Propyzamide – Independent Laboratory Validation of an Analytical Method for the Determination of Residues of Propyzamide and its Relevant Metabolites in Soil by Liquid Chromatography with Tandem Mass Spectrometry

ABSTRACT

This study was conducted to provide independent laboratory validation data for the determination of residues of propyzamide and its relevant metabolites in soil, following the analytical method, Dow AgroSciences LLC, Study Number 110586, "Validation of an Analytical Method for the Determination of Residues of Propyzamide and its Relevant Metabolites in Soil by Liquid Chromatography with Tandem Mass Spectrometry Detection" (reference 1). The validated limit of quantification of the method was 0.005 mg/kg. The final extracts were analyzed for residues of propyzamide and its relevant metabolites by liquid chromatography with triple quadrupole mass spectrometric detection (MS/MS).

The analytical procedure was demonstrated to be applicable for use in the quantative determination of residues of propyzamide and its relevant metabolites in loamy sand soil over a concentration range of 0.005 to 0.05 mg/kg.

INTRODUCTION

An analytical method was developed and validated for the determination of propyzamide and its relevant metabolites in soil. The method is identified as Dow AgroSciences Study Number 110586, "Method Validation Study for the Determination of Residues of Propyzamide and its Relevant Metabolites in Soil by Liquid Chromatography with Tandem Mass Spectrometry" (reference 1). This method is referenced as AGR/MOA/PROPIZ-2 at Eurofins Agroscience Services Chem SAS for the independent validation study.

The method was found to be suitable for the determination of residues of propyzamide and its relevant metabolites in soil over the concentration range of 0.005 to 0.05 mg/kg (LOQ to 10 LOQ) equivalent to 0.03 to 0.5 ng/mL in the final extract as prepared for analysis. The validated limit of quantification of the method was confirmed to be 0.005 mg/kg.

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.

An independent laboratory validation of the analytical method was conducted on loamy sand soil to satisfy the requirements of the European Commission Guidance Document on Residue Analytical Methods, SANCO/825/00 rev. 8.1 (Reference 2). The study was also conducted to satisfy the requirements of U.S. EPA Guideline OCSPP 850.6100 (Reference 3), PR Notice 96-1 (Reference 4). The report was formatted following the US EPA PR Notice 2011-3 formatting guidelines (Reference 5).

ANALYTICAL

Preparation and Storage of Samples

The independent laboratory validation was carried out on Loamy sand soil type (Appendix A). The soil was supplied as a standard soil 2.2 (batch Sp2.2 2811) according to GLP, by Lufa Speyer, Obere Laggasse 40, D-67346 Speyer.

Specimen	EAS Chem SAS Sample Reference Number
Loamy sand soil	233

Upon receipt, the specimens were stored at a temperature set at -20 °C.

Characterisation of Samples

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

Specimen	Loamy sand 2.2
Sampling date	15.07.11
Org. C in %	1.87 ± 0.20
Nitrogen in %N	0.17 ± 0.02
Particles <0.02 mm in %	13.9 ± 2.2
pH-Value (0.01 M CaCl2)	5.5 ± 0.2
Cation Exchange Capacity	9.9 ± 0.7
Particle Size According to USDA (%)	
< 0.002 mm	6.8 ± 1.3
0.002 – 0.05 mm	12.6 ± 1.7
0.05 – 2.0 mm	80.6 ± 2.6
Soil Type	Loamy sand
Water Holding Capacity (g/100g)	44.4 ± 6.0
Weight per Volume (g/1000 mL)	1257 ± 43

Preparation of Solutions and Standards

Reference items 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:	Propyzamide (RH-23315)
Supplier:	Sponsor
Reference Number:	TSN301352
Batch/Lot no:	YC2-106153-79
Purity:	98.8%
Expiry date:	22 Sep 2015
Storage:	Temperature set at 20°C (at EAS Chem SAS)

Test Substance/ Analytical Standard:	RH-24644
Supplier:	Sponsor
Reference Number:	TSN029409-0001
Batch/Lot no:	V43-037424-64
Purity:	99%
Expiry date:	28 Sep 2014
Storage:	Temperature set at 4°C (at EAS Chem SAS)

Test Substance/ Analytical Standard:	RH-24580
Supplier:	Sponsor
Reference Number:	TSN103029
Batch/Lot no:	WDW60:19A
Purity:	99.3%
Expiry date:	01 Sep 2014
Storage:	Temperature set at 20°C (at EAS Chem SAS)

The certificate of analysis was provided by Dow AgroSciences LLC (Appendix B).

