INTRODUCTION

Dow AgroSciences (DAS) residue analytical method 110356, "Method Validation Study for the Determination of Residues of Methoxyfenozide and its A-ring Phenol Metabolite and B-ring Mono Acid Metabolite in Surface Water, Ground Water and Drinking Water by Liquid Chromatography with Tandem Mass Spectrometry" (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 drinking water, surface water and ground water over the concentration range of 0.015 to 0.50 µg/L. The validated limit of quantification of the method was 0.05 µg/L.

An independent laboratory validation following DAS Study titled 'Method Validation Study for the Determination of Residues of Methoxyfenozide and its A-ring Phenol Metabolite and B-ring Mono Acid Metabolite in Surface Water, Ground Water and Drinking Water by Liquid Chromatography with Tandem Mass Spectrometry' was conducted on three types of water 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), PR Notice 96-1 (7).

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 sample analysis. The independent laboratory used all of its own equipment and supplies, so that there was no common link between Dow AgroSciences and the Eurofins Agroscience ILV analysis. 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 drinking water was obtained from a local water supply at EAS, Slade Lane, Wilson, Melbourne, Derbyshire, DE73 8AG, untreated surface water was obtained from the River Trent by S. Jones on 07 Jan 2012 and untreated ground water was purchased from a local supermarket. Upon receipt at EAS, the water samples were assigned EAS identification numbers. Characterization data for drinking, surface and ground water used for analysis is provided in Appendix B. Following arrival at EAS, all water samples were stored ambient until analysis. The samples required no preparation prior to the analysis.

Preparation of Solutions and Standards

Reagents used were of equivalent specifications as described in DAS Study titled 'Method Validation Study for the Determination of Residues of Methoxyfenozide and its A-ring Phenol Metabolite and B-ring Mono Acid Metabolite in Surface Water, Ground Water and Drinking Water by Liquid Chromatography with Tandem Mass Spectrometry'. 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	B-ring monoacid metabolite of methoxyfenozide
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	99.0	99.0
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 January 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 titled 'Method Validation Study for the Determination of Residues of Methoxyfenozide and its A-ring Phenol Metabolite and B-ring Mono Acid Metabolite in Surface Water, Ground Water and Drinking Water by Liquid Chromatography with Tandem Mass Spectrometry' 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 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. The disposal will be authorised by the Study Monitor.

Fortification of Recovery Samples

Two ILV trials for the determination of methoxyfenozide in drinking and surface water and for the determination of the A-ring phenol metabolite in the ground water and one ILV trial for the determination of methoxyfenozide in ground water, for the determination of the A-ring phenol metabolite in drinking and surface water and for the determination of the B-ring mono acid metabolite in drinking, ground and surface water following DAS Study titled 'Method Validation Study for the Determination of Residues of Methoxyfenozide and its A-ring Phenol Metabolite and B-ring Mono Acid Metabolite in Surface Water, Ground Water and Drinking Water by Liquid Chromatography with Tandem Mass Spectrometry' were attempted with each trial consisting of the following treatments:

1 reagent blank (containing no matrix or analyte)

2 unfortified control samples for each of the water types

5 control samples of each water type were fortified with a mixed solution of the test substances at 0.05 µg/L (the LOQ of the method).

5 control samples of each water type were fortified with a mixed solution of the test substances at 0.5 µg/L (10X the LOQ of the method).

Sample Extraction, Purification and Analysis

Untreated and fortified water samples were assayed according to the analytical method described in DAS Study titled 'Method Validation Study for the Determination of Residues of Methoxyfenozide

and its A-ring Phenol Metabolite and B-ring Mono Acid Metabolite in Surface Water, Ground Water and Drinking Water by Liquid Chromatography with Tandem Mass Spectrometry' with minor variations due to different laboratory equipment and practices.

In brief, residues of methoxyfenozide and its major metabolites were extracted from water by vortex mixing with 1.0 N hydrochloric acid solution. The resultant extract was purified using SPE and the eluate was evaporated to dryness, re-dissolved in acetonitrile/water (30/70, v/v) solution containing 0.1% formic acid and mixed thoroughly by vortex mixing prior to the analysis by LC-MS/MS.

