

## 2.0 INTRODUCTION

E.I. du Pont de Nemours and Company has commissioned Charles River to conduct a trial of the analytical method by an independent laboratory. This is required before it is submitted to the U.S. Environmental Protection Agency (EPA). This study was designed to demonstrate the utility, ruggedness, efficiency and any inherent weakness in the subject method as written.

All work was conducted in accordance with EPA Ecological Effects Test Guidelines OPPTS 850.7100, Data Reporting for Environmental Chemistry Methods.

The analytical work described in this report was conducted in the Department of Chemistry, Charles River Laboratories, Tranent, Edinburgh, EH33 2NE, UK according to the following schedule:

Study Initiation Date:	15 April 2008
Experimental Start Date:	05 May 2008
Experimental Completion Date:	10 June 2008
Study Completion Date:	See Good Laboratory Practice Statement page for date of Study Director's signature

Experiments were conducted in accordance with Charles River Laboratories Protocol No. 213825, Amendment 1 and Amendment 2.

Originals of all raw field and analytical data and pertinent information, including the original study plan, any amendments and the final report will be archived at Charles River Scientific Archive for 2 years and then transferred to the Sponsor where the material will be retained at:

E.I. du Pont de Nemours and Company  
 DuPont Crop Protection  
 Stine-Haskell Research Center  
 Newark  
 Delaware 19714-0030  
 USA

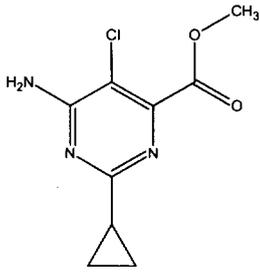
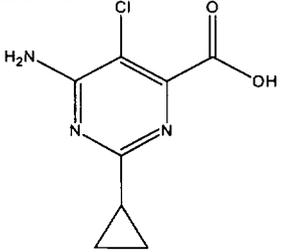
Laboratory specific or site specific raw data such as personnel files, instrument, equipment, refrigerator, and/or freezer raw data will be retained at the facility where the work was done.

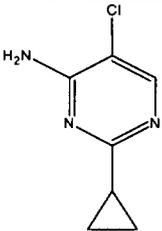
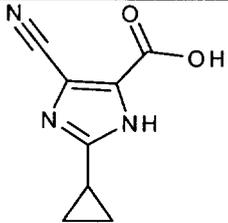
### 3.0 MATERIALS AND METHODS

#### 3.1 Test Substance

Reference analytical standards of DPX-KJM44, DPX-MAT28, IN-LXT69 and IN-QFH57 were supplied by E.I. du Pont de Nemours and Company, DuPont Crop Protection, Newark, Delaware. Information pertaining to the characterization and certification records of the test substance is archived by E.I. du Pont de Nemours and Company, DuPont Crop Protection, Global Technology Division, Stine-Haskell Research Center, Newark, Delaware, 19714-0030. All test items were stored at an ambient temperature when not in use.

The structures and specific information for DPX-KJM44, DPX-MAT28, IN-LXT69 and IN-QFH57 are shown below:

	<p><b>DuPont Code:</b> DPX-KJM44  <b>CAS Name:</b> Methyl 6-amino-5-chloro-2-cyclopropyl-4-pyrimidinecarboxylate  <b>CAS Registry Number:</b> 858954-83-3  <b>Formula:</b> C<sub>9</sub>H<sub>10</sub>ClN<sub>3</sub>O<sub>2</sub>  <b>Molecular Weight:</b> 227.65  <b>Monoisotopic Weight:</b> 227 Da  <b>pKa:</b> To be confirmed</p>
	<p><b>DuPont Code:</b> DPX-MAT28  <b>CAS Name:</b> 6-amino-5-chloro-2-cyclopropyl-4-pyrimidinecarboxylic acid  <b>CAS Registry Number:</b> 858956-08-8  <b>Formula:</b> C<sub>8</sub>H<sub>8</sub>ClN<sub>3</sub>O<sub>2</sub>  <b>Molecular Weight:</b> 213.62  <b>Monoisotopic Weight:</b> 213 Da  <b>pKa:</b> 4.65</p>

