2.0 Background Information

DPX-HGW86 is an experimental insecticide being developed for control of piercing and sucking insects such as aphids, thrips, whiteflies, and other pests on a wide variety of crops. The purpose of the current study is to develop a method for DPX-HGW86 and its significant photoproducts in soil with a target limit of quantitation (LOQ) of 1.0 ppb. The method is intended to meet SANCO/825/00 rev.7 and EPA Guidance OPPTS. 850.7100 (Reference 1 and Reference 2).

The method consists of soil samples extracted twice with a 90:10 acetone:1.0M formic acid solution and, after sequential clean-up through NH2 and ENV-SPE cartridges, they were analyzed by LC/MS/MS. The structures of DPX-HGW86 and its photoproducts IN-NXX70, IN-QKV54, and IN-RNU71 can be found in Appendix 2.

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

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	Corning® 50 mL Disposable Centrifuge Tube, Cat. No. 05-538-55A Kimble® 50-mL conical glass centrifuge tube, Cat. No. 05-538-41A BD™ Syringe, 60 mL, luer slip tip, Cat. No. 14-823-43	Fisher Scientific (Fairlawn, NJ)
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	Fisher Scientific (Fairlawn, NJ)
Pipettes	100-1000 µL Microman M1000 micropipettor 50-250 µL Microman M250 micropipettor	Gilson (Middleton, WI)

Equipment Description	Product ID	Supplier
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)

3.2 Reagents and Standards

3.2.1 Reagents

The equivalency/suitability of substituted reagents should be verified.

Reagents	Product Description	Product ID	Supplier
Formic Acid	99%	A0251444 A0273151	Acros (Fairlawn, NJ)
Methanol	Optima®, ACS	095121 095587 096610	Fisher Scientific (Fairlawn, NJ)
Acetone	HPLC	095016	Fisher Scientific (Fairlawn, NJ)
Acetonitrile	HPLC	090871 091849 B00J0596	Fisher Scientific (Fairlawn, NJ) Acros (Fairlawn, NJ)
DMSO	HPLC	B0514866	Acros (Fairlawn, NJ)
Water	In-house De-Ionized	NA	Labconco (Kansas City, MO)

REAGENTS	PRODUCT DESCRIPTION	PRODUCT ID	SUPPLIER
	OmniSolv®	49242 49126 49273	EM Science (G bbstown, NJ)
Ammonium Hydroxide	Certified, ACS	B00J0596	Acros (Fairlawn, NJ)

3.2.2 *Reference Analytical Standards*

Reference analytical standards of the following were used:

COMPOUND	DASH NO.	PURITY (%)
DPX-HGW86	307	99.2
IN-NXX70	001	98.2
IN-QKV54	001	98.3
IN-RNU71	001	93.8

The standards were synthesized at E. I. du Pont de Nemours and Company, DuPont Agricultural Products, Wilmington, DE. Characterization data are archived by DuPont Agricultural Products, E. I. du Pont de Nemours and Company, Wilmington, DE.

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*

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 3-4 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 30-40°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.

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 glass- and plastic ware 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 and Stability of Reagent Solutions*

The following procedures may be adjusted to prepare different volumes.

90:10 Acetone:1.0 M Formic Acid (Extraction Solution)

Add 900 mL acetone to 100 mL of 1 M formic acid. The solution may be stored at room temperature and should be stable for up to 1 year.

10% Acetonitrile (SPE Wash Solution)

Add 100 mL acetonitrile to 900 mL of purified water. The solution may be stored at room temperature and should be stable for up to 3 months.

1 M Formic Acid in Water

Add 4.5 mL of concentrated formic acid to approximately 80 mL of de-ionized water, and dilute to 100 mL with de-ionized water. The solution may be stored at room temperature and should be stable for 6 months.

1 mM Formic Acid in Water

Add 1.0 mL of 1 M formic acid solution to approximately 800 mL of de-ionized water, and dilute to 1 L with de-ionized water. The solution may be stored at room temperature and should be stable for 6 months.

0.02 M Formic Acid in Water

Add 20 mL of 1 M formic acid solution to approximately 800 mL of de-ionized water, and dilute to 1 L with de-ionized water. The solution may be stored at room temperature and should be stable for 6 months.

0.02 M Ammonium Hydroxide in Acetonitrile

Add 20 mL of 1 M ammonium hydroxide solution to 980 mL of acetonitrile. The solution may be stored at room temperature and should be stable for 6 months.

50:50 Methanol:0.02 M Formic Acid (Dilution Solution)

Add 500 mL methanol to 500 mL of 0.02 M formic acid solution. The solution may be stored at room temperature and should be stable for up to 1 year.

