

## **ANALYTICAL METHOD FOR THE DETERMINATION OF DPX-E2Y45 AND DEGRADATION PRODUCTS IN WATER USING LC/MS/MS**

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### **1.0 SUMMARY**

The purpose of this study was to develop and validate an analytical method for the detection, quantitative analysis, and confirmation of DPX-E2Y45 and potential degradation products IN-F9NO4, IN-EQW78, IN-GAZ70, IN-ECD73, and IN-F6L99 in water.

DPX-E2Y45, IN-F9NO4, IN-EQW78, IN-GAZ70, and IN-ECD73 were analyzed by measuring 50 mL of water into a 50-mL glass centrifuge tube. The samples were transferred to a 250-mL separatory funnel and partitioned with 100 mL of 50:50 hexane:ethyl acetate. The organic layer was collected in a 250-mL graduated mixing cylinder. The water sample was partitioned a second time with 50 mL of 50:50 hexane:ethyl acetate. The organic layers were transferred into the same mixing cylinder and diluted to 150 mL with ethyl acetate. A 50-mL aliquot of the organic layer was transferred to a 50-mL glass centrifuge tube and evaporated to dryness under a flow of nitrogen. The extract was reconstituted in 1.0 mL of acetonitrile and diluted to 2 mL using a 0.01 M aqueous formic acid solution. The extracts were analyzed using atmospheric pressure chemical ionization (APCI) LC/MS/MS system.

The analysis of IN-F6L99 was completed using solid phase extraction (SPE) followed by (APCI) LC/MS/MS detection. A 25-mL water sample was acidified and filtered through a Waters Oasis HLB SPE cartridge, which concentrated IN-F6L99 on the packing. IN-F6L99 was eluted from the SPE using 30 mL of acetone. The extract was evaporated to dryness, reconstituted in methanol, and diluted in 0.01 M aqueous formic acid solution. The extract was syringe filtered and analyzed using APCI-LC/MS/MS.

The separation of the analysis into two procedures was required to compensate for the range of solubility and polarity of the analytes.

The Limit of Quantitation (LOQ) was 0.10 ng/g (ppb) for all analytes in all water types. The Limit of Detection (LOD) was estimated to be 0.03 ng/g (ppb) for the least sensitive analyte IN-EQW78.

## 2.0 INTRODUCTION

The structure, CAS name, CAS registry number, and various physical properties of DPX-E2Y45, IN-F9NO4, IN-GAZ70, IN-EQW78, IN-ECD73, and IN-F6L99 can be found in Appendix 1. The method was validated on ground, surface, and drinking water.

DPX-E2Y45, IN-F9NO4, IN-GAZ70, IN-EQW78, and IN-ECD73 were analyzed by partitioning water samples two times with 50:50 hexane:ethyl acetate in a separatory funnel. The organic layers from the partition were combined and a 1/3 aliquot was removed. The aliquot was evaporated to dryness, reconstituted in acetonitrile, and diluted with 0.01 M aqueous formic acid solution.. The extracts were analyzed using atmospheric pressure chemical ionization (APCI) LC/MS/MS system.

The analysis of IN-F6L99 was completed using solid phase extraction (SPE) followed by APCI-LC/MS/MS detection.

The Limit of Quantitation (LOQ) was 0.10 ng/g (ppb) for all analytes in all water types. The Limit of Detection (LOD) was estimated to be 0.03 ng/g (ppb) for the least sensitive analyte IN-EQW78. During method validation, acceptable recoveries for water samples fortified at the LOQ and 10× LOQ were generated.

Confirmation of possible residues using LC/MS/MS was based on the detection and relative ratios of two MS/MS ion fragments. Confirmation criteria and examples are discussed in this report.

### 3.0 MATERIALS

Equivalent equipment and materials may be substituted unless otherwise specified. Note any specification 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*

##### Instrumentation

LC System, HP1100 with Temperature Controlled Autosampler  
(Agilent, Wilmington, DE)

Mass Spectrometer System, Quattro II with APCI Interface  
(Micromass Inc., Altrincham, UK)

##### Chromatographic Supplies

HPLC Column: 4.6 mm i.d. × 15 cm, Phenomenex Luna C-18 Analytical Column with 3 µm Diameter Packing, Part No. 00F-4251-E0 (Phenomenex, Torrance, CA)

HPLC Vials, Target DP Amber Kit, T/S/T Septa, 100 PK, Part No. 5182-0556  
(Agilent, Wilmington, DE)

##### Labware

Separatory Funnels with Stopper, Kimex Brand Metric Scale, 250-mL Capacity,  
Cat. No. 30352-200 (VWR Scientific Co., Bridgeport, NJ)

Mixing Cylinder with Stopper, Kimex Brand Metric Scale, 250-mL Capacity,  
Cat. No. 24763-109 (VWR Scientific Co., Bridgeport, NJ)

