

1. BACKGROUND AND SUMMARY

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

This method of analysis is suitable for the determination of the total extractable residues of NNI-0001, NNI-0001-des-iodo, NNI-0001-3-OH, NNI-0001-3-OH-hydroxyperfluoroalkyl and NNI-0001-benzoic acid in soil and sediment. The lower limit of quantitation for each analyte has been set at 0.5 µg/kg (0.5ppb).

The method is based on a previous method and validation study³ and incorporates modifications suggested by an independent laboratory validation⁴. Minor modifications also include the use of disposable glassware and labware items and minor changes to the microwave extraction to provide better temperature control independent of the type of glassware and stir bars used.

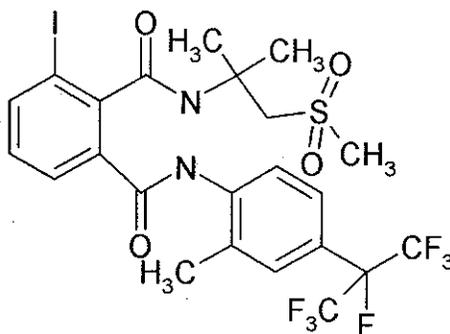
Residues of NNI-0001 and its metabolites in soils are extracted from soil or sediments using a temperature-controlled microwave oven. A 20g sample aliquot is extracted with 40 mL of 50/50 acetonitrile / 1% aqueous acetic acid with six minute extraction time at a maximum of 50°C and a maximum energy of 350 watts. After centrifugation the final extract is analyzed by LC/MS/MS. Quantification of residues is based on the use of isotopically labeled internal standards and comparison of peak areas with those of known standards.

The data generated during the method validation study³ demonstrated that the limits of quantitation (LOQ's) were at or below the target LOQ's of 0.5 µg/kg (0.5ppb) for NNI-0001, NNI-0001-des-iodo, NNI-0001-3-OH, NNI-0001-3-OH-hydroxyperfluoroalkyl and NNI-0001-benzoic acid, and that the MDL's were at or below 0.15 µg/kg.

2. TEST SUBSTANCES AND TEST SUBSTANCE STRUCTURES

NNI-0001:

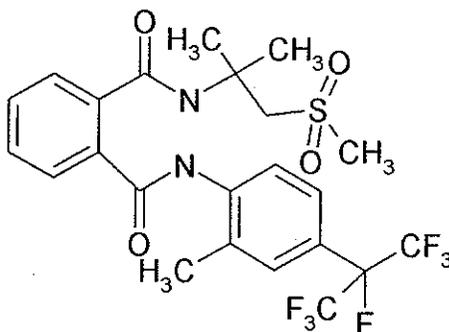
Structural formula:



Common name:	not available
Code name:	NNI-0001
Chemical code:	AE 1302996
Chemical name:	3-Iodo-N ² -(2-methanesulfonyl-1,1-dimethyl-ethyl)- N ¹ -[2-methyl-4-(1,2,2,2-tetrafluoro-1-trifluoromethyl-ethyl)-phenyl]-phthalamide
Empirical formula:	C ₂₃ H ₂₂ F ₇ I N ₂ O ₄ S
Molecular weight:	682.4 g/mol

NNI-0001-des-iodo:

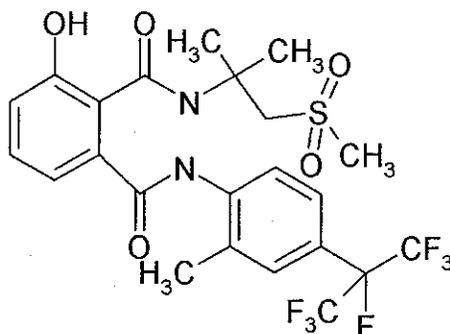
Structural formula:



Common Name:	not available
Code name:	NNI-0001-des-iodo
Chemical codes:	AE 1303002, A-1
Chemical name:	N ² -(1,1-dimethyl-2-methylsulfonyl-ethyl)- 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

NNI-0001-3-OH:

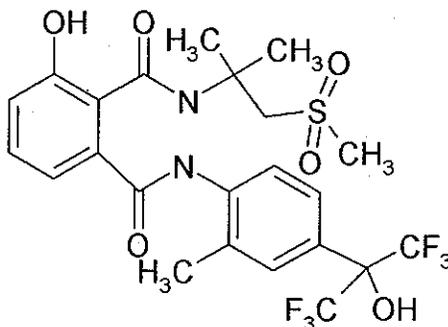
Structural formula:



Common Name: not available
 Code name: NNI-0001-3-OH
 Chemical codes: AE 1423032, A-2
 Chemical name: 3-hydroxy-N²-(2-mesyloxy-1,1-dimethyl-ethyl)-N¹-{2-methyl-4-[1,2,2,2-tetrafluoro-1-(trifluoromethyl)ethyl]phenyl}-phthalimide
 Empirical formula: C₂₃ H₂₃ F₇ N₂ O₅ S
 Molecular weight: 572.5 g/mol

NNI-0001-3-OH-hydroxyfluoroalkyl:

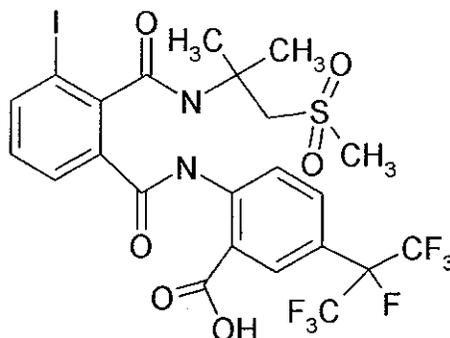
Structural formula:



Common name: not available
 Code name: NNI-0001-3-OH-hydroxyperfluoroalkyl
 Chemical codes: AE 1423031, A10, dihydroxy NNI-0001
 Chemical name: 3-hydroxy-N²-(2-mesyloxy-1,1-dimethyl-ethyl)-N¹-{2-methyl-4-[2,2,2-trifluoro-1-hydroxy-1-(trifluoromethyl)ethyl]phenyl}-phthalimide
 Empirical formula: C₂₃ H₂₄ F₆ N₂ O₆ S
 Molecular weight: 570.5 g/mol

NNI-0001- benzoic acid:

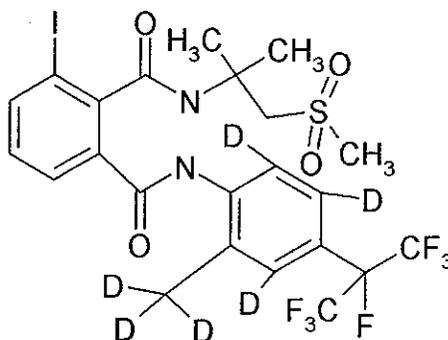
Structural formula:



Common name: not available
 Code name: NNI-0001-benzoic-acid
 Chemical codes: AE 1651796, A-18
 Chemical name: 2-({2-[(1,1-dimethyl-2-mesylethyl)carbamoyl]-3-iodo-benzoyl} amino)-5-[1,2,2,2-tetrafluoro-1-(trifluoromethyl)ethyl]benzoic acid
 Empirical formula: C₂₃ H₂₀ F₇ I N₂ O₆ S
 Molecular weight: 712.4 g/mol

NNI-0001-d6 (used as internal standard for the active analyte NNI-0001):

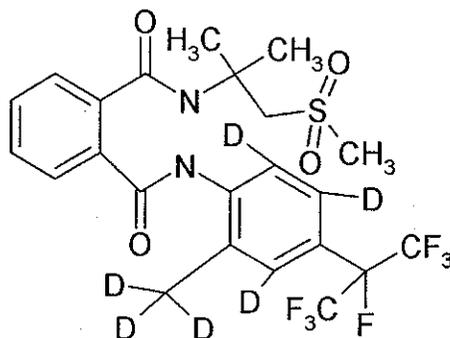
Structural formula:



Code name: NNI-0001-d6
 Chemical name: 3-iodo-N²-(2-mesyl-1,1-dimethyl-ethyl)- N¹-[2-(methyl-D₃)-4-(1,2,2,2-tetrafluoro-1-trifluoromethyl-ethyl)-phenyl-D₃]-phthalamide
 Empirical formula: C₂₃ H₁₆ D₆ F₇ I N₂ O₄ S
 Molecular weight: 688.4 g/mol

NNI-0001-des-iodo-d6 (internal standard for the metabolite NNI-0001-des-iodo):

Structural formula:



Code name:

NNI-0001-des-iodo-d6

Chemical code:

D6-A-1

Chemical name:

N^2 -(1,1-dimethyl-2-methylsulfonyl ethyl)- N^1 -{2-[D₃]methyl-4-[1,2,2,2-tetrafluoro-1-(trifluoromethyl)ethyl]phenyl}[3,5,6-D₃]phenyl}-phthalamide

Empirical formula:

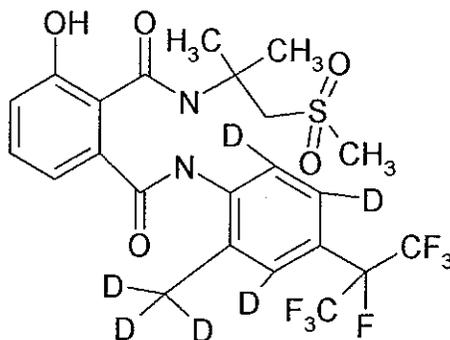
C₂₃ H₁₇ D₆ F₇ N₂ O₄ S

Molecular weight:

562.5 g/mol

NNI-0001-3-OH-d6 (used as internal standard for the metabolite NNI-0001-3-OH):

Structural formula:



Code name:

NNI-0001-3-OH-d6

Chemical code:

D6-A-2

Chemical name:

N^2 -(1,1-dimethyl-2-methylsulfonyl ethyl)-3-hydroxy- N^1 -{2-[D₃]methyl-4-[1,2,2,2-tetrafluoro-1-(trifluoromethyl)ethyl]-3,5,6-D₃]phenyl}-phthalamide

Empirical formula:

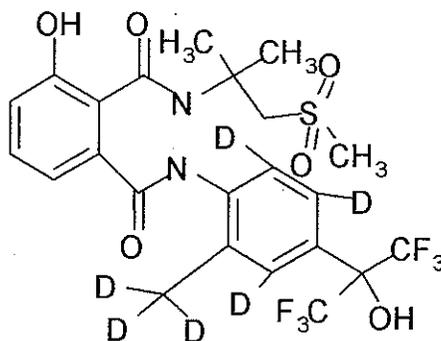
C₂₃ H₁₇ D₆ F₇ N₂ O₅ S

Molecular weight:

578.5 g/mol

NNI-0001-3-OH-hydroxyperfluoroalkyl-d6**(used as internal standard for the metabolite NNI-0001-3-OH-hydroxyperfluoroalkyl):**

Structural formula:



Code name:

NNI-0001-3-OH-hydroxyperfluoroalkyl-d6

Chemical code:

D6-A-10

Chemical name:

N²-(1,1-dimethyl-2-methylsulfonyl-ethyl)-3-hydroxy- N¹-(4-[1-hydroxy -2,2,2-trifluoro-1-(trifluoromethyl)-ethyl]- 2-[D₃]-methyl[3,5,6-D₃]phenyl}phthalamide

Empirical formula:

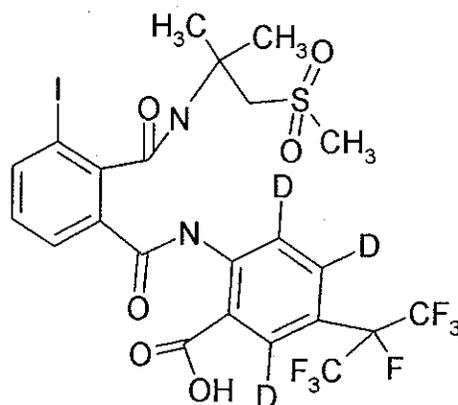
C₂₃ H₁₈ D₆ F₆ N₂ O₆ S

Molecular weight:

576.6 g/mol

NNI-0001- benzoic acid-d3**(used as internal standard for the metabolite NNI-0001-benzoic-acid):**

Structural formula



Code name:

NNI-0001-benzoic-acid-d3

Chemical code:

D3-A-18

Chemical name:

2-{[2-[(1,1-dimethyl-2-methylsulfonyl)amino]carbonyl]-3-iodophenyl}carbonyl]amino}-5-[1,2,2,2-tetrafluoro-1-(trifluoromethyl)ethyl][3,4,6-D₃]benzoic acid

Empirical formula:

C₂₃ H₂₀ D₃ F₇ I N₂ O₆ S

Molecular weight:

715.37 g/mol

3. MATERIALS AND APPARATUS

Brands listed are suggestions. Unless otherwise noted, equivalent brands and/or suppliers can be used.

