1. INTRODUCTION

The objective of this study was to independently validate the ISK Biosciences Corporation residue method H-855 for the determination of SL-573 and one metabolite, MT-2153, in soil. The independent laboratory validation (ILV) was conducted using untreated control samples of soil, which were considered representative for the intent of the method.

The method was found to be suitable for the determination of SL-573 and MT-2153 in soil over the concentration range 0.0250 ng/mL to 10.0 ng/mL with a validated limit of quantitation (LOQ) of 0.00100 µg/g (0.100 ng/mL).

This ILV 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. 8.1 (2, 3, 4). The study was also conducted to satisfy the requirements of U.S. EPA Guideline OPPTS 850.7100 (5) and PR Notice 96-1. This validation report presents the results of the ILV for SL-573 and MT-2153 in soil.

The ILV conducted at Pyxant Labs Inc. confirmed that an independent group having no prior experience with the method can achieve results meeting the requirements of the U.S. EPA and the European Commission for General Health and Consumer Protection.

2. ANALYTICAL

2.1 Sample Receipt, Labeling and Storage

The test system consists of untreated soil from North Dakota shipped in bulk by Agvise Research, 3671 5th Ave. NE, Northwood, ND 58267. When received at Pyxant Labs Inc., the control sample was assigned unique master logbook (MLB) number 24438265 and stored frozen (approximately -10°C to -20°C).

2.2 Preparation of Solutions and Standards

The analytical reference standards/test substances utilized during the independent method validation are summarized below. The reference standards were received from the Sponsor, assigned unique MLB numbers, and stored frozen (-20°C), protected from light. The Certificates of Analysis are included in Appendix B.

Standard	MLB Number	Percent Purity	Expiration Date	Lot Number
SL-573	00008169	99.81%	April 18, 2014	20110128
MT-2153	00008172	99.8%	May 30, 2015	20120125

Standard solutions and calibration standard solutions were prepared in glass containers as described below and refrigerated (2-8°C) when not in use.

The following stock solution was prepared in acetonitrile to obtain a nominal concentration of 1000 µg/mL:

Analyte	Solution Type	Solution Lot Number	Weight [mg]	Dissolve In [mL]	Obtain [µg/mL]*
SL-573	Standard	N779P01-1	14.0	10	1400

*Resulting concentration after correcting for purity

The following stock solution was prepared in acetonitrile to obtain a nominal concentration of 1000 µg/mL:

Analyte	Solution Type	Solution Lot Number	Weight [mg]	Dissolve In [mL]	Obtain [µg/mL]*
MT-2153	Standard	N779P01-2	11.2	10	1120

*Resulting concentration after correcting for purity

Stock solutions were diluted in acetonitrile:

From Solution Lot Number	Concentration [µg/mL]	Pipette [µL]	Dilute To [mL]	Obtain Total [µg/mL]	Final Solution Lot Number
N779P01-1	1400	7143	100	100	N779P02-1
N779P01-2	1120	8929	100	100	N779P02-2

Fortification solutions were prepared in acetonitrile:

From Solution Lot Number	Concentration [µg/mL]	Pipette [µL]	Dilute To [mL]	Obtain Total [µg/mL]	Final Solution Lot Number
N779P02-1	100	20.0	100	20.0	N779P04-1
N779P02-2	100	20.0	100	20.0	N779P04-2

Mixed fortification solutions were prepared in 80/20 acetonitrile/water:

From Solution Lot Number	Concentration of Stock Solution [ng/mL]	Aliquot of Stock Solution [mL]	Final Solution Volume [mL]	Calibration Solution Final Concentration [µg/mL]	Final Solution Lot Number
N779P02-1	10.0	1.00	100	1.00	N779P05-1
N779P02-2	10.0	1.00			
N779P05-1	1.00	10.0	100	0.100	N779P05-2

Mixed calibration standards were prepared in 75/25 water/acetonitrile:

From Solution Lot Number	Concentration of Stock Solution [ng/mL]	Aliquot of Stock Solution [mL]	Final Solution Volume [mL]	Calibration Solution Final Concentration [ng/mL]	Final Solution Lot Number
N779P05-2	100	1.00	10.0	10.0	N779P19-1
N779P05-2	100	0.0200	10.0	2.00	N779P19-2
N779P19-1	10.0	1.00	10.0	1.00	N779P20-1
N779P19-2	2.00	1.00	10.0	0.200	N779P20-2
N779P20-1	1.00	1.00	10.0	0.100	N779P21-1
N779P20-1	1.00	0.500	10.0	0.0500	N779P21-2
N779P20-1	1.00	0.250	10.0	0.0250	N779P22-1

2.3 Fortification of Recovery Samples

The ILV trial of the method was performed for SL-573 and MT-2153 in soil. The trial was comprised of one batch, which consisted of the following samples:

1 (one) solvent blank (containing no matrix or analyte)

1 (one) reagent blank (containing no matrix or analyte)

2 (two) unfortified control samples

5 (five) control samples fortified with SL-573 and MT-2153 at 0.001 µg/g, the LOQ of the method

5 (five) control samples fortified with SL-573 and MT-2153 at 0.10 µg/g, or 100×LOQ

For preparation of recovery control specimens, appropriate volumes of the fortification standards were added as indicated below:

Specimen Portion (dry weight)	Nominal Target Fortification Level [µg/g]	Aliquot of Fortification Solution [mL]	Fortification Solution Concentration [µg/mL]
20 g	0.00100	0.200	0.100
	0.100	0.100	20.0

2.4 Sample Analysis

The ILV trial was conducted as described in the Ishihara Sangyo Kaisha, Ltd. analytical method, "Validation of the Residue Analytical Method for the Determination of SL-573 and Its Metabolite MT-2153 in Soil" dated March 26, 2012 (1).

Soil samples were extracted using methanol:water (80:20, v/v) with ammonium formate (0.1 M), citric acid (0.05 M), and hydrochloric acid (0.5%, v/v) and a reciprocal shaker. The extracts were cleaned by solid phase extraction and diluted with acetonitrile:water (50:50, v/v). Analysis was performed by liquid chromatography with electrospray tandem mass spectrometry (LC/MS/MS).

For more specific details, refer to the analytical method (Appendix A).

2.5 Analytical Instrumentation and Equipment

The following instruments and equipment were utilized in the conduct of the independent laboratory validation of the residue analytical method:

2.5.1 Instrumentation

Typical HPLC Conditions

HPLC System:	Waters Acquity UPLC
Column:	Acquity BEH C18 2.1x50 mm, 1.7 µm, S/N 020732142157-71
Guard Column:	VanGuard BEH C18 2.1x50 mm, 1.7 µm (optional)
Column Temperature:	45°C
Injection Volume:	30 µL for SL-573 and 25 µL for MT-2153
Mobile Phase:	Solvent A: 10 mM Ammonium Acetate
	Solvent B: Methanol
Flow Rate:	0.500 mL/min for SL-573 and 0.700 mL/min for MT-2153

SL-573	**Isocratic:**	A:B, 35:65, v/v

		Time (min)	**% A**	**% B**
MT-2153	**Gradient:**	0	95	5
		0.2	95	5
		2.0	5	95
		2.5	5	95
		3.0	95	5
		4.0	95	5

Typical MS Conditions

Mass Spectrometer: Applied Biosystems API 4000 LC/MS/MS System
Detector Mode: Positive-ion electrospray
Source Temperature: 500°C
Ions Monitored:

Compound	**Transition (amu)**	**Declustering Potential (Volts)**	**Collision Energy (volts)**	**Cell Exit Potential (volts)**
SL-573	485.2 → 382.9	71	17	12
	485.2 → 110.8		71	10
MT-2153	383.1 → 324.7	63	24	9.8
	383.1 → 110.6		59	11.5

2.5.2 Equipment

Top loading balance, Sartorius, model number BA 2100S, serial number 20303446
Analytical balance, Sartorius, model number AC 120S, serial number 20103137
Orbit shaker, model 3520, serial number 208
Centrifuge, Beckman, model number TJ-6, serial number 12189
Turbovap, Zymark, model number N/A, serial number TV0338N11918
SPE manifold, Burdick and Jackson
SPE cartridges, Waters, lot #109A32234A

