DETERMINATION OF NF-149 (CYFLUFENAMID) AND ITS METABOLITES IN SOIL

Reason for Revision:

To add, as appendices, method validation data and example chromatography produced under Study No. AA050702, "Terrestrial Field Soil Dissipation of Cyflufenamid," Nippon Soda Co., Ltd., MRID No. 47638209 (Reference 1).

1 PRINCIPLE

The method described herein is capable of determining NF-149 (cyflufenamid) and its relevant metabolites (149-F11, 149-F6, 149-F1, and 149-F) in soil. It is based on a method developed previously for the sponsoring company and modifications made to that method (References 2 and 3).

Soil samples are extracted twice with acetone followed by a single extraction with 2M ammonium chloride/methanol (50:50, v/v). The organic solvent is removed by evaporation, then sodium chloride and 1M HCl are added to the remaining aqueous phase which is partitioned twice with ethyl acetate. The aqueous phase is then basified with 10M sodium hydroxide and partitioned a further two times with ethyl acetate. The combined ethyl acetate extracts are concentrated and made to a fixed volume with methanol/water (50:50, v/v) prior to analysis by LC-MS/MS. Aliquots of the final extract (of suitable volume, depending on the ionization mode of analysis) are transferred to two test tubes. One aliquot is submitted directly to negative-ion electrospray LC-MS/MS analysis for NF-149 and metabolites 149-F6 and 149-F11. The other aliquot is basified with 10% ammonia solution, then submitted to positive-ion electrospray LC-MS/MS analysis for metabolites 149-F and 149-F1. The target limit of quantitation (LOQ) for all analytes is 2 ppb.

2 EQUIVALENCE STATEMENT

During the conduct of this analysis, comparable apparatus, solvents, glassware, and techniques (such as sample extract evaporation) may be substituted for those described in this method, except where specified otherwise. In the event a substituted piece of equipment or technique is used, its use will be documented in the study records.

3 APPARATUS AND EQUIPMENT

Assorted laboratory glassware

Autosampler vials:	Glass, 2.0 mL, clear vials, part # 40011 and black polypropylene caps, part# 49185 (Thomson Inst. Co., Oceanside, CA)

Balances:	Analytical balance capable of weighing to ±0.1 mg
	Top-loading balance capable of weighing to ±0.1 g
Beakers:	Glass, various sizes
Bottles:	Polyethylene, 500 mL, with screw cap closures
Centrifuges:	IEC Model HN-SII (Damon IEC Division, Needham Hts., MA)
	IEC clinical centrifuge (Damon IEC Division, Needham Hts., MA)
	Centrific™ centrifuge (Fisher Scientific, Fair Lawn, NJ)
Centrifuge bottles:	250-mL VWR® HDPE (high-density polyethylene) bottles with screw cap closures
Centrifuge tubes:	50-mL, VWR® polypropylene tubes with screw cap closures
Erlenmeyer flasks:	Glass, 1000 mL, with glass stopper
Evaporation flasks:	Round or flat bottom, glass; 500 and 125 mL
Evaporator:	Rotary evaporator equipped with a Dewar condenser (Labconco Corp., Kansas City, MS)
Funnels:	Glass, 75 mm diameter
Graduated cylinders:	Glass; various sizes
Graduated mixing cylinders:	Glass; 500, 250, and 50 mL
HPLC/MS systems:	PE Sciex API 2000 LC/MS/MS system with a Perkin Elmer series 200 autosampler, an integrated Shimadzu chromatograph consisting of (2) LC-10AD*vp* Liquid Chromatograph units, a DGU-14A Degasser, and a SCL-10A*vp* System Controller. The system is controlled and data processed by PE Sciex Analyst Software.
	Applied BioSystems/MDX Sciex API 4000 LC/MS/MS system with a Shimadzu SIL-HTA autosampler, an integrated Shimadzu chromatograph consisting of (2) LC-10AD*vp* Liquid Chromatograph units and a DGU-14A