Analytical standard stock solutions, calibration standard solutions and fortification solutions were prepared as described in the analytical method. Details of these materials are presented in Appendix C of the report along with details of the preparation of all analytical and

fortification standards prepared from the primary reference items. The test/reference item and specimens will be retained until expiry and then disposed of with the approval of the Study Monitor.

Fortification of Recovery Samples

The control specimens were fortified as described below with propyzamide and its relevant metabolites:

Matrix	Untreated Control Specimen Reps	Replicates at Fortification Level (LOD)*	Replicates at Fortification Level (LOQ)**	Replicates at Fortification Level (10 LOQ)
Loamy sand soil	2	1 at 0.0015 mg/kg	5 at 0.005 mg/kg	5 at 0.05 mg/kg

*LOD – Limit of Determination
**LOQ – Limit of Quantification

One sample was fortified to achieve a fortification level of 0.0015 mg/kg (LOD), five samples were fortified to achieve the fortification level of 0.005 mg/kg (LOQ) and five samples were fortified to achieve the upper fortification level of 0.05 mg/kg (10 LOQ) for loamy sand soil. The fortification solution was fortified directly onto the matrix.

Sample Extraction, Purification and Analysis

Specimens were assayed according to the Dow AgroSciences analytical method, Study Number 110586, "Method Validation Study for the Determination of Residues of Propyzamide and its Relevant Metabolites in Soil by Liquid Chromatography with Tandem Mass Spectrometry" (reference 1). The method was internally referenced at Eurofins Agroscience Services Chem SAS under the number AGR/MOA/PROPIZ-2.

The soil matrix samples were extracted several times using an acetonitrile/ultra-pure water (80/20, v/v) extraction solution. An aliquot of final extract was diluted 5x with ultra-pure water/acetonitrile (80/20, v/v) and transferred into an auto sampler vial in order to be injected into LC-MS/MS tandem mass spectrometry. The resulting samples were analysed for the quantitative determination of propyzamide, RH-24580, and RH-24644 by liquid chromatography with positive-ion electrospray ionization (ESI) tandem mass spectrometry (LC-MS/MS).

Full extraction details:

1. Five-g (± 0.05 g) portions of soil sample was measured into separate 50-mL centrifuge tubes.

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

Concentration of Fortified Sample (mg/kg)	Volume of Spiking Solution (µL)	Concentration of Spiking Solution (µg/mL)
0.0015	75	0.1 µg/mL
0.005	250	0.1 µg/mL
0.05	250	1.0 µg/mL

3. Twenty five-mL of acetonitrile/ultra-pure water (80/20, v/v) extraction solution was added to the sample tube.

4. The samples were vortex mixed for 10 seconds.

5. The sample was shaken for 30 minutes on a reciprocating shaker at approximately 180 excursions/minute.

6. The sample tube was centrifuged for 5 minutes at 2000 rpm.

7. The extract was transferred into a new 50 mL centrifuge tube.

8. A fresh 20 mL portion of acetonitrile/ultra-pure water (80/20, v/v) extraction solution was added to the remaining soil sample from Step 7, and the sample was vortex mixed for 10 seconds.

9. The sample was again shaken for 30 minutes on a reciprocating shaker at approximately 180 excursions/minute.

10. The sample tube was centrifuged for 5 minutes at 2000 rpm.

11. The extract from Step 10 was combined in the same 50 mL centrifuge tube as the extract from Step 7, and the volume was adjusted to a final volume of 50 mL using additional acetonitrile/ultra-pure water (80/20, v/v) extraction solution. The combined sample is mixed well.

12. The final extract was centrifuged for 5 minutes at 4000 rpm.

13. Two hundred µL of the final extract was transferred into an auto sampler vial and 800 µL of the acetonitrile/ultra-pure water (20/80, v/v) extraction solution was added, diluting the sample 5 fold. The samples were vortex mixed for 10 seconds.

14. The samples were ready for final determination by LC-MS/MS tandem mass spectrometry.

Analytical Instrumentation and Equipment

- HPLC pump (Shimadzu LC20AD-XR)
- HPLC injector (Shimadzu SIL20AC-XR)
- HPLC oven (Shimadzu CTO-20AC)
- LC-MS/MS API 5500 (Sciex)
- HPLC column Zorbax SB-C8, 4.6 mm x 75 mm, 3.5 µm (Agilent, ref. 866953-906)
- Flatbed shaker (Fisher Bioblock, 74404)
- Laboratory centrifuge (Thermo Scientific)
- Polypropylene centrifugation tubes (BD Falcon, ref. 352070)
- Precision balance (Mettler XS203S)
- Centrifuge (Thermo Scientific)
- Standard laboratory glassware (volumetric flasks, measuring cylinders)
- Ultrasonic bath (Bioblock)
- Various pipettes (Thermo Scientific)
- Vortex (VWR Vortex Genie-2)