2.5.3 Materials

Adjustable pipettes, various sizes
Class A volumetric pipettes, various sizes
Class A volumetric flasks, various sizes
HDPE screw-top bottle, 250 mL
Glass mixing cylinders, 100 mL
Glass autosampler vials, capped, 2 mL
Glass graduated cylinders, 250 mL
Plastic centrifuge tubes, 50 mL

2.5.4 Chemicals

Acetic Acid, HPLC grade, lot number 51327, VWR, EMD Chemicals, Aurora, CO 80011-3366
Acetonitrile, HPLC grade, lot number 123455, 52242, DG445, EMD Chemicals, VWR, Aurora, CO 80011-3366
Methanol, HPLC grade, lot number 52209, EMD Chemicals, VWR, Aurora, CO 80011-3366
Hydrochloric acid, lot number 51327, EMD Chemicals, VWR, Aurora, CO 80011-3366
Citric acid monohydrate, lot number MKBG18631, Sigma-Aldrich, St. Louis, MO 63103
Ammonium formate, lot number MKBH2404V, Sigma-Aldrich, St. Louis, MO 63103
Ammonium acetate, lot number MKBK4437V, Sigma-Aldrich, St. Louis, MO 63103
HPLC grade water, lot number 52213, 52241, DG465-6, DG226-A, EMD Chemicals, VWR, Aurora, CO 80011-3366
UHP water, in house

2.6 Calculations

Calculations were not modified from the original analytical method except that the analyte concentration was calculated in ng/mL by the Analyst® software program and was then converted to µg/g in Excel. Using the calibration curve calculated by linear regression with 1/x weighting, the calculated analyte concentration in the sample extracts in ng/mL was calculated using Equation 1:

$$y = mx + b \quad (1)$$

Where:

- x = Analyte concentration in ng/mL
- y = Analyte peak area
- m = Slope, calculated by the Analyst® software program
- b = y-intercept, calculated by the Analyst® software program

Equation 1 was rearranged as Equation 2 to solve for the analyte concentration.

$$x = \frac{(y - b)}{m} \times DF \quad (2)$$

Where:

- DF = Dilution factor

The analyte concentration found in the final extract (µg/g), calculated by Excel, is given by Equation 3:

$$AC = \frac{(x \times V)}{W} \times \frac{1\,\mu g}{1000\,ng} \quad (3)$$

Where:

AC = Analyte concentration found in final extract (µg/g)
x = Analyte concentration in ng/mL
V = Volume of final extract (200 mL)
W = Sample weight (20 g)

The percent recovery of the fortified samples was calculated using Equation 4:

$$\% \text{ Recovery} = \frac{x}{FC} \times 100 \quad (4)$$

Where:

x = Analyte concentration in µg/g
FC = Concentration fortified in µg/g (0.00100 µg/g at the LOQ and 0.100 µg/g at 100×LOQ)

As an example, the 100×LOQ quality control sample, Pyxant ID P2460B01-010 (Table 1, Figure 13) resulted in a SL-573 recovery of 85%. The calculations for this sample are demonstrated below as a representative example of how all the sample results were calculated for this study.

The linear regression analysis of the calibration curve used in the analysis of SL-573 residues in soil samples from Trial 1 was determined to have the following regression coefficients: m = 9.12E+04 and b = -5.65E+02 (Figure 1). The analyte peak area (y) was 7.71E+04; therefore the concentration of SL-573 in the final extract of this sample was calculated using Equation 2:

$$x = \frac{(7.71E+04 + 5.65E+02)}{9.12E+04} \times 10 = 8.52 \text{ ng/mL} \quad (2)$$

The final concentration of SL-573 found in the sample in µg/g was calculated in Excel using Equation 3:

$$AC = \frac{(8.52 \text{ ng/mL} \times 200 \text{ mL})}{20 \text{ g}} \times \frac{1 \text{ µg}}{1000 \text{ ng}} = 0.0852 \text{ µg/g} \quad (3)$$

