2.0 INTRODUCTION

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

The subject method is applicable for the quantitation of DPX-KJM44, DPX-MAT28, IN-LXT69, or IN-QFH57 in water, as described in DuPont-22042 (Reference 3). Pond water was chosen to validate the analytical method as a representative matrix.

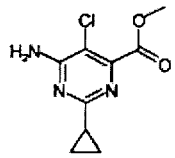
Fortification levels in this study were chosen to provide method performance data at the method LOQ and 10×LOQ for the matrix examined. The stated method LOQ was 0.10 ppb for all analytes in water.

The analytical method was performed without any significant modifications. The method was successfully validated for DPX-KJM44, DPX-MAT28 and IN-LTX69 in water in one trial, and for IN-QFH57 in two. This independent laboratory validation study demonstrated that the analytical method DuPont-22042 is acceptable for the quantitation of DPX-KJM44, DPX-MAT28, IN-LXT69, and IN-QFH57 in water, according to guidelines set forth by US EPA Ecological Effects Guidelines, OPPTS 850.7100 "Data Reporting for Environmental Chemistry Methods" (Reference 1) and EU Guidance document SANCO/825/00 rev. 7 (Reference 2).

3.0 MATERIALS AND METHODS

3.1 Test and Reference Substances

The *Chemical Abstracts* structures (if available) and chemical names of the analytes are shown below:



Test Substance: DPX-KJM44

Common Name: Aminocyclopyrachlor Methyl

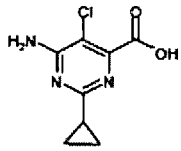
***Chemical Abstracts* Name**: Methyl 6-amino-5-chloro-2-cyclopropyl-4-pyrmidinecarboxylate

CAS Registry No.: 858954-83-3

Lot No.: 034

Purity: 99.9%

Storage: Ambient desiccator



Test Substance: DPX-MAT28-012

Common Name: Aminocyclopyrachlor

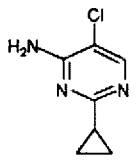
***Chemical Abstracts* Name**: 6-Amino-5-chloro-2-cyclopropyl-4-pyrimidinecarboxylic acid

CAS Registry No.: 858956-08-8

Lot No.: D100095-073

Purity: 98.0%

Storage: Ambient desiccator



Test Substance: IN-LXT69-003

Common Name: Not available

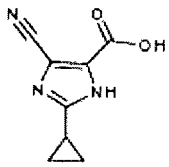
***Chemical Abstracts* Name**: Not available

CAS Registry No.: Not available

Lot No.: D100884-025

Purity: 99.8%

Storage: Ambient desiccator



Test Substance: IN-QFH57-002

Common Name: Not available

***Chemical Abstracts* Name**: Not available

CAS Registry No.: Not available

Lot No.: E112392-37C

Purity: 98.8%

Storage: Ambient desiccator

The test substances were supplied by E. I. du Pont de Nemours and Company, DuPont Agricultural Products, Stine Haskell Research Center, Newark, DE. Information pertaining to the characterization and stability of the test substances is archived by DuPont Crop Protection, E. I. du Pont de Nemours and Company, Newark, DE. Characterization data were provided by DuPont Agricultural Products, E.I. du Pont de Nemours and Company, Wilmington, DE. Certificates of Analysis, including lot numbers and purity, are included with the study raw data file that will be archived by E. I. du Pont de Nemours and Company.

3.2 Test System

The subject method is applicable for the quantitation of DPX-KJM44, DPX-MAT28, IN-LXT69, and IN-QFH57 in water. Pond water was chosen to validate the analytical method because it is expected to be one of the more difficult water sources to analyze.

All control matrices were acquired from a local Colorado pond. The samples were stored refrigerated and processed prior to being analyzed to verify that the control was free of interferences at the appropriate retention times.

3.3 Equipment

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

3.3.1 Instrumentation/Chromatography

MDS Sciex API 5000 LC-MS/MS System, comprised of:

MDS Sciex API 5000 MS/MS, Serial No. AG22340805 (Applied Biosystems Group, Foster City, CA), equipped with a TurboIonSpray interface and Analyst software version 1.4.2

HPLC Column: 4.6 mm i.d. × 15 mm, Phenomenex® Luna Phenyl-Hexyl, Serial No. 458403-6, 3-µm diameter packing Part No. 00F-4256-E0 (Phenomenex, Torrance, CA)

Shimadzu LC-20AD HPLC pumps, Serial Nos. L201046 52734 and L201046 52735 (Shimadzu US Manufacturing Inc., Columbia, MD)