Typical HPLC Operating Conditions

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

Pump + Autosampler:	LC20AD-XR, Shimadzu + SIL20AC-XR, Shimadzu or HTC
Oven:	CTO-20AC, Shimadzu

Column HPLC:	Agilent Zorbax SB-C8 4.6 x 75 mm, 3.5 µm
Column temperature:	Room temperature (about 22 °C)
Retention time:	approximately 3.9 minutes for propyzamide approximately 3.7 minutes for RH-24580 approximately 4.7 minutes for RH-24644
Injection volume::	25 µL
Flow:	0.8 mL/minute (splitted to about 0.3 mL/min)
Mobile phase:	Solvent A: Ultra-pure water + 0.1 % formic acid Solvent B: Acetonitrile + 0.1 % formic acid

Gradient:

Time (minute)	% A	% B
0:01	75	25
3:00	10	90
5:00	10	90
5:15	75	25
8:00	75	25

Flow Diverter Program:

Time (minute)	Position
0.0 – 1.0	to waste
1.0 – 6.0	to mass spectrometer
6.0 – 8.0	to waste

Typical Mass Spectrometry Operating Conditions

Instrumentation:	MDS SCIEX API 5500 LC-MS/MS Analyst 1.5.1 data system
Polarity:	Positive
Interface:	Electrospray
Scan Type:	MRM
Resolution:	Q1 – unit, Q3 – unit
Curtain Gas (CUR):	15 psi
Collision Gas:	4 Medium (API 5500 QTrap)
Temperature (TEM):	550°C

Ion Source Gas 1: 50 psi
Ion Source Gas 2: 50 psi
Dwell Time: 100 ms
Ion Spray Voltage: 2500

Analyte	Parent ion (*m/z*)	Daughter ion (*m/z*)	DP (V)	EP (V)	CXP (V)	CE (eV)
Propyzamide	256.0	190.0 (quantification)	51	10	8	20
	258.0	192.0 (confirmatory)	46	10	8	21
RH-24580	274.1	145.1 (quantification)	80	10	8	51
	274.1	109.0 (confirmatory)	58	10	8	75
RH-24644	256.0	145.0 (quantification)	56	10	8	53
	256.0	109.0 (confirmatory)	56	10	8	67

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

Calculation of Results

For each analytical batch, a range of 7 calibration standards at increasing concentrations was injected over the range 0.03 to 20 ng/mL, equivalent to samples over the concentration range of 0.0015 to 1.00 mg/kg (30% LOQ to 20000 LOQ). A calibration curve was prepared for propyzamide and its relevant metabolites by plotting the quantification peak area obtained versus the analyte concentration (in ng/mL).

Example: propyzamide recovery at 0.005 mg/kg in loamy sand soil. This calculation can be applied for quantification and confirmatory modes for any analytes.
A linear calibration curve was calculated using the method of least squares (with $1/x^2$ weighting):

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

Y = detector response for propyzamide = 11337.6

A = slope of the linear least squares fit of the calibration curve = 112298.5

C_a = Analyte concentration determined from the standard curve

B = Y-intercept of the linear least squares fit of the calibration curve = -15.9

The concentration determined from standard curve is $C_a = \frac{(Y-B)}{A} = 0.100818$ ng/mL

The residue of propyzamide in each test specimen is calculated as follows:

$$\text{Residue (mg/kg)} = \frac{V_f \times V_1}{M \times V_2 \times f} \times n = \frac{1 \times 50}{5 \times 0.2 \times 1000} \times 1$$

Where:

V_1 (mL)	=	total extraction volume (50 mL)
V_2 (mL)	=	aliquot volume (0.2 mL)
V_f (mL)	=	final volume (1 mL)
M (g)	=	Sample weight (5 g)
f	=	Conversion factor from µg/mL to ng/mL (1000)
n	=	final dilution

Extract concentration = 0.100818 ng/mL

Residue = 0.0050409 mg/kg

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

$$\text{Recovery } (\%) = \frac{A}{S} \times 100 = \frac{0.0050409}{0.005} \times 100 = 101\%$$

Where:

A = concentration of propyzamide found in spiked sample = 0.0050409 mg/kg.

S = concentration of propyzamide added in spiked sample = 0.005 mg/kg.

Recovery = 101% (calculation performed on unrounded values)
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

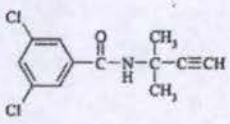
The LC-MS/MS method is highly selective for the determination of residues of propyzamide and its relevant metabolites in soil by virtue of the chromatographic separation and selective detection system used. To demonstrate further confirmation, a second MS/MS ion transition was monitored for propyzamide and its relevant metabolites. Calculations of %Recovery and %RSD were carried out on the confirmatory transition data in addition to the quantitative transition (Table 7).

