SUMMARY

PTRL West conducted a successful Independent Laboratory Validation of a method to determine NF-149 (Cyflufenamid) and its relevant metabolites 149-F, 149-F11, 149-F1 and 149-F6 in soil. The method validated is that reported by Gary Q. Westberg in 2006 under the title "Determination of NF-149 (Cyflufenamid) and its Metabolites in Soil" (Reference 1) with slight modifications described in this report.

Cyflufenamid and its metabolites were extracted from soil matrix twice with acetone followed by a single extraction with 2M-ammonium chloride/methanol (1:1 v:v). The organic solvent was removed by rotary film evaporation. The aqueous extract was then acidified with HCl and partitioned with ethyl acetate and sodium chloride. The aqueous portion was then basified with sodium hydroxide and partitioned with ethyl acetate. The ethyl acetate portions from both the acidic and basic partitioning were combined and 1-mL of water was added. The ethyl acetate was evaporated by rotary film evaporation and then brought to a final known volume in methanol:water (1:1, v:v). A portion of the final sample was readied for HPLC/MS/MS positive-ion electrospray analysis of 149-F and 149-F1 by basifying with a solution of ammonia. Another aliquot was readied for HPLC/MS/MS negative-ion electrospray analysis of NF-149, 149-F6 and 149-F11 by diluting with methanol:water (1:1, v:v). For each analyte, at least two ion transitions were monitored. One ion transition was used for quantitation purposes with the other transitions serving as confirmation. Quantitation was accomplished by external standard calibration and 1/x weighted least squares linear regression of concentration vs. peak area response.

Two communications were documented between PTRL West and the originator of the method where 149-F chromatography issues were discussed. Minor modifications to the original method were required to achieve acceptable chromatography and acceptable recovery for 149-F.

Based on the sample size and current methodology, the limit of quantitation (LOQ) was equal to the lowest fortification level or 2 ng/g for each analyte in soil. The limit of detection (LOD) was defined as the lowest calibrant concentration used or 0.25 ng/mL for the negative ion analysis and 1 ng/mL for the positive ion analysis. An equivalent LOD expressed in ng/g was 1 ng/g for both the negative ion and positive ion analysis analytes.

MATERIALS AND METHODS

Test/Reference Substances

All test/reference substances for this study were supplied by Nippon Soda Co., Ltd. (via Wildlife International, Ltd.). Cyflufenamid (code name NF-149) and relevant metabolite Test/Reference substance standards were supplied with the following lot numbers (see Appendix B for certificates of analyses and structures) and PTRL designations:

Code Name	Lot No.	PTRL No.	Purity (%)	Expiration Date
NF-149	KS-0244	1711W-002	99.7%	July 16, 2010
Chemical Name: (*Z*)-*N*-[[(cyclopropylmethoxy)amino][2,3-difluoro-6-(trifluoromethyl)phenyl]methylene]benzeneacetamide				
149-F	31-9109-AO	1711W-003	>99.9%	May 12, 2020
Chemical Name: *N*-Cyclopropylmethoxy-2,3-difluoro-6-trifluoromethylbenzamidine				
149-F11	31-8143-TS	1711W-004	99.1%	February 19, 2020
Chemical Name: (*Z*)-*N*-(α-cyclopropylmethoxyimino)-2,3-difluoro-6-(trifluoromethylbenzyl)malonic acid				
149-F1	31-8144-TS	1711W-005	97.3%	September 14, 2013
Chemical Name: 2,3-difluoro-6-trifluoromethylbenzamidine				
149-F6	31-8141-TS	1711W-006	>99.9%	April 1, 2019
Chemical Name: 2,3-difluoro-6-trifluoromethylbenzamide				

All neat standards were stored at < 0°C. All stock and working solutions were stored at < 10°C in amber bottles with Teflon®-lined screw-top caps. All solutions were prepared using class A volumetric flasks, syringes and pipettes. The reference substances were concluded to be stable throughout the study period based on qualitative comparisons of standard peak shapes and responses generated over the study period.