Analytical Instrumentation and Equipment

The instrument conditions used during the ILV trial were as described in DAS Study 'Method Validation Study for the Determination of Residues of Methoxyfenozide and its A-ring Phenol Metabolite and B-ring Mono Acid Metabolite in Surface Water, Ground Water and Drinking Water by Liquid Chromatography with Tandem Mass Spectrometry', 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, 2.7 µm
Column Temperature:	40 °C
Injection Volume:	15 µL
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 –0.1% formic acid in water B – acetonitrile

Gradient:

Time (min)	Flow Rate (mL/min)	Solvent A (percent)	Solvent B (percent)
00:01	0.40	70	30
01:00	0.40	70	30
08:00	0.40	10	90
09:00	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 1.4.1 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
IonSpray Voltage (IS):	5500 volts
Acquisition Time Delay:	0.00 minutes
Period Duration:	12.0 minutes
Dwell Time:	100 ms

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	149.2	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	343.2	75	8	11	10
confirmation	399.3	149.0	75	8	23	10

Adaptations to the Analytical Method

Adaptations to the analytical method described in DAS Study 'Method Validation Study for the Determination of Residues of Methoxyfenozide and its A-ring Phenol Metabolite and B-ring Mono Acid Metabolite in Surface Water, Ground Water and Drinking Water by Liquid Chromatography with Tandem Mass Spectrometry' are listed below:

a) Calibration standards were made up with 70:30 water:acetonitrile, instead of 70:30 water:acetonitrile containing 0.1% formic acid.

b) During extraction, a driblock (40 °C) was used to evaporate samples to dryness instead of Turbo Vap evaporator.

c) Different column and LC-MS/MS conditions were used

Column:	Ascentis Express C18 50 x 21 mm i.d 2.7µm 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)
00:00:01	0.40	70	30
00:01:00	0.40	70	30
00:08:00	0.40	10	90
00:09:00	0.40	70	30
00:12:00	0.40	70	30

d) Bracketing standards technique was used for the calculation of residues rather than interspersed standards

e) The HPLC column temperature was set at 40 °C rather than ambient.

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 (% RSD), was calculated by dividing the standard deviation by the mean, and then multiplying by 100.

Used in Study N° S11-04020

10/1/12

Methoxyfenozide Water Method Development
Date: Monday, May 23, 2011
Time: 2:29:54 PM
Darcy D. Shackelford

9. DETERMINATION OF RECOVERY OF METHOXYFENOZIDE, THE A-RING PHENOL METABOLITE AND THE B-RING MONO ACID METABOLITE OF METHOXYFENOZIDE IN DRINKING WATER, SURFACE WATER AND GROUND WATER

9.2. Sample Preparation

No sample preparation was required for the water samples prior to analysis. Samples were stored refrigerated at approximately 4 °C after their time of sampling and during the course of the method validation study, except when they were removed for taking aliquots for sample analysis.

9.3. Sample Analysis

9.3.1. Using a pipet, measure 10-mL portions of water (drinking water, surface water or ground water—HPLC grade water is used for the reagent blank sample) into individual 11-dram (45-mL) glass vials equipped with PTFE-lined caps (or 50-mL polypropylene centrifuge tubes equipped with caps). (Acceptable stopping point if sample is kept refrigerated.)

9.3.2. For recovery samples, add appropriate aliquots of the mixed spiking solutions to obtain concentrations ranging from 0.05-1.0 µg/L for methoxyfenozide, the A-ring phenyl metabolite and the B-ring mono acid metabolite. Refer to table below for example fortification levels to obtain this concentration range.

Sample Description	Spiking Volume µL	Spiking Solution µg/mL	Fortification Level µg/L[a]
CONTROL	---	---	---
LOD	15	0.010	0.015
LOQ	50	0.010	0.05
MID (2 x LOQ)	100	0.010	0.10
HIGH (20 x LOQ)	100	0.100	1.00

[a] Based on fortifying a 10.0-mL initial water sample.