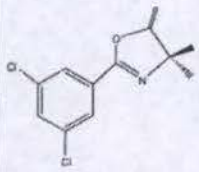
1 Materials and Method

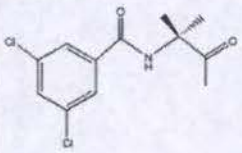
Reference Items

An aliquot of the certified reference item was supplied by the Sponsor.

All specifications of purity and composition of the reference item are provided by the Sponsor.

Common name:	**Propyzamide (RH-23315)**
Chemical name (IUPAC):	3,5-Dichloro-N-(1,1-dimethylprop2ynyl) benzamide
DAS reference number:	TSN301352
CAS-Registry-No.:	[*23950-58-5*]
Structure	
Molecular mass:	256.13 g/mol
Lot Number	YC2-106153-79
Purity	98.8%
Expiry Date	22 Sep 2015
Storage Conditions at Test Facility	20°C

Common name:	**RH-24644**
Chemical name (IUPAC):	2-(3,5 dichlorophenyl)-4,4-dimethyl-5-methylene-2-oxazoline
DAS reference number:	TSN029409-0001
CAS-Registry-No.:	[*29918-40-9*]
Structure	
Molecular mass:	256.13 g/mol
Lot Number	V43-037424-64
Purity	99%
Expiry Date	28 Sep 2014
Storage Conditions at Test Facility	4°C

Common name:	**RH-24580**
Chemical name (IUPAC):	3,5 dichloro N (1,1 dimethyl 2 oxopropyl) benzamide
DAS reference number:	TSN103029
CAS-Registry-No.:	[*29918-41-0*]
Structure	
Molecular mass:	274.15 g/mol
Lot Number	WDW60:19A
Purity	99.3%
Expiry Date	01 Sep 2014
Storage Conditions at Test Facility	20°C

All specifications of purity and composition of the test item are provided by the Sponsor.

Depending on the availability of the present test item, other batches could be used during this study. Their characteristics should be equivalent and documented in the final study report.

Test System(s), Origin, Preparation and Storage

Matrix (Commodity)
Loamy sand Soil (USDA classification)

Untreated specimens will be purchased at Lufa Speyer. Characterisation details of the soil will be presented in the study report (GLP)

APPENDIX C: ANALYTICAL METHOD

Preparation and use of the standard solutions

Stock solutions of propyzamide, RH-24644 and RH-24580
Between 2 to 50 mg was accurately weighed into a brown glass flask.
Adequate volume of acetonitrile was added using a burette in order to obtain a stock solution of 1000 µg/mL as mentioned below. This solution was sonicated until total dissolution.
The standard solutions were stored at a temperature set at -20°C Stability within these conditions were assessed for 135 days.

	Propyzamid	RH-24644	RH-24580
Solution a	3.69 mg	2.39 mg	3.41 mg
	3.65 mL	2.37 mL	3.39 mL
Solution b	3.65 mg	2.54 mg	2.39 mg
	3.61 mL	2.51 mL	2.37 mL

Fortification solutions
For fortifications, appropriate dilutions of the stock solutions were performed in acetonitrile to obtain solutions at 0.1, 1.0, 10.0 and 100 µg/mL.
The standard solutions were freshly prepared.

Calibration solutions
Appropriate dilutions of the stock solutions for propyzamide and its relevant metabolites were performed in acetonitrile to obtain solution at 0.1 µg/mL.
For calibration, appropriate dilutions of the solution at 0.1µg/mL were performed in acetonitrile/ultra-pure water (20/80, v/v) to obtain solutions at:

0.03 – 0.1 – 0.5 – 1.0 – 5.0 – 10.0 and 20 ng/mL

The standard solutions were freshly prepared.

Reagents and chemical compounds used

All solvents were HPLC-grade.

- Acetonitrile
- Acetone
- Formic acid 98%
- Hydrochloric acid 37%
- Ultra-pure water

Preparation of the reagents

- Acetonitrile/ultra-pure water (80/20, v/v), RPA 043
  Acetonitrile (800 mL) with ultra-pure water (200 mL) were mixed in a 1L flask using volumetric cylinders.
  Stopper flask securely and mix thoroughly by shaking.

- Acetonitrile/ultra-pure water (20/80, v/v), RPA 013
  Acetonitrile (200 mL) was mixed in ultra-pure water (800 mL) in a 1L flask using volumetric cylinders. Stopper flask securely and mix thoroughly by shaking.