3.1 Reagents/Solvents

Glacial acetic acid, (Fisher Cat. No. A490-212)

Acetonitrile Omni-Solv, (EM Science, Cat. No. AX0142)

Water Omni-Solv, HPLC Grade (EM Science, Cat. No. WX0004)

Certified analytical reference standards of NNI-0001, NNI-0001-des-iodo, NNI-0001-3-OH, NNI-0001-3-OH-hydroxyperfluoroalkyl and NNI-0001-benzoic acid

Deuterated standards of NNI-0001, NNI-0001-des-iodo, NNI-0001-3-OH, NNI-0001-3-OH-hydroxyperfluoroalkyl and NNI-0001-benzoic acid

3.2 Solutions

Acetonitrile / water - 80 / 20 v / v (for NNI-0001-benzoic acid stock standards): Combine 800 mL of acetonitrile and 200 mL of water in a 1 liter mixing cylinder. Stopper and mix well by inverting several times.

Acetonitrile / water / acetic acid - 400/ 600/ 1 v / v / v (for working standards): Combine 400 mL of acetonitrile, 600 mL water, and 1 mL glacial acetic acid in a 1 liter mixing cylinder. Stopper and mix well by inverting several times. (working standards)

Acetic acid, 1%, in ~50/50 v / v water / acetonitrile, (extraction solvent): Pour approximately 250 mL water into 500 mL cylinder. Add 10 mL concentrated acetic acid and fill to the 500 mL mark with water. Decant into a glass 1L container. Measure 500 mL of acetonitrile with a graduated cylinder and transfer into same glass container. Cap or stopper container and mix well by swirling or inverting.

1.5% glacial acetic acid in HPLC grade water (HPLC mobile phase component): Pour approximately 500 mL of water into a mixing cylinder. Add 15 mL of glacial acetic acid. Fill to 1L volume with water. Stopper and mix by inverting several times. Do not shake, to prevent dissolving more air into the solution. Sonicate for 2 to 5 minutes under vacuum to degas or use an in-line degasser.

Mobile phase is produced by high pressure mixing of the above solution with pure acetonitrile to produce the mobile phase gradient as outlined in the instrument conditions below. It has not been found necessary to sonicate the acetonitrile.

3.3 Instrumentation:

Microwave Extractor: Milestone, Ethos E, with fiber optic cable temperature probe and custom or flat tray carousel (See Section 6.3 for modification details.)

Sciex API 3000 LC/MS/MS System (Applied Biosystems)

Sciex Turbo IonSpray Electrospray Interface.

Shimadzu LC-10AD VP HPLC Pumps (2)
with 250 μ L High Pressure Mixer
and Shimadzu SCL-10A VP Pump Controller

Gilson 215 Autosampler

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

3.4 Equipment and Supplies

Balance for analytical standards: Accuracy \pm 0.1 mg, Mettler AT 201

Balance for samples: Accuracy \pm 0.01 g, Precisa 1000C - 3000D

Weighing spatulas

Ultrasonic bath: Branson

Centrifuge, Marathon 10K

Disposable pipettes

Glass Class A graduated cylinders, pipets, and volumetric flasks

Micropipetter, adjustable, 100-1000 μ L Eppendorf with disposable tips

Disposable 4 oz. glass jars with lids (Fisher catalog # 02-911-731)

Disposable, 1"-long, 5/16"-diameter magnetic stir bars
(Fisher catalog # 1451394)

HPLC vials and caps

Superspher 100 RP 18 HPLC column, (75mm x 4 mm x 4 μ m, pore size 100 nm)
Merck Cat. No.: 1.50980.0001, distributed in the US by VWR

Upchurch, ultra-low volume, inline, precolumn filter, Cat. No. A-318, with
A-102x, 0.5 μ m frits.

4. FORTIFICATION AND CALIBRATION SOLUTIONS

Note: All standard solutions must be stored in amber glass bottles.

All stock standard solutions will be stored in a freezer at $\leq -8^{\circ}\text{C}$ when not in use. All working and calibration standard solutions will be stored in a refrigerator at $4^{\circ}\text{C} \pm 3^{\circ}\text{C}$ when not in use. Solutions should be allowed to warm to room temperature prior to use. The following is an example of a procedure to follow in preparing standard solutions. Alternate or additional standards of appropriate concentration and volume may be prepared as needed.

Note: All reusable glassware should be baked in a muffle oven at $\sim 400^{\circ}\text{C}$ for at least 2 hours to remove possible contamination before use.

4.1 Standard Stock Solutions

Native, non-isotopically labeled materials:

1 mg/mL stock solution of NNI-0001:

Weigh approximately 10.0 mg (corrected for purity) NNI-0001 into a 10-mL volumetric flask. Dilute to volume with acetonitrile.

1 mg/mL stock solution of NNI-0001-des-iodo:

Weigh approximately 10.0 mg (corrected for purity) NNI-0001-des-iodo into a 10-mL volumetric flask. Dilute to volume with acetonitrile.

1 mg/mL stock solution of NNI-0001-3-OH:

Weigh approximately 10.0 mg (corrected for purity) NNI-0001-3-OH into a 10-mL volumetric flask. Dilute to volume with acetonitrile.

1 mg/mL stock solution of NNI-0001-3-OH-hydroxyperfluoroalkyl:

Weigh approximately 10.0 mg (corrected for purity) NNI-0001-3-OH-hydroxyperfluoroalkyl into a 10-mL volumetric flask. Dilute to volume with acetonitrile.

1 mg/mL stock solution of NNI-0001-benzoic acid:

Weigh approximately 10.0 mg (corrected for purity) NNI-0001-benzoic acid into a 10-mL volumetric flask. Dilute to volume with acetonitrile/water 80/20 (v/v).

Isotopically labeled materials:

1 mg/mL stock solution of NNI-0001-d6:

Weigh approximately 5.0 mg NNI-0001-6 into a 5-mL volumetric flask. Dilute to volume with acetonitrile.

1 mg/mL stock solution of NNI-0001-des-iodo-d6:

Weigh approximately 5.0 mg NNI-0001-des-iodo-d6 into a 5-mL volumetric flask. Dilute to volume with acetonitrile.

1 mg/mL stock solution of NNI-0001-3-OH-d6:

Weigh approximately 5.0 mg NNI-0001-3-OH-d6 into a 5-mL volumetric flask. Dilute to volume with acetonitrile.

1 mg/mL stock solution of NNI-0001-3-OH-hydroxyperfluoroalkyl-d6:

Weigh approximately 5.0 mg NNI-0001-3-OH-hydroxyperfluoroalkyl-d6 into a 5-mL volumetric flask. Dilute to volume with acetonitrile.

1 mg/mL stock solution of NNI-0001-benzoic acid-d3:

Weigh approximately 5.0 mg NNI-0001-benzoic acid-d3 into a 5-mL volumetric flask. Dilute to volume with acetonitrile/water 80/20 (v/v).

