2.0 INTRODUCTION

Independent laboratory validation of soil matrix methods is required to fulfill the requirements under U.S. EPA OPPTS 850.7100, PR Notice 96-1.

The environmental analytical method for DPX-V9360, IN-V9367 and IN-J0290 in soil as described in DuPont-33143, entitled "Analytical Method for the Determination of Nicosulfuron (DPX-V9360), IN-V9367 and IN-J0290 in Soil Using HPLC/ESI-MS/MS" is applicable for the quantitation of DPX-V9360, IN-V9367 and IN-J0290 in soil.

DPX-V9360, IN-V9367 and IN-J0290 were extracted from soil samples immersed in 0.1 M ammonium carbonate/acetone (9:1, v/v) using a wrist-action shaker at room temperature. Following centrifugation, sample extracts were passed through an Isolute® NH_2 solid-phase extraction (SPE) cartridge. Extracts were further purified and concentrated with stacked SPE cartridges of Oasis™ HLB (top) and ENV+® (bottom). The eluate was evaporated and reconstituted. The purified extract was analyzed by reverse phase HPLC/ESI-MS/MS. Two transitions were monitored per analyte, and all were detected by positive ion electrospray MS/MS.

The analytical method was designed to achieve a LOQ of 1.0 µg/kg (ppb) for all analytes, and the Limit of Detection (LOD) was estimated to be 0.3 µg/kg (ppb). The independent validation thus evaluated DPX-V9360, IN-V9367 and IN-J0290 recoveries of samples fortified at 1x and 10x the LOQ level. The method was used as written.

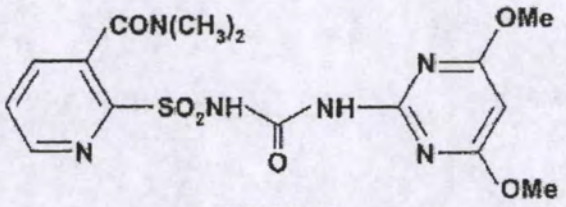
3.0 MATERIALS AND METHODS

3.1 Test Substance

The reference analytical standards (test substances) used for this study were:

DuPont code: DPX-V9360 (Nicosulfuron)

Chemical Structure:



DPX-V9360

CAS Name: 2-[[[[(4,6-Dimethoxy-2-pyrimidinyl)amino]carbonyl] amino]-sulfonyl]-*N,N*-dimethyl-3-pyridinecarboxamide monohydrate

Molecular weight: Average, 410.41 amu; monoisotopic, 410.10 amu

Formula: $C_{15}H_{18}N_6O_6S$

Source: E. I. du Pont de Nemours and Company

CAS Number: 111991-09-4

Batch/Lot Number: E98949-133

Purity: 97.9%

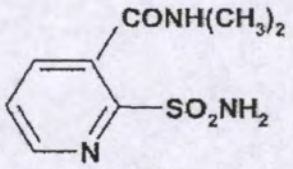
Receipt date: 23 January, 2012

Expiration date: 20 December, 2015

Storage: Ambient temperature

DuPont code: IN-V9367

Chemical Structure:



IN-V9367

CAS Name: 2-(Aminosulfonyl)-N,N-dimethyl-3-pyridinecarboxamide

Molecular weight: Average, 229.26 amu; monoisotopic, 229.05 amu

Formula: $C_8H_{11}N_3O_3S$

Source: E. I. du Pont de Nemours and Company

CAS Number: 112006-75-4

Batch/Lot Number: AG0132-068

Purity: 99.7%

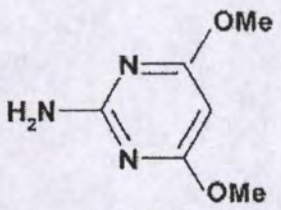
Receipt date: 23 January, 2012

Expiration date: 13 August, 2012

Storage: Ambient temperature

DuPont code: IN-J0290

Chemical Structure:



IN-J0290

CAS Name: 4,6-dimethoxy-2-pyrimidinamine

Molecular weight: Average, 155.16 amu; monoisotopic, 155.07 amu

Formula: $C_6H_9N_3O_2$

Source: E. I. du Pont de Nemours and Company

CAS Number: 36315-01-2

Batch/Lot Number: 9964-098

Purity: 99.9%

Receipt date: 23 January, 2012

Expiration date: 11 September, 2017

Storage: Ambient temperature

DPX-V9360, IN-V9367 and IN-J0290 were supplied by E. I. du Pont de Nemours and Company, DuPont Crop Protection, Newark, DE. Information pertaining to the characterization and stability of the test substances is archived by DuPont Crop Protection, E. I. du Pont de Nemours and Company, Newark, Delaware.

