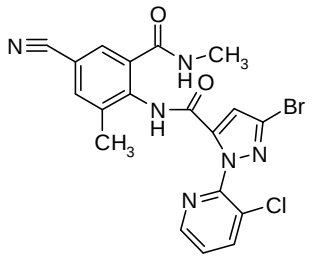
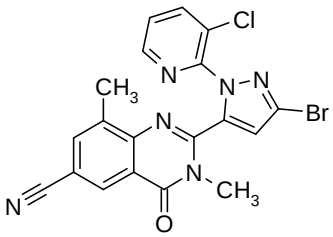
2.0 INTRODUCTION

The objective of this study was to perform an independent laboratory validation (ILV) of residue analytical method DuPont-15440 for the determination of DPX-HGW86 and metabolites in soil by LC/MS/MS, to achieve a target limit of quantitation (LOQ) of 1 ng/g (µg/kg) per analyte.

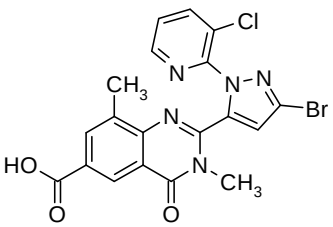
DPX-HGW86



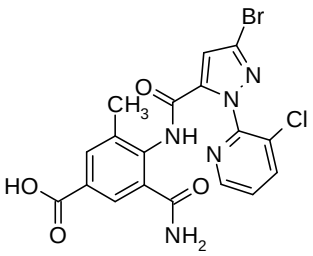
IN-J9Z38



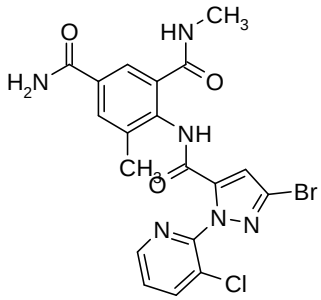
IN-K5A78



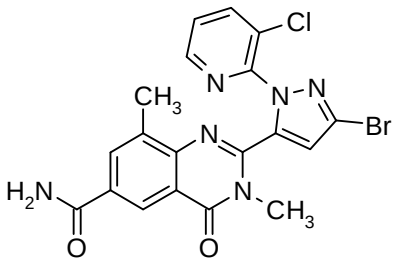
IN-K5A79



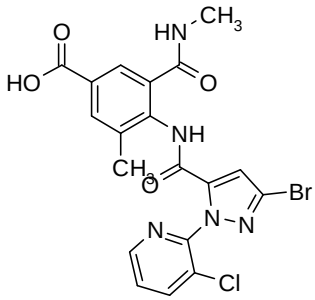
IN-JCZ38



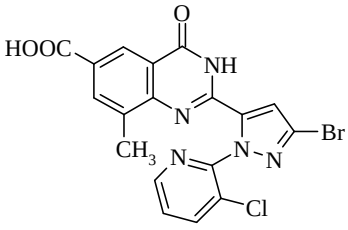
IN-K5A77



IN-JSE76



IN-PLT97



3.0 MATERIALS

3.1 Equipment and Materials

The following lists the equipment and material used in the present study.

3.1.1 Balances

Analytical: Sartorius RC 210D
Specimens and reagents: Sartorius ED 2202S-CW

3.1.2 Method, Extraction and Clean-up Equipment

Ultrasonic bath: Transsonic 700 (Elma)
Centrifuge: Hettich Rotixa 50 S
Rotary vacuum evaporators: Rotavapor R 200 V800, R-114 (Büchi).
Rotary vacuum evaporators: Laborota 4002 (Heidolph).
Horizontal shaker: HS 260 B (JKA)
SPE station: Baker SPE-100.
Vortex mixer: REAX (Heidolph).
Filter: PFTE-filter (Macherey & Nagel).

Assorted labware, volumetric pipettes and typical laboratory equipment.

