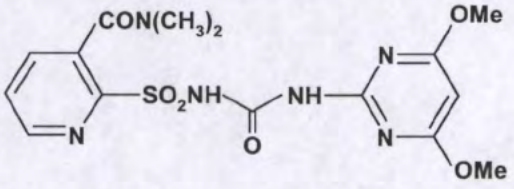
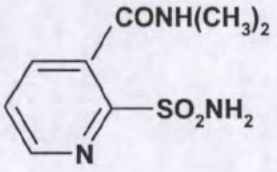
2.0 INTRODUCTION

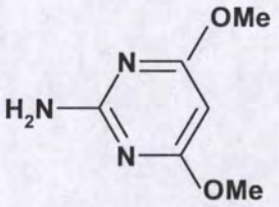
Nicosulfuron (DPX-V9360) is a sulfonylurea herbicide used for post-emergence control of broadleaf and grass weeds during the production of corn. In soil, nicosulfuron degrades aerobically into IN-V9367, IN-J0290 and other minor metabolites (References 1 and 2). The chemical structures and pertinent information of the test substances are shown below (References 1 and 3).



DuPont Code: DPX-V9360
Trivial Name: Nicosulfuron
IUPAC Name: 1-(4,6-Dimethoxypyrimidin-2-yl)- 3-(3-dimethylcarbamoyl-2-pyridylsulfonyl)urea
***Chemical Abstracts* Name**: 2-[[[[(4,6-Dimethoxy-2-pyrimidinyl)amino]carbonyl] amino]= sulfonyl]-*N,N*-dimethyl-3-pyridinecarboxamide monohydrate
***CAS* Registry Number**: 111991-09-4
Molecular Formula: $C_{15}H_{18}N_6O_6S$
Molecular Weight: Average, 410.41 amu; monoisotopic, 410.10 amu
pKa (25°C): 4.6
Solubility (g/kg), 25°C: Buffered Water: 3.59 (pH 5); 12.2 (pH 7); 39.2 (pH 9)
Organic: 18 (acetone); 4.5 (ethanol); 23 (acetonitrile); <0.02 (hexane); 160 (dichloromethane)
Stability: Relatively stable at pH7 and pH 9. Hydrolysis DT_{50} 15 days (pH 5)



DuPont Code: IN-V9367
***Chemical Abstracts* Name:** 2-(Aminosulfonyl)-N,N-dimethyl-3-pyridinecarboxamide
***CAS* Registry Number**: 112006-75-4
Molecular Formula: $C_8H_{11}N_3O_3S$
Molecular Weight: Average, 229.26 amu; monoisotopic, 229.05 amu



DuPont Code: IN-J0290
***Chemical Abstracts* Name:** 4,6-dimethoxy-2-pyrimidinamine
***CAS* Registry Number**: 36315-01-2
Molecular Formula: $C_6H_9N_3O_2$
Molecular Weight: Average, 155.16 amu; monoisotopic, 155.07 amu

This analytical method for nicosulfuron and its metabolites IN-V9367 and IN-J0290 in soil, at an LOQ of approximately 1.0 µg/kg (ppb), was developed to satisfy the requirements of the U.S. EPA Pesticide Assessment Guidelines Subdivision N and the EU Annex II 4.2.2.

Nicosulfuron and its metabolites were extracted from a 10-g soil sample by shaking with 0.1 M ammonium carbonate/acetone (9:1, v/v) solution at ambient temperature. Following centrifugation, an extract aliquot was purified on stacked SPE cartridges of Oasis™ HLB (top) and ENV+® (bottom). The purified extract was analyzed by reversed-phase HPLC/ESI-MS/MS.

The confirmatory method for the HPLC/ESI-MS/MS method was based on detection and the relative ratios of the two MS/MS parent-to-daughter ion transitions monitored during the validation.

3.0 MATERIALS

Equivalent equipment and materials may be substituted unless otherwise specified; note any specifications in the following descriptions before making substitutions. Substitutions should only be made if equivalency/suitability has been verified with acceptable control and fortification recovery data.