The percent recovery of the sample was calculated using Equation 4:

$$\% \text{ Recovery} = \frac{0.0852 \text{ µg/g}}{0.100 \text{ µg/g}} \times 100 = 85\% \quad (4)$$

3.4 Method Modifications

Several modifications were made to the original method:

- The ratio of acetonitrile to water for the preparation of the calibration standards was reduced to 75/25 water/acetonitrile (v/v). In addition, a solvent exchange was performed to reduce the organic content during the injection of the metabolite. The eluate from the SPE cleanup was evaporated to half volume (5 mL) and reconstituted to 10 mL with water. Due to lack of sensitivity for MT-2153, a more aqueous solution was needed in both instances to account for the re-injection of a higher volume.

- Intermediate standard solutions prepared in acetonitrile, rather than 80/20 acetonitrile/water (v/v), were used to fortify the high concentration QC samples. The fortification solutions specified in the method are for an LOQ and 10x LOQ fortification standard. To fortify at the 100x LOQ level using these solutions would have required a very large fortification volume. Therefore, a higher concentration intermediate standard was used for fortification, rather than the fortification standard outlined in the method.

3.5 Critical Steps

There were no steps encountered with the methodology that were found to need to be followed so exactly that they were considered critical to the success of the method for the determination of SL-573 and MT-2153 in soil.

3.6 Sample Analysis Time Requirements

One batch of 13 samples required approximately five person hours over one calendar day to complete the extraction and clean-up. Analysis of soil samples on the instrument was performed over a one-to-two hour period. Data manipulation required an additional one person hour. Initial solution preparation required approximately six person hours.

1 OBJECTIVE

The objective of this study was to validate a residue analytical method for the determination of SL-573 and its metabolite MT-2153 in soil.

2 CONDUCT OF STUDY

The study was conducted at Ishihara Sangyo Kaisha, Ltd., Central Research Institute, Safety Science Research Laboratory, Environmental Sciences Group, 3-1, 2-Chome, Nishi-shibukawa Kusatsu-shi, Shiga-ken, 525-0025 Japan. The experimental start and termination dates were January 23, 2012 and March 19, 2012, respectively.

3 MATERIAL AND METHOD

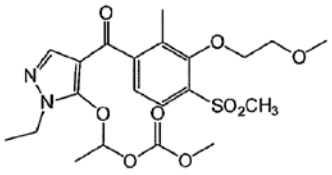
3.1 Analytical standards

3.1.1 SL-573

Common name: SL-573

Chemical name: 1-[[1-ethyl-4-[3-(2-methoxyethoxy)-2-methyl-4-(methylsulfonyl)benzoyl]-1H-pyrazol-5-yl]oxy]ethyl methyl ester (CA)

Structure:



Molecular weight: 484.52

Lot No.: 20120131

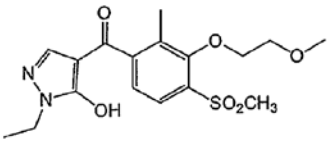
Purity: 99.82%

3.1.2 MT-2153

Common name: MT-2153

Chemical name: (5-Hydroxy-1-ethylpyrazol-4-yl)(3-(2-methoxyethoxy)-2-methyl-4-(methylsulfonyl)phenyl)methanone (CA)

Structure:



Molecular weight: 382.43

Lot No.: 20120125

Purity: 99.8%

3.2 Test soil

Two different type soils were used in the study. These soils were supplied from Huntingdon Life Sciences Ltd (HLS) in December 2011. Soil properties are shown in table below.

	Unit	Elmton	North Carolina (NC)
Origin		UK	US
pH		7.7	5.7
Sand 0.05 - 2.00 mm	% w/w	52	76
Silt 0.002 - 0.05 mm	% w/w	26	17
Clay <0.002 mm	% w/w	22	7
Cation Exchange Capacity	meq/100g	25.9	5.1
Organic Carbon	% w/w	3.7	0.8
Textural Classification [USDA]		Sandy Clay Loam	Loamy Sand

Note: This information was provided by HLS.

3.3 Reagents and apparatus

All reagents were of analytical, HPLC, or LC/MS/MS grade.