Remarks:

The standard stock solutions must be ultrasonicated for about one minute to achieve complete dissolution of the test substance.

4.2 Working Standard Solutions

Note: The working standard solutions are also used for sample fortification.

- (5MIX) 5 µg/mL solution of NNI-0001, NNI-0001-des-iodo, NNI-0001-3-OH, NNI-0001-3-OH-hydroxyperfluoroalkyl and NNI-0001-benzoic acid:
Pipette 0.5 mL each of the 1mg/mL stock standards of the native, non-deuterium-labeled analytes into a 100-mL volumetric flask and dilute to volume with acetonitrile/water/acetic acid (400/600/1, v/v/v).
- (250MIX) 250 ng/mL solution of NNI-0001, NNI-0001-des-iodo, NNI-0001-3-OH, NNI-0001-3-OH-hydroxyperfluoroalkyl and NNI-0001-benzoic acid:
Pipette 5 mL of (5MIX) into a 100-mL volumetric flask and dilute to volume with acetonitrile/water/acetic acid (400/600/1, v/v/v). (Use for 10xLOQ fortification)
- (100MIX) 100 ng/mL solution of NNI-0001, NNI-0001-des-iodo, NNI-0001-3-OH, NNI-0001-3-OH-hydroxyperfluoroalkyl and NNI-0001-benzoic acid:
Pipette 2 mL of (5MIX) into a 100-mL volumetric flask and dilute to volume with acetonitrile/water/acetic acid (400/600/1, v/v/v).

- (25MIX) 25 ng/mL solution of NNI-0001, NNI-0001-des-iodo, NNI-0001-3-OH, NNI-0001-3-OH-hydroxyperfluoroalkyl and NNI-0001-benzoic acid: Pipette 0.5 mL of (5MIX) into a 100-mL volumetric flask and dilute to volume with acetonitrile/water/acetic acid (400/600/1, v/v/v). (Use for fortification at LOQ)
- (2.5MIX) 2.5 ng/mL solution of NNI-0001, NNI-0001-des-iodo, NNI-0001-3-OH, NNI-0001-3-OH-hydroxyperfluoroalkyl and NNI-0001-benzoic acid: Pipette 5 mL of (25MIX) into a 50-mL volumetric flask and dilute to volume with acetonitrile/water/acetic acid (400/600/1, v/v/v).
- (10MIXd) 10 µg/mL solution of NNI-0001-d6, NNI-0001-des-iodo-d6, NNI-0001-3-OH-d6, and NNI-0001-3-OH-hydroxyperfluoro-alkyl-d6: Pipette 1.0 mL each of the 1mg/mL stock standards of the native, non-deuterium-labeled analytes into a 100-mL volumetric flask and dilute to volume with acetonitrile/water/acetic acid (400/600/1, v/v/v).
- (1MIXd) 1 µg/mL solution of NNI-0001-d6, NNI-0001-des-iodo-d6, NNI-0001-3-OH-d6, and NNI-0001-3-OH-hydroxyperfluoro-alkyl-d6: Pipette 5 mL of solution 10MIXd into a 50-mL volumetric flask and dilute to volume with acetonitrile/water/acetic acid (400/600/1, v/v/v).

4.3 Calibration Standard Solutions

Note: The concentration of the internal standards is 5ng/mL in each calibration solution.

(5 CAL)

5 ng/mL solution of NNI-0001, NNI-0001-des-iodo, NNI-0001-3-OH, NNI-0001-3-OH-hydroxyperfluoroalkyl and NNI-0001-benzoic acid containing 5ng/mL of NNI-0001-d6, NNI-0001-des-iodo-d6, NNI-0001-3-OH-d6 and NNI-0001-3-OH-hydroxyperfluoroalkyl-d6: Pipette 5.0 mL of (100MIX) and 0.5 mL of (1MIXd) into a 100-mL volumetric flask and dilute to volume with acetonitrile/water/acetic acid (400/600/1, v/v/v).

(1 CAL)

1 ng/mL solution of NNI-0001, NNI-0001-des-iodo, NNI-0001-3-OH, NNI-0001-3-OH-hydroxyperfluoroalkyl and NNI-0001-benzoic acid containing 5ng/mL of NNI-0001-d6, NNI-0001-des-iodo-d6, NNI-0001-3-OH-d6 and NNI-0001-3-OH-hydroxyperfluoroalkyl-d6: Pipette 1.0 mL of (100MIX) and 0.5 mL of (1MIXd) into a 100-mL volumetric flask and dilute to volume with acetonitrile/water/acetic acid (400/600/1, v/v/v).

(0.5 CAL)

0.5 ng/mL solution of NNI-0001, NNI-0001-des-iodo, NNI-0001-3-OH, NNI-0001-3-OH-hydroxyperfluoroalkyl and NNI-0001-benzoic acid containing 5ng/mL of NNI-0001-d6, NNI-0001-des-iodo-d6, NNI-0001-3-OH-d6 and NNI-0001-3-OH-hydroxyperfluoroalkyl-d6:

Pipette 2.0 mL of (2.5MIX) and 0.5 mL of (1MIXd) into a 100-mL volumetric flask and dilute to volume with acetonitrile/water/acetic acid (400/600/1, v/v/v).

(0.2 CAL)

0.2 ng/mL solution of NNI-0001, NNI-0001-des-iodo, NNI-0001-3-OH, NNI-0001-3-OH-hydroxyperfluoroalkyl and NNI-0001-benzoic acid containing 5ng/mL of NNI-0001-d6, NNI-0001-des-iodo-d6, NNI-0001-3-OH-d6 and NNI-0001-3-OH-hydroxyperfluoroalkyl-d6:

Pipette 8.0 mL of (2.5MIX) and 0.5 mL of (1MIXd) into a 100-mL volumetric flask and dilute to volume with acetonitrile/water/acetic acid (400/600/1, v/v/v).

(0.1 CAL)

0.1 ng/mL solution of NNI-0001, NNI-0001-des-iodo, NNI-0001-3-OH, NNI-0001-3-OH-hydroxyperfluoroalkyl and NNI-0001-benzoic acid containing 5ng/mL of NNI-0001-d6, NNI-0001-des-iodo-d6, NNI-0001-3-OH-d6 and NNI-0001-3-OH-hydroxyperfluoroalkyl-d6:

Pipette 4.0 mL of (2.5MIX) and 0.5 mL of (1MIXd) into a 100-mL volumetric flask and dilute to volume with acetonitrile/water/acetic acid (400/600/1, v/v/v).