	Degasser. The system is controlled and data processed by Applied BioSystems/MDX Sciex Analyst Software.
HPLC column:	15 cm × 2.0 mm i.d. Phenomenex Luna C8 (2), 3 µ particle size (Phenomenex, Torrance, CA)
Microliter syringes:	Various sizes, (Hamilton Co., Reno, NV)
Pasteur pipets:	Glass, 9 inch and 5 ¾ inch, disposable
Pipets:	Glass, graduated, serological; various sizes Glass, volumetric; various sizes
Reciprocating shaker:	Eberbach Model 6000 (Eberbach Corp., Ann Arbor, MI)
Test (culture) tubes:	Glass; 16 × 100 mm and 13 × 100 mm, with Teflon-lined screw caps
Ultrasonic bath:	Branson Model 2210 ultrasonic bath (VWR Scientific, Bridgeport, NJ) Aquasonic Model 550 T (VWR Scientific, Bridgeport, NJ)
Volumetric flasks:	Glass; 1000, 100 and 50 mL

4 REAGENTS AND MATERIALS

Acetic acid:	Glacial, OmniSolv® (EM Science, Gibbstown, NJ) Glacial, HPLC grade (Fisher Scientific, Fairlawn, NJ)
Acetone:	OmniSolv® (EM Science, Gibbstown, NJ)
Acetonitrile:	OmniSolv® (EM Science, Gibbstown, NJ) B&J Brand High Purity solvent (Burdick and Jackson, Muskegon, MI)
Ammonia hydroxide:	GR, ACS, 28.0-30% (as NH_3) (EMD Chemicals, Inc., Gibbstown, NJ)
Ammonium acetate:	HPLC grade (Fisher Scientific, Fairlawn, NJ)

Ammonium chloride:	AR®, ACS, crystal (Mallinckrodt Baker, Inc., Paris, Kentucky)
Ethyl acetate:	OmniSolv® (EM Science, Gibbstown, NJ)
Glass wool	Pyrex® fiber glass (Corning Inc., Corning, NY)
Hydrochloric acid:	A.C.S. Reagent, concentrated, 36.5-38% (J.T. Baker Chemical Co., Phillipsburg, NJ)
Methanol:	OmniSolv® (EM Science, Gibbstown, NJ) HPLC grade (Fisher Scientific, Fairlawn, NJ)
Reference standards	
NF-149 (cyflufenamid):	Analytical grade
149-F11:	Analytical grade
149-F6:	Analytical grade
149-F1:	Analytical grade
149-F	Analytical grade
Sodium chloride:	GR, ACS (EMD Chemicals, Inc., Gibbstown, NJ)
Sodium hydroxide:	Pellets, A.C.S. Reagent, 98.7% (J.T. Baker Chemical Co., Phillipsburg, NJ)
Water:	HPLC grade (Fisher Scientific, Fairlawn, NJ) Deionized (DI) water (Polymetrics System, Morse Laboratories, Inc.)

4.1 Reagents and Materials to be Prepared (including typical preparation instructions)

4.1.1 Acetonitrile:water (50:50, v/v): To a 500-mL graduated mixing cylinder, add 250 mL of OmniSolv® acetonitrile. Bring to a final volume of 500 mL with DI water. Mix well.

4.1.2 2M ammonium chloride solution: Weigh out 53.5 g of ammonium chloride crystals and transfer to a 500-mL graduated mixing cylinder. Add ~400 mL of DI water, mix to dissolve. Adjust to a final volume of 500 mL with DI water. Mix well.

4.1.3 2M ammonium chloride:methanol (50:50, v/v): To a 500-mL graduated mixing cylinder, add 250 mL of 2M ammonium chloride solution. Bring to a final volume of 500 mL with OmniSolv® methanol. Mix well.

4.1.4 1M HCl: To a 250-mL graduated mixing cylinder, add 183.5 mL of DI water, followed by 16.5 mL of concentrated HCl. Mix well.