3.1 Equipment

Balances

Mettler Analytical Balance, Model AE240, for weighing solid standards (Mettler Instrument Corporation, Hightstown, NJ)

Mettler Top-Loading Balance, Model PM600, for weighing soil samples and salts (Mettler Instrument Corporation)

Extractor

Wrist Action Shaker, Model 75 (Burrell, Pittsburgh, PA)

VWR Disposable Skirted Centrifuge Tube, 50-mL, Polypropylene, cs. of 500, Part # 21008-480 (VWR International)

HPLC/MS System

HP Series 1100 Liquid Chromatograph with G1332A degasser, G1312A binary pump, G1313A chilled autosampler, G1316A column compartment (Hewlett-Packard, Little Falls, DE)

Luna® Phenyl-Hexyl analytical column, 4.6 mm i.d. × 15 cm with 3-µm diameter packing, Part No. 00F-4256-E0 (Phenomenex, Torrance, CA)

API4000 triple quadrupole mass spectrometer using an electrospray interface (ESI) and Analyst Version 4.1 software (Applied Biosystems, Framingham, MA) with Valco zero-dead volume 3-port connector for 1/10 splitflow to mass spectrometer.

HPLC Vials – Hewlett Packard Target Dual-Purpose Vials with Teflon/Silicone/Teflon Septa, amber, 2-mL, Catalog No. 5182-0056 (Hewlett-Packard)

Nitrogen Evaporator - N-Evap Model 111 (Organomation Assoc., Berlin, MA)

Pipettes

Biohit Proline® Electronic Pipettors, variable volume, with tip ejector, 10-250 L and 50-1000 L, Catalog No. 53495-210 and 53496-205 (VWR Scientific)

Disposable Pasteur Pipets, Borosilicate glass, 9-inch length, Catalog No. 14673-043 (VWR Scientific)

EDP Electronic Digital Pipette, Catalog No. EP-10 mL (Rainin Instrument Co., Inc., Woburn, MA)

Pipette Tips

Sorenson™ Multifit Research Pipet Tips, 5-200 µL and 100-1000 µL, Catalog No. 53550-076 and 53503-076 (VWR Scientific)

Rainin Certified Disposable Pipette Tip, 10 mL, Catalog No. RC-10 mL (Rainin Instrument Co.)

Sample Collection Vials

Disposable Centrifuge Tube, 50-mL, pkg. of 72, Part # 73785 (VWR Scientific)

Glass Centrifuge Tubes – Pyrex® Conical Centrifuge Tubes, graduated, 50-mL capacity, Catalog No. 21048-050 (VWR Scientific)

Solid-Phase Extraction Apparatus

Visiprep™ SPE Manifold, Catalog No. 5-7030M (Supelco, Inc., Bellefonte, PA)

Solid-Phase Extraction Disposable Flow Control Valve Liners - for the Visiprep™, Catalog No. 57059 (Supelco, Inc.)

Solid-Phase Extraction Cartridges

Isolute® NH_2, 1.0-g/6-mL, Catalog No. 470-0100-C (Argonaut Technologies, Inc., Redwood, CA)

Oasis™ HLB cartridge, 1.0-g/20-mL, Part No. 186000117, (Waters, Milford, MA)

Isolute® ENV+ cartridge, 200-mg/6-mL, Catalog No. 915-0020-C (Argonaut Technologies, Inc.)

Solid Phase Extraction Plastic Reservoirs – 25-mL size, Catalog No. 1213-1012 (Varian Inc.)

Syringes - 3cc Disposable plastic syringes, Catalog No. 309585 (Becton Dickinson, Franklin Lakes, NJ)

Syringe Filters

Disposable 1.0-µm GMF-150 filter, polypropylene housing, Whatman No. 6783-2510, VWR Cat No. 28137-950 (VWR International)

Acrodisc® CR PTFE disposable filter, 0.2-45-µm pore size, 25-mm diameter Catalog No. 4225T and 4219 (VWR Scientific)

Ultrasonicator -Bransonic® Ultrasonic Cleaner, 0.75-gallon capacity, Model 2200, Catalog No. 952-214 (Branson Ultrasonics Corp., Danbury, CT)

Vortex mixer - Fisher Vortex Genie®, Catalog No. 12-812 (Fisher Scientific Co., Pittsburgh, PA)

3.2 Reagents and Standards

Acetonitrile - OmniSolv® #AX0142-1, HPLC grade (EM Scientific, Gibbstown, NJ)

Ammonium Carbonate – Powder, #0650-01 (J.T. Baker, Phillipsburg, NJ)

Formic Acid – Suprapur®, 98-100%, #11670-1 (EM Chemicals, Inc.)