(0.075 CAL)

0.075 ng/mL solution of NNI-0001, NNI-0001-des-iodo, NNI-0001-3-OH, NNI-0001-3-OH-hydroxyperfluoroalkyl and NNI-0001-benzoic acid containing 5ng/mL of NNI-0001-d6, NNI-0001-des-iodo-d6, NNI-0001-3-OH-d6 and NNI-0001-3-OH-hydroxyperfluoroalkyl-d6:

Pipette 3.0 mL of (2.5MIX) and 0.5 mL of (1MIXd) into a 100-mL volumetric flask and dilute to volume with acetonitrile/water/acetic acid (400/600/1, v/v/v).

(0.025 CAL)

0.025 ng/mL solution of NNI-0001, NNI-0001-des-iodo, NNI-0001-3-OH, NNI-0001-3-OH-hydroxyperfluoroalkyl and NNI-0001-benzoic acid containing 5ng/mL of NNI-0001-d6, NNI-0001-des-iodo-d6, NNI-0001-3-OH-d6 and NNI-0001-3-OH-hydroxyperfluoroalkyl-d6:

Pipette 1.0 mL of (2.5MIX) and 0.5 mL of (1MIXd) into a 100-mL volumetric flask and dilute to volume with acetonitrile/water/acetic acid (400/600/1, v/v/v).

4.4 Stability of the Calibration Standard Solutions

Stock standard solutions are considered stable for at least four months when stored in a refrigerator at $4^{\circ}\text{C} \pm 3^{\circ}\text{C}$ when not in use. Optionally, stock standards may be stored in a freezer at $\leq -8^{\circ}\text{C}$, for added protection. Working and calibration standard solutions are considered stable for at least four months when stored in a refrigerator at $4^{\circ}\text{C} \pm 3^{\circ}\text{C}$ when not in use.⁵ It is recommended that each batch of new standards be compared to previous batches to confirm that there has been no significant degradation.

5. SAFETY PRECAUTIONS

All available appropriate Material Safety Data Sheets should be available to the study personnel during the conduct of the method. General laboratory safety precautions should be taken.

6. PREPARATION AND EXTRACTION OF SOIL OR SEDIMENT SAMPLES

A method flow chart is presented in Appendix 3, and a summary of the analytical method parameters is presented in Table 6.

6.1 Sample Preparation

Samples should be thoroughly homogenized and stored frozen until sampled for extraction. Untreated control soil may be kept at ambient temperature. Fortified samples will be stored as other samples being analyzed.

6.2 Fortification

Concurrent recoveries may be obtained by analyzing control samples and control samples fortified with NNI-0001, NNI-0001-des-iodo, NNI-0001-3-OH, NNI-0001-3-OH-hydroxy-perfluoroalkyl and NNI-0001-benzoic-acid prior to the extraction procedure at and above the limit of quantitation.

Sample fortification is done by adding a certain amount of a fortification standard solution to 20 g of soil or sediment. After a waiting time of one hour between fortification and beginning of the extraction to allow for the standard to be soaked into the soil, the samples are extracted according to the procedure described in Section 6.3.

The following fortification levels may be used for concurrent recoveries:

- LOQ: 0.5 $\mu\text{g}/\text{kg}$: addition of 400 μL of (25MIX, 25ng/mL) to 20 g of soil or sediment
- 10x LOQ: 5 $\mu\text{g}/\text{kg}$: addition of 400 μL of (250MIX, 250ng/mL) to 20 g of soil or sediment

The preparation of the fortification standards is described in Section 4.2.

6.3 Extraction

Microwave Carousel/Rotor Modifications for Atmospheric Pressure Extraction with Temperature Feedback and Control:

Use one of the sample extraction jars, typically containing an untreated control sample, to hold the microwave fiber optic cable temperature control probe. Details of these modifications are as follows:

Temperature probe:

Take the cap off of a disposable 4 oz. glass jar and drill a hole on one side of the cap just big enough so that the temperature probe holder (thermo-well) fits tightly down into the sample solution, to be held just above the action of the stir bar. Drill another hole opposite from the first as a pressure vent.

Or...

Use a Teflon cover that fits over all the sample jars, with caps off, and has a hole over one of the jars just big enough so that the temperature probe holder (thermo-well) fits tightly down into the sample solution, to be held just above the action of the stir bar.

Carousel/Rotor:

Obtain from the manufacturer or modify an existing carousel/rotor to be a flat tray with center pivot (eg. a piece of Plexiglas cut to fit the high pressure carousel). The sample containers should be arranged evenly spaced around the center of the tray.

Notice: It is imperative to loosen the caps of the jars or use a loose fitting cover over the open jars before starting the microwave to allow for pressure relief.

1. Weigh 20 g of each soil or sediment sample to be analyzed into disposable 4 oz. glass jars containing a magnetic bar and having a lid.
2. Prepare at least one untreated control sample in one of the jars for use with the temperature probe.
3. Add 40 mL of a mixture of acetic acid, 1%, in ~50/50 v / v water / acetonitrile to each jar.
4. Place the jars into the microwave extractor, evenly spaced around the center of the carousel.
5. Insert the thermo-well into the untreated control sample and insert the fiber optic temperature probe into the thermo-well.
6. With the door open, turn on the manual magnetic stirrer control and check to see that the stir bars are turning but without splashing the samples. The manual stirrer will override any program stirring rates.

7. Close the microwave door, and program the microwave with the following oven method:

Ethos E Method	Setting
Time 1 (T) ₁ (ramp from ambient to 50°C)	4 minutes
Temperature (T1) ₁	50°C
Watts (E) ₁	350 max
Control for T1	
Time 2 (T) ₂	2 minutes
Temperature (T1) ₂ (hold)	50°C
Watts (E) ₂	350 max
Ventilation Time	1 minute
Stirrer speed setting	NA: manual control overrides

8. Leave the manual stirrer control on during ventilation / cool-down. After samples have cooled enough to handle and while still on the carousel, add 200 µL of internal standard solution (1MIXd, 1µg/mL) into each sample and stir for one minute on the oven stirrer.
9. Remove the jars from the oven and place them in the centrifuge. Centrifuge the samples at 2000 rpm for 5 minutes. (Higher rpm may cause the jars to break.)
10. Transfer a portion of the sample solution into an HPLC vial for analysis. Instead of filtering each sample, an inline, ultra-low volume, precolumn filter is used. (See Equipment, Section 3.4 above.)

7. ANALYSIS BY HPLC/MS/MS

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.

7.1 Liquid Chromatographic Conditions

Column: Superspher 100 RP-18
 Length 75 mm, 4 mm i.d.
 Particle size 4 µm, pore size 100 nm
 Cat. No.: 1.50980.0001
 Merck, distributed in the US by VWR

Column oven temperature: Ambient

Inline filter: Upchurch, ultra-low volume, precolumn filter, Cat. No. A-318,
 with A-102x, 0.5µm frits.