9.3.3. Add 1.0 mL of a 1.0N hydrochloric acid solution to the sample vial.

9.3.4. Cap the sample vial with a PTFE-lined cap, pulse vortex mix for about 10 seconds.

9.3.5. Purify samples using the following SPE procedure:

a. Place a Phenomenex Strata-X 33 µm polymeric sorbent reversed phase SPE cartridge (60-mg, 3-mL, catalog number 8B-S100-UBJ) on a vacuum manifold box.

b. Condition the SPE cartridge with 3 mL of methanol followed by 3 mL of water, discarding the eluates. Apply full vacuum (approximately -380 mm Hg) for about 10 seconds between solvent additions.

c. Transfer the entire sample solution from Step 9.3.4 to the SPE cartridge. Pull the sample through the SPE cartridge at approximately 1 mL/min, discarding the eluate. Dry the plate under full vacuum for 10 seconds after sample has eluted.

d. Wash the sample vial with a 1.0-mL aliquot of a 60% water/40% methanol/0.1% formic acid solution and transfer to the top of the SPE cartridge. Pull the solution through the SPE cartridge at approximately 1 mL/min, discarding the eluate. Dry the SPE cartridge under full vacuum for about 5 minutes.

e. Elute the methoxyfenozide and its two metabolites from the SPE cartridge at approximately 1 mL/min with two 1.0-mL aliquots of acetonitrile, collecting the eluate in a 12-mL (16 x 100 mm) culture tube.

9.3.6. Evaporate the acetonitrile to dryness at approximately 40 °C using nitrogen (approximately 10 psi) on a TurboVap evaporator.

9.3.7. Reconstitute the samples using a positive displacement pipet to add 1.0 mL of a 70% water/30% acetonitrile solution containing 0.1% formic acid to the tube. Pulse vortex for about 10 seconds to mix well.

9.3.8. Transfer a portion of each sample that is ready for analysis to a 96-deep well plate.

9.3.9. 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.)

9.3.10. Analyze the calibration standards and samples by positive-ion ESI LC/MS/MS, injecting the calibration standards interspersed with the samples throughout the run.

Method Departure No.005

9.3.11. Calculate the percent recovery found for each analyte.

9.3.12. 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:

a. 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.

Method Departure No.004

b. 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 0.05 µg/L of methoxyfenozide, the A-ring phenyl metabolite and the B-ring mono acid metabolite in the water sample).

9.3.13. 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. 001

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)
Column Temperature:	Ambient
Injection Volume:	30 µL
Injection Wash Program:	Autosampler loop and needle washed with: 1) 700 µL of a 50% MeOH/50% water 2) 2 x 700 µL of MeOH 3) 2 x 700 µL 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 µL 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 °C
Ion Source Gas 1 (GS1):	50
Ion Source Gas 2 (GS2):	50

Period 1	
Pre-acquisition Delay:	0.0 min
Acquisition Time	8.0 min
IonSpray Voltage (IS):	5500 volts
Entrance Potential (EP):	10 volts

Analytes:	Precursor Ion Q1	Product Ion Q3	Dwell Time (ms)	Collision Energy (CE)	Declustering Potential (DP)	Cell Exit Potential (CXP)
Methoxyfenozide (quantification)	369.1	313.2	150 ms	11 V	46 V	12 V
Methoxyfenozide (confirmation)	369.1	149.2	150 ms	23 V	46 V	18 V
A-ring phenyl (quantification)	355.0	299.2	150 ms	11 V	66 V	10 V
A-ring phenyl (confirmation)	355.0	135.2	150 ms	23 V	66 V	12 V
B-ring mono acid (quantification)	399.1	343.1	150 ms	11 V	76 V	12 V
B-ring mono acid (confirmation)	399.1	149.2	150 ms	21 V	76 V	12 V

Methoxyfenozide Water Method Development
Date: Monday, May 23, 2011
Time: 2:29:54 PM
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~~Method Departure No. 003~~

7. PREPARATION OF STANDARDS

7.1. Preparation of Methoxyfenozide, the A-ring Phenyl Metabolite and the B-ring Mono Acid Metabolite Stock Solutions

7.1.1. Weigh 0.1000 g of methoxyfenozide and quantitatively transfer to a 100-mL volumetric flask with acetonitrile. Dilute to volume with acetonitrile to obtain a stock solution containing 1000 µg/mL of methoxyfenozide.