Centrifuge Tubes, Glass with Stopper, Pyrex 50-mL Capacity, Cat. No. 21048-050  
(VWR Scientific Co., Bridgeport, NJ)

##### Solid-Phase Extraction Equipment

Visiprep 12 Port SPE Vacuum Manifold, Part No. 5-7030  
(Supelco, Bellefonte, PA)

Oasis<sup>®</sup> HLB Cartridge, 500 mg/6 cc, Part No. 186000115  
(Waters, Milford, MA) - **Do Not Substitute**

Miscellaneous

Syringe Filter - Acrodisc PTFE 0.20  $\mu$ m, 13 mm Diameter Filter Unit,  
Cat. No. 28143-982 (VWR Scientific Co., Bridgeport, NJ)

Evaporator - N-Evap<sup>®</sup> Model 111 Laboratory Sample Evaporator/Nitrogen Manifold  
Fitted with Teflon<sup>®</sup>-Coated Needles (Organomation Associates, South Berlin, MA)  
This unit is attached to a dry, clean nitrogen source.

VWR Brand Vortex Geni 2 Mixer, Cat. No. 58815-178  
(VWR Scientific Co., Bridgeport, NJ)

Balances - Mettler AE160 Analytical and PE600 Top-Loading Balances  
(Mettler Instrument Corp., Hightstown, NJ)

Biohit Proline Electronic Pipettors, Variable Volume with Tip Ejector, Vanguard,  
5.0-100  $\mu$ L, Cat. No. 53495-200; 50-1000  $\mu$ L, Cat. No. 53495-205;  
and 0.10-5.0 mL, Cat. No. 53495-290 (VWR Scientific Co., Bridgeport, NJ)

**3.2 Reagents and Standards**

Equivalent reagents may be substituted for those listed below.

Acetic Acid - Baker Analyzed Glacial acetic acid, #9524-00  
(J.T. Baker, Inc. Danvers, MA)

Acetone - EM Omni Solv<sup>®</sup>, GC-grade acetone, #AX0110-1  
(EM Science, Gibbstown, NJ)

Acetonitrile (ACN) - EM Omni Solv<sup>®</sup>, HPLC-grade acetonitrile, #AX0142-1  
(EM Science, Gibbstown, NJ)

Dimethyl Sulfoxide (DMSO) - EM Omni Solv<sup>®</sup>, 99.9% purity, #EM-MX1456-6  
(EM Science, Gibbstown, NJ)

Formic Acid - Guaranteed Reagent 98% minimum, #FX0440-5  
(EM Science, Gibbstown, NJ)

Ethyl Acetate - EM Omni Solv<sup>®</sup>, HPLC-grade ethyl acetate, #EX0241-1  
(EM Science, Gibbstown, NJ)

Hexanes - EM Omni Solv<sup>®</sup>, #HX0296-1 (EM Science, Gibbstown, NJ).

Water - EM Omni Solv<sup>®</sup>, HPLC-grade water, #WX0004-1  
(EM Science, Gibbstown, NJ)

DPX-E2Y45 Lot 100 (99.2% Pure) Reference substances used for sample analysis:  
Analytical standard grade reagents (prepared by DuPont Crop Protection, Global  
Technology Division, E.I. du Pont de Nemours and Company)

IN-F9NO4 Lot 1 (95.6% Pure) Reference substance used for sample analysis:  
Analytical standard grade reagents (prepared by DuPont Crop Protection, Global  
Technology Division, E. I. du Pont de Nemours and Company)

IN-GAZ70 Lot 1 (96% Pure) Reference substance used for sample analysis:  
Analytical standard grade reagents (prepared by DuPont Crop Protection, Global  
Technology Division, E. I. du Pont de Nemours and Company)

IN-EQW78 Lot 2 (97.2% Pure) Reference substances used for sample analysis:  
Analytical standard grade reagents (prepared by DuPont Crop Protection, Global  
Technology Division, E.I. du Pont de Nemours and Company)

IN-ECD73 Lot 3 (99.8% Pure) Reference substances used for sample analysis:  
Analytical standard grade reagents (prepared by DuPont Crop Protection, Global  
Technology Division, E.I. du Pont de Nemours and Company)

IN-F6L99 Lot 3 (97.7% Pure) Reference substances used for sample analysis:  
Analytical standard grade reagents (prepared by DuPont Crop Protection, Global  
Technology Division, E.I. du Pont de Nemours and Company)

### 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 used. DMSO should be handled with special care. Appropriate personal protective equipment must be worn whenever handling DMSO. An MSDS sheet for the analytes is available from DuPont Crop Protection, Global Technology Division, E. I. du Pont de Nemours and Company.