Mobile phase: A: 1.5% glacial acetic acid in HPLC grade water
 B: 100% acetonitrile

Flow rate (column): 1.0 mL/min

Flow rate (interface): 1.0 mL/min, no split

Table 1: HPLC Gradient Parameters

Time [min]	0	5	6	8	8.01	10
% A (1.5% acetic acid)	50	45	10	0	50	50
% B (acetonitrile)	50	55	90	100	50	50

Injection volume: 50 µL (Depending on sensitivity of the instrument.)

Run time: About 11 minutes between injections.

Table 2: Divert Valve Timing

Time [min]	Setting
0 to 1.00	Stream to waste
1.10 to 7.0	Stream to interface
7.1 to end	Stream to waste

Retention times: NNI-0001 (AE 1302996): approx. 5.9 min
 NNI-0001-des-iodo (AE 1303002): approx. 4.2 min
 NNI-0001-3-OH (AE 1423032): approx. 3.9 min
 NNI-0001-3-OH-hydroxyperfluoroalkyl (AE 1423031): approx. 1.6 min
 NNI-0001-benzoic-acid (AE 1651796): approx. 3.2 min *

* In extracts from different soils the retention time and peak shape of NNI-0001-benzoic acid may vary slightly due to matrix effects, depending on the soil pH or other characteristics.

7.2 Mass Spectrometry - Principle of Measurement

Substances introduced into the mass spectrometer are ionized using a TurboIonSpray interface. Sample ions are accelerated by an adequate voltage regulation and separated by mass in the first quadrupole (Q1). The most abundant ions (the protonated and deprotonated ions) of the analyte (parent ions) are collided with nitrogen in the collision cell (Q2). Fragments of these ions (daughter ions) are separated by mass in the third quadrupole (Q3) and detected. The mass spectrometric parameters for the analytes and the selected ions are listed in Table 3 below.

7.3 Mass Spectrometric Parameters

The parameters reported in Table 3 below are examples for an optimal adjustment of the mass spectrometer. They may vary between instruments used. These parameters should be adjusted if necessary.

Table 3: MS/MS Operating Parameters

Nebulizer Gas Setting	15
Curtain Gas Setting	9
Collision Gas Setting	5
Turbo Gas [L/min]	8.5
Turbo Gas Temperature [°C]	500

Compound dependent:	NNI-0001	NNI-0001-d6	NNI-0001-des-iodo	NNI-0001-des-iodo-d6	NNI-0001-3-OH	NNI-0001-3-OH-d6
Q1 Mass [amu]	681	687	555	561	571	577
Q3 Mass [amu]	254	259	254	259	296	296
Dwell [msec]	250	250	250	250	250	250
Ionization Mode	ESP-	ESP-	ESP-	ESP-	ESP-	ESP-
IS [V]	-4300	-4300	-4300	-4300	-4300	-4300
EP [V]	-10	-10	-10	-10	-10	-10
DP [V]	-46	-46	-41	-41	-51	-51
FP [V]	-210	-210	-190	-190	-220	-220
CE [V]	-32	-32	-30	-30	-22	-22
CXP [V]	-24	-24	-22	-22	-24	-24

Table 3 (continued): MS/MS Operating Parameters

	NNI-0001-3-OH- hydroxyper- fluoroalkyl	NNI-0001-3-OH- hydroxyper- fluoroalkyl-d6	NNI-0001- benzoic-acid	NNI-0001- benzoic-acid-d3
Q1 Mass [amu]	569	575	711	714
Q3 Mass [amu]	296	296	304	307
Dwell [msec]	250	250	500	500
Ionization Mode	ESP-	ESP-	ESP-	ESP-
IS [V]	-4300	-4300	-4300	-4300
EP [V]	-10	-10	-10	-10
DP [V]	-46	-46	-36	-36
FP [V]	-190	-190	-180	-180
CE [V]	-24	-24	-32	-32
CXP [V]	-22	-22	-26	-26

IS: Ion Spray Voltage

DP: Declustering Potential

FP: Focusing Potential

EP: Entrance Potential

CE: Collision Energy

CXP: Collision Cell Exit Potential

ESP-: electrospray, negative ion mode, i.e. production of negative ions

Product ions of NNI-0001, NNI-0001-d6, NNI-0001-des-iodo, NNI-0001-des-iodo-d6, NNI-0001-3-OH, NNI-0001-3-OH-d6, NNI-0001-3-OH-hydroxyperfluoroalkyl, NNI-0001-3-OH-hydroxyperfluoroalkyl-d6, NNI-0001-benzoic-acid and NNI-0001-benzoic-acid-d3 from Reference 3 are presented in [Appendix 2](#).

8. CALCULATIONS

For calculation of the concentrations linear regression calibration curves are used. These curves may be calculated using the instrument quantitation software or equivalent statistical spreadsheet or analytical software.

Matrix effects for NNI-0001, NNI-0001-des-iodo, NNI-0001-3-OH, NNI-0001-3-OH-hydroxyperfluoroalkyl and NNI-0001-benzoic acid are eliminated by using an internal standard solution of the isotopically labeled reference substance. This solution is added to the sample solutions before analysis.

The linear equation is expressed as:

$$y = \text{Intercept} + \text{Slope} \cdot x$$

$$y = \text{Area}, x = \text{Concentration}$$

If an internal standard is used:

$$y = \frac{\text{Area}_{\text{Standard}}}{\text{Area}_{\text{Internal Standard}}} = \text{Int. Ratio} \quad \text{and} \quad x = \frac{\text{Conc}_{\text{Standard}}}{\text{Conc}_{\text{IS}}} = \text{Conc}_{\text{Ratio}}$$

<i>Int. Ratio:</i>	<i>intensity ratio</i>
<i>Conc_{Standard}:</i>	<i>concentration of standard solution [µg/L]</i>
<i>Conc_{IS}:</i>	<i>concentration of internal standard solution [µg/L]</i>
<i>Conc_{ratio}:</i>	<i>concentration ratio</i>

Since the concentrations of the isotopically labeled internal standards were the same in all solutions that were injected into the HPLC instrument, their concentrations usually could be neglected for calculations. But if they were taken into account in the formula for $\text{Conc}_{\text{ratio}}$, they must also appear in the formula for $\text{Conc}_{\text{Soil (Sediment) Wet}}$.

The concentrations of the internal standards in the calibration solutions should be in the range of the concentrations in the sample solutions.

