1. BACKGROUND

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

The purpose of this study was to demonstrate that "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"[3], which is presented in Appendix 6, can be performed with acceptable recoveries for determination of the compounds NNI-0001 and NNI-0001-des-iodo at an independent laboratory having no prior experience with the method. The method was developed by Bayer CropScience AG, Development-Residues, Operator and Consumer Safety, at their laboratory in Monheim, Germany and reported by Bjoern Brumhard.

On initially reviewing the analytical method it was noted that parts of the method did not meet the criteria in OPPTS 860.1340 (The Residue Analytical Method), specifically: there were no estimates of the practical MDL and LOQ, and the method as written required the use of a sample of the untreated matrix as a blank for use in preparation of the matrix-matched standards.

As OPPTS 850.7100 (d)(2)(i) states that the laboratory conducting the ILV must use the method exactly as it is written, the analysis was performed as described in the method and then the samples were reanalyzed using calibration solutions prepared in deionized water. Section 12 of the analytical method states that without using matrix-matched standards recoveries of ~80% were obtained. If similar results are obtained in this study, the ILV will be considered successful.

A method detection limit (MDL) and calculated LOQ was determined using the data generated from the analyses using calibration solutions prepared in deionized water.

The study was performed in accordance with United States Environmental Protection Agency (EPA) Pesticide Assessment Guidelines and Good Laboratory Practices (and Ecological Effects Test Guidelines OPPTS 850.7100[1] and Residue Chemistry Test Guidelines, OPPTS 860.1340[2]). This validation fulfils the requirement that properly validated methods of analysis be utilized for the generation of pesticide residue data and for tolerance enforcement.

2. EXPERIMENTAL DETAILS

This study was conducted following an approved protocol. All amendments to the protocol were signed and dated by the Study Director and the Sponsor's Representative.

This study was initiated on January 31, 2006. The experimental phase of the study began on January 31, 2006 and concluded on February 9, 2006.

The following personnel were involved in the conduct of this study:

Derek J. Netzband
Senior Scientist
Environmental Research
17745 South Metcalf Avenue
Stilwell, Kansas 66085

Jessica Yin
Sr. Associate Scientist
Environmental Research
17745 South Metcalf Avenue
Stilwell, Kansas 66085

2.1 Test Substances

The test substances for this study were NNI-0001 and NNI-0001-des-iodo. See Appendix 3 for complete nomenclature and chemical structures.

2.2 Analytical Reference Substances

The test substances also served as the analytical reference substances. See Appendix 3 for complete nomenclature, chemical structures and reference information for the reference substances. The test and reference substances were stored in a freezer until used to prepare fortification and calibration solutions. The stock solutions were stored in a freezer at an average temperature of -23°C and the calibration and fortification solutions were stored refrigerated at an average temperature of 9°C when not in use.

2.3 Test System

The test system will be sub-samples of drinking (finished water) and surface water (raw water) obtained from Bayer CropScience Study Number 04RAOAY001[4], Surface Water Monitoring for Residues of Oxadiazon in High Use Areas in the United States. Characterization data for the water is presented in Appendix 5.

Spiked waters were maintained in a refrigerator except when removed to prepare for analysis.

2.4 Method Summary

Each analytical set included one reagent blank, two unfortified control samples, five samples fortified at the LOQ (0.05ng/mL(ppb)) and five samples fortified at 10x LOQ (0.5ng/mL)

Twenty milliliters of the water sample was pipetted into a 50-mL disposable centrifuge tube. Fortified samples were prepared by adding 1.0 mL of a mixed standard for each fortification level: 0.05 and 0.50ng/mL. Samples were then diluted to 24.0 mL by pipetting 3.0 mL of acidified acetonitrile with 0.08% acetic acid into each centrifuge tube. After mixing well, an aliquot of the sample was transferred into an HPLC vial for analysis by electrospray LC/MS/MS.

2.5 Instrumentation

- Sciex API 4000 LC/MS/MS System (Applied Biosystems)
- Shimadzu LC-10AD VP HPLC Pumps (2) with a High Pressure Mixer and Shimadzu SCL-10A VP Pump Controller
- Gilson 215 Series Autosampler

2.6 HPLC Conditions

Column: Phenomenex Aqua™ 5µ C18 125Å,
Length 150 mm x 4.6 mm i.d.,
Particle size 5 µm,
Part. No.: 00F-4299-E0

Column oven temperature: Ambient

Injection Volume: 250 µL (to fill 200µL loop)
Mobile phase: A: water / acetonitrile / acetic acid (900/100/0.1; v/v/v)
B: acetonitrile / acetic acid (1000/0.1; v/v)

Flow rate (column): 1.0 mL/min

Retention times:
NNI-0001: approx. 7.8 min
NNI-0001-des-iodo: approx. 7.2 min

HPLC Gradient Parameters

Time [min]	% A	% B
0	60	40
1	60	40
8	10	90
10	10	90
10.1	System controller	Stop

2.7 MS/MS Conditions

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 (IS) [V]	4800	4800
Entrance Potential (EP) [V]	8.6	8.6
Declustering Potential (DP) [V]	70	70
Collision Energy (CE) [V]	35	18
Collision Cell Exit Potential (CXP) [V]	7.00	7.53

2.8 Calculations

An example calculation for NNI-0001 from sample drinking water LOQ spike 3, which was analyzed during the study, is shown below. This sample was fortified with 0.05ppb NNI-0001 and NNI-0001-des-iodo. The chromatogram used in this example is presented in Appendix 2 (Chromatogram 6).