4.1.5 10M NaOH: Weigh out 80 g of NaOH pellets and transfer to a 250-mL graduated mixing cylinder. Add ~150 mL of DI water, mix to dissolve. Adjust to a final volume of 200 mL with DI water. Mix well.

4.1.6 Methanol:water (50:50, v/v): To a 250-mL graduated mixing cylinder, add 100 mL of HPLC-grade methanol. Bring to a final volume of 200 mL with HPLC-grade water. Mix well.

4.1.7 10% ammonia solution: To a 50-mL graduated mixing cylinder, add ~30 mL of HPLC-grade water. Add 5.0 mL of ammonium hydroxide. Bring to a final volume of 50 mL with HPLC-grade water. Mix well.

4.1.8 Methanol:water (50:50, v/v), basified with ammonia solution: To a 1000-mL glass stoppered Erlenmeyer flask, add 288 mL of HPLC-grade water, 12 mL of 10% ammonia solution, and 300 mL of HPLC-grade methanol. Mix well.

4.1.9 HPLC mobile phases:

0.01M ammonium acetate + 0.1% acetic acid: Weigh 0.771g ammonium acetate (Fisher, HPLC Grade) into a 1-liter volumetric flask. Add approximately 900 mL of HPLC grade water to the flask (Fisher, HPLC grade), and 1.0 mL of acetic acid (Fisher HPLC Grade, Glacial) using a volumetric pipet. Bring to volume with HPLC grade water. Mix thoroughly.

0.01M ammonium acetate + 2% ammonia (10%) solution: Weigh 0.771g ammonium acetate (Fisher, HPLC Grade) into a 1-liter volumetric flask. Add approximately 900 mL of HPLC grade water to the flask (Fisher, HPLC grade), and 2.0 mL of 10% ammonia solution using a volumetric pipet. Bring to volume with HPLC grade water. Mix thoroughly.

0.01M ammonium acetate + 0.1% acetic acid:acetonitrile (90:10, v/v): Measure 900 mL of 0.01M ammonium acetate + 0.1% acetic acid into a 1-liter graduated cylinder. Measure 100 mL of acetonitrile (B&J Brand High Purity Solvent) in a 100-mL graduated cylinder, then add to the ammonia acetate solution. Transfer to a 1-liter mobile phase reservoir. Mix thoroughly.

0.01M ammonium acetate + 0.1% acetic acid:acetonitrile (10:90, v/v): Measure 900 mL of acetonitrile (B&J Brand High Purity Solvent) into a 1-liter graduated cylinder. Measure 100 mL of 0.01M ammonium acetate + 0.1% acetic acid in a 100-mL graduated cylinder, then add to the acetonitrile. Transfer to a 1-liter mobile phase reservoir. Mix thoroughly.

0.01M ammonium acetate + 0.2% ammonia (10%) solution:acetonitrile (90:10, v/v): Measure 900 mL of 0.01M ammonium acetate + 0.2% ammonia (10%) solution into a 1-liter graduated cylinder. Measure 100 mL of acetonitrile (B&J Brand High Purity Solvent) in a 100-mL graduated cylinder, then add to the ammonia acetate solution. Transfer to a 1-liter mobile phase reservoir. Mix thoroughly.

0.01M ammonium acetate + 0.2% ammonia (10%) solution:acetonitrile (10:90, v/v): Measure 900 mL of acetonitrile (B&J Brand High Purity Solvent) into a 1-liter graduated cylinder. Measure 100 mL of 0.01M ammonium acetate + 0.2% ammonia (10%) solution in a 100-mL graduated cylinder, then to the ammonia acetate solution. Transfer to 1-liter mobile phase reservoir. Mix thoroughly

5 STANDARD PREPARATION

5.1 Stock Standard Solutions

Typically, 50.0 mg (corrected for purity) of each analytical standard is accurately weighed and quantitatively transferred to a separate 50-mL volumetric flask. Each is brought to volume with acetonitrile. The resulting concentration of each solution is 1000 µg/mL. These solutions are to be stored at 1 to 8°C when not in use.