Formic Acid, Ammonium Salt – A.C.S. reagent, #M530-08 (J.T. Baker)

Glacial Acetic Acid - OmniSolv® #AX0073-6, reagent grade, (EM Scientific)

Methanol - OmniSolv® #MX0488-1, HPLC grade (EM Scientific)

Ultrapure Water – OmniSolv® #WX0004-1, HPLC grade, (EM Scientific) ; Milli-Q water

Reference standards (DuPont Crop Protection, Newark, DE):
DPX-V9360-116 (Nicosulfuron), 97.9% purity
IN-V9367-03 analytical standard, 99.7% purity
IN-J0290-017 analytical standard, 99.6% purity

3.3 Safety and Health

No unusually hazardous materials are used in this method. All appropriate material safety data sheets should be read and followed, and proper personal protective equipment should be used.

4.0 METHODS

4.1 Principle of the Analytical Method

Nicosulfuron and its metabolites IN-V9367 and IN-J0290 were extracted from a 10-g soil sample with 0.1 M ammonium carbonate/acetone (9:1, v/v) solution at ambient temperature using a wrist-action shaker. One-fourth aliquot of extract was separated and passed through an Isolute® NH_2 solid-phase extraction (SPE) cartridge. The partially purified extract was evaporated in a nitrogen-evaporator (N_2-vap) system at 30°C to remove acetone and then acidified with 1 M formic

acid to adjust the pH to 3. It was further purified and concentrated by passing through stacked SPE cartridges of Oasis™ HLB (top) and ENV+® (bottom). After the cartridges were washed with water, the analytes were eluted using 9:1 methanol/1M NH_4OH solution and methanol. The eluate was evaporated to about 3 mL in an N_2-vap at 30°C, brought to a final volume of 5 mL by adding 5 mM ammonium formate/methanol (19:1, v/v) solution, and then filtered. The purified extract was analyzed by reversed-phase HPLC using a Phenomenex® Luna phenyl-hexyl (4.6 x 150 mm, 3-µm particle) column and a mobile phase of 0.1 mM formic acid in 0.01 mM ammonium formate (aq) and methanol. (Prior to the analysis, 10 µL of 1M formic acid was added 990 µL of each of the standards and investigative samples.) Detection of the analytes was by electrospray mass spectrometry/mass spectrometry (ESI-MS/MS) in the positive ion mode. Two parent-to-daughter ion transitions per analyte were monitored during analysis.

Purification of DPX-V9360 and its metabolites by SPE would require only Isolute® NH_2 and Oasis™ HLB cartridges.

Addition of 1 M formic acid (10 µL) to all HPLC samples was necessary to keep the pH of the investigative samples close to that of calibration standards, as well as keep its peak shape narrow in the investigative samples.

Only low concentrations of formic acid and ammonium formate in the mobile phase was necessary to obtain good MS response for all analytes. Formic acid assisted in the formation of molecular ions; ammonium formate, in reducing sodium adducts of most analytes.

Extraction efficiency of the method was evaluated by analyzing laboratory-aged soil samples, which were fortified with nicosulfuron and its metabolites and then aged for three days. The experiment was designed for a high fortification level of 50 µg/kg per analyte and the resulting extract would only be diluted and analyzed immediately by HPLC/ESI-MS/MS; no more purification and concentration by SPE. Freshly fortified samples were extracted and analyzed simultaneously with the laboratory-aged samples. The recoveries of the analytes from the aged soil samples were compared to that of the freshly fortified samples to obtain normalized recoveries. A clay-loam soil sample with a pH of 5.2 and organic matter and clay contents of 4.3 and 29.2%, respectively, was used.

The confirmatory method for the HPLC/ESI-MS/MS method was based on detection and the relative ratios of the two MS/MS parent-to-daughter ion transitions monitored during the validation.

During method validation, post-fortified samples were analyzed for each soil type to determine if matrix effect, suppression or enhancement, influenced percent recovery of nicosulfuron and its metabolites. The post-fortified samples, in this study, were extracts of control soil samples that were purified and prepared in the same manner as with the other samples, but fortified with the analytes prior to HPLC/ESI-MS/MS analysis.

4.2 Analytical Procedure

4.2.1 Glassware & Equipment Cleaning Procedures

The effectiveness of any cleaning procedure used should be demonstrated by preparation and analysis of reagent blanks. In general, all reusable glassware and

plasticware should be washed in hot tap water with laboratory grade, non-phosphate detergent, rinsed several times with tap water, rinsed several times with deionized water, rinsed once with acetone, and allowed to fully dry before use. Care should be taken to avoid working with high levels of the analyte being monitored in the same laboratory where samples are being extracted and analyzed.

