2.0   INTRODUCTION

Independent laboratory validation of enforcement methods are required by the U.S. EPA OPPTS 850.7100 (Reference 1) and EU Guidance document SANCO/825/00 rev. 7 (Reference 2).

The subject method is applicable for the quantitation of IN-NXX70, IN-QKV54, or IN-RNU71 in soil, as described in DuPont-29747 (Reference 3). Loamy sand soil was chosen to validate the analytical method as a representative matrix.

Fortification concentrations in this study were chosen to provide method performance data at the method LOQ and 10×LOQ for the matrix examined. The stated method LOQ was 1.0 ppb for all analytes in soil.

The analytical method was performed without any significant modifications using the extraction with SPE clean-up. The method was successfully validated for IN-NXX70, IN-QKV54, and IN-RNU71 in soil in one trial.

3.0   MATERIALS AND METHODS

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   Test and Reference Substances

The IN-NXX70, IN-QKV54, and IN-RNU71 analytical standards were received from DuPont ambient, assigned a unique ABC Laboratories identification code, and stored at room temperature. The analytical standards were used to prepare mixed fortification and calibration standards for LC/MS/MS instrumental analyses. The following lots were used for method verification for this Independent Laboratory Validation study.

TEST SUBSTANCE	GRADE	SOURCE	DATE RECEIVED	ABC LABS ID#	% PURITY	LOT OR CODE	EXP DATE	PHYSICAL APPEARANCE
IN-NXX70	Technical	DuPont	21 May 09	PS-23670	98.2	E112180-119F	01 Apr 12	Solid
IN-QKV54	Technical	DuPont	12 Jan 10	PS-9000621	98.3	D101698-53	05 Mar 13	Solid
IN-RNU71	Technical	DuPont	12 Jan 10	PS-9000622	93.8	E115107-39A	05 Mar 13	Solid

IN-NXX70

Stock Item/Batch No.	4008307 (001)
CAS Registry No.:	NA
Molecular Weight:	437.3
Empirical Formula:	$C_{19}H_{13}BrN_6O_2$
Chemical Structure:	
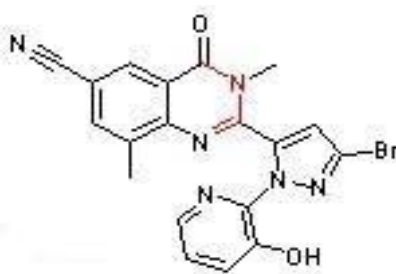
IN-QKV54

Stock Item/Batch No.	4010024 (001)
CAS Registry No.:	NA
Molecular Weight:	344.2
Empirical Formula:	$C_{14}H_{10}BrN_5O$
Chemical Structure:	
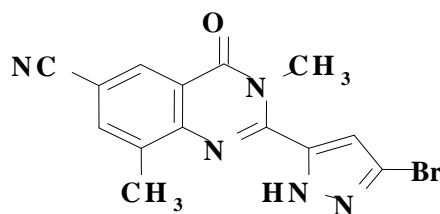
IN-RNU71

Stock Item/Batch No.	4010002 (001)
CAS Registry No.:	NA
Molecular Weight:	437.3
Empirical Formula:	$C_{19}H_{13}BrN_6O_2$
Chemical Structure:	
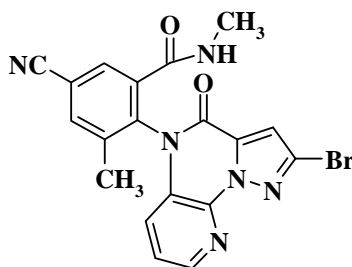
Information pertaining to the characterization and stability of the test substances is archived by E. I. du Pont de Nemours and Company, DuPont Crop Protection, Newark, Delaware. Characterization data were provided by E.I. du Pont de Nemours and Company, DuPont Agricultural Products, Newark, Delaware. Certificates of Analysis, including lot numbers and purity, are included with the study raw data file that will be archived by E. I. du Pont de Nemours and Company.

3.2   Test System

The subject method is applicable for the quantitation of IN-NXX70, IN-QKV54, and IN-RNU71 in soil. In this study, the analytical method was validated in loamy sand from California, a representative soil matrix previously sampled as part of an earlier study which did not include analysis of DPX-HGW86 or its photoproducts.