3.2 ***Test System***

In this study, the analytical method was validated in soil. Soil samples were taken from a field located at 17 Lee Blvd, Malvern, PA 19355. The soil control sample was dried by exposure in the chemical hood overnight and then ground before use.

Fortifications of the samples were made using 10 g of soil spiked with 0.10 µg/mL and 1.0 µg/mL standard solutions. The samples were assigned unique identification by the laboratory, an alpha-numeric sample ID along with additional designations such as "control" and "LOQ", as appropriate.

3.3 *Equipment*

Equipment used was either the same as that specified in the analytical method or equivalent. A Phenomenex® Luna Phenyl-Hexyl 3.0x150 mm 3-µm column was used at a flow rate of 0.425 mL/min, instead of a Phenomenex® Luna Phenyl-Hexyl 4.6x150 mm 3-µm column at a flow rate of 1.0 mL/min, as described in the method. This change was approved by the study monitor and demonstrated as equivalent to that specified in the method.

3.4 *Reagents*

Reagents used were either the same as that specified in the analytical method or equivalent grade of quality.

3.5 *Principles of the Analytical Method*

The analyses in this study followed the analytical method for Nicosulfuron (DPX-V9360), IN-V9367 and IN-J0290 in soil, as described in the method for DuPont-33143. The following is a summary of the method conducted at Alliance Pharma. The complete description of the method is described in the original method (DuPont-33143).

Nicosulfuron and its metabolites IN-V9367 and IN-J0290 were extracted from a 10 gram soil sample. Those requiring fortification were fortified with the appropriate standard solution and allowed to sit in a hood for 15-20 minutes. Thirty milliliters of 0.1 M ammonium carbonate/acetone (9:1, v/v) were added to each sample. The samples were then shaken via a wrist action shaker for 15-20 minutes then centrifuged for 10-15 minutes at approximately 3000 rpm with no refrigeration. One-fourth aliquot of extract was separated and passed through an Isolute® NH_2 SPE cartridge. The partially purified extract was evaporated in a nitrogen-evaporator system to remove acetone and then acidified with 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). The purified extract was analyzed by reverse-phase HPLC using a Phenomenex® Luna phenyl-hexyl 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 1 M formic acid was added to 990 µL of each of the standards and fortified 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. The confirmatory method was based on the relative ratios of the two MS/MS ion transitions during the validation.

Method validation was accomplished by analyzing each of the three analytes in validation sets all consisting of 2 blank control specimens, 5 replicate specimens fortified at the LOQ, and 5 replicate specimens fortified at 10xLOQ.

3.6 *Modifications, Interpretations, and Critical Steps*

The analytical method was run exactly as written, except a Phenomenex® Luna Phenyl-Hexyl 3.0x150 mm 3-µm column was used at a flow rate of 0.425 mL/min, instead of a Phenomenex® Luna Phenyl-Hexyl 4.6x150 mm 3-µm column at a flow

rate of 1.0 mL/min, as described in the method. The study monitor was notified of and approved this substitution. The substitution was demonstrated to be equivalent to that specified in the method. There was no one critical step that appeared to impact the analytical results.