	<p><b>DuPont Code:</b> IN-LXT69  <b>CAS Name:</b> To be confirmed  <b>CAS Registry Number:</b> To be confirmed  <b>Formula:</b> C<sub>7</sub>H<sub>8</sub>ClN<sub>3</sub>  <b>Molecular Weight:</b> 169.61  <b>Monoisotopic Weight:</b> 169 Da  <b>pKa:</b> To be confirmed</p>
	<p><b>DuPont Code:</b> IN-QFH57  <b>CAS Name:</b> To be confirmed  <b>CAS Registry Number:</b> To be confirmed  <b>Formula:</b> C<sub>8</sub>H<sub>7</sub>N<sub>3</sub>O<sub>2</sub>  <b>Molecular Weight:</b> 177.16  <b>Monoisotopic Weight:</b> 177 Da  <b>pKa:</b> To be confirmed</p>

Copies of the Certificates of Analysis for all analytical standards are presented in Appendix 1.

### 3.2 *Test System*

The analytical method, DuPont-22043 and Supplement No.1 were independently validated by Charles River for the determination of DPX-KJM44, DPX-MAT28, IN-LXT69 and IN-QFH57 residues in soil using LC-MS/MS. Prepared, frozen, control soil [685499/S1/009/III (Charles River Laboratories ID: 05-159)] and [685499/S1/011/III (Charles River Laboratories ID: 05-165)] from Charles River Laboratories Project No. 685499 (DuPont-14436) was used for analysis. Control soil matrix was chosen from Charles River Laboratories Project No. 685499 (DuPont-14436) since the trial location of this study is in Lleida, Spain. Prior to sample preparation, the samples were stored at *ca.* -20°C. The samples were air dried, homogenized by hand mixing and sieved and were stored at *ca.* -20°C prior to analysis and when not in use. The characteristics of the soil samples are presented in Appendix 2. These characteristics apply to the sample before preparation.

3.3 **Equipment**

Equipment Description	Product I.D.	Supplier
Freezer	CF6 168	Scandinova
Refrigerator	Elan Turbo	LEC
Analytical Balance	AE163	Mettler Toledo
Analytical Balance	PL602-S	Mettler Toledo
Analytical Evaporator	DB3A Dri-Block	Techne
Sonic Bath	FS400B	Decon
Vortex mixer	Whirlimixer	Fisherbrand (Fisons)
SPE System	Oasis <sup>®</sup> MCX cartridges, 6 cc/500 mg	Waters
	20 port Vac-Elut SPE vacuum manifold mounted with flow control valves and stainless steel needles	Flashvac
Vacuum Pump	MZ-2C	VacuuBrand
Centrifuge	GR422	Jouan
Labware	Standard Analytical Glassware	Fisher
Labware	Microman <sup>®</sup> mechanical positive displacement pipette (M25, M50, M250, and M1000)	Gilson
Labware	Pasteur pipette, borosilicate	BDH / Fisher Scientific
Flat-Bed Shaker	HS501	IKA

**LC/MS/MS System**

HPLC	Pump series 200, column oven series 200, degasser series 200,	Perkin Elmer
Autosampler	HTS PAL	CTC Analytics
Triple quadrupole MS	Applied Biosystems API 5000 mass spectrometer using a Turbolon Spray ionization source and Analyst 1.4.2 software	Applied Biosystems
Valco Valve	-	Vici AG International
Analytical Column	Luna <sup>®</sup> Phenyl-Hexyl, 150 x 4.6 mm, 3 $\mu$ m	Phenomenex
Guard Column	C18 (ODS) 4 x 2 mm	Phenomenex

### 3.4 *Reagents*

Reagents	Product Description	Supplier
Ammonium Acetate	Sigmaultra	Sigma-Aldrich
Ammonium Hydroxide	ACS Reagent	Aldrich
Formic Acid	-	Fluka
Formic Acid	Analytical	Fisher
Water	HPLC	BDH
Acetonitrile	HPLC	Rathburn
Methanol	HPLC	Rathburn

### 3.5 *Principles of the Analytical Method*

The analytical method DuPont-22043 and Supplement No.1 were developed for the determination of DPX-KJM44, DPX-MAT28, IN-LXT69 and IN-QFH57 in soil matrices to support regulatory studies. The method was independently validated at Charles River Laboratories at a target LOQ of 1.0 µg/kg (ppb).

### 3.6 *Modifications, Interpretations, and Critical Steps*

Modifications to the analytical method DuPont-22043 and Supplement No.1 were minor.