5.2 Intermediate/Fortification Standard Solutions

Typically the following concentrations of all analytes are prepared. Suitable mixtures may be prepared accordingly. All solutions are stored at 1 to 8°C when not in use.

Prepared as individual solutions:

100 µg/mL: Transfer 5.0 mL of a 1000 µg/mL standard solution to a 50-mL volumetric flask. Bring to volume in acetonitrile:water (50:50, v/v). Mix well.

Prepared as mixtures:

Negative-ion mix (containing NF-149, 149-F6, 149-F11) and positive-ion mix (containing 149-F, 149-F1)

10 µg/mL: Transfer 5.0 mL of each applicable 100 µg/mL standard solution to a 50-mL volumetric flask. Bring to volume in acetonitrile:water (50:50, v/v). Mix well.

2.5 µg/mL: Transfer 2.5 mL of each applicable 100 µg/mL standard solution to a 100-mL volumetric flask. Bring to volume in acetonitrile:water (50:50, v/v). Mix well.

1.0 µg/mL: Transfer 500 µL of each applicable 100 µg/mL standard solution to a 50-mL volumetric flask. Bring to volume in acetonitrile:water (50:50, v/v). Mix well.

0.1 µg/mL: Transfer 5.0 mL of each applicable 1.0 µg/mL standard solution to a 50-mL volumetric flask. Bring to volume in acetonitrile:water (50:50, v/v). Mix well.

5.3 HPLC (Calibration) Standard Solutions

All standard solutions prepared in this section are stored at 1 to 8°C when not in use. Typically the following concentrations of HPLC standard solution *mixtures* are prepared:

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

12.5 ng/mL: Transfer 500 µL of a 2.5 µg/mL mixed standard solution to a 100-mL volumetric flask. Bring to volume in methanol:water (50:50, v/v). Mix well.

5.0 ng/mL: Transfer 200 µL of a 2.5 µg/mL mixed standard solution to a 100-mL volumetric flask. Bring to volume in methanol:water (50:50, v/v). Mix well.

2.0 ng/mL: Transfer 200 µL of a 1.0 µg/mL mixed standard solution to a 100-mL volumetric flask. Bring to volume in methanol:water (50:50, v/v). Mix well.

0.50 ng/mL: Transfer 500 µL of a 0.1 µg/mL mixed standard solution to a 100-mL volumetric flask. Bring to volume in methanol:water (50:50, v/v). Mix well.

0.25 ng/mL: Transfer 250 µL of a 0.1 µg/mL mixed standard solution to a 100-mL volumetric flask. Bring to volume in methanol:water (50:50, v/v). Mix well.

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

50 ng/mL: Transfer 2.0 mL of a 2.5 µg/mL mixed standard solution to a 100-mL volumetric flask. Bring to volume in methanol:water (50:50, v/v), basified with ammonia solution. Mix well.

10 ng/mL: Transfer 400 µL of a 2.5 µg/mL mixed standard solution to a 100-mL volumetric flask. Bring to volume in methanol:water (50:50, v/v), basified with ammonia solution. Mix well.

5.0 ng/mL: Transfer 200 µL of a 2.5 µg/mL mixed standard solution to a 100-mL volumetric flask. Bring to volume in methanol:water (50:50, v/v), basified with ammonia solution. Mix well.

2.0 ng/mL: Transfer 4.0 mL of a 50 ng/mL mixed standard solution to a 100-mL volumetric flask. Bring to volume in methanol:water (50:50, v/v), basified with ammonia solution. Mix well.

1.0 ng/mL: Transfer 2.0 mL of a 50 ng/mL mixed standard solution to a 100-mL volumetric flask. Bring to volume in methanol:water (50:50, v/v), basified with ammonia solution. Mix well.