0.1% Formic Acid in Water (Mobile Phase A)

Add 2 mL concentrated formic acid (99%) to approximately 1.5 L of de-ionized water, and dilute to 2 L with de-ionized water. The solution may be stored at room temperature and should be stable for 3 months.

1:1:1 Acetonitrile:Methanol:Water (Needle Rinse Solution)

Add equal parts of acetonitrile, methanol, and water. Mix well and sonicate. The solution may be stored at room temperature and should be stable for 3 months.

***Note**:* The expiration dates of the above listed solvents and reagents may be extended if their suitability has been verified with acceptable control and fortification recovery data.

4.2.3 Stock Standard Preparation and Stability

Individual stock solutions are required for each analyte. Use an analytical balance that provides a weight precision to at least three significant figures. To prepare a stock solution of 100 µg/mL, weigh approximately 10.0 mg (adjusted for purity) of the analyte in a tarred 100-mL volumetric flask. Add approximately 100 mL of acetonitrile and sonicate to dissolve. **IN-QKV54 and IN-RNU71** may require dimethyl sulfoxide (DMSO) to dissolve. If analyte does not go into solution with acetonitrile, add DMSO in 10 mL increments and sonicate after each addition. Once analyte dissolves, dilute to a total volume of 100 mL with acetonitrile. These solutions are stored in a refrigerator at approximately 4°C and are stable for at least six months. Stock standards use may be extended if supported by stability test data.

4.2.4 Standard Preparation and Stability

The following standard preparation procedures are examples and may be adjusted to prepare different volumes. The individual stock standards are combined in the fortification standards.

Prepare fortification solutions from dilutions of the individual stock solutions. Prepare a 1.0-µg/mL, 0.10-µg/mL, and 0.010-µg/mL fortification solutions for sample fortification at the 10 × LOQ and LOQ, respectively. Alternative concentrations may be prepared as needed for other fortification levels. Store fortification solutions at or below 4°C and replace monthly.

1.0 μg/mL Fortification Solution

Dilute 1.0 mL of the stock solution for each analyte into a common 100-mL volumetric flask and fill to line with acetonitrile, cap and mix well.

0.10 μg/mL Fortification Solution

Dilute 10.0 mL of the 1.0-μg/mL fortification standard into a 100-mL volumetric flask and fill to line with acetonitrile, cap and mix well.

0.010 μg/mL Fortification Solution

Dilute 1.0 mL of the 1.0-μg/mL fortification standard into a 100-mL volumetric flask and fill to line with acetonitrile, cap and mix well.

4.2.5 Calibration Standard Preparation and Stability

LC Calibration standards are prepared from dilutions of fortification standards with methanol and 0.02 M formic acid. Five or more calibration standards are recommended. The concentration of sample fortified at the LOQ and carried through the extraction is equivalent to a final concentration of 1.0 ng/mL for the each analyte. Keep calibration standards refrigerated and they should be stable for up to a week.

Calibration standards can be prepared according to the following table (alternative or additional standards may be prepared as needed). Prepare weekly.

Initial Standard (μg/mL)	Volume of Initial Standard (mL)	Volume of Methanol (mL)	Volume of 0.02 M Formic Acid (mL)	Final Concentration (ng/mL)
0.10	0.50	2.00	2.50	10.0
0.10	0.25	2.25	2.50	5.0
0.10	0.15	2.35	2.50	3.0
0.10	0.10	2.40	2.50	2.0
0.010	0.50	2.00	2.50	1.0
0.010	0.40	2.10	2.50	0.80
0.010	0.30	2.20	2.50	0.60
0.010	0.25	2.25	2.50	0.50

4.2.6 *Source and Characterization of Samples*

Samples were characterized under GLP by AGVISE Laboratories of Northwood, ND. Results of the characterization are listed below:

Matrix	Soil Classification	pH	% Organic Matter	C.E.C. (meq/100 g)	Bulk Density
Soil (0-2 inch)	Sandy Clay Loam	7.9	1.2	19.4	1.19

4.2.7 *Storage of Samples*

In order to obtain a homogeneous representative sample the entire soil sample should be homogenized using a Hobart processor (or equivalent). Dry ice should be added to keep sample frozen during processing.

4.2.8 *Sample Fortification Procedure*

All fortifications were made directly to the soil sample in the centrifuge tubes after measuring the sample. Ten gram samples were fortified with the 1-µg/mL and 0.1-µg/mL multi-analyte fortification standards.