#### 3.6.1 *Sample Extraction*

All samples were extracted as detailed in analytical method DuPont-22043, Supplement No.1 with the following modifications. In step 4.2.9.5 of analytical method DuPont-22043, Supplement No.1, a wrist action shaker is used. Since no wrist action shaker was available at the independent laboratory, a flat bed shaker was substituted for this step.

For the Analyte Purification Procedure, section 4.2.10, there were two minor modifications to the analytical method. At step 4.2.10.1, all 15 mL aliquots were transferred to 30 mL glass vials instead of 50 mL propylene centrifuge tubes, which would not fit in the N-Evap holders and in step 4.2.10.11, the purified extracts were not filtered through 0.45 µm PTFE filters.

### 3.6.2 Calibration Standards

All calibration standards were prepared as detailed in analytical method DuPont-22043, Supplement No.1, section 4.2.3 through section 4.2.5, with the following additional standard. A *ca.* 0.03 ng/mL (DPX-KJM44, DPX-MAT28 and IN-LXT69) and *ca.* 0.30 ng/mL (IN-QFH57) mixed calibration standard was prepared as follows:

CALIBRATION STANDARD ID	STANDARD SOLUTION USED	STANDARD SOLUTION AMOUNT	FINAL VOLUME
0.030 ng/mL KJM44, MAT28, LXT69; 0.30 ng/mL QFH57	0.50 ng/mL KJM44, MAT28 LXT69; 5.0 ng/mL QFH57	0.060 mL	1.0 mL

### 3.6.3 Quantitation/Calculations

A calibration curve was determined for each analyte by weighted least squares linear regression analysis (1/x) of the plot of peak area versus the concentration of DPX-KJM44, DPX-MAT28, IN-LXT69 and IN-QFH57 in each calibration standard. The concentration of each sample was then calculated as detailed in section 3.8. This is a modification from analytical method DuPont-22043, Supplement No.1 which uses the average response factor (defined as peak area response/analyte concentration).

### 3.6.4 Confirmatory Procedure

No confirmatory procedure calculations were performed in this study at the request of the Sponsor prior to analysis. Confirmatory ions were routinely monitored, however they have not been reported.

## 3.7 Instrumentation

### 3.7.1 Description

The independent laboratory validation of the analytical method for DPX-KJM44, DPX-MAT28, IN-LXT69 and IN-QFH57 in soil matrices was conducted using 2 single Perkin Elmer Series 200 micro-pumps connected to an Applied Biosystems API 5000 mass spectrometer using a TurboIon Spray interface. HPLC components were: a CTC Analytics HTS PAL autosampler, a Perkin Elmer Series 200 column oven and degasser, and valco switching valve. Data acquisition and system control was by Analyst version 1.4.2 software. The API 5000 was operated in LC-MS/MS positive ionization mode for DPX-KJM44, DPX-MAT28 and IN-LXT69 and negative ionization mode for IN-QFH57 with MRM detector output for quantitative and confirmatory analysis.

3.7.2 Operating Conditions**HPLC Operating Conditions:**

Injection Volume ( $\mu\text{L}$ ): 60  $\mu\text{L}$   
 Guard Column: Phenomenex, C18 (ODS) 4 x 2 mm  
 Column: Luna Phenyl-Hexyl, 150 x 4.6 mm, 3  $\mu\text{m}$   
 Column Temperature: 30°C  
 Solvent A: 0.1% formic acid in HPLC-grade water  
 Solvent B: HPLC-grade methanol

TIME	FLOWRATE (ML/MIN)	%A	%B	COMMENTS
0.00	1.000	95	5	
5.00	1.000	41	59	
8.00	1.000	1	99	
10.00	1.000	1	99	
10.10	1.000	95	5	
14.50	1.000	95	5	End Run

## Approximate Analyte Retention Times:

IN-LXT69 = 3.6 min  
 DPX-MAT28 = 4.7 min  
 IN-QFH57 = 7.5 min  
 DPX-KJM44 = 8.1 min

**Post-column Split:** ~100  $\mu\text{L}/\text{min}$  to MS and ~900  $\mu\text{L}/\text{min}$  to waste

**Triple Quadrupole MS Operating Conditions**

Interface: electrospray (ESI)  
 Mode: MRM  
 Resolution Q1: Unit  
 Resolution Q3: Unit  
 ESI Source Voltage: 5.5 kV for DPX-KJM44, DPX-MAT28 and IN-LXT69 and -4.5 kV for IN-QFH57  
 Divert Valve: 0.0–3.0 min to waste  
 3.0–9.5 min to source  
 9.5–14.5 min to waste