Shimadzu SiL-20AC-HT Autosampler, Serial No. L203546 55317 (Shimadzu US Manufacturing Inc., Columbia, MD)

Shimadzu CTO-20AC Column Oven, Serial No. L202143 50331 (Shimadzu US Manufacturing Inc., Columbia, MD)

Shimadzu DGU-20A3 Degasser, Serial No. SSI-3-0911 (Shimadzu US Manufacturing Inc., Columbia, MD)

Phenomenex C18 Guard Cartridge, 4×3 mm, Part No. AJO-4287

3.3.2 *General Lab Equipment/Devices*

Cahn Microbalance, Model No. C-34/35, Serial No. C1066/C2251 (Orion Research Inc., Beverly, MA 01915)

Sartorius Top-Loading Balance, Model No. BA2100S, Serial No. 20303446 (Brinkmann Instruments Co., Westbury, NY 11590)

Acculab Top-Loader Balance, Model No. AC1205 (Huntington Valley, PA 19006)

Purelab Classic UV UHP Water System, Model No. PL5232, ELGA (Lowell, MA 01851)

Fisher brand Vortex Geni 2 Mixer, Catalog No. 12-812 (Scientific Industries, Inc., Bohemia, NY 11716)

N-Evap 112, Nitrogen Evaporator, Model 8125, Serial No. 50266 (Organomation Associates Inc. Berlin, MA 01503) This unit is attached to a dry, clean nitrogen source.

Microman Positive Displacement Pipettes, various sizes (Gilson, Middleton, WI 53562)

Air Displacement Pipettes, various sizes (Eppendorf Research, Westbury, NY 11590)

3.3.3 *Solid-Phase Extraction Equipment/Supplies*

24-port SPE vacuum manifold (Burdick and Jackson, Muskegon, MI)

Oasis MCX SPE cartridges, 500 mg/6 mL, Cat. No. 1860000776, Lot Nos. 002537200A, 003539131A (Waters Corporation, Millford, MA)

3.3.4 *Labware*

15 mL Polypropylene Centrifuge Tubes, Part No. 20171-024 (VWR, West Chester, PA 19380)

50 mL Polypropylene Centrifuge Tubes, Part No. 89004-367
(VWR, West Chester, PA 19380)

Borosilicate glass scintillation vials with cap, 20 mL Part No. 986546
(Wheaton, Millville, NJ 08332)

Nunc U96 Deepwell plate and cover, 2.0 mL Polypropylene
(Nalge Nunc International, Rochester, NY 14625)

Disposable Transfer Pipettes, 3 mL, Part No. 16001-176
(VWR, West Chester, PA 19380)

Pyrex graduated cylinders, 50 and 100 mL, Part Nos. CLS2982250 and CLS3022250
(Sigma-Aldrich, St. Louis, MO 63103)

HDPE widemouth polypropylene bottles, 250 mL, with linerless cap,
Part No. 209548SP (Wheaton, Millville, NJ 08332)

Syringe filter, Nylon 0.45 µm, 30-mm diameter filter unit, Part. No. F2500-1
(National Scientific, Rockwood, TN 37854)

3.4 Reagents

Ammonium Hydroxide – 28% NH_3 in water 99.99+% pure (Sigma-Aldrich,
St. Louis, MO 63103)

Formic acid, 99.0 % pure, Fluka, Catalog No. 06440 (Sigma-Aldrich, St. Louis, MO 63103)

Methanol – HPLC-grade Catalog No. MX0475-1 (EMD Chemicals, Gibbstown, NJ)

Water – Ultra high purity, obtained from Purelab Classic UV UHP Water System

3.5 Principles of the Analytical Method

Sample aliquots (20 mL) were measured into 50-mL propylene centrifuge tubes and acidified by adding 60 µL of concentrated formic acid. Purification was performed with Oasis® MCX solid phase extraction (SPE) cartridges, where the analytes were retained. The analytes were eluted with 15.0 mL of 75 mM ammonium hydroxide in methanol into tubes containing 1.0 mL of 0.2% formic acid (aq) and evaporated under nitrogen gas flow to 2 mL in a water bath at 40°C, and then diluted with 0.01% formic acid (aq) to a final volume of 5.0 mL. The purified samples were filtered through 0.45-µm PTFE filters and placed in clean 15-mL propylene centrifuge tubes. Approximately 1 mL of each sample was placed into an autosampler vial and analyzed for IN-QFH57 by LC/MS/MS. A 10-fold dilution was made by mixing 900 µL of 0.01% formic acid (aq) with 100 µL of each sample in a clean glass autosampler vial. The 10-fold diluted samples were analyzed for DPX-KJM44, DPX-MAT28 and IN-LXT69 by LC/MS/MS.