## 4.0 **METHOD**

### 4.1 *Analytical Method*

DPX-E2Y45 was analyzed by measuring 50 mL of water into a 50-mL glass centrifuge tube. The use of glass tubes is important since IN-GAZ70 has been shown to adhere to some plastic surfaces. The samples were transferred to a 250-mL separatory funnel and partitioned with 100 mL of 50:50 hexane:ethyl acetate. The organic layer was collected in a 250-mL graduated mixing cylinder and the water layer was partitioned a second time with 50-mL of 50:50 hexane:ethyl acetate. The organic layers were transferred into the same mixing cylinder and diluted to 150 mL with ethyl acetate. If a small amount of water is present on the bottom of the tube, remove it using a pipette. Additional water will bias the volume of the aliquot removed, resulting in high recoveries or an over estimation on the concentration determined. A 50-mL aliquot was transferred to a 50-mL glass centrifuge tube and evaporated to dryness under a flow of nitrogen. The extract was reconstituted in acetonitrile and diluted to 2 mL using 0.01 M aqueous formic acid solution. The extracts were analyzed using atmospheric pressure chemical ionization (APCI) LC/MS/MS system.

The analysis of IN-F6L99 was completed using solid phase extraction (SPE) followed by (APCI) LC/MS/MS detection. A 25-mL water sample was acidified and filtered through a Waters Oasis HLB SPE cartridge, which concentrated IN-F6L99 on the packing. IN-F6L99 is a relatively polar molecule and was difficult to concentrate on some SPE cartridges. Therefore, other cartridges should not be substituted for the Waters Oasis HLB cartridge. Poor recoveries were observed for some water types when acid was not added prior to the SPE step. IN-F6L99 was eluted from the SPE using 30 mL of acetone. The extract was evaporated to dryness, reconstituted in methanol, and diluted in 0.01 M aqueous formic acid solution. The extract was syringe filtered and analyzed using APCI-LC/MS/MS.

The separation of the analysis into two procedures was required to compensate for the range of solubility and polarity of the analytes. IN-F6L99 would not partition into the organic layer and the elution of IN-ECD73 from the Oasis cartridge requires strong organic solvents.

The analysis was completed using an APCI ion source. APCI was chosen since IN-GAZ70 was not detected using an electrospray ion source.

## 4.2 *Analytical Procedure*

### 4.2.1 Glassware and Equipment

#### *Cleaning*

Glassware should be scrubbed with a brush using a laboratory soap solution, rinsed two to five times with tap water, rinsed with distilled or deionized water, and finally rinsed with acetone or another suitable solvent and allowed to air dry prior to each use.

Due to the tendency of the analytes to adhere to surfaces when in water, it is extremely important not to wash analyte-contaminated glassware, such as stock standard volumetric flasks in common wash areas. Contaminated glassware must be thoroughly rinsed with acetonitrile prior to following normal glassware cleaning procedures.

### 4.2.2 Preparation of Solutions

The following solutions should be prepared weekly and stored at room temperature unless stated otherwise:

**0.010 M Aqueous Formic Acid (Mobile Phase A)** - Add 0.460 mL of formic acid to 950 mL of HPLC-grade water in a 1-liter volumetric flask. Dilute to 1000 mL.

**50:50 Acetonitrile:0.01 M Aqueous Formic Acid** - Add 250 mL of 0.010 M Aqueous Formic Acid to 250 mL of acetonitrile, and mix thoroughly.

#### 4.2.3 Preparation and Stability of Stock Standards

Use volumetric flasks when preparing standard solutions.

##### DPX-E2Y45, IN-GAZ70, IN-EQW78, and IN-ECD73

Prepare standard stock solutions of each compound by accurately weighing  $10.00 \pm 0.1$  mg into a 10-mL volumetric flask using an analytical balance. Record the accurate weight of the standard. Dissolve the standards in approximately 8.0 mL of DMSO. After dissolving, bring the solution to a volume of 10.0 mL, using DMSO, and invert the volumetric flask to mix the solution to homogeneity. These standard solutions are stable for approximately 1 month, when stored in a refrigerator at approximately 4°C immediately after each use. The concentration in solution is 1000 µg/mL.

Prepare 100 µg/mL standard stock solutions by accurately pipetting 1.0 mL of the 1000 µg/mL stock standard into a 10-mL volumetric flask, dilute to 10.0 mL with acetonitrile, and mix to homogeneity. This standard solution is stable for approximately 6 months, when stored at approximately 4°C immediately after each use.

##### IN-F9NO4 and IN-F6L99

Prepare standard stock solutions of each compound by accurately weighing  $10.00 \pm 0.1$  mg into a 100-mL volumetric flask using an analytical balance. Record the accurate weight of the standard. Dissolve the standards in approximately 50 mL of acetonitrile. After dissolving, bring the solution to a volume of 100.0 mL using acetonitrile, and invert the volumetric flask to mix the solution to homogeneity. These standard solutions are stable for approximately 6 months, when stored at approximately 4°C immediately after each use. The concentration in solution is 100 µg/mL.

#### 4.2.4 Preparation and Stability of Intermediate and Fortification Standards

Use Class A volumetric flasks when preparing standard solutions.