4.2.2 *Preparation & Stability of Reagent Solutions*

0.1 M Ammonium Carbonate (aq)

Dissolve 9.6 grams of ammonium carbonate ($(NH_4)_2CO_3$)) in a 1.0-L volumetric flask that is partially filled with ultrapure water. Adjust final volume to the mark with water and mix to homogeneity. This solution is stored capped at room temperature and stable for 2-3 months.

1.0 M Ammonium Formate (aq)

Dissolve 63.06 grams of ammonium formate ($HCOONH_4$) in a 1.0-L volumetric flask that is partially filled with ultrapure water. Adjust final volume to the mark with water and mix to homogeneity. This solution is stored capped at room temperature and stable for 2-3 months.

1.0 M Ammonium Hydroxide (aq)

To a 100-mL volumetric flask that is partially filled with ultrapure water, add 7 mL of concentrated ammonium hydroxide (28% - 30% NH_3) and mix. Bring to the mark with ultrapure water and mix to homogeneity. This solution is stored capped at room temperature and should be prepared monthly.

1.0 M Formic Acid (aq)

To a 100-mL volumetric flask that is partially filled with ultrapure water, add 385 µL of concentrated formic acid. Bring to the mark with ultrapure water and mix to homogeneity. This solution is stored capped at room temperature and should be prepared monthly.

0.1 % Formic Acid (aq)

Mix 0.5 mL of 1.0 M formic acid and 500 mL of ultrapure water in a 500-mL glass storage bottle. This solution is stored capped at room temperature and should be prepared monthly.

Extraction Solution (9:1 0.1M Ammonium Carbonate/Acetone)

Mix 900 mL of 0.1M ammonium carbonate (aq) and 100 mL acetone in a 1-L glass storage bottle. This solution is stored capped at room temperature and should be prepared monthly.

Elution Solution (9:1 Methanol/1.0 M Ammonium Hydroxide)

Mix 450 mL of methanol and 50 mL of 1.0 M ammonium hydroxide solution in a 500-mL glass storage bottle. This solution is stored capped at room temperature and should be prepared weekly.

Sample Diluent (19:1 5 mM Ammonium Formate/Methanol)

Mix 237 mL of ultrapure water, 1.19 mL of 1.0 M ammonium formate, and 12.5 mL of methanol in a 250 mL glass storage bottle. This solution is stored capped at room temperature and should be prepared monthly.

Mobile Phase A (0.1 mM formic acid in 0.01 mM ammonium formate (aq))

To a 1-L volumetric flask that is partially filled with ultrapure water, add 0.10mL of 0.10 M ammonium formate and 0.10 mL 1.0 M formic acid. Dilute to the mark with ultrapure water and mix to homogeneity. This solution is stored capped at room temperature and should be prepared monthly.

4.2.3 *Stock and Intermediate Standards Preparation and Stability*

Five milligrams each of nicosulfuron (DPX-V9360) and metabolites IN-V9367 and IN-J0290 analytical standards are accurately weighed into separate 50-mL volumetric flasks, dissolved, and diluted to the mark using acetonitrile to make stock standard solutions of approximately 100 µg/mL.

Prepare the first intermediate mix standard of 10-µg/mL (DPX-V9360, IN-V9367 and IN-J0290) by adding 1.0 mL each of the 100-µg/mL of DPX-V9360, IN-V9367, and IN-J0290, solutions into a 10-mL volumetric flask. Dilute to the mark with methanol and mix.

Solutions of the stock standards and intermediate mix standards are stored in the freezer maintained at a temperature below ≤-10°C and are stable for six months.

4.2.4 *Fortification Standard Preparation and Stability*

Using 10-mL volumetric flasks, dilute 1.0 mL and 0.10 mL of the 10-µg/mL intermediate mix standards to the mark with methanol to prepare the fortification mix standards of 1.0- and 0.10-µg/mL (DPX-V9360, IN-V9367, and IN-J0290) respectively.

These solutions are stable for at least 6 months when stored at ≤ -10°C.

4.2.5 *Chromatographic Standard Preparation and Stability*

Prepare a calibration standard solution by adding 8 mL of methanol and 7 mL of water into a 25-mL volumetric flask. Dilute to the mark with 5mM ammonium formate/methanol (19:1, v/v) solution and mix to homogeneity.