Sample Identification	Amount (g)	Fortification Solution		Fortification (ppb)
		µg/mL	mL	
LOQ Fort	10 ± 0.1	0.10	0.10	1
10×LOQ Fort	10 ± 0.1	1.0	0.10	10

4.2.9 *Analyte Extraction Procedure*

1. Weigh 10 ± 0.1 grams of soil into a 50-mL centrifuge tube. Fortify sample if necessary.
2. Add 25 mL of soil extraction solution (90:10 Acetone:1.0 M Formic Acid) and 3 to 4 steel ball bearings. Shake on a wrist action shaker at high speed for 15 minutes.
3. Centrifuge sample for 5 minutes at approximately 3000 RPM in a bench top centrifuge. Decant supernatant into a clean 50-mL centrifuge tube.
4. Repeat Steps 2 and 3 combining the extracts in the 50-mL centrifuge tube. Adjust extract volume to 50 mL with extraction solution and mix well.

4.2.10 *Analyte Purification Procedure*

1. Pipet a 10-mL aliquot of the extract from Step 4 of the extraction procedure into a clean 50-mL centrifuge tube; add 40 mL of purified water and mix well.
2. Condition an (6-cc/500-mg) NH2 Cartridge with 5 mL of methanol followed by 5 mL of purified water allowing the eluate to go to waste. Attach a 60-mL reservoir to the NH2 Cartridge; an additional 5 mL of water may be added to keep the cartridge wet during sample loading. Add the sample to the reservoir and pull through the column at approximately 1-5 mL/min.
3. Collect eluate. (Use vacuum if needed to maintain a flow of 1-2 drops per second). After the sample has gone through the NH2 cartridge, wash with 8 mL of 10% acetonitrile in water and collect the eluate. The sample is now ready for the ENV SPE clean-up.
4. Condition an (6-cc/500-mg) ENV cartridge for clean-up using 5 mL of methanol followed by 5 mL of 1 mM formic acid (aq) allowing the eluate to go to waste. Attach a 60-mL reservoir to the NH2 Cartridge; an additional 5 mL of 1mM formic acid (aq) may be added to keep the cartridge wet during sample loading.
5. Load the sample from Step 3 through the ENV cartridge using gravity flow. After the sample is through the column, discard the 60-mL reservoir and wash the ENV cartridge with 5 mL of water. Apply vacuum for approximately 20-30 seconds to remove any excess solution. Analytes are retained on the ENV cartridge.
6. Elute the ENV cartridges into a 50-mL glass centrifuge tube with 3 x 5 mL of 0.02 M ammonium hydroxide in acetonitrile using gravity flow.
7. Evaporate to dryness on an N-Evap using a heated water bath (Recommended temperature is around 40°C). Reconstitute the extracts in 1.0 mL of methanol. Mix the sample for approximately 20 seconds using a vortex mixer, and then sonicate for approximately 2 minutes. Add 1.0 mL of 0.02 M formic acid (aq); mix the sample for approximately 20 seconds using a vortex mixer, and then sonicate for approximately 2 minutes.
8. Filter a portion of the sample through a 13 mm 0.2-µm PTFE filter into an autosampler vial and submit for LC/MS/MS analysis.

4.3 Instrumentation

4.3.1 *Analysis of DPX-HGW86 and Photoproducts*

This method uses gradient-elution reversed-phase UPLC on a polar-RP column. The column choice reflects experimental results indicating optimum chromatographic separation from co-extractants. Alternative chromatographic conditions can be used provided the analytical method is validated and acceptable recoveries are obtained. UPLC instrument parameters are located in Appendix 4. HPLC instrumental conditions for DPX-HGW86 and metabolites in soil can be found in DuPont-15440. Similar conditions should be suitable for DPX-HGW86 and photoproducts in soil.

4.3.2 UPLC Operating Conditions

System:	Waters Acquity
Column:	50 × 3.00 mm, Synergi Polar-RP analytical column with 2.5-µm diameter packing.
Column Temperature:	30°C
Injection Volume:	3.0 µL
Autosampler Temperature:	10°C
Flow Rate:	0.500 mL/min
Conditions:	Time / %A / %B / Flow Rate (mL/min): 0.00 / 50.0 / 50.0 / 0.500; 3.00 / 30.0 / 70.0 / 0.500; 6.00 / 30.0 / 70.0 / 0.500; 7.00 / 5.0 / 95.0 / 0.500; 8.00 / 5.0 / 95.0 / 0.500; 8.10 / 50.0 / 50.0 / 0.500; 10.00 / 50.0 / 50.0 / 0.500. A: 0.1% Formic acid in Water; B: Methanol
Approximate Retention Times	(minutes)
DPX-HGW86	2.82
IN-NXX70	4.19
IN-QKV54	4.57
IN-RNU71	2.68
Total Run Time:	10.0 minutes

Conditions gradient:

Time	%A	%B	Flow Rate (mL/min)
0.00	50.0	50.0	0.500
3.00	30.0	70.0	0.500
6.00	30.0	70.0	0.500
7.00	5.0	95.0	0.500
8.00	5.0	95.0	0.500
8.10	50.0	50.0	0.500
10.00	50.0	50.0	0.500

