Method Validation Study for the Determination of Residues of Propyzamide and its Relevant Metabolites in Soil by Liquid Chromatography with Tandem Mass Spectrometry

INTRODUCTION

Scope

This method is applicable for the quantitative determination of residues of 3,5-dichloro-N-(1,1-dimethylpropynyl)benzamide (RH-23315, propyzamide) and its relevant metabolites, 2-(3,5-dichlorophenyl)-4,4-dimethyl-5-methylene-2-oxazoline (RH-24644) and 3,5-dichloro-N-(1,1-dimethyl-2-oxo-n-propyl)benzamide (RH-24580), in soil. The method was validated over the concentration range of 0.005-0.5 µg/g with a validated limit of quantitation of 0.005 µg/g. The common and chemical names, molecular formulas, and nominal masses for the analytes are given in Table 1.

This study was conducted to fulfill data requirements outlined in the U. S. EPA Residue Chemistry Test Guidelines, OPPTS 860.1340 (*1*); the European Commission Guidance Document, SANCO/3029/99 rev.4 (*2*), SANCO/825/00 rev.8.1 (*3*); and PMRA Residue Chemistry Guidelines as Regulatory Directive Dir98-02 (*4*).

Method Principle

Residues of RH-23315 (propyzamide), RH-24580, and RH-24644 are extracted from soil matrices using an acetonitrile/water (80/20, v/v) extraction solution. A 5.0 g soil sample is weighed into a 50 mL centrifuge tube and 25.0 mL of extraction solution is added to the sample and vortexed for 10 seconds. The sample is shaken for 30 minutes on a reciprocating shaker at approximately 180 excursions/minute and then centrifuged for 5 minutes at 2000 rpm. The extract is transferred into a clean 50 mL centrifuge tube. A volume of 20.0 mL extraction solvent is added to the original 5.0 g soil sample and vortexed for 10 seconds. The sample is shaken for 30 minutes on a reciprocating shaker at approximately 180 excursions/minute and then centrifuged for 5 minutes at 2000 rpm. The extract is combined with the first extract solution and the volume is adjusted to 50 mL with additional acetonitrile/water (80/20, v/v). The combined extract solution is centrifuged for 5 minutes at 4000 rpm. The total extract solution is diluted 5x with acetonitrile/water (80/20, v/v) and transferred into an autosampler vial. The resulting sample along with the calibration standards containing RH-23315, RH-24580, and RH-24644 are analyzed for the quantitative determination of RH-23315, RH-24580, and RH-24644 by liquid chromatography with positive-ion electrospray ionization (ESI) tandem mass spectrometry (LC-MS/MS).

The method is validated by evaluating the recoveries of RH-23315, RH-24644, and RH-24580 fortified in four control soil types. Soil information for the four soil types, representing diverse environmental conditions and soil consistencies, can be found in Table 2.

Test Substances / Reference Compounds/ Analytical Standards

Test Substance	TSN	Percent Purity	Recertification Date	Reference
RH-23315	TSN105825	98.2%	23-July-2013	FAPC09-227036
RH-24644	TSN029409-0001	99.0%	28-Sep-2014	FAPC12-000440
RH-24580	TSN103029	99.3%	01-Sep-2014	FAPC10-267039

The Certificates of Analysis for the test substances can be found in Figures 25-27. The above standards may be obtained free of charge from Dow AgroSciences LLC, 9330 Zionsville Road, Building 304, Indianapolis, IN 46268-1054.

EXPERIMENTAL

Sample Origin, Numbering, Preparation, Storage, and Characterization

Untreated control samples of the soils were obtained from the Dow AgroSciences LLC Sample Management Group. All samples were tracked in the Dow AgroSciences LLC Regulatory Labs Information Management System (RLIMS) database. Unique sample numbers were assigned to the samples to track them during receipt, storage, and analysis. Complete source documentation was included in the study file.

During the course of the study, the samples were stored in temperature-monitored freezers at approximately -20 to -10 °C, except when removed for analysis. Information for the four soil types are listed in Table 2.