Prepare a 1.0 µg/mL multi-analyte intermediate standard by pipetting 1.0 mL of each 100.0 µg/mL stock standard into a 100-mL volumetric flask. Bring to volume using HPLC-grade acetonitrile and mix to homogeneity.

Prepare a 0.10 µg/mL multi-analyte fortification standard by pipetting 1.0 mL of the 1.0 µg/mL intermediate standard into a 10-mL volumetric flask. Bring to volume using HPLC-grade acetonitrile and mix to homogeneity.

All standard solutions prepared in acetonitrile are stable for approximately 6 months, if stored at approximately 4°C immediately after each use.

#### 4.2.5 Preparation and Stability of Calibration Standards

##### DPX-E2Y45, IN-F9NO4, IN-GAZ70, IN-EQW78, and IN-ECD73

##### Calibration Standards

Prepare the calibration standards by pipetting the volumes of the 1.0 µg/mL and 0.10 µg/mL DPX-E2Y45, IN-F9NO4, IN-GAZ70, IN-EQW78, IN-ECD73, and IN-F6L99 fortification standard solutions, shown in the following table, into separate volumetric flasks (alternative or additional standards may be prepared as needed):

STANDARD CONCENTRATION (NG/ML)	VOLUME OF 1.0 µG/ML STANDARD REQUIRED (ML)	VOLUME OF 0.10 µG/ML STANDARD REQUIRED (ML)	FINAL VOLUME (ML)
20.0	0.200	-	10.0
10.0	0.100	-	10.0
5.0	0.050	-	10.0
1.0	-	0.100	10.0
0.50	-	0.050	10.0

Add the appropriate amount of the 50:50 acetonitrile:0.01 M aqueous formic acid solution to the volumetric flasks to dilute to 10.00 mL. These standard solutions should be prepared weekly. Each of the calibration standards was vortexed for 30 seconds prior to injection.

##### IN-F6L99 Calibration Standards

Prepare the calibration standards by pipetting the volumes of the 1.0 µg/mL and 0.10 µg/mL DPX-E2Y45, IN-F9NO4, IN-GAZ70, IN-EQW78, IN-ECD73, and IN-F6L99 fortification standard solutions, shown in the following table, into separate volumetric flasks (alternative or additional standards may be prepared as needed):

STANDARD CONCENTRATION (NG/ML)	VOLUME OF 1.0 µG/ML STANDARD REQUIRED (ML)	VOLUME OF 0.10 µG/ML STANDARD REQUIRED (ML)	FINAL VOLUME (ML)
20.0	0.200	-	10.0
10.0	0.100	-	10.0
5.0	0.050	-	10.0
1.0	-	0.100	10.0
0.5	-	0.050	10.0

Dilute the standards to volume using 0.01 M aqueous formic acid solution, cap, and mix thoroughly. These standard solutions should be prepared weekly and stored approximately 4°C. Each of the calibration standards was vortex mixed for 30 seconds prior to injection.



#### 4.2.6 Source of Samples

The ground and surface waters were collected from local bodies of water and the drinking water was purchased from a grocery store. Water characterization data is provided in Appendix 5.

#### 4.2.7 Storage and Preparation of Samples

Water samples should be stored at approximately 4°C. The water samples were shaken by hand prior to use to ensure homogeneity. No additional filtration or purification was performed prior to sample processing.

In the event water samples contain sediment, the sample must be centrifuged and the water decanted into a clean container prior to analysis.

#### 4.2.8 Sample Fortification Procedures

##### For DPX-E2Y45, IN-F9NO4, IN-GAZ70, IN-EQW78, and IN-ECD73

All fortifications were made directly to the water following the measurement of the sample. Fortified 50.0-mL samples were prepared using the 0.10 µg/mL and 1.0 µg/mL fortification standard.

FORTIFICATION LEVEL (PPB)	VOLUME OF 0.10 µG/ML STANDARD (ML)	VOLUME OF 1.0 µG/ML STANDARD (ML)
0.10	0.050	-
1.0	-	0.050

##### For IN-F6L99

All fortifications were made directly to the water following the measurement of the sample. Fortified 25.0-mL samples were prepared using the 0.10 µg/mL and 1.0 µg/mL fortification standard.

FORTIFICATION LEVEL (PPB)	VOLUME OF 0.10 µG/ML STANDARD (ML)	VOLUME OF 1.0 µG/ML STANDARD (ML)
0.10	0.025	-
1.0	-	0.025

#### 4.2.9 Analysis of DPX-E2Y45, IN-F9NO4, IN-GAZ70, IN-EQW78, and IN-ECD73

1. Accurately measure 50 mL ( $\pm$  1.0 mL) of water into a 50-mL glass centrifuge tube. Fortify sample if necessary. Mix the sample using a vortex mixer. Transfer the sample to a 250-mL separatory funnel.