A: 0.1% Formic acid in Water

B: Methanol

Triple Quadrupole MS Operating Conditions

Splitter: None (all flow from column goes to source)

Interface: Turbospray

Mode: MRM

Resolution: Unit

TIS Source: Positive

Analyte	Q1 (m/z)	Q3 (m/z)	Dwell (msec)	CUR (psi)	GS1 (psi)	GS2 (psi)	TEM (°C)	Ihe	IS (V)	CAD (psi)	DP (V)	EP (V)	CE (V)	CXP (V)
DPX-HGW86	475.3	444.1	100	30	60	40	500	on	5500	6	60	10	24	17
	475.3	286.1											20	24
IN-NXX70	437.2	344.0	100	30	60	40	500	on	5500	6	60	10	43	27
	437.2	405.9											36	34
IN-QKV54	344.0	236.1	100	30	60	40	500	on	5500	6	60	10	45	15
	344.0	186.0											47	
IN-RNU71	437.1	300.2	100	30	60	40	500	on	5500	6	60	10	50	20
	437.1	406.0											36	35

4.3.3 *Calibration Procedure and Sample Analysis*

A chromatographic standard should be analyzed prior to the start of analyses to establish that the instrument is working properly. Operating parameters must be tailored to the particular instrument used, especially if it is to be an alternate vendor's instrument, and should be checked daily. Note that it may be necessary to add some ion channels other than those used for development of this method when utilizing this method on other instrumentation. Each ion channel used for sample analysis/ quantitation must be checked to insure it is free of interference. A control will be used to demonstrate that baseline interference is less than signal-to-noise 3:1. Begin each sample set by injecting a minimum of 2 calibration standards. The first injection of a calibration standard should always be disregarded.

4.4 *Calculations*

4.4.1 *Methods*

The recoveries of each analyte can be calculated using the following equation:

ppb analyte Found =

$$\frac{[\text{Peak Area - b}] \div \text{m} \times [\text{Aliquot Factor x Final Vol.(mL) x Dilution Factor}]}{\text{Sample Volume (g)}}$$

Where:

Extraction Volume = 50 mL

Aliquot Volume = 10 mL

Aliquot Factor = Extraction Volume / Aliquot Volume = 5

Final Sample Volume (Final Vol.) = 2 mL

Sample Weight = 10 g

The percent recovery found was calculated as follows:

$$\%\text{ Recovery} = \frac{(\text{ppb found})}{(\text{Fortification level, ppb})} \text{ x } 100\%$$

4.4.2 *Example*

The calculation below shows the concentration of DPX-HGW86 from a soil sample fortified at 1.0 ppb, (65534-MV03) in the validation set. See chromatogram, (Figure 3) and Residue Summary Sheet (Appendix 3) for values to substitute into calculations:

m (slope) = 10622.92

b (Intercept) = 55.30443

Peak Area HGW86 Fortified Sample = 9783

Extraction Volume = 50 mL

Aliquot Volume = 10 mL

Aliquot Factor = Extraction Volume/Aliquot Volume = 5

Final Sample Volume (Final Vol.) = 2 mL

LC Dilution Factor = 1

ppb HGW86 Found =

$$\frac{[9783 - 55.30443] \div 10622.92 \ \text{x} \ [5 \ \text{x} \ 2 \ \text{mL} \ \text{x} \ 1]}{10 \ \text{g}} = 0.9157 \ \text{ppb}$$

$$\% \ \text{Recovery} = \frac{0.9157 \ \text{ppb}}{1.0 \ \text{ppb}} \ \text{x} \ 100\% = 92\%$$

APPENDIX 2 STRUCTURES OF DPX-HGW86 AND PHOTOPRODUCTS

IDENTIFIER	DESCRIPTION	STRUCTURE	IUPAC
DPX-HGW86	Parent M.W. 473.72		3-Bromo-N-[4-cyano-2-methyl-6-(methylcarbamoyl)phenyl]-1-(3-chloropyridin-2-yl)-1*H*-pyrazole-5-carboxamide
IN-NXX70	Photoproduct M.W. 437.26		2-[3-Bromo-1-(3-hydroxypyridin-2-yl)-1H-pyrazol-5-yl]-3,8-dimethyl-4-oxo-3,4-dihydroquinazoline-6-carbonitrile
IN-QKV54	Photoproduct M.W. 344.17		2-(5-Bromo-1H-pyrazol-3-yl)-3,4-dihydro-3,8-dimethyl-4-oxo-6-quinazolinecarbonitrile
IN-RNU71	Photoproduct M.W. 437.26		2-(2-Bromo-4-oxopyrazolo[1,5-a]pyrido[3,2-e]pyrazin-5 (4H)-yl)-5-cyano-N,3-dimethylbenzamide