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 peak area

The calibration points were weighted 1/x, and the intercept forced through zero to provide better fit near the limit of detection. The example shown below is for the calculation of NNI-0001 residues. NNI-0001-des-iodo residues are calculated in a similar fashion.

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

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

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

V_1	V_2	Y	M	B
20mL	24mL	9538.1	208000	0

From the above equations:

Dilution Factor (D) = $\frac{24}{20}$ = 1.2

NNI-0001 found = $\frac{(9538.1-0) \times 1.2}{208000}$ = 0.05503ng/mL

Therefore, when analyzed against calibration solutions prepared using deionized water, sample drinking water LOQ spike 3 contains 0.05503ng/mL NNI-0001.

As the sample was fortified with known amounts of analyte prior to extraction, the percent recovery was determined using the following equation:

$$\% \text{ Recovery} = \frac{(\text{analyte found in spike(ppb)} - \text{analyte found in control (ppb)}) \times 100}{\text{analyte added (ppb)}}$$

Therefore, for sample drinking water LOQ spike 3, the NNI-0001 recovery may be calculated as follows:

$$\% \text{ Recovery} = \frac{0.05503 - 0 \times 100}{0.05} = 110\%$$

Remark: Calculations were performed using the LC/MS/MS software *Analyst (version 1.4.1)*. The example calculation was performed using the area values reported by the instrument. The instrument software carries additional figures not shown in the intermediate results. The instrument software calculated a recovery of 0.05505ng/mL and a percent recovery of 110% for this sample. The *Analyst* results for this analytical set are presented in Appendix 4.

3. RESULTS

3.1 LC-MS/MS Verification

An Applied Biosystems API 4000 MS was used in place of the MS system specified in the original method. The API 4000 conditions used for this study are recorded in the raw data and Section 2.7 of this report. Calibration standards were injected prior to the method validation trial to determine the analyte retention times and instrumental sensitivity.

3.2 Method Trial Phase

The first independent laboratory trial was performed on surface water on January 31, 2006 and followed the method as written except for instrument specific parameters. The first analyses were performed using a 50µL injection volume as opposed to the 220µL volume specified in the method and erratic results were obtained. The reason for the erratic recoveries was hypothesized to be an insufficient response on the MS. In order to improve the analyte response, the analyses were repeated using the same samples with an increased injection volume (80µL) and was reinjected on February 1, 2006. The results from this analysis also showed significant variability. The samples were injected for a third time on February 2, 2006 after replacing the 100µL injection loop on the Gilson Autosampler with a 200µL injection loop and increasing the injection volume to 250µL (i.e. a sufficient volume to fill the loop). The results from this analysis were found to give acceptable recoveries using calibration solutions prepared with surface water. Inspection of the results for surface water using calibration solutions prepared in deionized showed that while acceptable recoveries were obtained for the fortified samples the calibration standard curve was poor. A new calibration curve was prepared using deionized water on February 3, 2006 and the surface water samples analyzed using the new calibration solutions on the same day. Acceptable recoveries were obtained from this analysis.

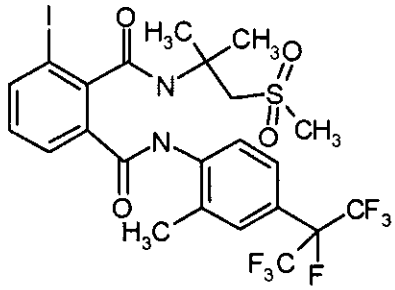
The first trial on drinking water was performed on February 6, 2006 using a 200µL injection loop and injecting 250µL of sample in order to fill the loop. The results from both sets of analyses were rejected due to poor calibration curves. The samples were reinjected on February 7, 2006 and while acceptable results were obtained with the calibration curve prepared using deionized water, the results using the calibration solutions prepared with drinking water were rejected due to a poor calibration curve. A new calibration curve was prepared using drinking water on February 8, 2006 and the drinking water samples analyzed using the new calibration solutions on the same day. Acceptable recoveries were obtained from this analysis.

A summary of the results obtained are presented below, and the complete results may be found in Tables 1 and 2.

Appendix 3 Identity and Purity of the Test and Reference Materials Used

NNI-0001:

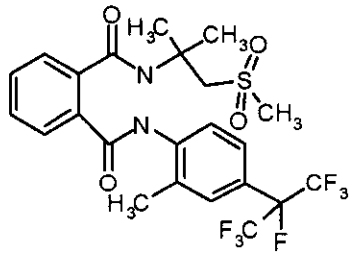
Structural formula:



CAS Number:	[272451-65-7]
Common name:	Flubendiamide
Chemical code:	AE 1302996
CAS 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:	$C_{23} H_{22} F_7 I N_2 O_4 S$
Molecular weight:	682.4 g/mol
Batch:	K-1155
Purity	98.5%
Expiration date	10/21/2006

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:	$C_{23} H_{23} F_7 N_2 O_4 S$
Molecular weight:	556.5 g/mol
Batch	K-1529
Purity	99.2%,
Expiration date	11/8/2008