Specimen origin

The soil specimen was obtained from a soil dissipation trial in California, 0-6" untreated control soil, -1 DAA1, from study# AA050702, Morse Laboratories, Inc. The specimen was received at PTRL West in frozen condition on October 9, 2007. The harvest date for the soil was May 18, 2005. The specimen was stored frozen at PTRL West when not in use.

Solvents and Reagents

All solvents were HPLC grade and all reagents were ACS grade or better where not specified.

Acetic acid:	Acros, Glacial, ACS
Acetone	
Acetonitrile	Burdick and Jackson
Ammonium hydroxide	J.T. Baker, 30% or Fisher ACS plus 29.1%
Ammonium acetate	Fisher (HPLC grade 97.8%), or Fluka (Ultra, >99%)
Ammonium chloride	Mallinckrodt AR (ACS grade)
Ethyl acetate	
Hydrochloric acid	Fisher, ACS plus, concentrated
Methanol	Fisher, Optima
Sodium chloride	EMD Chemicals, GR ACS
Sodium hydroxide	EMD Chemicals, GR ACS
Water	Deionized or Burdick and Jackson high purity (for mobile phases)

Glassware and Equipment

Balances, various models

Beakers, various sizes

Bottles, 250 mL and 500 mL, high density polyethylene (HDPE)

Centrifuge, Mistral 3000E

Centrifuge tubes, polypropylene, 50 mL

Evaporation flasks, round or flat bottom, glass; 500 and 125 mL

Funnels, glass

Glass wool

Graduated cylinders, various sizes including 500 mL

Hamilton micro liter syringes, various volumes

Pasteur pipettes, 5 inch, 9 inch

Vials, Amber glass (2 mL capacity) with Teflon®-lined crimp or snap caps

Vacuum evaporator, Büchi Model RE111 with temperature-controlled bath, (Brinkmann Instruments, Burlingame, CA)

Volumetric pipette, class-A, various volumes

Volumetric flask, class-A, various volumes

Vortex Mixer

Weighing dishes, aluminum

Wrist-action shaker, Burrell Scientific model 75

Preparation of Reagents and Materials

Preparation of reagents and materials for extraction and analysis followed the instructions detailed in Reference 1. See also Appendix A, Protocol Appendix 1 for the cited method details. Note that the cited method refers to a "10% ammonia solution" that is used for preparing positive ion analysis mobile phases and is used to basify the positive ion calibrants and final samples. This "10% ammonia solution" is really a 3% ammonia solution (a 10 fold dilution of a ~30% ammonia solution). It is hereafter referred to as a 3% ammonia solution in this report.

Preparation of Stock Standard Solutions

Individual stock standard solutions of each of the five analytes were prepared. Approximately 50 mg of each analytical standard was weighed and quantitatively transferring to a 50 mL volumetric flask. The flask was brought to volume with acetonitrile and additional acetonitrile was added as necessary after accounting for the weight and purity of the standard to achieve a 1.00 mg/mL stock standard solution. The individual stock standard solutions were stored in a refrigerator (< 10°C) when not in use.

Preparation of Intermediate Standard Solutions

Individual Intermediate solutions each at 100 µg/mL were prepared by transferring 5.0 mL of a 1,000 µg/mL stock standard solution to a 50 mL volumetric flask and bringing to

volume with acetonitrile:water (1:1, v:v). The 1.0 µg/mL Intermediate solutions were prepared by transferring 5.0 mL of a 100 µg/mL intermediate standard solution to a 50 mL volumetric flask and bringing to volume with acetonitrile:water (1:1, v:v).

