2. BACKGROUND

NNI-0001 is an insecticide currently being developed by Bayer CropScience with potential uses in several crops including vegetables and orchards.

An analytical method was developed for the analysis of NNI-0001 and its metabolite NNI-0001-des-iodo in water. The method was initially validated in analytical method 00838 (MR-134/03): "Determination of NNI-0001 and NNI-0001-des-iodo In Drinking and Surface Water by HPLC-MS/MS, 2004"[1]. This method used matrix-matched calibration standards and during the independent laboratory validation study[2] (ILV) in addition to validating the method as written, method 00838 was also validated using calibration solutions prepared with deionized water.

This method, based on analytical method 00838, was prepared on completion of the ILV study and uses calibration solutions prepared with deionized water.

The structures for these compounds are presented in Appendix 2.

Typical recovery results are presented in Appendix 3, and the data shown were obtained from the independent laboratory validation study.

3. PRINCIPLE

Before analysis water samples are diluted with acidified acetonitrile, and analyzed by LC/MS/MS. The LC/MS/MS technique allows quantitation of all analytical targets with a high inherent specificity and without the need of derivatization for the more polar analytes.

Additional summaries outlining the method parameters and method characteristics are presented in Table 1 and Table 2.

4. APPARATUS

Use as a guide; equivalent apparatus may be substituted.

- VWR Pyrex® Brand volumetric pipets, glass class A (Assorted Volumes)
- Rainin Microman® Classic positive-displacement pipettes (Cat. No.:M-50 and M-250)
- VWR Pyrex® Brand graduated cylinder (Cat. No.: 24760-100)
- VWR Pyrex® Brand volumetric flasks, glass class A (Assorted Volumes)
- VWR Pyrex® Brand disposable Pasteur pipets (Cat. No.: 53283-910 & 53283-914)
- National Scientific LC vials, Snap-Its (Cat. No.: C4011-5)
- National Scientific LC vial Snap-It Seals, (Cat. No.: C4011-55)
- Phenomenex Aqua™ 5µ C18 150 x 4.6 mm Column (Part No.: 00F-4299-E0)
- Applied Biosystems PE Sciex 4000 LC/MS/MS System using Analyst Version 1.4.1.
- Applied Biosystems Turbo Ionspray Interface.
- Shimadzu LC-10AD VP HPLC pumps (two), Shimadzu SCL-10AVP Controller with a Gilson 215 Liquid handler and Gilson 819 Valve Actuator.
- Analytical Balance with accuracy of ± 0.0001 grams for analytical standards.
- VWR Centrifuge tubes,50mL, 25x20 PP (Cat. No. 21008-169)

5. REAGENTS

Use as a guide; equivalents or different manufactures (brands) may be substituted.

- Acetonitrile, EM Science Omnisolv, (VWR Cat. No.: EM-AX0145-1)
- Deionized Water filtered through a Milli-Q water system or:
- Water, EM Science Omnisolv, (VWR Cat. No.: EM-WX0004-1)
- Acetic Acid, Guaranteed Reagent, (VRW Cat. No.: EM-AX0073-14)
- Certified analytical reference standards of NNI-0001 and NNI-0001-des-iodo.

6. PREPARATION OF ANALYTICAL STANDARDS

Note: *The following procedure is an example description of how standard solutions may be prepared. Standards may be prepared as mixed solutions by dilution from individual stock solutions or prepared individually. Alternate or additional standards of appropriate weight and volume may be prepared as needed.*

Class "A" volumetric glassware or calibrated pipets should be used in the preparation of all analytical standards. All standard solutions should be stored in amber glass bottles at or below 10°C when not in use. Solutions should be allowed to warm to room temperature prior to use.

6.1 Primary Stock Standard Solutions

Prepare individual 100 µg/mL stock solutions of NNI-0001 and NNI-0001-des-iodo by placing 0.0100 grams of each analyte in separate 100 mL volumetric flasks. Dilute to volume with acetonitrile.

Note: Corrections for standard purities should be applied when expressing standard concentrations. For Example: If an analytical standard material has a purity of 98.5%, then 0.0102 grams (0.0100 g / 0.985) would be required to prepare a 100 µg/mL stock solution.

The stock standard solutions are stable for a minimum of 6 months when stored in the dark at ≤-18°C.

6.2 Fortification Standard Solutions

- Prepare a stock 10 µg/mL solution containing a mixture of NNI-0001 and NNI-0001-des-iodo by taking a 10.0 mL aliquot of each stock solution and diluting to 100 mL with acetonitrile.