AB SCIEX API-5000 ACQUISITION PARAMETERS (ESI INTERFACE, MRM MODE)												
ANALYTE	Q1 M/Z	Q3 M/Z	DWELL (MSEC)	CUR (PSI)	GS1	GS2	TEM (°C)	CAD (PSI)	DP (V)	EP (V)	CE (V)	CXP (V)
DPX-KJM44	228.2	68.1	100	20	50	50	325	8	180	10	38	15
	228.2	168.1									25	12
DPX-MAT28	214.2	68.1	100	20	50	50	325	8	75	10	33	10
	214.2	101.0									35	14
IN-LXT69	170.1	76.0	100	20	50	50	325	8	215	10	46	13
	170.1	103.1									33	15
IN-QFH57	176.0	132.1	100	20	50	50	325	8	-40	-10	-12	-8
	176.0	105.0									-35	-14

### 3.8 Calculations

#### 3.8.1 Interpolation from the Calibration Line

System linearity is established by injecting mixed calibration standards prepared as detailed in section 3.6.2. Calibration standards are run on each analytical occasion to demonstrate system linearity.

The calibration line should be linear with an intercept approaching zero and a percentage difference from the line not greater than  $\pm 15\%$  at all levels except for the lowest calibration level, where  $\pm 20\%$  is acceptable.

#### 3.8.2 Expression of Results

The DPX-KJM44, DPX-MAT28, IN-LXT69 and IN-QFH57 peak areas are calculated for each of the calibration standards, quality control samples and controls and unknown test samples. A calibration curve is then obtained by weighted least squares linear regression analysis ( $1/x$ ) of the plot of peak area versus the concentration of DPX-KJM44, DPX-MAT28, IN-LXT69 and IN-QFH57 in each calibration standard. A smoothing factor of 2 was applied to all chromatographic peaks. The calibration curve should not be forced through zero.

The calculated concentration in each sample is corrected for the proportion of sample taken through the extraction, sample weight and the sample final volume. The calculation is detailed below with an example calculation on the following page:

Concentration of DPX-KJM44, DPX-MAT28, IN-LXT69 and IN-QFH57 in sample:-

$$\text{Sample A} = \frac{\text{PR A} - c}{m} \times \text{Correction Factor}$$

where PR A = Peak response for analyte in Sample A

c = Intercept on y-axis from regression analysis

m = Slope of the line from regression analysis

Correction Factor:

$$\frac{\left(\frac{75}{15}\right)^+ \times 5^{++}}{10^{+++}}$$

+ Procedural Cut in Method (mL)

++ Final volume (mL)

+++ Sample weight (g)

Correction factor = 2.5 if no further dilutions performed.

The analytical method is considered to be acceptable if the mean batch recoveries are between 70-110% of the theoretical value and there is no significant interference from the control.

### 3.8.3 Example Calculation

The calculation shown below is for the calculation of percentage recovery. The percentage recovery values are presented in Table 1 to Table 3.

$$\% \text{ Recovery} = [\text{ppb Found} / \text{Fortification Level (ppb)}] \times 100$$

For example, control sample with Charles River Laboratories reference 05-159 fortified at 0.9950 ppb (reported as 1 ppb). Details in Study Record Notebook 213825, RES 0466, P54. Dataset for extraction and analysis on 09 June 2008 for DPX-KJM44, Rec 1.0 A dil 1:10.

Concentration	=	(Y - -4190) ÷ 1932452
Where Y = peak area		
peak area for ion 228.2/68.1	=	68970
Final Volume	=	50 mL (i.e. 5 mL extract diluted 1:10)
Sample wet weight	=	10 g
Correction Factor	=	25
Sample µg/kg	=	[(68970 - -4190) ÷ 1932452] x 25
µg/kg (ppb) found	=	0.94646594
ppb added	=	0.9950
Recovery for DPX-KJM44	=	0.94646594 ppb × 100% ÷ 0.9950 ppb
		= 95.12562814 %, rounded to 95.1% (shown in Table 3)