Preparation of Mixed Fortification Standard Solutions

Separate mixtures at various concentrations were prepared for the negative-ion analytes (NF-149, 149-F6 and 149-F11) and the positive-ion analytes (149-F and 149-F1) as follows:

Fortification Solution Concentration (each analyte)	Intermediate Standard Solution Concentration	Volume of Intermediate Solution Added (each)	Final Volume (acetonitrile:water, 1:1, v:v)
10 µg/mL	100 µg/mL	5.0 mL	50.0 mL
2.5 µg/mL	100 µg/mL	2.5 mL	100 mL
1.0 µg/mL	100 µg/mL	500 µL	50.0 mL
0.1 µg/mL	1.0 µg/mL	5.0 mL	50.0 mL

Mixed fortification solutions were prepared using volumetric flasks, syringes and pipets. Solutions were stored in a refrigerator (< 10°C) when not in use.

Preparation of Calibration Standard Solutions

Calibration standard solutions were prepared using volumetric pipets, micro liter syringes and volumetric flasks. Calibrant solutions containing equal concentrations of NF-149, 149-F6 and 149-F11 were prepared for the negative ion analysis in methanol:water (1:1, v:v).

Negative-ion Calibrants:

Calibration Standard Concentration (each analyte)	Mixed Standard Solution Concentration (NF-149, 149-F6 and 149-F11)	Volume Mixed Standard Solution Added	Final Volume (methanol:water 1:1, v:v)
12.5 ng/mL	2.5 µg/mL	500 µL	100 mL
5.0 ng/mL	2.5 µg/mL	200 µL	100 mL
2.0 ng/mL	1.0 µg/mL	200 µL	100 mL
0.50 ng/mL	0.1 µg/mL	500 µL	100 mL

Calibration Standard Concentration (each analyte)	Mixed Standard Solution Concentration (NF-149, 149-F6 and 149-F11)	Volume Mixed Standard Solution Added	Final Volume (methanol:water 1:1, v:v)
0.25 ng/mL	0.1 µg/mL	250 µL	100 mL

Calibrant solutions containing equal concentrations of 149-F and 149-F1 were prepared for the positive ion analysis in methanol:water (1:1, v:v) basified with ammonia solution. The basified methanol:water (1:1, v:v) was prepared by mixing 288 mL of water with 12 mL of 3% ammonia solution and 300 mL of methanol.

Positive-ion Calibrants:

Calibration Standard Concentration (each analyte)	Mixed Standard Solution Concentration (149-F and 149-F1)	Volume Mixed Standard Solution Added	Final Volume (basified methanol:water 1:1, v:v)
50 ng/mL	2.5 µg/mL	2.0 mL	100 mL
10 ng/mL	2.5 µg/mL	400 µL	100 mL
5.0 ng/mL	2.5 µg/mL	200 µL	100 mL
2.0 ng/mL	50 ng/mL	4.0 mL	100 mL
1.0 ng/mL	50 ng/mL	2.0 mL	100 mL

All standard solutions were stored in amber glass bottles with PTFE lined screw caps and placed in a refrigerator (< 10°C) when not in use.

Sample Fortification

A 20.0-gram portion of a well-mixed control (untreated) soil specimen was weighed into a 250 mL HDPE centrifuge bottle with screw cap enclosure. The soil sample was fortified with the appropriate amount of fortification solution and then extracted according the extraction method. For samples fortified at 2 ng/g, 40 µL of each of the 1.0 µg/mL positive and negative fortification solutions was added using a 50 µL syringe. For samples fortified at 20 ng/g, 400 µL of each of the 1.0 µg/mL positive and negative fortification solutions was added using a 500 µL syringe.

ILV Sample Set

The independent laboratory validation sample set consisted of a reagent blank sample, two control (unfortified) specimens and five fortified control specimens at each of two fortification levels.

Extraction Method Modifications

The extraction method used was followed as closely as possible to that described in Appendix A, Protocol Appendix 1 with the following modification:

Step #2 & #12; used a wrist action shaker (set at "10") instead of a reciprocating shaker.