Calculation of Standard Calibration Curve

Calculation of a standard curve was achieved with the injection of a series of calibration standards described in Appendix I and acquisition of peak areas for the following analytes. (Typical calibration curves can be found in Figures 1-6). Refer to Tables 22-27 for calibration data and Tables 28-32 for analytical set parameters for all data sets performed during the validation.

RH-23315 (Propyzamide)	*m/z* Q1/Q3 256/190 (quantitative) *m/z* Q1/Q3 258/192 (confirmatory)
RH-24580	*m/z* Q1/Q3 274/145 (quantitative) *m/z* Q1/Q3 274/109 (confirmatory)
RH-24644	*m/z* Q1/Q3 256/145 (quantitative) *m/z* Q1/Q3 256/109 (confirmatory)

In order to generate a standard curve, plot the analyte concentration on the abscissa (x-axis) and the respective peak area on the ordinate (y-axis) in Analyst. Using regression analysis, determine the equation for the curve with respect to the abscissa.

$$Y = \text{slope} \times X + \text{intercept}$$

$$X = \left(\frac{Y - \text{intercept}}{\text{slope}}\right)$$

Where

X = analyte concentration
Y = analyte peak area

$$\text{Analyte concentration} = \left(\frac{\text{analyte peak area} - \text{intercept}}{\text{slope}}\right)$$

For example, using linear regression with $1/x^2$ weighting, for analyte RH-23315 (256/190) in sample set 110586_S01,

$$\text{RH-23315 (gross ng/mL)} = \left(\frac{\text{RH-23315 peak area} - \text{intercept}}{\text{slope}}\right)$$

$$\text{RH-23315 (gross ng/mL)} = \left(\frac{\text{RH-23315 peak area} - 1331.29}{191182}\right)$$

Calculation of Percent Recovery

Determine the gross concentration in each recovery sample by substituting the corresponding analyte peak area in the sample into the above equation and solve for the concentration.

Determination of gross concentration and calculation of percent recovery for each analyte is done similarly; therefore, only a single example calculation will be presented here.

For example, the gross concentration in ng/mL of analyte RH-23315 (256/190) is determined for the sample 110586-001-0001A3 + 0.005 µg/g of the analytical set 110586_S01 as follows. The chromatogram is shown in Figure 12.

$$\text{RH-23315 (gross ng/mL)} = \left(\frac{\text{RH-23315 peak area - intercept}}{\text{slope}}\right)$$

$$\text{RH-23315 (gross ng/mL)} = \left(\frac{17730 - 1331.29}{191182}\right)$$

$$\text{RH-23315 (gross ng/mL)} = 0.0858 \text{ ng/mL}$$

Convert the gross concentration (ng/mL) of RH-23315 in the processed sample to the gross concentration (µg/g) of RH-23315 in the original sample as follows:

$$\text{RH-23315 (gross µg/g)} = \text{RH-23315 (gross ng/mL)} \times (MF \times DF \times UC)$$

Where

MF = Method Factor
DF = Dilution Factor = 1
UC = Unit Conversion = 1.0 µg/1000 ng

$$MF = \frac{\text{Final Volume} \times \text{Extraction Solution Volume}}{\text{Aliquot Factor} \times \text{Nominal Weight}}$$

Where

Final Volume = 1.0 mL
Ext. Sol. Vol. = 50 mL
Aliquot Factor = 0.2 mL
Nominal Weight = 5.0 g

Then

$$MF = 50 \text{ mL/g}$$

$$\text{RH-23315 (gross } \mu\text{g/g)} = \text{RH-23315 (gross ng/mL)} \times 50 \text{ (mL/g)} \times 1.0 \times 0.001$$

$$\text{RH-23315 (gross } \mu\text{g/g)} = 0.0858 \text{ ng/mL} \times 0.050 \frac{\text{mL} \times \mu\text{g}}{\text{g} \times \text{ng}}$$

$$\text{RH-23315 (gross } \mu\text{g/g)} = 0.0043 \ \mu\text{g/g}$$

Determine the percent recovery by dividing the gross concentration (found concentration) of each recovery sample by the theoretical concentration added as follows:

$$\text{Recovery} = \frac{\text{Concentration Found}}{\text{Concentration Added}} \times 100\%$$

$$\text{Recovery} = \frac{0.0043 \ \mu\text{g/g}}{0.005 \ \mu\text{g/g}} \times 100\%$$

$$\text{Recovery} = 86 \ \%$$

Confirmation of Residue Identity

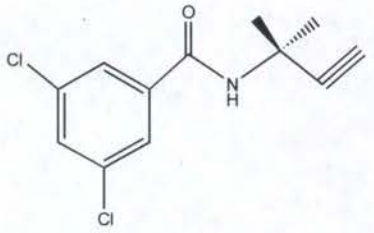
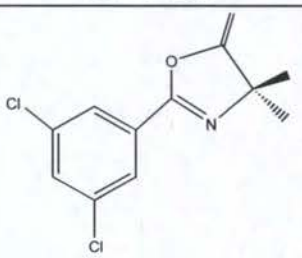
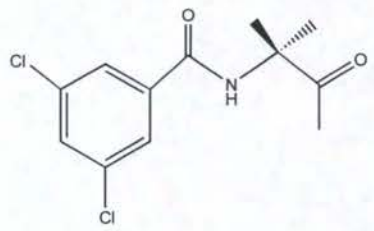
The method is specific for the determination of RH-23315, RH-24580, and RH-24644 by virtue of the chromatographic separation and selective detection system used. To demonstrate further confirmation, an additional MS/MS ion transition was monitored for each analyte.
A series of calibration standards were injected and peak areas were acquired for the following analytes.

RH-23315 (Propyzamide)	*m/z* Q1/Q3 256/190 (quantitative) *m/z* Q1/Q3 258/192 (confirmatory)
RH-24580	*m/z* Q1/Q3 274/145 (quantitative) *m/z* Q1/Q3 274/109 (confirmatory)
RH-24644	*m/z* Q1/Q3 256/145 (quantitative) *m/z* Q1/Q3 256/109 (confirmatory)

Statistical Treatment of Data

Statistical treatment of data included the calculation of regression equations, correlation coefficients (r) for describing the linearity of calibration curves, and means, standard deviations, and relative standard deviations of the results for the fortified recovery samples.

Table 1. Identities and Structures of RH-23315, RH-24644, and RH-24580

Identifying information	Chemical Structure
Common Name of Compound: Propyzamide (Pronamide, RH-23315) Chemical Formula: $C_{12}H_{11}Cl_2NO$ Molecular Weight: 256.13 Nominal Mass: 255 CAS Number: 23950-58-5	 3,5-dichloro-N-(1,1-dimethylpropynyl) benzamide
Common Name of Compound: RH-24644 Chemical Formula: $C_{12}H_{11}Cl_2NO$ Molecular Weight: 256.13 Nominal Mass: 255 CAS Number: 29918-40-9	 2-(3,5-dichlorophenyl)-4,4-dimethyl-5-methylene-2-oxazoline
Common Name of Compound: RH-24580 Chemical Formula: $C_{12}H_{13}Cl_2NO_2$ Molecular Weight: 274.15 Nominal Mass: 273 CAS Number: 29918-41-0	 3,5-dichloro-N-(1,1-dimethyl-2-oxo-n-propyl)benzamide

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Safety Precautions

Each analyst must be acquainted with the potential hazards of the equipment, reagents, products, solvents, and procedures used in this method before commencing laboratory work. Sources of information include: operation manuals, material safety data sheets, literature, and other related data. Safety information should be obtained from the supplier. Disposal of waste materials, reagents, reactants, and solvents must be in compliance applicable governmental requirements. Acetonitrile is flammable and should be used in well-ventilated areas away from ignition sources. Formic acid is corrosive and can cause severe burns. It is imperative that proper eye and personal protection equipment be worn when handling these reagents.