By means of the linear equation, the compounds NNI-0001, NNI-0001-des-iodo, NNI-0001-3-OH, NNI-0001-3-OH-hydroxyperfluoroalkyl and NNI-0001-benzoic acid contents in dry soil or dry sediment can be calculated as follows:

$$\text{Dilution}_{\text{Factor}} = \frac{\text{Volume}_{\text{Extraction}}}{\text{Weight}}$$

$$\text{Conc}_{\text{Analyte}} = \frac{\text{Int. Ratio} - \text{Intercept}}{\text{Slope}}, \quad \text{Int. Ratio} = \frac{\text{Area}_{\text{Analyte}}}{\text{Area}_{\text{Internal Standard}}}$$

$$\text{Conc}_{\text{Soil (Sediment) Wet}} = \text{Conc}_{\text{Analyte}} \times \text{Dilution}_{\text{Factor}} \times \text{Conc}_{\text{IS}}$$

$$\text{Conc}_{\text{Soil (Sediment) Dry}} = \text{Conc}_{\text{Soil (Sediment) Wet}} \times \frac{100\%}{100\% - \text{Moisture}}$$

<i>Volume_{Extraction}:</i>	<i>volume of the extraction solvent [L]</i>
<i>Weight:</i>	<i>weight of the soil (sediment) sample [kg]</i>
<i>Intercept:</i>	<i>intercept of the linear regression curve</i>
<i>Slope:</i>	<i>slope of the linear regression curve</i>
<i>Area_{Analyte}:</i>	<i>area of the analyte in the sample solution</i>
<i>Moisture:</i>	<i>water content of the sample [kg/100kg]</i>
<i>Conc_{Soil (Sediment) Wet}:</i>	<i>concentration of the analyte in wet soil (sediment) [µg/kg]</i>
<i>Conc_{Soil (Sediment) Dry}:</i>	<i>concentration of the analyte in dry soil (sediment) [µg/kg]</i>

The recovery is calculated according to the following equation:

$$\text{Recovery} = \frac{\text{Conc}_{\text{Soil (Sediment) Wet}} \times 100\%}{\text{Conc}_{\text{Soil (Sediment) Spiked}}}$$

Conc_{Soil (Sediment) Spiked}: concentration of the reference substance spiked [$\mu\text{g}/\text{kg}$]

Example calculation (taken from Reference 3) for a 100 $\mu\text{g}/\text{kg}$ recovery of NNI-0001 of soil Höfchen (sample: soil Höfchen recovery 6,100 $\mu\text{g}/\text{kg}$)

$$\text{Dilution}_{\text{Factor}} = \frac{0.04 \text{ L}}{0.02 \text{ kg}} = 2.0 \frac{\text{L}}{\text{kg}}$$

$$\text{Int. Ratio} = \frac{138236.6}{46621.19} = 2.965102$$

$$\text{Conc}_{\text{Analyte}} = \frac{2.965102 - 0.00177339}{1.17054} = 2.53159$$

$$\text{Conc}_{\text{Soil Wet}} = 2.53159 \times 2.0 \frac{\text{L}}{\text{kg}} \times 20.03 \frac{\mu\text{g}}{\text{L}} = 101.415 \frac{\mu\text{g}}{\text{kg}}$$

$$\text{Recovery} = \frac{101.415 \frac{\mu\text{g}}{\text{kg}} \times 100\%}{100.2 \frac{\mu\text{g}}{\text{kg}}} = 101.2\%$$

Remark: The results given are rounded values. Thus, rounding deviations may occur if recalculations are made using the rounded figures.

9. DETECTOR LINEARITY

The detector linearity was examined in Reference 3. Standard solutions containing NNI-0001, NNI-0001-des-iodo, NNI-0001-3-OH, NNI-0001-3-OH-hydroxyperfluoroalkyl and NNI-0001-benzoic acid were measured in a concentration range of about 0.074 to 100 $\mu\text{g}/\text{L}$ corresponding

to a concentration in soil of 0.15 - 200 µg/kg. In this concentration range the mass spectrometric detector showed linear correlation between concentration and peak area. (See Table 4 below.)

Table 4 Correlation Between Concentration and Intensity Ratio³

Compound	Concentration range [µg/L]	Correlation coefficient r
NNI-0001	0.07 – 100	0.9999
NNI-0001-des-iodo	0.07 – 100	0.9998
NNI-0001-3-OH	0.07 – 100	0.9992
NNI-0001-3-OH- hydroxyperfluoroalkyl	0.07 – 100	0.9996
NNI-0001-benzoic acid	0.07 – 100	0.9999

10. LIMIT OF QUANTITATION AND LIMIT OF DETECTION

Acceptable recoveries at the targeted limits of quantitation for NNI-0001, NNI-0001-des-iodo, NNI-0001-3-OH, NNI-0001-3-OH-hydroxyperfluoroalkyl and NNI-0001-benzoic acid of 0.5µg/kg were validated in References 3 and 4. The limit of detection of the method was shown to be equal to or less than 0.15µg/kg for NNI-0001, NNI-0001-des-iodo, NNI-0001-3-OH, NNI-0001-3-OH-hydroxyperfluoroalkyl and NNI-0001-benzoic acid as determined in Reference 3.

11. CRITICAL STEPS AND OBSERVATIONS

During the independent laboratory validation of the original method it was found that controlling the factors that affect the microwave energy absorbed into the samples is essential for obtaining good recoveries.⁴ These factors included the size and type of stir bars used, the type, size and number of sample containers used and the extraction time. The placement of the samples in the oven was also critical. The number of samples had to be the same, and untreated control samples or solvent blanks had to be prepared to occupy any unused positions.

Using the fiber optic cable temperature probe and energy control software with the Milestone Model Ethos E microwave oven, temperature feedback may be used to control the amount of microwave energy applied to the samples. Therefore, the factors affecting energy absorbed are less critical, because the temperature is held at a set point while the energy input can vary. Then, for example, the stir bars will be only important for stirring, not for energy absorption, and any bars that properly stir the samples may be used.

By using temperature feedback and energy control, inexpensive disposable stir bars and disposable glass jars may be used without changing the amount of energy absorbed into the samples. There is also more latitude for varying the number of samples in the oven since the energy is reduced or increased, up to the method maximum of 350 watts, depending on the sample load, while the temperature specified in the method is maintained for the specified time period.

It is recommended that before proceeding with any analyses using a new or modified microwave oven setup, the analyst should fortify five or six soil samples with the test compounds, and analyze the fortified samples in accordance with this analytical method to ensure that there is no problem with the procedure or oven setup.