LC/MS/MS Analysis

LC Instrument:

Pump: Agilent 1100 series, model G1312A

Autosampler: Agilent 1100 series, model G1329A

Micro-Degasser: Agilent 1100 series, model G1379A

Column Compartment: Agilent 1100 series, model G1316A

Column: Phenomenex Luna C8(2), 150 mm x 2.0 mm I.D., 3 µm particle size

HPLC Operating Conditions:

Negative-ion analysis (NF-149, 149-F6, 149-F11):

A) 0.01M ammonium acetate + 0.1% acetic acid in water :acetonitrile (90:10, v:v)

B) 0.01M ammonium acetate + 0.1% acetic acid in water:acetonitrile (10:90, v:v)

Time (min.)	Flow Rate (µL/min.)	% A	% B
0.0	200	90	10
2.0	200	50	50
10.0-15.0	200	20	80
16.0-17.0	200	0	100
18.0-23.0	200	90	10

Injection Volume: 20 µL

Column temperature 35°C

Positive-ion analysis (149-F and 149-F1):

A) 0.01M ammonium acetate containing 0.2% ammonia (3%) solution:acetonitrile (90:10, v:v)

B) 0.01M ammonium acetate containing 0.2% ammonia (3%) solution:acetonitrile (10:90, v:v)

Time (min.)	Flow Rate (µL/min.)	% A	% B
0.0	200	90	10
6.0-9.0	200	0	100
10.0-16.0	200	90	10

Injection Volume: 5µL

Column temperature 35°C

Mass Spectrometry Operating Conditions:

MS Instrument: Applied Biosystems MDS/SCIEX API 4000 LC/MS/MS system. This instrument was used with turbo ion spray in both positive and negative ionization mode. The Triple Quadrupole Mass Spectrometer was used in MRM-multiple reaction monitoring mode.

Negative-ion analysis (NF-149, 149-F6, 149-F11):

NebulizerTemperature (°C): 300
Nebulizer Gas (GS1): 90 psi
Turbo Gas (GS2): 60 psi
Curtain Gas (CUR): 20 psi
Collision Activated Dissociation (CAD): 10
IonSpray Voltage (IS): -4500
Interface Heater (ihe): on

Ion transitions monitored:

Analyte	Ion, *m/z*		Time, *ms*	CE, *v*
	Q1	Q3		
NF-149 (both Z & E isomers)	411.30	217.90	150	-30
	411.30	203.90	150	-50
	411.30	391.10	150	-13
149-F11	379.40	242.90	150	-24
	379.40	223.10	150	-38
	379.40	315.00	150	-12
149-F6	224.10	180.90	150	-25
	224.10	160.90	150	-36

Retention times:
149-F6: ~ 6.9 minutes
149-F11: ~ 7.4 minutes
NF-149 E isomer: ~ 13.7 minutes
NF-149 Z isomer: ~ 14.8 minutes

Positive-ion analysis (149-F, 149-F1):

NebulizerTemperature (°C): 500
Nebulizer Gas (GS1): 70 psi
Turbo Gas (GS2): 60 psi
Curtain Gas (CUR): 25 psi
Collision Activated Dissociation (CAD): 12
IonSpray Voltage (IS): 4500
Interface Heater (ihe): on

Ion transitions monitored:

Analyte	Ion, *m/z*		Time, *ms*	CE, *v*
	Q1	Q3		
149-F	294.80	241.00	150	22
	294.80	223.00	150	20
	294.80	203.00	150	36
149-F1	224.60	165.00	150	42
	224.60	185.00	150	30
	224.60	205.00	150	25

Retention times: 149-F: ~ 9.1 minutes
149-F1: ~ 6.3 minutes

Sample Analysis

Separation of the analytes from the sample matrix was achieved by high performance liquid chromatography (HPLC). The analytes were identified by the coincidence of their retention times with the calibration standards. One ion transition for each analyte was used for quantitation. The other transitions served as confirmation. For each ionization mode analysis, a calibration curve was generated from five calibration standards interspersed throughout the injection sequence with soil samples. Each analytical sequence was ended with one or more QC injections (repeat injection of calibrant solutions at end of sequence) that were used to compare instrument response at the end of the sequence to calibrant standard response in the middle of the sequence.

