0.01	1.0	100	0.10	0.0016

^a The equivalent sample concentration of methoxyfenozide, the A-ring phenyl metabolite, and the B-ring mono acid metabolite is based on taking a 1.0-mL initial aliquot of the 40-mL sample extract, diluting it with 2.0 mL of water, purifying one third of the diluted extract on an SPE cartridge, and reconstituting the eluate to a final volume of 1.0 mL with using a 70% water/30% acetonitrile solution containing 0.1% formic acid (equivalent to 0.0625 g of soil matrix per mL of final sample as prepared for assay).