7.1.2. Weigh 0.1000 g of the A-ring phenyl metabolite and quantitatively transfer to a 100-mL volumetric flask with acetonitrile. Dilute to volume with acetonitrile to obtain a stock solution containing 1000 µg/mL of the A-ring phenyl metabolite.

7.1.3. Weigh 0.1000 g of the B-ring mono acid metabolite and quantitatively transfer to a 100-mL volumetric flask with acetonitrile. Add approximately 50 mL of acetonitrile to the flask plus 5 mL of HPLC grade water. Mix well and equilibrate to room temperature. Dilute to volume with acetonitrile to obtain a stock solution containing 1000 µg/mL of the B-ring mono acid metabolite.

7.1.4. Accurately pipet 10.0 mL of each of the 1000-µg/mL stock 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 stock solution containing 100.0 µg/mL of each analyte in a 70% water/30% acetonitrile solution.

7.2. Preparation of Methoxyfenozide, the A-ring Phenyl Metabolite and the B-ring Mono Acid Metabolite Mixed Fortification Solutions

7.2.1. Pipet 10.0 mL of the 100-µg/mL mixed stock solution prepared in Step 7.1.4 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 10.0-µg/mL mixed fortification stock solution.

7.2.2. Pipet 10.0 mL of the 10-µg/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 1.0-µg/mL mixed fortification solution.

7.2.3. Pipet 10.0 mL of the 1.0-µg/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 0.10-µg/mL mixed fortification solution.

7.2.4. Pipet 10.0 mL of the 0.10-µg/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 0.01-µg/mL mixed fortification solution.

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 fortification solutions (Steps 7.2.2-7.2.4) using a 70% water/30% acetonitrile solution containing 0.1% formic acid according to the following table:

Method Departure No. 02

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/L
1.00	5.0	100	50.0	5.00
1.00	3.5	100	35.0	3.50
1.00	2.0	100	20.0	2.00
1.00	1.0	100	10.0	1.00
0.10	5.0	100	5.00	0.500
0.10	1.0	100	1.00	0.100
0.01	5.0	100	0.50	0.050
0.01	1.5	100	0.15	0.015

[a] The equivalent sample concentration of methoxyfenozide, the A-ring phenyl metabolite and the B-ring mono acid metabolite is based on taking a 10.0-mL initial aliquot of the water sample, concentrating and purifying it on an SPE cartridge, evaporating the eluate and reconstituting the sample to a final volume of 1.0 mL with using a 70% water/30% acetonitrile solution containing 0.1% formic acid.

Concentrations of methoxyfenozide, the A-ring phenol metabolite and the B-ring mono acid metabolite in the final extracts (resulting in µg/kg residue results) were determined using the bracketing standards method.

µg/mL in extract = analyte peak area/standard peak area x bracketing standard concentration

µg/L found = (µg/mL x FV)/FW

Where:

FV = final extract vol (mL)

VM = volume of matrix in final extract (g)

For example:

Peak area of the B-ring mono acid metabolite for the soil sample L11-04019-L1-005 (343 *m/z*) =5085

Average peak area of 0.001µg/kg standard = 12033.5

FV = 1mL

WM = 0.01L

Fortification level = 0.05mg/kg

µg/mL in extract = 5085/12033.5 x 0.001 = 0.0004226

mg/kg found = (0.0004226µg/mL x 1mL)/0.01L = 0.0423

% recovery = 0.0423mg/kg / 0.05 mg/kg x 100 = 85