Analysis Method Modifications

The following modifications to the analytical method cited in Reference 1 (See also Appendix A, Protocol Appendix 1) were made in conducting this independent laboratory validation:

1. Positive-ion analysis: An Applied BioSystems/MDS Sciex API 4000 LC/MS/MS instrument was used instead of an API 2000.

2. Positive-ion analysis: The injection volume for analysis of analyte 149-F1 was reduced from 20 µL to 5 µL.

3. Positive-ion analysis: For the analysis of 149-F1, the re-equilibration period of the LC gradient (time 10 minutes to 13 minutes) was extended to 6 minutes (10 minutes to 16 minutes) with an isocratic mixture of 90% mobile phase A and 10% mobile phase B.

Methods of Calculation

The methods of calculation are equivalent to those described in Reference 1 (see also Appendix A, Protocol Appendix 1). Weighted curve statistics (1/x weighting) were generated by Excel® software. A single ion transition for each analyte was used for quantitation with additional ion transitions used as confirmation only.

For negative-ion analysis:

Analyte Found (ng/g) = (FE-Conc x TE-Vol x FE-Vol1 x FE-Vol2 x DF) ÷ (SW x EA-Vol1 x EA-Vol2)

Where:

FE-Conc	= Final Extract Concentration (ng/mL)
	= (peak area –curve intercept) ÷ curve slope
TE-Vol	= Total Extract Volume (mL) = 300 mL
FE-Vol1	= Final Extract Volume 1 (mL) = 10 mL
FE-Vol2	= Final Extract volume 2 (mL) = 4 mL
DF	= Dilution Factor = 1
SW	= Sample Weight (g) = 20 g
EA-Vol1	= Extract Aliquot Volume 1 (mL) = 150 mL
EA-Vol2	= Extract Aliquot Volume 2 (mL) = 1 mL

For positive-ion analysis:

Analyte Found (ng/g) = (FE-Conc x TE-Vol x FE-Vol x DF) ÷ (SW x EA-Vol)

Where:

FE-Conc	= Final Extract Concentration (ng/mL)
	= (peak area –curve intercept) ÷ curve slope
TE-Vol	= Total Extract Volume (mL) = 300 mL

FE-Vol = Final Extract Volume (mL) = 10 mL
DF = Dilution Factor = 1
SW = Sample Weight (g) = 20 g
EA-Vol = Extract Aliquot Volume 1 (mL) = 150 mL

% Recovery for positive or negative-ion analysis:

= [(Analyte found (ng/g) – analyte found in control (ng/g)) ÷ Fortification level (ng/g)] x 100

Example Calculations

An example of 149-F6 quantitation of a 2 ng/g-fortified sample (F1A) from a negative-ion analysis follows (See Also Figure 31).

1/x weighted calibration curve statistics:

Slope:	2,808
Intercept:	-73
R^2:	0.9999

The calculations for analyte found (ng/g) and percent recovery (for a fortified sample) are:

Final Extract Conc. (ng/mL) = [(1,327 – (-73)) ÷ 2,808]
= 0.499 ng/mL

Analyte Found (ng/g) = (0.499 ng/mL x 300 mL x 10 mL x 4 mL x 1)
÷ (20 g x 150 mL x 1 mL)
= 2.00 ng/g

% Recovery = [(2.00 ng/g – 0 ng/g) ÷ 2.0 ng/g] x 100
= 100%

An example of 149-F quantitation of a 2 ng/g-fortified sample (F1A) from a positive-ion analysis follows (See Also Figure 69).