3.1.3 LC-MS/MS System

Agilent 1200 SL Series HPLC system (vacuum solvent degasser, binary HPLC pump, column oven), and CTC Analytics HTC-Pal Autosampler.

Applied Biosystems MDS Sciex API 5500 triple quadrupole LC-MS/MS system with Turbo IonSpray ESI source. Analyst 1.5 Instrument control and data acquisition software.

HPLC Column:

Phenomenex Luna C_8 column
(length: 150 mm, i.d.: 2.0 mm, particle size: 5 μm)
Pre-column: Phenomenex C_{18} (length: 4 mm, i.d.: 3.0 mm)

3.2 Reagents and Test Items

3.2.1 Solvents

Acetonitrile, acetone, methanol, all pesticide or HPLC grade (Promochem)
Millipore water (supply at PTRL Europe)

3.2.2 Chemicals

Formic acid, 98-100 % (Riedel de Haën)
Dimethyl sulfoxide, 99 % (J.T. Baker)
Ammonium hydroxide, 28-30 % (Sigma-Aldrich)
Ammonium formate (Sigma-Aldrich)
Bond Elut™ NH_2 SPE cartridge, 6 mL/0.5 g (Varian)
Bond Elut™ ENV SPE cartridge, 6 mL/0.5 g (Varian)

3.2.3 *Test / Reference Items*

(Data as provided by the Sponsor)
The test/reference items of DPX-HGW86 and its soil metabolites IN-J9Z38, IN-K5A78, IN-K5A79, IN-JCZ38, IN-K5A77, IN-JSE76, and IN-PLT97 were provided by DuPont Crop Protection, Newark, Delaware, U.S.A., with a Certificate of Analysis (see Appendix 1 for details).

DPX-HGW86

Chemical Name:	3-Bromo-*N*-[4-cyano-2-methyl-6-(methylcarbamoyl)phenyl]-1-(3-chloropyridin-2-yl)-1*H*-pyrazole-5-carboxamide
Molecular Mass:	473.72 g/mol

IN-J9Z38

Chemical Name:	2-[3-bromo-1-(3-chloropyridin-2-yl)-1*H*-pyrazol-5-yl]-3,8-dimethyl-4-oxo-3,4-dihydroquinazoline-6-carbonitrile
Molecular Mass:	455.70 g/mol

IN-K5A78

Chemical Name:	2-[3-bromo-1-(3-chloropyridin-2-yl)-1*H*-pyrazol-5-yl]-3,8-dimethyl-4-oxo-3,4-dihydroquinazoline-6-carboxylic acid
Molecular Mass:	474.70 g/mol

IN-K5A79

Chemical Name:	4-({[3-bromo-1-(3-chloropyridin-2-yl)-1H-pyrazol-5-yl]carbonyl}amino)-3-carbamoyl-5-methylbenzoic acid
Molecular Mass:	478.69 g/mol

IN-JCZ38

Chemical Name:	4-({[3-bromo-1-(3-chloropyridin-2-yl)-1H-pyrazol-5-yl]carbonyl}amino)-N3,5-dimethylisophthalamide
Molecular Mass:	491.73 g/mol

IN-K5A77

Chemical Name:	2-[3-bromo-1-(3-chloropyridin-2-yl)-1H-pyrazol-5-yl]-3,8-dimethyl-4-oxo-3,4-dihydroquinazoline-6-carboxamide
Molecular Mass:	473.72 g/mol

IN-JSE76

Chemical Name:	4-({[3-bromo-1-(3-chloropyridin-2-yl)-1H-pyrazol-5-yl]carbonyl}amino)-3-methyl-5-(methylcarbamoyl)benzoic acid
Molecular Mass:	492.72 g/mol

IN-PLT97

Chemical Name:	2-[3-bromo-1-(3-chloropyridin-2-yl)-1H-pyrazol-5-yl]-8-methyl-4-oxo-3,4-dihydroquinazoline-6-carboxylic acid
Molecular Mass:	460.68 g/mol

All appropriate material safety data sheets should be read and followed, and proper personal protective equipment should be used.