6 SAMPLE FORTIFICATION

1. Weigh 20.0 g of a well-mixed soil sample into a 250-mL HDPE centrifuge bottle with screw cap enclosure.

2. Fortify the sample with the appropriate amount of fortification solution. Use a volume of fortification solution ≤1.0 mL. Allow approximately 10 minutes for the fortification solution solvent to evaporate.

3. Proceed with Step 7.2.

7 SAMPLE EXTRACTION

Procedure:

1. Weigh 20.0 g of a well-mixed soil sample into a 250-mL HDPE centrifuge bottle with screw cap enclosure. As applicable, fortify appropriate samples at this time.

2. Add 100 mL of acetone. Cap the bottle and shake on a reciprocating shaker at ~100 excursions/minute for ~30 minutes (placing the bottle on its side).

3. Centrifuge the mixture for ~10 minutes at ~2000 rpm.

4. Decant the supernatant through a funnel containing a glass wool plug into a 500-mL polyethylene bottle.

5. With the solids remaining in the 250-mL centrifuge bottle, repeat steps 2 through 4 with 100 mL acetone. Combine the filtered supernatant with the first in the same 500-mL polyethylene bottle from Step 4.

6. With the solids remaining in the 250-mL centrifuge bottle, repeat steps 2 through 4 with 80 mL of 2M ammonium chloride:methanol (50:50, v/v). Combine the

filtered supernatant with the two acetone extracts in the same 500-mL polyethylene bottle from Step 5. Discard the solids remaining in the 250-mL centrifuge bottle.

7. Add 10 mL DI water to the combined extracts, invert to mix, then pour contents into a 500-mL graduated mixing cylinder and bring to a final volume of 300 mL with OmniSolv® methanol. Mix well to ensure that any visible solid material is dissolved. Return the measured solution to the 500-mL polyethylene bottle. (Use this bottle as an extract storage container.)

8. Transfer 150 mL of the extract from Step 7 to a 500-mL evaporation flask.

9. Concentrate the extract on a rotary evaporator, set at ≤40 °C, to the aqueous phase. The volume at this point should be ~30 mL.

10. Transfer the aqueous remainder to a 50-mL polypropylene centrifuge tube, using a 10-mL ethyl acetate rinse (with sonication) of the evaporation flask to aide in the transfer.

11. Add ~2 g of sodium chloride and 1 mL of 1M HCl solution to the contents of the tube.

12. Cap the tube and shake on a reciprocating shaker at ~150-200 excursions/minute for ~1 minute.

13. Centrifuge the mixture for ~2-3 minutes at ~2000 rpm.

14. Draw off the upper (ethyl acetate) phase, using a Pasteur pipet, and transfer to a 125-mL evaporation flask.

15. Extract the remaining aqueous phase, as before, one additional time with 10 mL ethyl acetate.

16. Centrifuge as before, then draw off the ethyl acetate layer and combine with first in the same evaporation flask from Step 14.

17. Add 2 mL of 10M NaOH solution to the remaining aqueous phase from Step 16.

18. Extract the basified solution, as in Step 12, two times with 10 mL each of ethyl acetate. After each extraction, centrifuge as before, then draw off the ethyl acetate layer and combine in the same evaporation flask from Step 16.

19. Add 1 mL HPLC water to the combined ethyl acetate extracts and concentrate on a rotary evaporator, set at ≤40 °C, until only aqueous remains. Approximately 5 mL of methanol can be added at this point to aid in the reduction of the aqueous phase to ~1 mL.

20. Quantitatively transfer the concentrate to a 16 × 100 mm test tube calibrated at 10.0 mL. Use 1 mL of HPLC-grade methanol, then 3-4 rinses of methanol:water (50:50, v/v) to assist in the transfer. Bring to a final volume of 10.0 mL with methanol:water (50:50, v/v). Invert to mix.