REAGENTS & APPARATUS	SUPPLIER	NOTE
Purified water	Millipore	MILLIPORE UF
Acetonitrile	Wako Pure Chemical	
Methanol	Nacalai Tesque	
Hydrochloric acid	Wako Pure Chemical	6 mol/L
Citric acid monohydrate	Wako Pure Chemical	
Ammonium formate	Wako Pure Chemical	
Acetic acid	Wako Pure Chemical	
Ammonium Acetate	Wako Pure Chemical	1 mol/L
HDPE screw-top bottle	Nalgene	250 mL
Volumetric flasks	Iwaki	various sizes
Volumetric pipettes	Iwaki	various sizes
Measuring cylinders	Iwaki	various sizes
Glass funnel	Iwaki	
µL-Pipettes	Nichiryo	various sizes
SPE cartridge	Waters	OASIS® HLB VAC RC (60mg)
SPE manifold	Waters	
HPLC vial	Waters	LC/MS certificated
Analytical balance	A&D	MODEL HA-202M
Laboratory balance	Shimadzu	MODEL UX4200S
Reciprocal shaker	TAIYO	MODEL SR-2
Centrifuge	KUBOTA	MODEL 6500
	TOMY SEIKO	MODEL LC06
Oven	ISUZU SEISAKUSHO	MODEL 2-2132

3.4 Extraction solvent

Methanol:water (80:20, v/v) containing ammonium formate (0.1 M), citric acid (0.05 M) and hydrochloric acid (0.5%, v/v) was used as an extraction solvent. Methanol (1600 mL) was mixed thoroughly with water (400 mL) and ammonium formate (12.6 g), citric acid (21 g) and 6 mol/L hydrochloric acid (20 mL) were added. The contents were mixed well.

3.5 Standard solutions

3.5.1 Stock solutions

10.0 mg of SL-573 and MT-2153 was weighed into separate 100 mL-Volumetric flask. Acetonitrile was added to make stock standard solutions with a concentration of 100 µg/mL.

3.5.2 Fortification solutions

The stock solutions were further diluted with acetonitrile:water (80:20, v/v) to obtain fortification solutions with a concentration of 1 and 0.1 µg/mL.

3.5.3 Calibration solutions

Calibration solutions, over the concentration range 0.025 to 10 ng/mL were prepared by serial dilution of the fortification solutions in acetonitrile:water (50:50, v/v).

3.6 Fortification

To demonstrate the validity of the method used, untreated soils were fortified with the following levels for SL-573 and MT-2153.

0.001 mg/kg	0.2 mL of the fortification solution (0.1 µg/mL) was added to 20 g (dry mass) soil.
0.05 mg/kg	1 mL of the fofrtification solution (1 µg/mL) was added to 20 g (dry mass) soil.

3.7 Analytical method

3.7.1 Extraction

20 g (dry mass) of the untreated soil sample was weighed into a 250 mL HDPE screw-top bottle. 100 mL of extraction solvent (see 3.4) was added to the soil sample. The sample was shaken for 30 minutes using a reciprocal shaker. The mixture was centrifuged at 3000 rpm for 10 minutes and supernatant was decanted. The soil residue was re-extracted with 80 mL of extraction solvent for 30 minutes. The mixture was centrifuged and decanted likewise. The extracts were combined in glass flask and diluted to volume (200 mL) with extraction solvent. And then, aliquot of extract was centrifuged at 3000 rpm for 5 minutes to remove the particle prior to SPE clean-up.

3.7.2 Sample clean-up on SPE

A SPE cartridge (OASIS® HLB VAC RC, 60 mg) was placed onto a SPE vacuum manifold and conditioned using methanol (4 mL) followed by 0.2% acetic acid (4 mL). 10 mL of the extract and 20 mL of 0.2% acetic acid were mixed and transferred into the SPE cartridge. The aqueous sample solution was sucked through the column followed by 2 mL of water. All eluates were discarded. SL-573 and MT-2153 were eluted with 8 mL of acetonitrile:water (50:50, v/v). The eluate was collected and then diluted to volume (10 mL) with acetonitrile:water (50:50, v/v).