**APPENDIX 3 COMMUNICATION**

<b>DATE</b>	<b>MEDIA</b>	<b>ORIGIN</b>	<b>CONTENT OF THE COMMUNICATION</b>
22 Feb 08	Secure Website	Sponsor	DuPont-22043 and Supplement No.1 (Draft) posted on secure website.
27 Feb 08	Secure Website	Sponsor	SIF posted on secure website for review.
28 Mar 08	Secure Website	Sponsor	DuPont-22043, Supplement 1 (Final) posted on secure website.
07 Apr 08	Secure Website	Charles River Laboratories	Draft Study Plan submitted to secure website for review.
09 Apr 08	Secure Website	Sponsor	Draft Study Plan reviewed by Sponsor Monitor with changes posted on secure website. Sponsor Monitor clarifies 'Confirmatory Procedure' not required.
10 Apr 08	Email	Charles River Laboratories	Request to Sponsor Representative for Soil Characterization Report.
10 Apr 08	Email	Sponsor	Soil Characterization Report provided by Sponsor.
10 Apr 08	Email/ Secure Website	Charles River Laboratories	Acknowledgement of changes to Study Plan. Draft Study Plan posted on secure website. Request for change to sample calculation in DuPont-22043.
14 Apr 08	Email	Sponsor	Request agreed by Sponsor.
23 Apr 08	Email	Charles River Laboratories	Request for updated expiry date for DPX-KJM44 and for updated COA for IN-QFH57.
23 Apr 08	Email	Sponsor	Updated expiry date for DPX-KJM44 confirmed.
23 Apr 08	Secure Website	Sponsor	Updated COA for IN-QFH57 provided via secure website
08 May 08	Email	Charles River Laboratories	Trial 1 results provided. Example chromatograms also provided.
08 May 08	Email	Sponsor	Request for telephone call to discuss Trial 1 results.
09 May 08	Telecon	Sponsor	Sponsor Monitor and Study Director discussed results of Trial 1. Sponsor Monitor confirmed acceptance of DPX-KJM44 and IN-LXT69 from Trial 1. Discussed re-injection of final extracts after re-tuning for DPX-MAT28 and IN-QFH57.
09 May 08	Email	Charles River Laboratories	Telecon minutes provided to Sponsor Monitor for confirmation of topics discussed.
09 May 08	Email	Sponsor	Sponsor Monitor confirmed minutes were accurate.
12 May 08	Email	Charles River Laboratories	Trial 1 re-analysis results provided.
12 May 08	Email	Sponsor	Sponsor Monitor advises Trial 2 should proceed for DPX-MAT28 and IN-QFH57

**APPENDIX 3 COMMUNICATION (CONTINUED)**

DATE	MEDIA	ORIGIN	CONTENT OF THE COMMUNICATION
15 May 08	Email	Charles River Laboratories	Trial 2 results provided.
15 May 08	Email	Sponsor	Request by Sponsor Monitor for Raw Data and chromatograms for Trial 2.
16 May 08	Email	Charles River Laboratories	Raw Data and chromatograms for Trial 2 provided.
19 May 08	Telecon	Sponsor	Discussion held concerning Trial 2 data. Sponsor Monitor requested confirmation of correction factors. Sponsor Monitor requested further method try-out before any Trial 3 attempts. It was also agreed an amendment would be issued to allow an attempt at DuPont-22043 if Trial 3 was unsuccessful.
19 May 08	Email	Charles River Laboratories	Telecon minutes provided to Sponsor Monitor for confirmation of topics discussed.
19 May 08	Email	Sponsor	Sponsor Monitor confirmed minutes were accurate. Sponsor Monitor also clarified that any additional work would be part of the ILV and would be reported in 213825 and would relate to DuPont-24563 not Supplement No. 1.
27 May 08	Email	Charles River Laboratories	Method tests results provided.
30 May 08	Secure Website	Charles River Laboratories	Draft Amendment 1 posted on secure website for review.
30 May 08	Secure Website	Sponsor	Updates to Draft Amendment 1 posted on secure website for review.
10 Jun 08	Email	Charles River Laboratories	Trial 3 results provided.
10 Jun 08	Email	Sponsor	Trial 3 results accepted by Sponsor Monitor for all analytes.
12 Jun 08	Email	Charles River Laboratories	Request for re-submission of Soil Characterization Report to Sponsor Monitor
12 Jun 08	Email	Sponsor	Soil Characterization Report provided by Sponsor Monitor
20 Jun 08	Secure Website	Charles River Laboratories	Unaudited Draft Report placed on secure website for review.
07 Jul 08	Secure Website	Charles River Laboratories	Audited Draft Report placed on secure website for review.
17 Jul 08	Secure Website	Sponsor	Audited Draft Report comments received from Sponsor.