2. Measure 50 mL of ethyl acetate using the same centrifuge tube. Mix the ethyl acetate, using a vortex mixer, and transfer the ethyl acetate to the same 250-mL separatory funnel.
3. Measure 50 mL of hexane using the same centrifuge tube. Mix the hexane, using a vortex mixer, and transfer the hexane to the same 250-mL separatory funnel. *Set aside the 50-mL centrifuge tube to collect the water after the partition step.*
4. Mix the separatory funnel for approximately 30 seconds by hand and allow the funnel to stand until a partition is formed. Transfer the lower layer (water) back into the original 50-mL centrifuge tube. Transfer the upper layer (hexane/ethyl acetate) into a 250-mL glass graduated mixing cylinder. *Care should be taken to avoid any water transferring into the mixing cylinder.*
5. Transfer the water layer back into the 250-mL separatory funnel. Using the 50-mL centrifuge tube, measure 25 mL of ethyl acetate and mix using a vortex mixer. Dilute the ethyl acetate to 50 mL with hexane, and transfer the ethyl acetate and hexane into the 250-mL separatory funnel.
6. Mix the separatory funnel for approximately 30-seconds by hand and allow the funnel to stand until a partition is formed. Discard the lower layer (water). Transfer the upper layer (hexane/ethyl acetate) into the same 250-mL glass graduated mixing cylinder as used following the first partition. *Care should be taken to avoid any water transferring into the mixing cylinder.* If any water is present in the bottom of the mixing cylinder, remove it using a pipette. Dilute the extract to 150 mL using ethyl acetate and mix the extract by hand.
7. Transfer a 50 mL aliquot of the extract to a clean glass 50-mL centrifuge tube and reduce to dryness using nitrogen in an N-Evap at 50-55°C. Reconstitute the extract in 1.0 mL of acetonitrile. Mix using a vortex mixer for 30 seconds and sonicate for 5 minutes. Add 1.0 mL of 0.01 M aqueous formic acid solution. Mix using a vortex mixer for 30 seconds.
8. Syringe filter an aliquot of the extract into an auto-sampler vial and analyze by APCI-LC/MS/MS.

Extracts will be stable for approximately 72 hours if stored at 4°C.

#### 4.2.10 Analysis of IN-F6L99

1. Accurately measure 25 mL ( $\pm$  1.0 mL) of water into a 50-mL glass centrifuge tube. Fortify sample if necessary. Mix the sample using a vortex mixer. Add 0.25 mL of acetic acid and mix the sample using a vortex mixer.
2. Place a 0.5-g Oasis HLB cartridge on an SPE manifold. Condition the Oasis cartridges with 5 mL of methanol followed by 10 mL of HPLC-grade water. **Do not let the cartridges go to dryness.**

3. Pass the 25-mL sample through the SPE, discarding the eluate. After the extract has passed through the cartridges, rinse the tube with  $2 \times 5$  mL of the water, which is passed through the cartridges. *Vacuum may be used to keep a steady slow flow of approximately 2-5 mL/minute. Do not allow steady streams.*
4. After the sample and all rinses have passed through the cartridges, dry the Oasis SPE using vacuum (15" Hg or 0.50 atmospheres) for 5 minutes.
5. Elute IN-F6L99 by the addition of 30 mL of acetone. Measure 30 mL of acetone using the original sample tube. Load the solution onto the cartridge and collect the eluate in a 50-mL glass centrifuge tube at a flow rate of 2-3 mL/minute. *Vacuum may be used to maintain the flow rate, no steady streams.*
6. Evaporate the extract to dryness using a flow of nitrogen in an N-Evap at approximately 50-55°C.
7. Add 0.5 mL of methanol into the centrifuge tube. Vortex and sonicate the centrifuge tube for 5 minutes. Pipette 1.5 mL of 0.01 M aqueous formic acid solution into the centrifuge tube and vortex the extract for 30 seconds.
8. Using a disposable syringe, filter the resulting solution through a 0.2- $\mu$ m Acrodisc filter into an HPLC vial. Analyze the solution by LC/MS/MS as described in the following section.

### 4.3 *Instrumentation for the Method*

#### 4.3.1 HPLC Conditions for DPX-E2Y45, IN-F9NO4, IN-GAZ70, IN-EQW78, and IN-ECD73

This method uses a gradient-elution, reversed-phase analysis on a C-18 column. The column choice reflects experimental results indicating preferred chromatography for separation from co-extractants. Alternative chromatographic conditions can be used, provided the analytical method is validated and provides acceptable recoveries.