- Prepare a 100 ng/mL solution containing a mixture of NNI-0001 and NNI-0001-des-iodo by taking a 1.0 mL aliquot of 10 µg/mL stock solution and diluting to 100-mL with acetonitrile.

- Prepare a 10.0 ng/mL fortification solution containing a mixture of NNI-0001 and NNI-0001-des-iodo by taking a 10.0 mL aliquot of the 100 ng/mL solution and diluting to 100 mL with acetonitrile.

- Prepare a 1.00 ng/mL fortification solution containing a mixture of NNI-0001 and NNI-0001-des-iodo by taking a 10.0 mL aliquot of the 10.0 ng/mL solution and diluting to 100 mL with acetonitrile.

The fortification standard solutions are stable for a minimum of 6 months when stored in the dark at ≤4°C.

6.3 Calibration Standard Solutions

Prepare working calibration solutions consisting of 0.01,0.025,0.04,0.05,0.1,0.5,1.0 and 5.0 ng/mL of NNI-0001 and NNI-0001-des-iodo diluted to 50mL with deionized water : acetonitrile : acetic acid (80:20:0.01 v/v/v).

Further calibration solutions may be prepared as needed.

7. SAMPLE PREPARATION

Note: Fortification experiments may be used for establishing and validating method efficiency as required.

7.1 Pipet 20.0 mL of the sample into a 50-mL disposable centrifuge tube.

7.2 Fortify the recovery samples at the desired fortification level with 1.0mL of the appropriate mixed standard solution prepared in acetonitrile (see Section 6.2 Fortification Standard Solutions).

7.3 Add by pipet 3.0mL of acetonitrile acidified with 0.08% acetic acid to the contents of the centrifuge tube

Note: The final volume of the solution should be 24.0mL. If the solution was fortified in 7.1 with a volume other than 1.0mL, adjust the volume added in 7.3 to bring the total volume to 24.0mL.

7.4 Mix well and transfer an aliquot into a LC vial for LC/MS/MS analysis

8. LC/MS/MS ANALYSIS

8.1 Sample Analysis

NNI-0001 and NNI-0001-des-iodo are analyzed by LC/MS/MS by external standard.

Install a 200µL injection loop on the autosampler, and inject a 250 µL aliquot (i.e. sufficient to fill the 200µL loop) of each test sample (or fortified sample matrix) from step 7.4 into the LC/MS/MS under the conditions presented in Appendix I. Variations in equipment or sample characteristics may require different injection volumes or slight modifications in the chromatographic or detector conditions listed in order to obtain adequate chromatographic peak shapes or sensitivity.

It is often beneficial to make several 'priming' injections of standards and/or samples prior to starting the LC/MS/MS analysis. Typically 4 to 6 priming injections are made. The results from these injections are not included in any calculations used in residue determinations. These injections help stabilize the LC/MS/MS response prior to running the analytical set.

8.2 LC/MS/MS Standard Calibration

Standardize the LC/MS/MS response under the conditions outlined in Appendix 1 by injecting a 250µL aliquot of each LC/MS/MS calibration solution interspersed with samples. Construct a standard curve for the analytes of interest by plotting the peak area vs. the standard concentration. Obtain the least square regression line of this data.

The equations used and example calculations are presented in Section 8.4.

8.3 Fortification Experiments

Note: Fortification experiments may be performed as needed to monitor method efficiency and reproducibility, but are not required when analysis of samples is performed for tolerance enforcement. Fortification experiments are intended to be used for data collection methods or establishing & validating method efficiency.

With each sample set, analyze an untreated control sample and one or more fortified control samples. Calculate recoveries using the following equation:

$$Recovery\ (\%) = \frac{(R - S)}{T} \times 100$$

Where: R = ppb of target analyte found in fortified sample
S = ppb of target analyte found in control sample, real or apparent
T = theoretical ppb in fortified sample

Recoveries are determined by analyzing fortified control samples alone or in conjunction with a sample set.

8.4 Calculations

NNI-0001 and NNI-0001-des-iodo residues were quantified using external standard linear regression analysis. A separate calibration curve was produced for each set of samples analyzed on the LC/MS/MS. A calibration curve was generated by linear regression of the standard peak area versus the standard concentrations in ng/mL using Applied Biosystems Analyst Software (Version 1.4.1), a computer-programmed data capturing system. The Analyst Software uses the MS/MS standard responses to calculate the regression coefficients M and B, respectively called slope and intercept, for each analytical set. The calibration points are weighted 1/x, and the intercept forced through zero to provide better fit near the limit of detection.