4.0 METHODS

4.1 Analytical Procedure

The independent laboratory validation within this study was performed according to residue analytical method DuPont-15440. Only minor adaptations due to slightly different technical equipment were made during preparation of calibration solutions, extraction and clean-up.

Modifications and observations are noted.

4.1.1 Glassware and Equipment Cleaning

All glassware is rinsed with de-ionized water (to remove detergents) and dried before use.

4.1.2 Preparation and Stability of Stock Solution

To prepare a stock solution of 100 µg/mL, weigh approximately 10.0 mg (adjusted for purity) of each analyte in a tared 100-mL volumetric flask. Add approximately 50 mL of acetonitrile sonicate to dissolve. If analyte does not go into solution add DMSO in 10 mL increments sonicate after each addition. IN-PLT97 may need up to 30 ml of DMSO. IN-JCZ38 and IN-K5A79 may need smaller amounts of DMSO. Once analyte dissolves, dilute to a total volume of 100 mL with acetonitrile.

Stored refrigerated. Stability has not been tested specifically. Recovery data indicate stability for all analytes for at least one week. Stability at or below 4°C for at least six months is specified in the original study DuPont-15440.

The actual weights and purities of the standards used to prepare the stock solutions in this study were as follows: 10.14 mg of DPX-HGW86 (purity 99.2%), 10.33 mg of IN-J9Z38 (purity 97.2%), 10.48 mg of IN-K5A78 (purity 96.1%), 11.85 mg of IN-K5A79 (purity 84.4%), 10.59 mg of IN-JCZ38 (purity 94.4%), 10.49 mg of IN-K5A77 (purity 95.3%), 10.55 mg of IN-JSE76 (purity 94.8%) and 10.78 mg of IN-PLT97 (purity 92.8%[1]).

4.1.3 *Preparation and Stability of Fortification Solutions*

Prepare mixed fortification solutions from dilutions of the individual stock solutions. Prepare 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, using volumetric pipettes and volumetric flasks as follows.

SOLUTION(S) USED	PIPETTE VOLUME [ML]	DILUTE TO [ML]	OBTAIN [µG/ML]
Stock solutions 100 µg/mL	0.10	10	1.0
Fortification solution 1.0 µg/mL	1.0	10	0.10
Fortification solution 0.10 µg/mL	1.0	10	0.010

Stored refrigerated. Stability has not been tested specifically. Recovery data indicate stability for all analytes for at least one week. Stability at or below 4°C for about one month is specified in the original study DuPont-15440.

4.1.4 *Preparation and Stability of Calibration Solutions in Solvent*

Intermediate calibration standard solutions including all analytes are prepared by diluting the fortification solutions with methanol/0.02 M formic acid (1/1, v/v) using a volumetric pipette and a volumetric flask.

LC calibration standards are prepared in methanol/0.02 M formic acid (1/1, v/v) from the intermediate calibration standards using volumetric pipettes and volumetric flasks as follows.

1 The IN-PLT97 standard was certified by the Sponsor during the study. A purity of 92.8% was obtained.

USE SOLUTION(S) WITH [NG/ML]	PIPETTE [µL]	DILUTE TO [ML]	OBTAIN [NG/ML]
100	400	1.0	40
100	200	1.0	20
100	100	1.0	10
100	50	1.0	5.0
10	200	1.0	2.0
10	100	1.0	1.0
10	50	1.0	0.50
10	20	1.0	0.20

These calibration solutions were used for external calibration.

Stored refrigerated. Stability has not been tested specifically. Recovery data and peak responses, using these standard solutions during the independent laboratory method validation procedure, indicate stability for the duration of the experimental part of the study (approximately one week). A stability of one week at refrigerated conditions is specified in the original study DuPont-15440.

4.1.5 *Source (& Characterization) of Specimens*

Sandy loam (5M) soil with a certificate was obtained from LUFA Speyer (for soil characteristic see Appendix 3).