Laboratory Equipment

Balance, analytical, Model AE100, Mettler-Toledo, Inc.
Balance, pan, Model BB2440, Mettler-Toledo, Inc.
Centrifuge, with rotor to accommodate 8 oz wide-mouth bottles, Model Legend XFR, Thermo International Equipment Company
Pipet, positive-displacement, 20-50 µL capacity, Model M50, Gilson Inc.
Pipet, positive-displacement, 10-100 µL capacity, Model M100, Gilson Inc.
Pipet, positive-displacement, 10-250 µL capacity, Model M250, Gilson Inc.
Pipet, positive-displacement, 100-1000 µL capacity, Model M1000, Gilson Inc.
Repeater, positive-displacement, 1-25 mL capacity, catalog number 022260201, Eppendorf.
Shaker, variable speed reciprocating with box carrier, Model 6000, Eberbach Corporation.
Vortex mixer, Model G-560, Scientific Industries, Inc.

Chromatographic/ Mass Spectrometer System

Column, analytical, Zorbax SB-C8, 4.6 mm x 75 mm, 3.5 µm particle size, catalog number 866953-906, Agilent Technologies.
Liquid chromatograph, Model Agilent 1290/1260, Agilent Technologies.

Mass spectrometer, Model QTRAP 5500, AB SCIEX.
Mass spectrometer data system, Analyst v.1.5.1, AB SCIEX.

Glassware and Materials

Combitips 10 mL, catalog number 022266501, Eppendorf.
Combitips 25 mL, catalog number 022266551, Eppendorf.
Disposable transfer pipette, catalog number 13-711-7M, Fisher Scientific.
Kimax Serialized Class A Mixing Cylinders, 1000 mL, catalog number 08-564-5E, Fisher Scientific.
Pipet tip, positive-displacement, 25 µL capacity, catalog number CP25, Gilson Inc.
Pipet tip, positive-displacement, 100 µL capacity, catalog number CP100, Gilson Inc.
Pipet tip, positive-displacement, 250 µL capacity, catalog number CP250, Gilson Inc.
Pipet tip, positive-displacement, 1000 µL capacity, catalog number CP1000, Gilson Inc.
Vial, autosampler, 2 mL, catalog number C4000-1W, National Scientific Company.
Vial cap, for autosampler vial, catalog number C4000-55B, National Scientific Company.
Volumetric glass pipette, 10 mL, catalog number 13-660-5, Fisher Scientific.
Volumetric glass disposable pipette, 5 mL, catalog number 13-678-25D, Fisher Scientific.
Volumetric flask, 20 mL, catalog number 10-205B, Fisher Scientific.
Volumetric flask, 100 mL, catalog number10-205C, Fisher Scientific.

Reagents

Acetone, Chromasolv for HPLC ≥ 99.9 %, catalog number 4391344L, Sigma Aldrich.
Acetonitrile, Chromasolv HPLC grade, catalog number 439134-4L, Sigma Aldrich.
Formic acid, OPTIMA LC/MS grade, ≥99.5% purity, catalog number A117-50, Fisher Scientific.
Hydrochloric acid, 1 N, certified concentration, catalog number SA48-1, Fisher Scientific.
Water, Chromasolv HPLC grade, catalog number 270733-4L, Sigma Aldrich.

Prepared Solutions

Acetonitrile/water (80/20, v/v) - Extraction Solution

Measure 800 mL of water using a graduated cylinder and transfer into a 4.0 liter glass bottle. Measure 3200 mL of acetonitrile using a graduated cylinder and transfer solvent into the 4.0 liter glass bottle. Mix the contents thoroughly.

HPLC Mobile Phase A - Water with 0.1% Formic Acid

Measure 2.0 liters of water using a graduated cylinder and transfer contents into a 2.0 liter mobile phase bottle. Remove 2.0 mL of water from the mobile phase bottle with a pipet. Transfer 2.0 mL of formic acid into the 2.0 liter mobile phase bottle and mix thoroughly.

HPLC Mobile Phase B - Acetonitrile with 0.1% Formic Acid

Measure 2.0 liters of acetonitrile using a graduated cylinder and transfer contents into a 2.0 liter mobile phase bottle. Remove 2.0 mL of acetonitrile from the mobile phase bottle with a pipet. Transfer 2.0 mL of formic acid into the 2.0 liter mobile phase bottle and mix thoroughly.