Using 2-mL volumetric flasks, dilute 1.0 mL of each of the 1.0-µg/mL and 0.1-µg/mL fortification mix standards with the calibration solution to the mark to prepare the 0.50- and 0.050-ng/mL intermediate calibration mix standards (DPX-V9360, IN-V9367, and IN-J0290), respectively. These solutions should be prepared fresh.

Prepare chromatographic standards ranging from 0.25 to 20.0 ng/mL (or in concentrations expected to cover the range of nicosulfuron and metabolites in the investigative samples) from the fortification mix standards diluted with the calibration solution. Keep all chromatographic standards at or below 4°C right after preparation. These standards should be prepared fresh for every set of investigative samples even they are stable for 48 hours. The tables below describe how standards were prepared in 2-mL volumetric flasks or 2-mL HPLC vials, respectively, for the validation work presented in this report:

Standard Concentration (ng/mL)	µL added	Intermediate Standard Used	Final Volume (mL)
20	80	0.50 µg/mL (int. cal. std)	2.0
10	40	0.50 µg/mL (int. cal. std)	2.0
5.0	200	0.050 µg/mL (int. cal. std)	2.0
1.0	200	10 ng/mL (chromatographic std)	2.0
0.50	200	5.0 ng/mL (chromatographic std)	2.0
0.25	100	5.0 ng/mL (chromatographic std)	2.0

Standard Concentration (ng/mL)	µL added	Intermediate Standard Used	Diluent Volume (µL)	Final Volume (mL)
20	40	0.50 µg/mL (int. cal. std)	960	1.0
10	20	0.50 µg/mL (int. cal. std)	980	1.0
5.0	100	0.050 µg/mL (int. cal. std)	900	1.0
1.0	100	10 ng/mL (chromatographic std)	900	1.0
0.50	100	5.0 ng/mL (chromatographic std)	900	1.0
0.25	50	5.0 ng/mL (chromatographic std)	950	1.0

Int. cal. std - intermediate calibration standard

Additional or alternative standards may be prepared if required.

4.2.6 *Source (& Characterization) of Samples*

Soil samples from five different sources were used for the method validation. The source of the soil samples and the pertinent physical characteristics are summarized in the following table. All soil samples was characterized at Harris Environmental Technologies (Lincoln, NE). Characterization records are maintained at DuPont Agricultural Products.

Soil Name (Location, Notebook No.)	Type	pH_w	Sand (%)	Silt (%)	Clay (%)	OM_{ash} (%)
Drummer #7 (Rochelle, Illinois, 2003-050)	Clay Loam	6.1	22.0	46.0	32.0	5.2
Mattapex #25 (Chesapeake, Maryland, 2002-30)	Loam	6.2	32.0	46.0	22.0	2.5
Nambsheim (Nambsheim, France, 2003-035)	Silty Loam	8.0	40.0	54.0	6.0	1.6
Speyer 2.2 (Speyer, Germany, 2000-031)	Sandy Loam	6.6	70.8	22.4	6.8	2.5
Fujishiro (Fujishiro ,Japan, 2002-037)	Clay Loam	5.2	27.6	43.2	29.2	4.3

4.2.7 *Storage & Preparation of Samples*

Soil samples were received frozen and stored in a freezer maintained at a temperature below 0°C prior to preparation for analysis.

4.2.8 *Sample Fortification Procedure*

One hundred microliters of the 0.10- and 1.0-µg/mL (DPX-V9360, IN-V9367, and IN-J0290) fortification solutions in methanol were spiked to 10.0-g soil samples for 1.0- and 10.0-µg/kg fortification levels, respectively.

4.2.9 *Analyte Extraction Procedure*

1. Weigh 10.0 (± 0.1) g of soil sample into a 50-mL polypropylene centrifuge tube. Fortify, if necessary. Let the sample sit under the hood for 15-20 minutes to evaporate the fortification solvent.
2. Add 30.0 (± 0.5) mL of extraction solution to the soil sample. Cap the tube and vortex for 10-15 seconds to mix the solution thoroughly with the soil.
3. Place the sample on a wrist action shaker set to maximum deflection. Shake the sample for 15-20 minutes.
4. Centrifuge the sample for 10-15 minutes at ~3000 rpm (refrigeration not necessary).
5. Attach a 1-µm disposable syringe filter to a solid-phase extraction vacuum manifold port. Connect a 75-mL reservoir (fitted with a 20-µm frit inside) to the filter. Place a calibrated 60-mL Dionex collection vial under the vacuum port to collect the filtered extract.