4.1.6 *Preparation of Specimens*

In order to obtain a homogeneous representative sample the soil sample was mixed manually with a spoon prior analysis.

4.1.7 *Specimen Fortification Procedure*

Fortifications were performed using the 1.0 and 0.10-µg/mL fortification solutions. 100-µL portions of these solutions were added to a 10.0 g sample for the 1.0 ng/g and 10 ng/g fortification levels, respectively.

4.1.8 *Analyte Extraction and Purification Procedure*

Independent laboratory validation was performed according to the procedures described in the original method validation report with minor modifications due to different technical equipment. Excerpts of the respective sections in the report of study DuPont-15440 are given below:

4.2.9 *Analyte Extraction Procedure*

1. Extract 10-gram soil sample in 50-mL centrifuge tube with 25 mL of the soil extraction solution on a wrist action shaker at high speed for 15 minutes (90% Acetone and 10% 1.0 M Formic Acid).
2. Centrifuge sample for 5 minutes at approximately 3000 RPM in a bench top centrifuge. Decant supernadent into a 50 mL centrifuge tube.
3. Repeat Steps 1 and 2 on a shaker for and combine extracts in a 50-mL centrifuge tube (bring volume to total of 50 mL with extraction solution, mix).

a: 100 mL measuring cylinder was used instead of a 50 mL centrifuge tube.

4.2.10 *Analyte Purification Procedure*

1. To a 10-mL aliquot of the extract from Step 3 of the extraction procedure, add 40 mL of water and mix thoroughly.
2. Condition a (6-cc/500-mg) NH2 Cartridge with 5 mL of methanol followed by 5 mL of water. Attach a 60-mL reservoir to the NH2 Cartridge; add 5 mL of water to it also. Add the sample before reservoir drains completely.
3. Collect eluent in a 60-mL centrifuge tube. (Use vacuum if needed to achieve 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. The sample is now ready for the ENV SPE clean up.
4. Condition a (6-cc/500-mg) ENV cartridge for clean-up using 5 mL of methanol followed by 5 mL of acidic water (100µL of 1 M Formic acid in 100 mL water). Attach a 60 mL reservoir; and add an additional 5mL of acidic water.
5. Pass 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 cartridges into a 60 mL glass centrifugetube with 3 x 5 mL of basic acetonitrile (2 mL of 1 M ammonium hydroxide in 100 mL of acetonitrile).
7. Evaporate to dryness on an N-Evap at approximately 40°C. Add 1.0 mL of methanol for 2 minutes. Homogenize the sample for 20 seconds using a vortex mixer, and then sonicate.
8. Add 1.0 mL of aqueous mobile phase. Homogenize the sample for 20 seconds using a vortex mixer, and then sonicate for 2 minutes. Instrument sensitivity may vary, the final volume may be adjusted to so that a signal to noise ratio of approximately 10 is achieved for the least sensitive analyte.
9. Filter sample with a 3-mL syringe and a -, 0.2-µm PTFE filter into an HPLC vial. Samples are ready for LC/MS/MS analysis.

b 25 mL pear shape flask was used instead of a 60 mL glass centrifuge tube.

c evaporators were used instead of a N-Evap

4.2 LC-MS/MS Determination

4.2.1 LC-MS/MS Method

The final extracts were analyzed by liquid chromatography with tandem mass spectrometric detection (LC-MS/MS).