Acetonitrile/water (80/20, v/v) – Sample Dilution Solution

Measure 800 mL of water using a graduated cylinder and transfer into a 4.0 liter glass bottle. Measure 3200 mL of acetonitrile using a graduated cylinder and transfer solvent into the 4.0 liter glass bottle. Mix the contents thoroughly.

Preparation of Spiking Solutions

1. Weigh 0.0200 g of RH-23315 analytical standard and quantitatively transfer to a 20 mL volumetric flask with acetonitrile. Dilute to volume to obtain a 1000 µg /mL RH-23315 stock solution. Repeat this step to produce 1000 µg /mL RH-24644 and RH-24580 metabolite stock solutions.

2. Pipet 2 mL of each of the 1000 µg/mL solutions into a 20 mL volumetric flask and dilute to volume with acetonitrile to produce a 100 µg/mL mixed standard solution.

3. Pipet 10 mL of the 100 µg/mL solution into a 100 mL volumetric flask and dilute to volume with acetonitrile to produce a 10 µg/mL mixed standard solution.

4. Pipet 10 mL of the 10 µg/mL solution into a 100 mL volumetric flask and dilute to volume with acetonitrile to produce a 1 µg/mL mixed standard solution.

5. Pipet 10 mL of the 1 µg/mL solution into a 100 mL volumetric flask and dilute to volume with acetonitrile to produce to produce a 0.1 µg/mL mixed standard solution.

Store the stock solutions prepared in acetonitrile for steps 1-5 above at -20 °C when not in use.

Preparation of Calibration Standards

Prepare calibration standard solutions by series dilution of the 0.1 µg/mL standard solution with acetonitrile/water (20/80, v/v) to obtain calibration standards over the concentration range 0.03-20.0 ng/mL; note that calibration standard solutions should be made fresh from stock solutions on the day of analysis. Prepare calibration standards as described in the following table:

Original Spiking Solution Conc.	Volume of Spiking Solution	Volume of Final Calibration Solution	Final Calibration Solution Conc.	Equivalent Sample Conc. [a]
ng/mL	µL	µL	ng/mL	µg/g
100	400	1600	20	1.0
20	1000	1000	10	0.5
10	1000	1000	5	0.25
5	400	1600	1	0.05
1	1000	1000	0.5	0.025
0.5	400	1600	0.1	0.005
0.1	300	700	0.03	0.0015

[a] Equivalent sample concentration is based on fortifying a 5 g sample.

Analysis Procedure

1. Weigh 5.0 ± 0.05 g of the sample into a 50 mL centrifuge tube. To prepare fortified samples containing RH-23315, RH-24644, and RH-24580, add the appropriate aliquots of the spiking solutions (mixed standard solutions) as described in the following table:

Description	Spiking Volumes	Spiking Solutions	Fortification Level
	µL	µg/mL	µg/g
Control	N/A	N/A	N/A
LOD	75	0.1	0.0015
LOQ	250	0.1	0.005
100x LOQ	250	10	0.5

2. Add 25 mL of the acetonitrile/water (80/20, v/v) extraction solution to the sample tube. Vortex for 10 seconds.

3. Shake the sample for 30 minutes on a reciprocating shaker at approximately 180 excursions/minute.

4. Centrifuge the sample tube for 5 minutes at 2000 rpm.

5. Transfer the extraction solution into a clean 50 mL centrifuge tube.

6. Add 20 mL of acetonitrile/water (80/20, v/v) extraction solution to the soil sample from Step 5 and vortex for 10 seconds.

7. Shake the sample for 30 minutes on a reciprocating shaker at approximately 180 excursions/minute.

8. Centrifuge the sample tube for 5 minutes at 2000 rpm.

9. Add the extraction solution from step 8 to the extraction solution from step 5, and adjust the volume in the centrifuge tube to 50 mL with additional acetonitrile/water (80/20, v/v).

10. Centrifuge total extract for 5 minutes at 4000 rpm.

11. Perform 5x dilution on extract with acetonitrile/water (20/80, v/v). Transfer the diluted sample aliquot into autosampler vials. Seal vials with pre-slit caps.