   Note: Dionex collection vial is calibrated with 60 mL of ultrapure water and marked at this volume.
6. Carefully pour off the extract from the sample into the 75-mL reservoir. Apply vacuum (as needed) to draw the extract through the filter and into the collection vial.
7. Repeat steps 2-4 and step 6 above for the sample. Use the same filter setup and collect the filtered extract into the same collection vial (the total volume of the two pooled extracts is ~50-55 mL).
8. Bring the final volume of the extract to 60 mL with the extraction solution.
9. Proceed to the next section to continue processing the samples. The sample extracts may be stored frozen (<-10°C) for 72 hours, if they are not to be processed immediately.

4.2.10 *Analyte Purification/Concentration Procedure*

1. Attach an Isolute® NH_2 (1-g/6-mL) SPE cartridge to an SPE vacuum manifold port. Condition the cartridge with 5 mL of methanol followed by 5 mL of extraction solution. Use vacuum as needed to achieve a flow rate of 1-2 drops/second. Do not let the cartridge go to dryness. Place a 40-mL Dionex collection vial under the cartridge.
2. Pass 15 mL (3x5 mL) of the soil sample extract through the NH_2 cartridge and collect the eluate. Use vacuum as needed to achieve a flow rate of 1-2 drops/second. Do not let the cartridge go to dryness.
3. After the entire extract has passed through, vacuum the cartridge dry for 10-15 seconds. Pass 3 mL of deionized water through the cartridge and collect with eluate (use vacuum as needed to achieve a flow rate of

1-2 drops/second). After the water has passed through, vacuum the cartridge dry for 10-15 seconds. Remove the Isolute® NH2 cartridge from the vacuum manifold and set aside for further processing later.

4. Mark the level of eluate in the tube. Evaporate the eluate to about 75-80% of its original volume using an N2-vap with a moderate flow of nitrogen and a water bath set at 30°C (~ 1-1.5 hours).
5. Stack an OasisTM HLB (1.0-g/20-mL) SPE cartridge above an ENV+ (0.2-g/6 mL) SPE cartridge using an adapter and attach this stack to the vacuum manifold. Condition both cartridges with 5-6 mL methanol followed by 15-20 mL 0.1% formic acid (use vacuum as needed to start flow and once flow has started, turn vacuum off and use gravity flow). Do not allow the cartridges to go to dryness. A thin film of liquid should be on top of the packing material (at least 0.5 mL) of the Oasis HLB. For ENV+, retain at least 1 mL of 0.1% formic acid.
6. Add 3 mL of 1 M formic acid to the evaporated extract from Step 4. Cap and sonicate the sample for 1 minute and then vortex mix for 5-10 sec. (Note: The sample extract foams and warms up upon addition of acid.) Pass the sample through the stacked OasisTM HLB/ENV+ cartridges (use vacuum as needed to establish a flow rate of 1-2 drops/sec). Do not let the cartridges to go to dryness. Discard eluate.
7. After the entire sample has passed through, wash the sample tube twice with 5 mL of 0.1% formic acid and pass rinse through the stacked cartridges. Then rinse the sample tube with twice with 5 mL of ultrapure water and pass this rinsate through the cartridges (use vacuum as needed to establish a flow rate of 1-2 drops/sec). Discard eluates. Vacuum both cartridges dry for 10-15 seconds.
8. Remove only the OasisTM HLB cartridge from the stack and set aside for further processing. Attach the Isolute® NH2 cartridge (from step 3) on top of the ENV+ cartridge. Use the rinsed 40-mL sample tube (from step 7) to collect the next eluate. Elute the cartridges with 10 mL 9:1 methanol/1 M ammonium hydroxide (use vacuum as needed to establish a flow rate of 1-2 drops/sec).
9. Remove the stacked NH2 /ENV+ cartridges from the vacuum manifold and replace them with the OasisTM HLB cartridge (from step 8). To the NH2 /ENV+ eluate (from Step 8), add 10 mL of methanol and mix to homogeneity (this is the eluent for the OasisTM HLB cartridge in Step 10).
10. Place a 50-mL graduated glass centrifuge tube under the OasisTM HLB cartridge. Elute the analytes with the 20 mL of the eluent from Step 9 (use vacuum as needed to establish a flow rate of 1-2 drops/sec). After all the elution solution has passed through the cartridge, apply a full vacuum for 10-15 seconds to remove all liquid.
11. Remove the eluate and evaporate to ≤3 mL in an N2-vap using a moderate stream of nitrogen and the water bath set at 30°C (about 1-1.5 hr.). Add ultrapure water, if necessary, to bring the volume to 3 mL.