LC System	Agilent 1200 HPLC system (vacuum solvent degasser, binary HPLC pump, column oven), and CTC Analytics HTC-Pal Autosampler.
LC Column	Phenomenex Luna C_8 column: Length: 150 mm, i.d.: 2.0 mm, particle size: 5 μm. Column oven temperature: 40 °C.
LC Injection Volume	15 μL.
LC Method	Solvent A: Water + 100 μL of 1 M formic acid + 100 μL of 0.1 mM ammonium formate Solvent B: Methanol + 100 μL of 1M formic acid Mobile Phase Composition: Time (min) / Flow rate (mL/min) / % A / % B 0.00 / 0.25 / 50 / 50 15.00 / 0.25 / 13 / 87 15.10 / 0.25 / 1 / 99 19.00 / 0.25 / 1 / 99 19.10 / 0.25 / 50 / 50 30.00 / 0.25 / 50 / 50
Approximate Retention Time	≈ 4.6 min for IN-JCZ38 ≈ 7.0 min for DPX-HGW86 ≈ 8.0 min for IN-K5A77 ≈ 11.5 min for IN-J9Z38 ≈ 11.6 min for IN-K5A79 ≈ 12.5 min for IN-JSE76 ≈ 15.0 min for IN-K5A78 ≈ 15.3 min for IN-PLT97
MS/MS System	Applied Biosystems MDS Sciex API 5500 triple quadrupole LC-MS/MS system with Turbo IonSpray (ESI) source, detection of positive ions
Ion Source Conditions	Source temperature: 400 °C GS1: 40 (arbitrary units) GS2: 70 (arbitrary units) Curtain gas: 30 (arbitrary units) Collision activated dissociation (CAD): Medium Declustering potential: 150 V Entrance potential: 10 V Cell exit potential: 12 V IonSpray voltage: 4500 V Resolution Q1: unit Resolution Q3: unit
Period 1: 0.0 – 9.0 min MS/MS Conditions	*IN-JCZ38:* Scan Type: MS/MS (MRM) 1st MS/MS transition: 493 m/z → 286 m/z Collision energy (CE): 25 V 2nd MS/MS transition: 493 m/z → 462 m/z Collision energy (CE): 25 V Dwell time per transition: 200 ms

Period 1 (cont.): 0.0 – 9.0 min MS/MS Conditions	*DPX- HGW86:*	
	Scan Type:	MS/MS (MRM)
	1st MS/MS transition:	475 m/z → 286 m/z
	Collision energy (CE):	30 V
	2nd MS/MS transition:	475 m/z → 444 m/z
	Collision energy (CE):	30 V
	Dwell time per transition:	200 ms
	IN-K5A77:	
	Scan Type:	MS/MS (MRM)
	1st MS/MS transition:	475 m/z → 188 m/z
	Collision energy (CE):	50 V
	2nd MS/MS transition:	475 m/z → 299 m/z
	Collision energy (CE):	50 V
	Dwell time per transition:	200 ms
Period 2: 9.0 – 30 min MS/MS Conditions	*IN-J9Z38:*	
	Scan Type:	MS/MS (MRM)
	1st MS/MS transition:	457 m/z → 188 m/z
	Collision energy (CE):	45 V
	2nd MS/MS transition:	457 m/z → 299 m/z
	Collision energy (CE):	45 V
	Dwell time per transition:	100 ms
	IN-K5A79:	
	Scan Type:	MS/MS (MRM)
	1st MS/MS transition:	480 m/z → 286 m/z
	Collision energy (CE):	25 V
	2nd MS/MS transition:	480 m/z → 463 m/z
	Collision energy (CE):	25 V
	Dwell time per transition:	400 ms
	IN-JSE-76:	
	Scan Type:	MS/MS (MRM)
	1st MS/MS transition:	494 m/z → 286 m/z
	Collision energy (CE):	30 V
	2nd MS/MS transition:	494 m/z → 463 m/z
	Collision energy (CE):	30 V
	Dwell time per transition:	400 ms
	IN-K5A78:	
	Scan Type:	MS/MS (MRM)
	1st MS/MS transition:	476 m/z → 188 m/z
	Collision energy (CE):	45 V
	2nd MS/MS transition:	476 m/z → 299 m/z
	Collision energy (CE):	45 V
	Dwell time per transition:	200 ms
	IN-PLT97:	
	Scan Type:	MS/MS (MRM)
	1st MS/MS transition:	462 m/z → 317 m/z
	Collision energy (CE):	51 V
	2nd MS/MS transition:	462 m/z → 286 m/z
	Collision energy (CE):	39 V
	Dwell time per transition:	400 ms

The pseudomolecular ions $[M+H]^+$ of the analytes were used as parent ions for MS/MS detection. The 1st MS/MS transitions to the daughter ions were used for quantification and the 2nd MS/MS transitions to the daughter ions were used for quantitative confirmation of the analytes.