12. Analyze the calibration standards and samples interspersed throughout the run by liquid chromatography with positive-ion electrospray ionization (ESI) tandem mass spectrometry (LC-MS/MS). Determine the suitability of the chromatographic system using the following performance criteria:

a. Standard curve linearity: determine that the correlation coefficient (r) equals or exceeds 0.994 for the least squares equation which describes the detector response as a function of standard curve concentration.
b. Peak resolution: visually determine that sufficient resolution has been achieved for the analyte relative to background interferences.
c. Appearance of chromatograms: visually determine the chromatograms 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 each analyte in the 0.1 ng/mL calibration standard.

13. For the field sample analysis, if the found concentration of any analyte exceeds 80% of the highest calibration standard concentration, re-analyze the sample using an appropriate dilution factor. Fortified samples with a concentration that encompasses the sample concentration should also be included in the analytical set with the appropriate dilution factor. After dilution, the analyte concentration in the processed sample should be within 80% of the highest calibration standard concentration.

Supplemental Notes

1. Equipment, glassware, materials, reagents, and chemicals considered to be equivalent to those specified may be substituted with the understanding that their performance must be confirmed by appropriate tests. Common laboratory glassware and supplies are assumed to be readily available. Unless specified otherwise, class A volumetric glassware or equivalent is used to prepare analytical standards, fortification solutions, and calibration standards.

2. The instrumental conditions may be modified to obtain optimal chromatographic separation and sensitivity.

3. Based on availability of material, weighing of the analytical standard can be modified and the subsequent solution preparation scheme adjusted.

4. Intermediate fortification levels of the residues can be prepared for analysis as needed.

5. The prepared solutions preparation scheme and volumes can be adjusted as needed.

Typical HPLC Operating Conditions

Instrumentation:	Agilent 1290/1260
Column :	Agilent Zorbax SB-C8 4.6 x 75 mm, 3.5 µm
Column Temperature:	Ambient (approximately 22 °C)
Injection Volume:	10 µL
Injection Wash:	Acetone, 10 sec
Run Time:	Approximately 8 min
Mobile Phase:	A – Water containing 0.1% formic acid B – Acetonitrile containing 0.1% formic acid
Mobile Phase Split:	approximately 300 µL/min split to source

Gradient:

Time (Min)	Flow Rate (mL/min)	Solvent A (%)	Solvent B (%)
0:01	0.8	75	25
3:00	0.8	10	90
5:00	0.8	10	90
5:15	0.8	75	25
8:00	0.8	75	25

Flow Diverter

Flow to Waste	0.0 min → 1.0 min
Flow to Source	1.0 min → 6.0 min
Flow to Waste	6.0 min → end of run

TYPICAL MASS SPECTROMETRY OPERATING CONDITIONS

Instrumentation:	AB SCIEX QTRAP 5500 MS System AB SCIEX Analyst version1.5.1 data system
Ionization Mode:	Electrospray
Polarity:	Positive
Scan Type:	MRM
Resolution:	Q1 – unit, Q3 – unit
Curtain Gas (CUR)	15 psi
Collision Gas (CAD):	medium
Ion Source Gas 1 (GS1)	50 psi
Ion Source Gas 2 (GS2)	50 psi
Temperature (TEM):	550 °C
Entrance Potential (EP):	10 volts
IonSpray Voltage (IS):	2500 volts
Acquisition Time Delay:	1.00 minutes
Period Duration:	6.00 minutes

Compound:	Precursor Ion Q1(*m/z*)	Product Ion Q3(*m/z*)	Dwell Time (ms)	DP/CE (V)
RH-23315 quantitation	256.0	190.0	100	51/20
RH-23315 confirmation	258.0	192.0	100	46/21
RH-24580 quantitation	274.1	145.1	100	80/51
RH-24580 confirmation	274.1	109.0	100	58/75
RH-24644 quantitation	256.0	145.0	100	56/53
RH-24644 confirmation	256.0	109.0	100	56/67