12. Bring the final volume to 5 mL by adding 2 mL of 5mM ammonium formate/MeOH (19:1, v/v). Vortex to mix the sample, sonicate for 2 minutes, and again vortex mix.
13. Filter the final purified sample extract using a 5-mL disposable syringe with a 25-mm, 0.45-µm PFTE filter into a 20-mL glass vial with screw cap.
14. Transfer 990 µL of the sample to an HPLC autosampler vial. Add 10 µL of 1 M formic acid to the sample vial and vortex for 10-15 seconds and analyze immediately. DO NOT add the formic acid, if samples are not to be analyzed immediately. This sample is stable for ~ 24 hours when kept at or below 4°C in the presence of formic acid. The sample is stable for 2 and 5 days without formic acid addition, when kept at or below 4°C and –10°C, respectively.
15. For the calibration standards, transfer 990 µL of each to an HPLC vial. Add 10 µL of 1 M formic acid to the sample vial and vortex for 10-15 seconds and analyze immediately together with the investigative samples.

4.3 Instrumentation

4.3.1 Description

Method validation data in this study were generated using an Agilent HP Series 1100 HPLC coupled to Applied Biosystems MDS SCIEX API 4000 (a triple quadrupole MS) with an electrospray ion source.

4.3.2 Operating Conditions

The HPLC and MS operating conditions used during method validations are summarized in the following tables:

HPLC Conditions:

System:	Agilent HP Series 1100 HPLC			
Columns:	Phenomenex® Luna Phenyl-hexyl, 4.6 mm x 150 mm, 3-µm dp			
Column Temperature:	40°C			
Autosampler Temperature:	4°C			
Injection Volume:	75 µL			
Mobile Phase Conditions:	Time	%A	%B	Flow (mL/min)
Solvent A:	00.00	95.0	5.0	1.0
0.1 mM Formic acid in	10.00	5.0	95.0	1.0
0.01 mM ammonium formate	13.00	5.0	95.0	1.0
Solvent B: Methanol	13.10	95.0	5.0	1.0
	17.00	95.0	5.0	1.0
Approximate Retention Time:	(min)			
IN-V9367	~5.7			
IN-J0290	~8.4			
DPX-V9360	~10.9			

Mass Spectrometer Conditions:

MS System:	Applied Biosystems MDS SCIEX API4000					
Analyte Monitored	Ions Monitored (amu)	DP[a] (V)	CE[b] (V)	CXP[c] (V)	Dwell Time (ms)	Acquisition Timing (min)
IN-V9367	230.0 → 78.0 ± 0.1	60	50	15	50	3.0 – 6.5
	230.0 → 106.0 ± 0.1		30	5	50	
IN-J0290	156.0 → 57.0 ± 0.1	50	35	5	50	6.5 - 11.5
	156.0 → 100.0 ± 0.1		25	15	50	
DPX-V9360	411.0 → 182.0 ± 0.1	80	30	20	50	6.5 - 11.5
	411.0 → 213.0 ± 0.1		25	40	50	
Scan type/Polarity:	Multiple Reaction Monitoring/Positive					
Ion Source Voltage:	ESI+, 4500 V					
Collision Gas (CAD):	5 psig					
Curtain Gas (CUR):	10 psig					
Nebulizer Gas (GS1):	35 psig					
Heater Gas (GS2):	35 psig					
Source Heater (TEM):	350°C					
Interface Heater (ihe):	ON					
Entrance Potential (EP):	10 V					
MS Flow Rate:	(Post-column split): 100-µL/min (approximately 10:1 split)					

[a]Declustering Potential [b]Collision Energy [c]Collision Exit Potential

A triple quadrupole MS instrument with an electrospray ionization (ESI) source was used for the detection of nicosulfuron and its metabolites. The response of each analyte was optimized initially by infusing the analyte into the ionization source. The flow rate and mobile phase were adjusted to the elution conditions of the analyte from the HPLC column. The positive molecular ion detected was fragmented in the MS/MS collision cell (Appendix 2). The tune file created was adjusted to maximize the response of the fragmented ions detected. Two parent-daughter ion transitions were monitored for each analyte.