21. Centrifuge the mixture for ~2-3 minutes at ~2000 rpm.

22. Transfer a 1.0-mL aliquot to a 13 × 100 mm test tube. Add 3.0 mL methanol:water (50:50, v/v). Mix well. Submit to HPLC for negative-ion analysis. 1 mL = 0.25 g of sample.

23. Transfer a 2.0-mL aliquot to a 13 × 100 mm test tube. To this, add 40 µL of a 10% ammonia solution. Mix well. Submit to HPLC for positive-ion analysis. 1 mL = 1.0 g of sample.

8 HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS

Note: The instrument (HPLC/MS/MS system), column and conditions stated in the method have been satisfactory for the matrices being analyzed. The specific instrument, column packing, mobile phase, column temperature and flow rate listed are typical conditions for this analysis. Alternate columns may be used depending on the need to resolve analyte and/or interfering responses. Specific instrument and chromatographic conditions used will be noted on each chromatographic run and will not otherwise be documented.

8.1 Operating Conditions

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

Note: NF-149 consists of two isomers. The Z-isomer represents the biologically active ingredient and is most prevalent, the E-isomer represents a degradate. Two chromatographic responses are thus potentially produced, in varying degrees of intensity.

Instrument: Applied BioSystems/MDS Sciex API 4000 LC/MS/MS system with a Shimadzu SIL-HTA autosampler, an integrated Shimadzu chromatograph consisting of (2) LC-10AD*vp* Liquid Chromatograph units and a DGU-14A Degasser. The system is

controlled and data processed by Applied BioSystems/MDS Sciex Analyst Software.

HPLC column: 15 cm × 2.0 mm i.d. Phenomenex Luna C8 (2), 3 µ particle size

Mobile phase: *Fisher* water, *Burdick and Jackson* acetonitrile, *Fisher* ammonium acetate, *Fisher* acetic acid

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

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

Gradient:

Time (min)	Mobile Phase A (%)	Mobile Phase B (%)
0.0	90	10
2.0	50	50
10.0-15.0	20	80
16.0-17.0	0	100
18.0-23	90	10

Divert valve: Programmed to divert LC flow from column to waste (bypassing detector) from 0 to 5.5 minutes and again from 8.2 to 12.0 minutes and 15.8 to 23 minutes. LC flow is directed to detector during the 5.5 to 8.2 minute and 12.0 to 15.8 minute windows. Diversion time settings can be adjusted as necessary depending on the retention times of the analytes.

Flow rate: 0.2 mL/min

Interface: TIS (turbo ion spray)

Ionization mode: Negative (−)

Acquisition mode: MRM

Source temperature: 300 °C

Curtain gas: Nitrogen @ 15

Collision gas: Nitrogen @ setting of "6"

Injection volume: 20 µL

Column temperature: 35 °C

Transitions monitored:

	Ion, *m/z* Q1	Ion, *m/z* Q3	Time, *ms*	CE,v	
NF-149 (both Z and E-isomers:	411.2	217.9	150	-30	(quantitation)
	411.2	204.0	150	-50	(confirmation)
	411.2	391.0	150	-14	(confirmation)
149-F11:	379.1	242.9	150	-20	(quantitation)
	379.1	222.9	150	-40	(confirmation)
	379.1	315.0	150	-12	(confirmation)
149-F6:	223.9	180.9	150	-24	(quantitation)
	223.9	160.9	150	-38	(confirmation)

Retention times:
149-F6: ~6.6 minutes
149-F11: ~7.2 minutes
NF-149 E-isomer: ~13.6 minutes
NF-149 Z-isomer: ~14.6 minutes

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

Instrument: PE Sciex API 2000 LC/MS/MS system with a Perkin Elmer series 200 autosampler, an integrated Shimadzu chromatograph consisting of (2) LC-10AD*vp* Liquid Chromatograph units, a DGU-14A Degasser, and a SCL-10A*vp* System Controller. The system is controlled and data processed by PE Sciex Analyst Software.