3.7 Instrumentation

HPLC Conditions

System:	Shimadzu LC-20AD / Sil-20AC Autosampler
Column:	Phenomenex® Luna Phenyl-Hexyl, 3 mm x 150 mm, 3-µm
Column Temperature:	40°C
Injection Volume:	25 µL
Autosampler Temperature:	5°C
Conditions:	A: 0.1 mM Formic Acid in 0.01 mM Ammonium Formate
	B: Methanol
	Flow in mL/minute
	Time %A %B Flow 0.00 95 5 0.425 10.0 5 95 0.425 13.0 5 95 0.425 13.1 95 5 0.425 17.0 95 5 0.425
Analyte Retention Times (minutes)	
DPX-V9360	~10.9
IN-V9367	~5.7
IN-J0290	~8.4

The detection method utilized was LC-MS/MS employing atmospheric pressure electrospray ionization (ESI) interface in the positive mode on a triple quadrupole instrument. The acquisition method was adjusted to maximize the response of the fragment ions detected. The ion transitions for DPX-V9360, IN-V9367 and IN-J0290 are shown below:

SYSTEM:	APPLIED BIOSYSTEMS 4000 Q-TRAP			
ANALYTES	IONS MONITORED	DECLUSTERING POTENTIAL (DP)	COLLISION ENERGY (CE)	EXIT POTENTIAL (CXP)
DPX-V9360	**411.0 → 182.0 AMU[1]**	**80**	**30**	**20**
	411.0 → 213.0 AMU	80	25	40
IN-V9367	**230.0 → 78.0 AMU[1]**	**60**	**50**	**15**
	230.0 → 106.0 AMU	60	30	5
IN-J0290	**156.0 → 57.0 AMU[1]**	**50**	**35**	**5**
	156.0→ 100.0 AMU	50	25	15
Ion Mode:	Positive			
Turbo Spray Voltage:	4500 V			
Source Temperatures:	350 °C			
CUR:	20 psig			
CAD:	Med			
GS1:	40 psig			
GS2:	50 psig			
Dwell	50 mseconds			

[1] Transition ion used for quantitation.

The instrument was operated in the MS/MS (MRM) positive ion mode for quantitative analysis. The ion chromatograms were integrated and the peak areas were used for quantitation.

For each analytical run, a six-point standard curve was prepared by injecting constant volumes of mixed standard solutions composed of each analyte of interest. Constant volume injections were used for sample extracts, as well. The relative ratio of the fragment ions was evaluated to confirm the presence of an analyte in an unknown sample.

3.8 Calculations

Residues of DPX-V9360, IN-V9367 and IN-J0290 were quantitated by external standards. A calibration curve for each analyte was generated by plotting the detector's response in peak area versus the amount (ng) of standard injected. The data system derived an equation for the fit of the standard curve, and this equation was used to calculate intercept and slope of the linear regression curve.

The calibration curve was obtained by direct injection of 25 µL of standard (ranging from 0.25 ng/mL to 20 ng/mL) into the LC-MS/MS for each analyte. In a given injection run, the same injection volume was used for all samples and standards.

Peak integration and quantitation were performed using Applied Biosystems' Analyst software version 1.4.2. Calculations of recovery results were computed for each set

of samples in a Microsoft Excel® spreadsheet. The equations used for quantitation are shown below.

$R = C_{End} X V_F / (AF*DF) / W$

Where:

R:	Analyte residue in µg/kg (ppb)
C_{End}:	Final concentration of analyte derived from calibration curve in ng/mL
AF:	Aliquot factor = Aliquot extraction volume ($V_{aliq\ Ex}$) / Total extraction volume ($V_{Total\ Ex}$)
DF:	Dilution factor = Aliquot volume (V_{aliq}) / Total volume (V_{Total})
V_F:	Final volume
W:	Soil matrix sample weight: 10 g

Recoveries (Rec.) were calculated for the fortified specimens as follows:

$Rec. = (R / R_{fortified}) \times 100\ \%$

Example: Table 1, Sample LOQ A, DPX-V9360, Soil, Fortified @ 1 ppb

$C_{End} = 0.557$ ng/mL

$R_{fortified} = 1$ ppb

$AF = V_{aliq\ Ex} / V_{Total\ Ex} = 15\ mL / 60\ mL = 0.25$

$DF = V_{aliq} / V_{Total} = 1\ mL / 1\ mL = 1$

$R = C_{End} X V_F / (AF*DF) / W$

$= 0.557\ (ng/mL) * (5\ mL) / (0.25*1) / 10\ g = 1.1$ ppb

$Rec. = (R / R_{fortified}) \times 100\ \% = (1.1 / 1) \times 100\% = 110\%$