1/x weighted calibration curve statistics:

Slope:	13,724
Intercept:	91
R^2:	0.9998

The calculations for analyte found (ng/g) and percent recovery (for a fortified sample) are:

Final Extract Conc. (ng/mL) = [(25,002 –91) ÷ 13,724]
= 1.82 ng/mL

Analyte Found (ng/g) = (1.82 ng/mL x 300 mL x 10 mL x 1) ÷ (20 g x 150 mL)
= 1.82 ng/g

% Recovery = [(1.82 ng/g – 0 ng/g) ÷ 2.0 ng/g] x 100
= 91%

Statistical Analysis

The residue data included the following statistical calculations: averages, standard deviations, relative standard deviations and linear regression analyses (with 1/x weighting).

Limit of Quantitation and Limit of Detection

The limit of quantitation (LOQ) was 2.0 ng each analyte/g soil as defined by the lowest fortification level successfully tested.

The limit of detection (LOD) was defined as the detector response (peak area) from the lowest linearity standard injection. The lowest linearity standard had a concentration of 0.25 ng/mL for each negative-ion analyte (NF-149, 149-F6 and 149-F11) and was 1.0 ng/mL for each positive-ion analyte (149-F and 149-F1). The lowest linearity standard is equivalent to a nominal residue concentration of 1 ng /g for all analytes.

Time Required for Analysis

Time required for one analyst per sample set, where a sample set consists of twelve (12) specimen samples and a reagent blank sample:

Extraction and Clean-up takes approximately 20 hours.

LC/MS/MS analysis takes approximately 6 hours for setup and data processing (plus unattended overnight LC/MS/MS operation)

TOTAL = approximately 26 hours (or ~ 3 calendar days)

PTRL West conducted the required assessment for an independent laboratory validation of a method to determine Cyflufenamid and four metabolites in soil using the validated method specified in Reference 1 (See also Appendix A, Protocol Appendix 1) with only slight modifications as follows:

1. Extraction Step #2 & #12; used a wrist action shaker (set at "10") instead of a reciprocating shaker. The wrist action shaker represents a comparable apparatus.

2. Positive-ion analysis: An Applied BioSystems/MDS Sciex API 4000 LC/MS/MS instrument was used instead of an API 2000. The use of the API 4000 represents a comparable apparatus.

3. Positive-ion analysis: The injection volume for analysis of analyte 149-F1 was reduced from 20 µL to 5 µL. As discussed in correspondence log (Appendix D).

4. Positive-ion analysis: For the analysis of 149-F1, the re-equilibration portion of the LC gradient (time 10 minutes to 13 minutes) was extended to 6 minutes (10 minutes to 16 minutes) with an isocratic mixture of 90% mobile phase A and 10% mobile phase B. As discussed in correspondence log (Appendix D).

The following changes were implemented to improve the chromatography of 149-F1:

1. Fresh reagents were used in preparing mobile phases resulting in a change in pH from 8.77 to 9.03. Fresh ammonia solution was also used to basify the soil extracts for positive-ion analysis.

2. Additional time was added for column re-equilibration at initial conditions after each gradient cycle (from 3 minutes to 6 minutes). It was noted the 149-F1 peak splitting was more pronounce when a fortified matrix sample was re-injected after the 3 minute re-equilibration as compared to the first injection that had excessive equilibration.

3. The injection volume was reduced from 20 µL to 5 µL to minimize the discrepancy between the solvent composition of the final samples and the solvent composition of the initial conditions of the LC gradient.

Note that a second Phenomonex Luna C8(2) column was obtained but the chromatography for 149-F1 on this column was considerably worse and was therefore not used. It is possible that a more appropriate or robust column could be used for the 149-F1 analyses, however this was not explored. Also note that adding additional ammonia to a fortified soil extract showed some improvement to peak shape but no improvement to % recovery, therefore this modification was not incorporated.