HPLC column: 15 cm × 2.0 mm i.d. Phenomenex Luna C8 (2), 3 µ particle size

Mobile phase: *Fisher* water, *Burdick and Jackson* acetonitrile, *Fisher* ammonium acetate, *EM Science* ammonia (10%) solution

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

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

Gradient:

Time (min)	Mobile Phase A (%)	Mobile Phase B (%)
0	90	10
6.0-9.0	0	100
10.0-13.0	90	10

Divert valve: Programmed to divert LC flow from column to waste (bypassing detector) from 0 to 4.2 minutes and again from 9.2 to 13 minutes. LC flow is directed to detector during the 4.2 to 9.2 minute window. Diversion time settings can be adjusted as necessary depending on the retention times of the analytes.

Flow rate: 0.2 mL/min

Interface: TIS (turbo ion spray)

Ionization mode: Positive (+)

Acquisition mode: MRM

Source temperature: 500 °C

Curtain gas: Nitrogen @ 35

Collision gas: Nitrogen @ setting of "8"

Injection volume: 20 µL

Column temperature: 35 °C

Transitions monitored:

	Ion, *m/z* Q1	Ion, *m/z* Q3	Time, *ms*	CE, *v*	
149-F:	295.0	241.1	150	19	(quantitation)
	295.0	222.5	150	17	(confirmation)
	295.0	202.7	150	41	(confirmation)
149-F1:	225.0	165.0	150	39	(quantitation)
	225.0	185.1	150	31	(confirmation)
	225.0	205.0	150	27	(confirmation)

Retention times: 149-F1: ~4.9 minutes
149-F: ~7.5 minutes

8.2 Sample Analysis

Prepare a five-point standard curve by injecting constant volumes of mixed standard solutions. Use constant volume injections for sample extracts as well. Sample responses not bracketed by the standard curve require dilution and reinjection. Inject a curve check standard every 3-5 sample injections.

9 CALCULATIONS

Calculations for instrumental analysis are conducted using a validated software application to create a standard curve based on linear regression. The regression functions are used to calculate a best fit line (from a set of standard concentrations in ng/mL versus peak response) and to determine concentrations of the analyte found during sample analysis from the calculated best fit line. Use 1/x weighting.

The equation used for the least squares fit is:

$$y = mx + b$$

where:

y	=	peak response of analyte (for NF-149, peak response is the sum of the peak responses for both Z and E isomers)
x	=	ng/mL found for peak of interest
m	=	slope
b	=	y-intercept

The calculations for ppb found and percent recovery (for fortified samples) are:

1. The amount of analyte (in ppb) found in the sample is calculated according to the following equation:

   - Analytes analyzed by positive-ion HPLC analysis:

$$ppb = ng/mL\ found \times \frac{HPLC\ final\ vol.\ 1\ (mL)}{sample\ wt.\ (g)} \times \frac{mL\ ext.\ solv.}{mL\ aliq.\ 1} \times HPLC\ dil.\ factor$$

- Analytes analyzed by negative-ion HPLC analysis:

$$ppb = ng/mL\ found \times \frac{HPLC\ final\ vol.\ 1\ (mL)}{sample\ wt.\ (g)} \times \frac{mL\ ext.\ solv.}{mL\ aliq.\ 1} \times \frac{HPLC\ final\ vol.\ 2\ (mL)}{mL\ aliq.\ 2} \times HPLC\ dil.\ factor$$

where:

ng/mL found	=	ng/mL of analyte found from standard curve
sample wt. (g)	=	gram weight of sample extracted (typically 20.0 g)
mL ext. solv.	=	final volume of extraction solvent (typically 300 mL)
mL aliq. 1	=	volume of sample extract processed through remainder of method (typically 150 mL)
HPLC final vol. 1	=	final volume of HPLC-ready extract for positive-ion analyses prior to basification (typically 10.0 mL)
mL aliq. 2	=	volume of positive-ion HPLC-ready extract further diluted for negative-ion HPLC analysis (typically 1.0 mL)
HPLC final vol. 2	=	final volume of HPLC-ready extract for negative-ion analysis (typically 4.0 mL)
HPLC dil. factor	=	dilution of sample extract required to produce an analyte response bracketed by standards