## HPLC Conditions:

System:	Agilent HP1100 HPLC				
Column:	4.6 mm i.d. × 15 cm, Phenomenex C-18 Analytical Column with 3- $\mu$ m Diameter Packing				
Column Temperature:	40°C				
Injection Volume:	0.10 mL				
Autosampler Temperature:	4°C				
Flow Rate:	1.0 mL/min				
Conditions:	<u>Time</u>	<u>%A</u>	<u>%B</u>	<u>Flow</u>	A: 0.01 M Aq. Formic Acid B: Methanol Flow in mL/min
	0.0	40	60	1.0	
	0.5	40	60	1.0	
	2.0	20	80	1.0	
	5.0	2	98	1.0	
	8.0	2	98	1.0	
8.1	40	60	1.0		
Approximate Retention Times:	Minutes				
IN-F9NO4:	4.7				
DPX-E2Y45:	4.9				
IN-GAZ70:	6.4				
IN-EQW78:	6.4				
IN-ECD73:	7.9				
Total Run Time:	11.5 Minutes				

A six-port, electronically activated switching valve was used to direct the flow to waste prior to and following the elution of the compounds of interest. The use of this valve reduces source contamination and enables additional samples to be analyzed prior to source cleaning. The valve switching times are given in the following table.

TIME (MINUTES)	COLUMN ELUATE FLOW
0.0-3.8	Waste
3.8-8.9	MS Source
8.9-End	Waste

#### 4.3.2 *HPLC Conditions for IN-F6L99*

This method uses a gradient-elution, reversed-phase analysis on a C-18 column. The column choice reflects experimental results indicating preferred chromatography for separation from co-extractants. Alternative chromatographic conditions can be used, provided the analytical method is validated and provides acceptable recoveries.

## HPLC Conditions:

System:	Agilent HP1100 HPLC				
Column:	4.6 mm i.d. × 15 cm, Phenomenex C-18 Analytical Column with 3- $\mu$ m Diameter Packing				
Column Temperature:	40°C				
Injection Volume:	0.075 mL				
Autosampler Temperature:	4°C				
Flow Rate:	1.0 mL/min				
Conditions:	<b>Time</b>	<b>%A</b>	<b>%B</b>	<b>Flow</b>	A: 0.01 M Aq. Formic Acid B: Methanol Flow in mL/min
	0.0	90	10	1.0	
	0.5	90	10	1.0	
	5.5	20	80	1.0	
	5.8	10	90	1.0	
	8.8	10	90	1.0	
9.0	90	10	1.0		
Approximate Retention Times:	Minutes				
IN-F6L99:	6.2				
Total Run Time:	11.0 Minutes				

A six-port, electronically activated switching valve was used to direct the flow to waste prior to and following the elution of the compounds of interest. The use of this valve reduces source contamination and enables additional samples to be analyzed prior to source cleaning. The valve switching times are given in the following table.

TIME (MINUTES)	COLUMN ELUATE FLOW
0.0-4.5	Waste
4.5-7.0	MS Source
7.0-End	Waste

#### 4.3.3 LC/MS/MS Analysis of DPX-E2Y45, IN-F6NO4, IN-GAZ70, IN-EQW78, and IN-ECD73

The detection method utilized was LC/MS/MS employing atmospheric pressure chemical ionization (APCI) in the positive ion mode on a triple quadrupole instrument. During the method development process, each analyte was infused into the APCI ion source. A tune file was created to maximize the response for each analyte. All MS experimental conditions are provided in Appendix 4. The flow rate and mobile phase were adjusted to approximate the elution conditions from the reversed phase column. The protonated parent compound, or in-source ion fragment detected for each analyte, was fragmented in the MS/MS collision cell. The tune file was adjusted to maximize the response of the fragment ions detected. The ion

transitions for each analyte are shown in the tables below. The ion response acquired in MS/full-scan and MS/MS-full scan modes are presented in Figure 1. The relative intensities of the multiple reaction monitoring (MRM) ions detected can be used for confirmation of DPX-E2Y45 or the DPX-E2Y45 degradation products in a water sample.

APCI-LC/MS/MS Conditions:

ANALYTES MONITORED	IONS MONITORED	CONE VOLTAGE	COLLISION ENERGY	DWELL TIME (SECONDS)	ACQUISITION TIMING (MIN.)
IN-F9NO4	<b>470.0</b> → <b>452.3 ± 0.5 AMU</b>	15 V	17 V	0.30	4.0-5.4
	470.0 → 285.1 ± 0.5 AMU	15 V	12 V	0.30	4.0-5.4
DPX-E2Y45	484.0 → 453.0 ± 0.5 AMU	12 V	15 V	0.30	4.0-5.5
	484.0 → 285.8 ± 0.5 AMU	12 V	11 V	0.20	4.0-5.5
IN-GAZ70	451.0 → 414.0 ± 0.5 AMU	25 V	32 V	0.30	4.0-5.5
	451.0 → 306.5 ± 0.5 AMU	25 V	22 V	0.30	4.0-5.5
IN-EQW78	465.9 → 187.8 ± 0.5 AMU	40 V	30 V	0.30	5.5-7.0
	465.9 → 75.8 ± 0.5 AMU	40 V	120 V	0.30	5.5-7.0
IN-ECD73	244.0 → 209.0 ± 0.5 AMU	50 V	25 V	0.30	7.0-8.5
	<b>279.0</b> → <b>243.8 ± 0.5 AMU</b>	<b>25 V</b>	<b>26 V</b>	<b>0.30</b>	<b>7.0-8.5</b>
	279.0 → 208.8 ± 0.1 AMU	25 V	35 V	0.30	7.0-8.5
Ion Mode	MRM				
APCI Probe Temp	600°C				
Detector Voltage	750 V				
Source Heater	150°C				
Gas Cell Pressure	2.9e-3 mBar (approximately)				
Nebulising Gas Flow	15 L/h				
Drying Gas Flow	300 L/h				