The standards were fit to the linear equation: $Y = MX + B$

where: X is the concentration of the reference standard in ng/mL
M is the calibration line slope
B is the calibration line intercept
Y is the area of the native peak

After regression coefficients were calculated, the residue in parts per billion was determined. The parts per billion (ppb) of NNI-0001 and NNI-0001-des-iodo in water was calculated using the following equation,

$$\text{NNI-0001 found (ppb)} = \frac{(Y-B) \times D}{M}$$

$$\text{Where Dilution Factor (D)} = \frac{\text{Final volume}(V_2)}{\text{Sample volume}\ (V_1)}$$

9. DISCUSSION

9.1 Independent Laboratory Validation (ILV)

This method is based on analytical method 00838 (MR-134/03)[2] which used matrix-matched calibration solutions. The ILV[1] was performed at Bayer CropScience, Stilwell, Kansas by using the method as written, and then reanalyzing the samples using calibration solutions prepared in deionized water. Acceptable results were obtained using both matrix-matched calibration solutions and calibration solutions prepared in deionized water.

The results from the ILV using calibration solutions prepared in deionized water are summarized in Appendix 3.

On completion of the ILV Bayer CropScience analytical method AM-003-W06-01 was issued, which was based on method 00838 (MR-134/03) and the results from the ILV.

9.2 Time Considerations

A set of thirteen samples can be prepared for analysis in 1 to 2 hours. The samples are analyzed overnight and the data processed the following working day.

10. REFERENCES

No.	Doc. No.	Report No.	**Author(s).** Title. Year.
1		RAAMX098	**Netzband, D.J.**, Independent Laboratory Validation of "Analytical Method 00838(MR-134/03) For The Determination Of NNI-0001 And NNI-0001-des-iod In Drinking and Surface Water by LC/MS/MS, 2006
2	P 684 037058	MR-134/03	**Brumhard, B.**, Analytical Method 00838 (MR-134/03) for the Determination Of NNI-0001 And NNI-0001-des-iodo In Drinking and Surface Water by HPLC-MS/MS, 2004

Appendix 1 Instrument Conditions

Equipment with equivalent or better sensitivity and performance may be substituted.

LC/MS/MS Parameters

NOTE: *As the LC/MS/MS system is used over time, system components slowly and gradually become contaminated which in turn decreases system performance. The chromatographic response and/or peak shape of one or more of the analytical targets may be gradually affected over time. Therefore, the given LC/MS/MS parameters listed below are guidelines of where to start. Each instrument has its own unique personality. Variations in equipment or sample characteristics may require slight modifications in the chromatographic or detector conditions listed in order to obtain adequate chromatographic peak shapes or sensitivity. These parameters should be optimized for the instrument and column actually used. Instrument parameters and mobile phase may be adjusted to improve separation from interfering peaks.*

Acquisition Parameters

Instrument Used:	Perkin Elmer Sciex API 4000 LC/MS/MS System
Interface:	PE Sciex Turbo Ion Spray Electrospray
Synchronization Mode:	LC Sync
AutoEquilibration:	Off
Acquisition Duration:	10.1 min. 0 sec.
Number of Scans:	1117
Periods in File:	1
Acquisition Module:	Acquisition Method
Software Version:	Analyst 1.4.1
Scan Type:	MRM
Polarity:	Positive
Scan Mode:	N/A
Resolution Q1:	Unit
Resolution Q3:	Unit
Intensity Threshold:	0 counts
Smart Settling:	Off
Settling Time:	0.0000 ms
MR Pause:	2.0000 ms
MCA:	No
Step Size:	0.00 amu

Appendix 1 (cont.)