All control matrices were acquired from sample 89.CA.BA.SL.1.A.9.0-2in. The control matrix was stored frozen and processed to verify that the control was free of interferences at the appropriate retention times.

SOIL NAME (LOCATION, DUPONT STUDY NO./NOTEBOOK NO.)	TYPE	PH$_W$	SAND (%)	SILT (%)	CLAY (%)	OM (%)
California, DuPont-26889	Loamy Sand	6.4	83	12	5	0.6

3.3   Equipment

The following equipment items were used in the conduct of this independent laboratory validation.

EQUIPMENT DESCRIPTION	PRODUCT ID	SUPPLIER
Analytical Balance	Mettler XP205DR Analytical Balance Mettler BB2440 Analytical Balance	Mettler Instrument Corp (Hightstown, NJ)
Analytical Evaporator	N-Evap® Model 111 with stainless steel luer fit needles with water bath	Organomation Assoc. (South Berlin, MA)
Bench Top Centrifuge	Beckman GP Centrifuge	Beckman coulter (Fullerton, CA)
Filtration	Xpertex® syringe filter, 0.2-μm PTFE, 13 mm diam. Cat. No. 9445601 BD™ Syringe, 3 mL, luer-lok™ tip, Cat. No. 14-823-40	PJ Cobert Associates (St. Louis, MO) Fisher Scientific (Fairlawn, NJ)
Labware	50 mL Disposable Centrifuge Tube, Kimble® 50-mL conical glass centrifuge tube,	Fisher Scientific (Fairlawn, NJ)

EQUIPMENT DESCRIPTION	PRODUCT ID	SUPPLIER
	Cat. No. 05-538-41A BD™ Syringe, 60 mL, luer slip tip, Cat. No. 14-823-43	
Pipettes	FisherBrand® Disposable 10 mL Pipettes, Cat. No. 13-678-31J FisherBrand® Disposable 5 mL Pipettes, Cat. No. 13-678-25D FisherBrand® 9" Disposable Pastuer Pipettes, Cat. No. 13-678-20D 100-1000 μL Hamilton pipettor	Fisher Scientific (Fairlawn, NJ)
Pipettes	100-1000 μL Microman M1000 micropipettor 50-250 μL Microman M250 micropipettor	Gilson (Middleton, WI)
Shaker	Eberbach Model 6010	Eberbach Corporation (Ann Arbor, MI)
Sonication	5200 Ultrasonic cleaner	Branson Ultrasonics Corp. (Danbury, CT)
Solid Phase Extraction	Visiprep DL™ SPE Manifold, Cat. No. 57250-U	Supelco (Bellefonte, PA)
Solid Phase Extraction	Bond Elut™ NH$_2$ SPE Cartridge, 6 mL/500 mg, Cat. No. 12256045, Bond Elut™ ENV SPE Cartridge, 6 mL/500 mg, Cat. No. 12255011	Varian, Inc. (Palo Alto, CA)
Steel Beads	Steel Shot F, 2/9 in. diameter Cat. No. SH1F	Ballistics Products (Corcoran, MN)
Vortex Mixer	Vortex Genie 2, cup head Cat. No. 58815-234	VWR International (Bridgeport, NJ)

UPLC/MS/MS System

EQUIPMENT DESCRIPTION	PRODUCT ID	SUPPLIER
UPLC	Waters Acquity System	Waters (Milford, MA)
Autosampler Vials	Target DP Snap-It Vials and caps with T/S/T Septa, Cat. No. 03-395C and 03-396M, respectively	Fisher Scientific (Fairlawn, NJ)
UPLC Column	Synergi Polar RP; 3 mm × 50 mm, 2.5 μm particle size diameter	Phenomenex® (Torrance, CA)
Switching Valve	Valco 6-Port electrically actuated valve, Cat. No. 1384	Valco Instruments, Inc. (Houston, TX)
Triple Quadrupole MS	API 5000 triple quadrupole mass spectrometer using Turbo Ion Spray (TIS) and Analyst version 1.5 software	Applied Biosystems/MDS Sciex (Foster City, CA)