In the original method it was noted that the retention time for the NNI-0001-benzoic acid varied with soil matrix.³ Additional experience with the method showed that the benzoic acid peak also became broader and the peak shape degraded for some soil matrices, especially if the soil pH was strongly alkaline. It was found that increasing the acetic acid from 0.1 to 1% in the extraction solvent improved the peak shape of the benzoic acid for the alkaline soils. This version of the method incorporates this modification.

12. REFERENCES

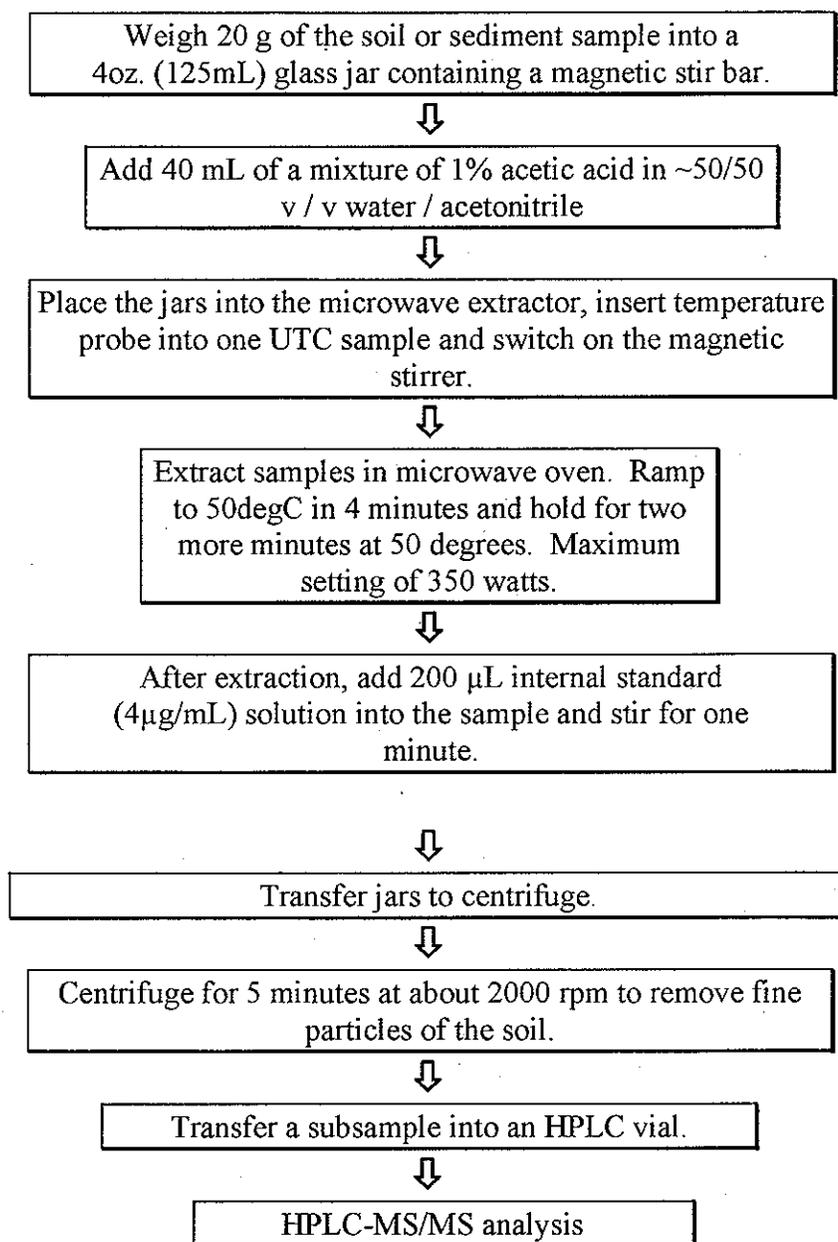
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3. Analytical Method 00849 for the Determination of Residues of NNI-0001, NNI-0001-des-iodo, NNI-0001-3-OH, NNI-0001-3-OH-hydroxyperfluoroalkyl and NNI-0001-benzoic acid in Soil and Sediment by HPLC-MS/MS, Bayer CropScience AG, Monheim, Germany, Laboratory Project ID P601030020, Method Number 00849, B. Brumhard, August 2, 2004.
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5. Storage Stability of NNI-0001, NNI-0001-des-iodo, NNI-0001-3-OH, NNI-0001-3-OH-hydroxyperfluoroalkyl and NNI-0001-benzoic acid, Bayer CropScience AG, Monheim, Germany, B. Brumhard, study in progress.

Table 5 NNI-0001 Physiochemical Properties
(Background information, not required for performing the method.)

Parameter	Value	Reference(s)
Melting point	259°C	Bayer CropScience ISIS Chemical Database, November 2004
Water solubility (g/L at ambient)	0.00003	Bayer CropScience ISIS Chemical Database, November 2004
Solvent solubility (g/L at ambient)	Not available	
Vapor pressure at 20°C	Not available	
Dissociation constant (pK _a)	Not available	
Octanol/water partition coefficient Log at 20°C(K _{ow})	2291	Bayer CropScience ISIS Chemical Database Document Number: MO-02-008799
UV/visible absorption spectrum	Not available	

Table 6 Analytical Method Summary Parameters

Analyte(s)	NNI-0001 (AE 1302996), NNI-0001-des-iodo (AE 1303002), NNI-0001-3-OH (AE 1423032), NNI-0001-3-OH-hydroxyper-fluoroalkyl (AE 1423031) and NNI-0001-benzoic-acid (AE 1651796)
Extraction solvent / Technique	Microwave extraction using solvent system of acetonitrile and 1% acetic acid in water
Cleanup	Centrifuge to remove fine soil particles
Instrument Detector Column	- Shimadzu LC-10AD VP HPLC pump with Gilson 215 Liquid handler and Gilson 819 Valve Actuator - Applied Biosystems API 3000 MS/MS - Superspher 100 RP 18 (75mm x 4 mm x 4 micron), Merck (distributed in the US by VWR)
Standardization Method	Multi point calibration curve (Internal standard)
Stability of Standard Solutions	Stock standard solutions are stable for a minimum of 4 months when stored in the dark at ≤-8°C Fortification and calibration standard solutions are stable for a minimum of 4 months when stored in the dark at 4°C ± 3°C
Retention times	AE 1302996 (4.9), AE 1303002 (3.9), AE 1423032 (3.8), AE 1423031 (1.9) and AE 1651796 (2.6)

Appendix 3 Flow Diagram of the Extraction Procedure

Appendix 4 Revision History

Method #	Revision Number	Description
00849	None	Method prepared on completion of validation study ³
AM001-S04-01	01	This method, prepared after ILV ⁴