LC/MS/MS Parameters (cont'd)

Acquisition Information (cont'd)

Nebulizer Gas Setting [L/min]	30
Curtain Gas Setting [L/min]	11
Collision Gas Setting [L/min]	8
Turbo Gas [L/min]	30
Turbo Gas Temperature [°C]	450
Resolution of Q1 and Q3	Unit (~0.7 amu)

Compound dependent:	NNI-0001	NNI-0001-des-iodo
Q1 Mass [amu]	408	557
Q3 Mass [amu]	274	282
Dwell [msec]	250	250
Ionization Mode	Positive	Positive
Ion Spray Voltage [V]	4800	4800
Entrance Potential [V]	8.6	8.6
Declustering Potential [V]	70	70
Collision Energy [V]	35	18
Collision Cell Exit Potential [V]	7.00	7.53

Auto Sampler Parameters

Autosampler Used:	Gilson 215 Autosampler
Syringe Size:	10000 µL
Injection Volume:	250 µL (fill 200 µL loop)
Pre-inject Flushes:	0
Post inject Flushes:	6
Air Cushion:	10 µL
Excess Volume:	10 µL
Sample Speed:	5.00 mL/min
Needle Level	5%
Inject Delay Time:	0.00 min
Needle Z-Direction Speed	Very Fast
Inject Time Delay	0.0 min
Loop Volume:	200 µL
Needle Flush Volume:	2000 µL
Flush Speed	5.00 mL/min
Port Flush Volume	2000 µL

Appendix 1 (cont.)

LC/MS/MS Parameters (cont'd)

HPLC Parameters

Pumps Used:	Two Shimadzu LC-10ADVP (High Pressure Mixer) pumps with a Shimadzu SCL-10 controller	
Minimum Pressure:	0.0 psi	
Maximum Pressure:	4000 psi	
Shutdown Time:	999.9 min.	
Column Temperature:	Ambient	
Column:	Manufacturer:	Phenomenex
	Type:	Aqua
	Phase:	C18
	Particle Size:	5 µM
	Diameter:	4.6 mm
	Length:	150 mm
	Mobile Phase A:	water/acetonitrile/acetic acid (900/100/0.1; v/v/v)
	Mobile Phase B:	acetonitrile/acetic acid (1000/0.1; v/v)

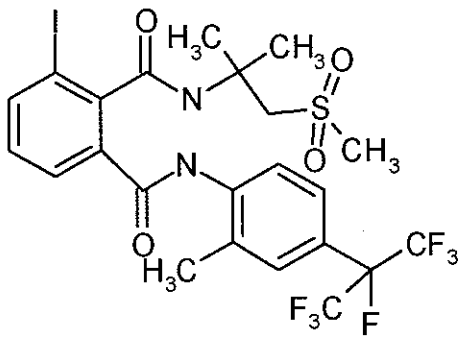
Gradient Program:

Time (min.)	Flow	A(%)	B(%)
0	1.0 mL/min.	60	40
1	1.0 mL/min	60	40
8	1.0 mL/min	10	90
10	1.0 mL/min	10	90
10.1	1.0 mL/min	60	40

Appendix 2 Structures

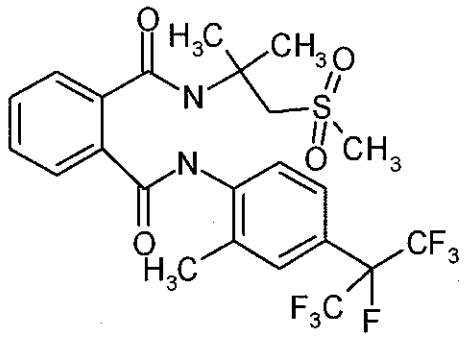
The structures for NNI-0001, its metabolite NNI-0001-des-iodo are presented below:

NNI-0001:
Structural formula:



CAS Number:	272451-65-7
Common name:	Flubendiamide
Code name:	NNI-0001
Chemical code:	AE 1302996
Chemical name:	*N*^{2}-[1,1-Dimethyl-2-(methylsulfonyl)ethyl]-3-iodo-*N*^{1}-[2-methyl-4-[1,2,2,2-tetrafluoro-1-(trifluoromethyl)ethyl]phenyl]-1,2-benzenedicarboxamide
Empirical formula:	$C_{23}H_{22}F_7IN_2O_4S$
Molecular weight:	682.4 g/mol

NNI-0001-des-iodo
Structural formula:



CAS Number:	not available
Common Name:	Deslodo Flubendiamide
Code name:	NNI-0001-des-iodo
Chemical codes:	AE 1303002, A-1
Chemical name:	N^2-(1,1-dimethyl-2-methylsulfonylethyl)- N^1-{2-methyl-4-[1,2,2,2-tetrafluoro-1-(trifluoromethyl)ethyl]phenyl}-phthalamide
Empirical formula:	$C_{23}H_{23}F_7N_2O_4S$
Molecular weight:	556.5 g/mo

Appendix 5 Revision History

Method #	Revision	Description
AM003-W06-01	01	Method prepared on completion of ILV[1] for method 00838[2]