The total ion current (TIC) was used for the quantitation of DPX-E2Y45, IN-GAZ70, and IN-EQW78. The ion transitions indicated in bold type were used for the quantitation of IN-F9NO4 and IN-ECD73. Additional or alternative MS/MS ion transitions may be used for analysis or confirmation.

#### 4.3.4 LC/MS/MS Analysis of IN-F6L99

##### APCI-LC/MS/MS Conditions:

ANALYTES MONITORED	IONS MONITORED	CONE VOLTAGE	COLLISION ENERGY	DWELL TIME (SECONDS)	ACQUISITION TIMING (MIN.)
IN-F6L99	<b>203.8</b> → 172.2 ± 0.5 AMU	14 V	20 V	0.30	5.0-7.0
	203.8 → 65.9 ± 0.5 AMU	14 V	36 V	0.30	5.0-7.0
Ion Mode	MRM				
APCI Probe Temp	600°C				
Detector Voltage	750 V				
Source Heater	150°C				
Gas Cell Pressure	2.9e-3 mBar (approximately)				
Nebulising Gas Flow	15 L/h				
Drying Gas Flow	300 L/h				

The instrument was operated in MS/MS-(MRM) positive ion mode for quantitative analysis. Quantitation of IN-F6L99 was performed using the ion indicated in **bold face print**. In the event of a background peak in one or more transitions, quantitation may be performed using the background-free transitions. The selective and specific MS/MS fragmentation of the protonated molecule can be used to confirm the identity of a peak eluting at the predetermined retention time. However, the relative ratio of the fragment ions must also be evaluated to confirm the presence of an analyte in an unknown field sample.

#### 4.3.5 Calibration Procedure and Sample Analysis

A 0.50 ng/mL chromatographic standard should be analyzed prior to the start of analyses to establish that the instrument is working properly. If a signal-to-noise ratio of at least approximately 5-10 to 1 is not attained, the instrument must be tuned or cleaned prior to sample analysis. 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 some ion channels other than those used for validation of this method may need to be added or eliminated when utilizing this method on other instrumentation. Each ion channel used for sample analysis/quantitation must be checked to ensure it is free of interference. The 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 should always be disregarded.

#### 4.4 Calculations

Recovery data was calculated using the average response factor for all standards analyzed concurrently.

4.4.1 Method

Average Response Factor ( $RF_{Ave}$ ) was calculated as follows:

$$RF_{Ave} = \frac{(\text{Conc. A} \div \text{Area A}) + (\text{Conc. B} \div \text{Area B}) + (\text{Conc. C} \div \text{Area C}) + (\text{Conc. D} \div \text{Area D}) + (\text{Conc. E} \div \text{Area E})}{\text{Total Number of Standards Injected}}$$

$$\text{ng/g Found} = \frac{(\text{Peak Area}) \times (RF_{Ave}) \times (\text{Aliquot Factor}) \times (\text{Final Volume})}{(\text{Sample Weight})}$$

$$\text{ppb Found} = \text{ng/g Found}$$

*In the event a peak was detected in the control, a corrected peak area was used to calculate ppm found for freshly fortified samples. The corrected peak area is the area of the fortified sample minus the area of the control sample.*

The percent recovery found was calculated as follows:

$$\% \text{ Recovery} = \frac{(\text{ppb Found})}{(\text{ppb Fortified})} \times 100$$

4.4.2 Example

For a Brandywine River sample fortified with DPX-E2Y45 at 0.10 ppb (9-December -2004 sample 2C), the concentration found was calculated as follows:

Average Response Factor was calculated as follows:

$$RF_{Ave} = \frac{(0.50 \text{ ng/mL} \div 231 \text{ AC}) + (1.0 \text{ ng/mL} \div 495 \text{ AC}) + (5.0 \text{ ng/mL} \div 2044 \text{ AC}) + (10.0 \text{ ng/mL} \div 4248 \text{ AC}) + (20.0 \text{ ng/mL} \div 8211 \text{ AC})}{5}$$

(AC  $\equiv$  Area Counts)

$$RF_{Ave} = 2.28414 \text{e-}3 \text{ ng/mL/AC}$$

ppm found was calculated as follows:

$$\text{ng/g Found} = \frac{(344 \text{ AC}) \times (2.28414 \text{e-}3 \text{ ng/mL/AC}) \times (2 \text{ mL}) \times (3)}{(50 \text{ g})}$$

$$\text{ppb found} = 0.094 \text{ ng/g}$$

The percent recovery found was calculated as follows:

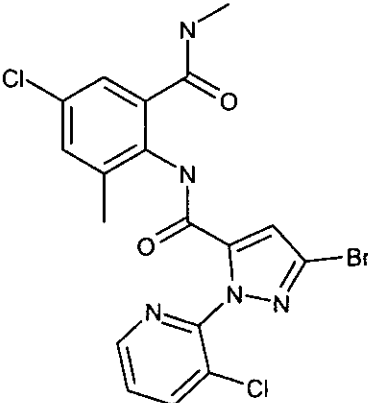
$$\% \text{ Recovery} = \frac{(0.094 \text{ ng/g})}{(0.10 \text{ ng/g})} \times 100$$

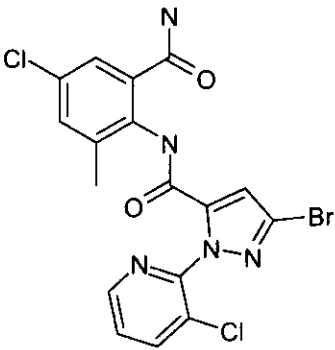
$$\% \text{ Recovery} = 94\%$$

(Percent recoveries are rounded to the nearest whole number in Table I, without rounding the concentration or ppm found.)

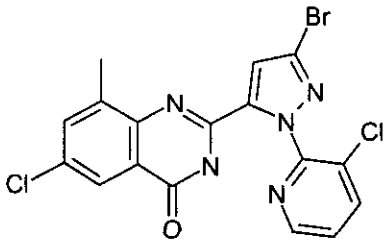


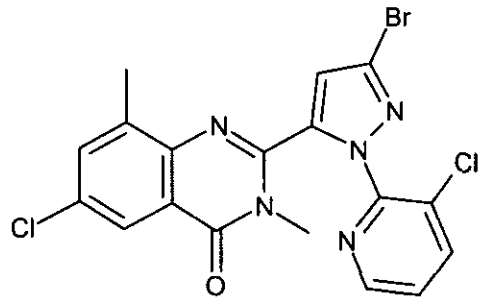
## APPENDIX 1 STRUCTURE AND PROPERTIES OF DPX-E2Y45

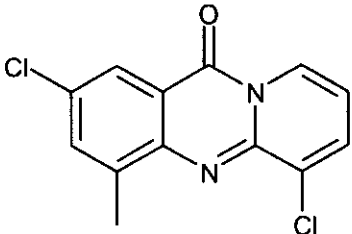
Common Name	None
Structure	
DPX Number	DPX-E2Y45
CAS Chemical Name	3-Bromo-N-[4-chloro-2-methyl-6-(methylamino)carbonyl]phenyl]-1-(3-chloro-2-pyridinyl)-1H-pyrazole-5-carboxamide
CAS Number	500008-45-7
Formula	C <sub>18</sub> H <sub>14</sub> BrCl <sub>2</sub> N <sub>5</sub> O <sub>2</sub>
Molecular Weight	483.1511
Monoisotopic Weight	480.9708

Common Name	None
Structure	
DPX Number	IN-F9NO4
Formula	C <sub>17</sub> H <sub>12</sub> BrCl <sub>2</sub> N <sub>5</sub> O <sub>2</sub>
Molecular Weight	469.1242
Monoisotopic Weight	466.9581

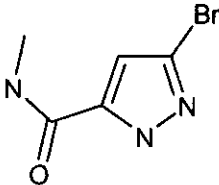
## APPENDIX 1 STRUCTURE AND PROPERTIES OF DPX-E2Y45 (CONTINUED)

Common Name	None
Structure	
DPX Number	IN-GAZ70
Formula	C <sub>17</sub> H <sub>10</sub> BrCl <sub>2</sub> N <sub>5</sub> O
Molecular Weight	451.1089
Monoisotopic Weight	448.9446

Common Name	None
Structure	
DPX Number	IN-EQW78
Formula	C <sub>18</sub> H <sub>12</sub> BrCl <sub>2</sub> N <sub>5</sub> O
Molecular Weight	465.1358
Monoisotopic Weight	462.9602

Common Name	None
Structure	
DPX Number	IN-ECD73
Formula	C <sub>13</sub> H <sub>8</sub> Cl <sub>2</sub> N <sub>2</sub> O
Molecular Weight	279.1248
Monoisotopic Weight	278.0014

**APPENDIX 1 STRUCTURE AND PROPERTIES OF DPX-E2Y45 (CONTINUED)**

Common Name	None
Structure	
DPX Number	IN-F6L99
Formula	C <sub>5</sub> H <sub>6</sub> Br N <sub>3</sub> O
Molecular Weight	204.0263
Monoisotopic Weight	202.9694