Nicosulfuron and its metabolites were each identified in soil by its retention time, the presence of two parent-daughter ion transitions with a signal-to-noise ratio greater than 5, and the ratio of the two ion transitions within an acceptable range as determined during the method validation. For quantitation, the ion chromatogram for each analyte was integrated and the peak area was used for quantitation. Quantitation was performed using the total ion current (TIC).

A six-port electronically activated switching valve was used to direct the HPLC column effluent to waste prior to and following the elution of analytes. The retention times of the analytes were within 5-11 minutes. The chromatographic run time is 17 minutes, but the MS sample collection time is 3.0 to 11.5 minutes. Outside of this sample collection time, the column effluent was directed to waste. This process reduced the ionization source contamination and allowed more samples to be analyzed prior to source cleaning.

Since the electrospray interface is optimal at low flow rates, the column effluent flow was split such that only 100-µL/min actually passed through the interface (approximately 10:1 split), the remainder going to waste.

4.3.3 *Calibration Procedures*

Prepare chromatographic standards that bracket the levels of nicosulfuron and its metabolites found in the soil samples to be analyzed. Preparation of standards is described in Section 4.2.5 of this report.

4.3.4 *Sample Analysis*

Each set of analytical samples should consist of calibration standards, at least one control (a sample without the analyte of interest and matches the analytical samples as closely as possible), and the investigative (treated/fortified) samples. In addition, at least one post-fortified sample of the control with nicosulfuron and its metabolites at a known level should be included to assess if matrix effect, if any, influence the residue levels found or percent recovery.

The calibration standard solution (see Section 4.2.5, p. 17) should be injected prior to the chromatographic runs of standards and samples in an analytical set. Then a standard can be analyzed, followed by a maximum of 4 samples (controls, fortified controls, or treated samples), followed by another standard, etc. The last injection should be a standard.

4.4 Calculations

4.4.1 *Methods*

The average response factor was calculated as follows:

$$\text{Response} = \frac{\text{Concentration (ng/mL) of Standard}}{\text{Peak Area Counts}}$$

$$Rf_{avg} = \frac{\sum \text{Standard Response}}{n}$$

where:

Rf_{ave} = Average Response Factor

n = total number of standards analyzed in a sample set

Concentration of nicosulfuron and metabolites in the fortified samples (ppb found) was then calculated using the equation below:

µg/kg (ppb) found =

$$\frac{A \times Rf_{ave}\text{ (ng/mL/area counts)} \times \text{Extract Volume (mL)} \times \text{Final Volume (mL)} \times \text{Dilution Factor}}{\text{Sample Weight (g)} \times \text{Aliquot Volume(mL)}}$$

where:

A = Corrected Peak Area Counts

= Peak Area Counts in sample – Peak Area Counts in control

Rf_{ave} = Average Response Factor

Percent Recovery was calculated as:

$$\% \text{ Recovery} = \frac{\text{Analyte Found (ppb)}}{\text{Fortification Level (ppb)}} \times 100$$

4.4.2 *Examples*

Calculation for the percent recovery of nicosulfuron (DPX-V9360) in Drummer, U.S.A. soil fortified at 1.0 ppb (sample DR-LOQ-A, Appendix 4), which was prepared and analyzed on September 9, 2004, is shown below.

Rf_{avg} of five DPX-V9360 standards = 1.67 x 10-6 ng/mL/area counts

Peak Area Counts (ac) for DPX-V9360, fortified sample = 310000

Peak Area Counts (ac) for DPX-V9360, control = 6260

Sample Weight = 10.0 grams

Final Volume = 5.0 mL

Fortification Level = 1.0 ppb

DPX-V9360 Found =

$$\frac{303740 \text{ ac} \times 1.67 \times 10^{-6} \text{ ng/mL/ac} \times 40 \text{ mL} \times 5.0 \text{ mL} \times 1}{10.0 \text{ g} \times 10 \text{ mL}}$$

$$= 1.01 \text{ ng/g} = 1.0 \text{ µg/kg} = 1.0 \text{ ppb}$$

$$\text{DPX-V9360 \%Recovery} = \frac{1.01 \text{ ppb}}{1.0 \text{ ppb}} \times 100 = 101\%$$