4.2.2 *Calibration Procedures*

Quantification was done by LC-MS/MS using two MS/MS transitions per analyte.

External standard calibration technique was used to quantify the amount of the analytes in specimen extracts. Representative standard calibration curves are presented in Figure 1 to Figure 8.

Calibration standard solutions of the analytes were injected during specimen injections (peak areas of characteristic MS/MS transitions). Calibration was based on linear regression calculation performed by the ANALYST 1.5 software.

Calibration levels ranged from 0.20 ng/mL to 40 ng/mL per analyte. Calibration functions calculated by regression analysis gave correlation coefficients r > 0.99. See Figure 9, Figure 10, Figure 14, Figure 15, Figure 19, Figure 20, Figure 24, Figure 25, Figure 29, Figure 30, Figure 34, Figure 35, Figure 39, Figure 40, Figure 44 and Figure 45 for representative chromatograms of standard calibration solutions.

The concentrations of the analytes in the extracts from control and recovery specimens were evaluated by external calibration. See Figure 11 to Figure 13, Figure 16 to Figure 18, Figure 21 to Figure 23, Figure 26 to Figure 28, Figure 31 to Figure 33, Figure 36 to Figure 38, Figure 41 to Figure 43 and Figure 46 to Figure 48 for representative chromatograms of specimen extracts.

4.3 Specimen Analysis for Independent Laboratory Validation

Specimen analysis was conducted as outlined below.

Control (untreated) soil specimens were analyzed in duplicate. A total of five specimens were analyzed for each fortification level. Each set of specimens analyzed for investigation purposes included two untreated control specimens with no residue (< 30 % of LOQ) present. Untreated specimens were fortified with the analytes at 1.0 ng/g (LOQ) and 10 ng/g (10×LOQ), and carried through the procedure to verify recoveries.

One specimen per fortification level was injected twice to demonstrate repeatability of LC-MS/MS determination.

4.4 Calculations

The following equation was used to calculate the individual residues R in ng/g:

$$R = C_{End} \times [(V_{Ex} \times V_{End}) / (V_1 \times W)]$$
$$= C_{End} \times \text{Multiplier M}$$

Where:

R:	Residue in ng/g or ppb.
C_{End}:	Final concentration of analyte in extract in ng/mL. (where multiple injections were evaluated: mean).
V_{Ex}:	Extraction volume: 50 mL.
V_1:	Aliquot of V_{Ex}: 10 mL.
V_{End}:	Final volume of extract: 2.0 mL.
W:	Weight used for extraction: 10.0 g.

Recoveries (Rec.) were calculated for the fortified specimens as follows:
Rec. = $(R / R_{fortified}) \times 100\ \%$

The calculation is exemplified with the soil specimen P1840-39 fortified at 1.0 ng/g (LOQ) with DPX-HGW86 and metabolites. The further calculation is exemplified for the LC-MS/MS determination of DPX-HGW86 using the mass transition 475 m/z → 286 m/z.

The final extract was examined by LC-MS/MS in run file P1840#028 (Figure 17) to give a final concentration C_{End} of 0.903 ng/mL (see Table 1).

Thus:

$R = C_{End} \times [(V_{Ex} \times V_{End}) / (V_1 \times W)]$
$= C_{End} \times$ Multiplier M
$= 0.903$ ng/mL × [(50 mL × 2.0 mL) / (10 mL × 10 g)]
$= 0.903$ ng/mL × 1 mL/g
$= 0.903$ ng/g

$Rec = (R / R_{fortified}) \times 100\ \%$
$= (0.903$ ng/g / 1.0 ng/g) x 100 % = 90%