3.7.3 Quantitation

Quantitation of SL-573 and MT-2153 concentration was performed by LC/MS/MS using the external standard method. The calibration standards at seven concentrations (0.025, 0.05, 0.1, 0.2, 1, 2 and 10 ng/mL) were used for construction of a calibration curve. The calibration curve was constructed by plotting the peak areas against the concentration of calibration standards. From the calibration curve, the concentration of SL-573 and MT-2153 in the injected solution was determined and the residue of SL-573 and MT-2153 in soil sample was calculated.

3.8 LC/MS/MS conditions

3.8.1 Analysis of SL-573

Part A; HPLC

Instrument:	ACQUITY UPLC System (Waters)
Column:	BEH C18 2.1×50 mm, 1.7 μm (Waters)
Guard column:	VanGuard BEH C18 2.1×5 mm, 1.7 μm (Waters)
Column temp.:	45°C
Mobile phase A:	10 mM ammonium acetate
Mobile phase B:	Methanol
Ratio (A:B):	35:65, v/v (Isocratic)
Flow rate:	0.5 mL/min
Injection volume:	4 μL
Retention time:	0.61 min

Part B; MS/MS

Instrument:	API4000QTRAP (AB sciex)				
Ionization mode:	ESI				
Scan mode:	MRM				
Ion polarity:	Positive $[M+H]^+$				
Mass resolution	Q1;unit, Q3;low				
Heater gas temp.:	500 °C				
Ion voltage:	5000 V				
Gas flow settings:	Gas1;60, Gas2;60, CUR;15, CAD;8				
Parameter:		DP	FP	CE	CXP
	Primary method;	86	10	17	10
	Confirmatory method;	86	10	73	16
Precursor ion (m/z):	485.0				
Product ion (m/z):	Primary method;	383.3			
	Confirmatory method;	111.1			

3.8.2 Analysis of MT-2153

Part A; HPLC

Instrument:	ACQUITY UPLC System (Waters)		
Column:	BEH C18 2.1×50 mm, 1.7 µm (Waters)		
Guard column:	VanGuard BEH C18 2.1×5 mm, 1.7 µm (Waters)		
Column temp.:	45°C		
Mobile phase A:	10 mM ammonium acetate		
Mobile phase B:	Methanol		
Gradient:	Time (min)	A%	B%
	0	95	5
	0.2	95	5
	2	5	95
	2.5	5	95
	3	95	5
	4	95	5
Flow rate:	0.5 mL/min		
Injection volume:	4 µL		
Retention time:	1.36 min		

Part B; MS/MS

Instrument:	API4000QTRAP (AB sciex)				
Ionization mode:	ESI				
Scan mode:	MRM				
Ion polarity:	Positive $[M+H]^+$				
Mass resolution	Q1;unit, Q3;low				
Heater gas temp.:	450 °C				
Ion voltage:	5000 V				
Gas flow settings:	Gas1;60, Gas2;80, CUR;25, CAD;6				
Parameter:		DP	FP	CE	CXP
	Primary method;	86	10	23	8
	Confirmatory method;	86	10	57	18
Precursor ion (m/z):	383.0				
Product ion (m/z):	Primary method;	325.1			
	Confirmatory method;	111.0			

3.9 Calculation

The residue of SL-573 and MT-2153 in soil was calculated according to equation 1.

$$R = \frac{X \times V_F \times D}{W \times 1000} \quad (1)$$

Where

R = Residue of SL-573 and MT-2153 in soil sample [mg/kg]
X = Concentration of injected solution [ng/mL]
V_F = Final Volume [10 mL]
D = Dilution Factor [if applicable]
W = Aliquot of sample [1 g]
1000 = Conversion factor from ng to µg

The recovery of SL-573 and MT-2153 in soil was calculated according to equation 2.

$$Rec = \frac{R \times 100}{F} \quad (2)$$

Where

Rec = Recovery of SL-573 and MT-2153 [%]
R = Residue of SL-573 and MT-2153 in soil sample [mg/kg]
F = Fortification level [mg/kg]