3.4 Reagents

REAGENTS	PRODUCT DESCRIPTION	PRODUCT ID	SUPPLIER
Formic Acid	99+%	A0255304 A0273151	Acros (Fairlawn, NJ)
Methanol	HPLC	0863444 093826 094346 095121 095587 096610 100360	Fisher Scientific (Fairlawn, NJ)
Acetone	HPLC	095617	Fisher Scientific (Fairlawn, NJ)
Acetonitrile	HPLC	086858 092333 090871 091849 B00J0596	Fisher Scientific (Fairlawn, NJ) Acros (Fairlawn, NJ)
DMSO	HPLC	B0514866	Acros (Fairlawn, NJ)
Water	In-house De-Ionized OmniSolv®	NA 49242 49203 50056	Labconco (Kansas City, MO) EM Science (Gibbstown, NJ)
Ammonium Hydroxide	Certified A.C.S. *PLUS*	072478	Fisher Scientific (Fairlawn, NJ)

3.5 Principles of the Analytical Method

A 10-g soil sample was prepared by adding twenty-five milliliters of soil extraction solution (90% acetone:10% 1.0 M formic acid) to the sample container along with 3 ball bearings. Samples were capped, shaken on high for 15 minutes on a platform shaker, and then centrifuged at approximately 3000 RPM for five minutes. The supernatant was decanted into a 50-mL centrifuge tube. This extraction procedure was repeated and the volume adjusted to 50 mL with extraction solution. Ten milliliters of the extract were removed and combined with 40 mL of water, and the sample was mixed thoroughly.

The soil samples were purified using NH2 and ENV solid phase extraction (SPE) cartridges. The cartridges were conditioned with 5 mL of methanol followed by 5 mL of water (NH2) or 5 mL of 1.0 mM formic acid in (aq) (ENV). Sixty milliliter reservoirs were attached to the cartridges, and additional water (NH2) or 1.0 mM formic acid (ENV) was added to keep SPE cartridges wet during sample loading. Samples were added to the NH2 reservoir and pulled through the column at approximately 1 to 5 mL/min. Eluate was collected and, after the samples had passed through the NH2 cartridges, the cartridges were washed with 8 mL of 10% acetonitrile in water. The wash was collected with the eluate. The samples were then added to the ENV reservoirs and pulled through the ENV cartridges using gravity flow. After the samples had passed through the column, the ENV cartridges were washed with 5 mL of water and vacuum was applied for approximately

20-30 seconds. The ENV cartridge was eluted into a 50-mL glass centrifuge tube with 3 x 5 mL of 0.02M ammonium hydroxide in acetonitrile, and then the samples were evaporated to dryness under a stream of N2 in a heated water bath set at approximately 40°C. One milliliter of methanol was added to each sample, the tubes were capped, and the samples were mixed using a vortex mixer and then sonicated for 2 minutes. One milliliter of 0.02 M formic acid solution was added, the tube was capped, and the samples were mixed using a vortex mixer and then sonicated for 2 minutes. Samples were filtered with a 3-mL syringe and 0.2-µm PTFE filter into an HPLC vial for analysis.

3.6   *Modifications, Interpretations, and Critical Steps*

DuPont-29747 analytical method was run as written.

3.7   *Instrumentation*

3.7.1   <u>Chromatography</u>

Reversed-phase liquid chromatography was used to separate the analytes from co-extractants. A Synergi Polar-RP (3.0×50 mm, 2.5-µm particle) column was used.

HPLC Conditions with SPE Cleanup

System:	Waters Acquity UPLC
Column:	Synergy Polar-RP (3.0 × 50 mm, 2.5 -µm particle)
Column Temperature:	30°C
Injection Volume:	3.0 µL
Autosampler Temperature	4°C
Conditions:	A: 0.1% formic acid in water
	B: methanol

Time	%A	%B	Flowrate (mL/min)	Comments
0.00	50	50	0.5	
3.00	30	70	0.5	
6.00	30	70	0.5	
7.00	5.0	95	0.5	
8.00	5.0	95	0.5	
8.10	50	50	0.5	
10.00	50	50	0.5	End Run