2. The percent recovery for fortified control samples is calculated as follows:

$$\%\ Recovery = \frac{ppb\ found\ in\ fortified\ control - ppb\ found\ in\ control}{fortification\ level\ (ppb)}$$

10 REFERENCES

1. Study No. AA050702, "Terrestrial Field Soil Dissipation of Cyflufenamid," Nippon Soda Co., Ltd., MRID No. 47638209.

2. Brewin, S.A., "NF-149 and Metabolites: Development and Validation of Methodology for the Determination of Residues in Soils from Three Sites in Southern France, Northern France and Germany and for the Determination of Residues in Soil and Water from a Site in the United Kingdom," Study Report RD-II 02006, Nippon Soda Co., Ltd., Tokyo, Japan, January 31, 2002.

3. Method Modifications to the analytical method found in Nippon Soda Co., Ltd. Study Report RD-II 02006 dated January 31, 2002, entitled "NF-149 and Metabolites: Development and Validation of Methodology for the Determination of Residues in Soils from Three Sites in Southern France, Northern France and Germany and for the Determination of Residues in Soil and Water from a Site in the United Kingdom," Morse Laboratories, Inc., October 21, 2005.

Method author: Gary L. Westberg

ANALYSIS FLOWCHART

Sample (20.0 g)

- weigh sample into 250 mL centrifuge bottle
- add 100 mL acetone

Shake

- using reciprocating shaker
- at ~100 excursions/minute for ~30 minutes
- centrifuge mixture at ~2000 rpm for ~10 minutes

Filter

- pass supernatant thru glass wool into a 500-mL polyethylene bottle

Re-extract

- solids remaining in centrifuge bottle 1x with 100 mL acetone, followed 1x with 80 mL of 2M NH_4Cl:MeOH, each using same reciprocating shaking procedure as before
- after each extraction, centrifuge at ~2000 rpm for ~10 minutes

Filter

- pass each supernatant thru glass wool into same 500-mL polyethylene bottle as above, combining all 3 extracts
- add 10 mL DI water, mix, transfer mixture to a 500-mL graduated mixing cylinder, bring to final volume of 300 mL with methanol
- return measured solution to polyethylene bottle. Transfer 150 mL to a 500-mL evaporation flask

Concentrate

- @ ≤40°C to aqueous phase (~30 mL) on rotary evaporator
- transfer concentrate to a 50-mL polypropylene tube

Aqueous (acidic)/ethyl acetate partition

- add ~2g NaCl and 1 mL 1M HCl to concentrate and extract 2x with 10 mL ethyl acetate each time.
- after each extraction, centrifuge at ~2000 rpm for ~2-3 minutes, then draw off each ethyl acetate layer and combine in a 125-mL evaporation flask

Aqueous (basic)/ethyl acetate partition

- add 2 mL 10M NaOH to aqueous remainder and extract resulting basic solution 2x with 10 mL ethyl acetate each time
- After each extraction, centrifuge at ~2000 rpm for ~2-3 minutes, then draw off each ethyl acetate layer and combine in the same 125-mL evaporation flask as above. Add 1 mL HPLC water.

Concentrate

- @ ≤40°C to aqueous phase (~1 mL w/ aid of MeOH) on rotary evap.
- transfer concentrate to 16 x 100 mm test tube and bring to final volume of 10 mL with MeOH and MeOH:water rinses
- centrifuge mixture @ ~2000 rpm for ~2-3 minutes

- transfer 2 mL supernatant to 13 x 100 mm test tube
- add 40 µL 10% ammonia solution
- mix well

HPLC/MS/MS (+)

- transfer 1 mL supernatant to 13 x 100 mm test tube
- add 3 mL MeOH;water (50:50, v/v)
- mix well

HPLC/MS/MS (−)