Analyte	Retention Time (min)
IN-NXX70:	4.4
IN-QKV54:	4.8
IN-RNU71:	2.7
Total Run Time:	10.00

3.7.2   <u>*LC/MS/MS Analysis*</u>

Analysis of IN-NXX70, IN-QKV54 and IN-RNU71 was performed using a Sciex API 5000 LC-MS/MS, equipped with a TurboIonSpray source, and operated in MRM, positive ion mode. Quantitation was based on an average response factor using peak areas supplied by Analyst software version 1.5. Calculations were performed using Microsoft Excel. A summary of representative experimental conditions is provided in the following table:

Sciex API 5000 LC/MS/MS Mass Spectrometer Conditions with SPE Cleanup

ANALYTES	IONS MONITORED (AMU)	CXP (COLLISION CELL EXIT POTENTIAL)	DP (DECLUSTERING POTENTIAL)	DWELL TIME (MSEC)	COLLISION ENERGY
IN-NXX70a	437.2 → 344.2	27V	70V	100	43V
IN-NXX70	437.2 → 406.1	27V	75V		36V
IN-QKV54	344.0 → 236.2	15V	65V	100	44V
IN-QKV54a	344.0→ 186.0	11V	70V		49V
IN-RNU71a	437.4 → 300.3	20V	70V	100	50V
IN-RNU71	437.4 → 406.3	14V			35V

Scan Type/Polarity: MRM/Positive
Ion Source Voltage: ESI, 5500
Collision Gas (CAD): 8 psig
Curtain Gas (CUR): 15 psig
Nebulizer Gas (GS1): 60 psig
Heater Gas (GS2): 50 psig
Source Heater (TEM): 500°C
Interface Heater (IHE): ON
Resolution Q1: Unit
Resolution Q3: Unit

3.7.3 *Calibration Procedure*

Calibration standards were interspersed throughout the batch with concentrations varying randomly. The response factor of each calibration standard was calculated by dividing the analyte concentration by the analyte peak area. The average response was calculated for calibration standards injected with each batch.

3.8 *Calculations*

A standard curve was determined by linear regression (y = mx+b) for each compound by plotting the peak area response obtained from IN-NXX70, IN-QKV54, and IN-RNU71 standards against the corresponding concentration (ng/mL) of each analyte in the standards. The correlation coefficient (R^2) was ≥0.98 for all analyses. The concentration (µg/kg) found in the samples was determined by the following calculation:

$$X = \frac{A \times Rf_{avg} \, (ng/mL / \, area \, counts) \times Extract \, Volume \, (mL) \times Final \, Volume \, (mL) \times Dilution \, Factor}{Sample \, Weight \, (g) \times aliquot \, Volume \, (mL)}$$

Where: A = Corrected Peak Area Counts

= Peak Area Counts in Sample (ac) – Peak Area Counts in Control (ac)

Rf_{avg} = Average Response Factor

= ($\sum$ Standard Response)/n

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

n   = total number of standards analyzed in a sample set

Percent Recovery was calculated as:

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

For example, the calculation for the concentration (µg/kg) found and percent recovery of IN-NXX70 in soil fortified at 1.0 µg/kg (sample LOQ-2), which was prepared and analyzed on 08 June 2010, is shown below:

RF_{avg} of eight standards   = 1.2007 x 10^{-4} ng/mL/area counts (ac)
Peak Area Counts of fortified sample = 8309
Peak Area Counts of Control sample = 0 (average)
Sample Weight   = 10 g
Total Extract Volume   = 50 mL
Aliquot Volume   = 10 mL
Final Volume   = 2.0 mL
Dilution Factor   = 1
Fortification Level   = 1.0 µg/kg

IN-NXX70 Found =

$$\frac{(8309 - 0) \times 1.2007\mathrm{e}^{-04} \times 50 \text{ mL} \times 2.00 \text{ mL} \times 1}{(10 \text{ g} \times 10 \text{ mL})}$$

$$= 0.99766 \text{ µg/kg} = 0.9977 \text{ µg/kg}$$

$$\% \text{ Recovery} = \frac{0.9977 \text{ mg/kg}}{1.0 \text{ µg/kg}} \times 100 = 100\%$$