3.6 Modifications, Interpretations, and Critical Steps

Some API 5000 LC/MS/MS instrumental parameters were modified to optimize sensitivity. DPX-KJM44, DPX-MAT28 and IN-LXT69 were injected with bracketing curves instead of standards embedded after every fourth sample.

For analysis of IN-QFH57-002, the modifications included decreasing injection volume from 60 µL to 35 µL and truncating the curve range from 0.2 ng/mL to 20 ng/mL to 0.2 ng/mL to 7.0 ng/mL.

3.7 Instrumentation

3.7.1 Chromatography

Reversed-phase liquid chromatography was used to separate the analytes from co-extractants. A Phenomenex Luna Phenyl-Hexyl column was used.

HPLC Conditions

System:	MDS Sciex API 5000 LC/MS/MS
Column:	Phenomenex Luna Phenyl-Hexyl, 4.6 × 15 mm, 3 µm diameter packing
Column Temperature:	30°C
Injection Volume:	60 µL
Conditions:	A: aqueous 0.1% formic acid, positive mode B: methanol

Time	%A	%B	Flowrate (mL/min)	Comments
0.01	95	5	1.00	Split 100 µL source/900 µL waste
5.00	41	59	1.00	
8.00	1	99	1.00	
10.00	1	99	1.00	
10.10	95	5	1.00	
14.50	95	5	1.00	End run

DPX-KJM44 Retention Time:	~ 8.24 min
DPX-MAT28 Retention Time:	~ 4.57 min
IN-LXT69 Retention Time:	~ 3.69 min
IN-QFH57 Retention Time:	~ 7.09 min
Total Run Time:	14.5 min

3.7.2 LC/MS/MS Analysis

Analysis of DPX-KJM44, DPX-MAT28, IN-LXT69, and IN-QFH57 was performed using a MDS Sciex API 5000 LC/MS/MS, equipped with a TurboIonSpray source, and operated in MRM, positive or negative ion mode. Quantitation was based on an average response factor using peak areas supplied by Analyst software version 1.4.2. Calculations were performed using Microsoft Excel. A summary of representative experimental conditions is provided in the following table:

MDS Sciex API 5000 MS/MS Mass Spectrometer Conditions Positive Ion Mode

Analytes	Ions Monitored	CXP (Collision Cell Exit Potential)	DP (Declustering Potential)	Dwell Time (msec)	Collision Energy
DPX-KJM44	227.6→ 68.1 AMU	15V	70V	100	40
DPX-KJM44	227.6→ 168.2 AMU	15V	51V	100	26
DPX-MAT28	214.2→ 68.0 AMU	15V	50V	100	36
DPX-MAT28	214.2→ 100.9 AMU	15V	50V	100	41
IN-LXT69	170.0→ 76.0 AMU	15V	62V	100	46
IN-LXT69	170.0→ 103.0 AMU	15V	90V	100	34

MDS Sciex API 5000 MS/MS Mass Spectrometer Conditions Negative Ion Mode

Analytes	Ions Monitored	DP (Declustering Potential)	Dwell Time (msec)	Collision Energy
IN-QFH57	176.0→ 131.9 AMU	-69V	100	-18
IN-QFH57	176.0→ 105.0 AMU	-62V	100	-36

Ion Mode	Ion Spray Voltage	CEM Voltage	Source Temperature	Curtain Gas Setting	GS1 Setting	GS2 Setting	CAD Setting
Positive	5000V	2000V	550°C	30	50	50	8
Negative	-2500V	2000V	550°C	50	60	40	8

3.7.3 *Calibration Procedure*

Calibration standards were analyzed at the beginning and end of each batch and progressed from low to high concentrations. The response factor of each calibration standard was calculated by dividing the analyte peak area of each standard by the analyte concentration for that standard. The average response was calculated for calibration standards injected with each batch.

3.8 ***Calculations***

Calculations from section 4.4.1 were used as written in the method.

APPENDIX 2 COMMUNICATION LOG

DATE	PERSONS INVOLVED	METHOD	REASON FOR CONTACT	RESULTING CHANGES TO METHOD	TIMING
2/16/09	Melissa Gaubatz, Del Koch, David Rockwell	Email	Clarify definition of "clean vials" as stated in method	None	Prior to Trial 1
2/17/09	Melissa Gaubatz, Del Koch, David Rockwell, Audrey Knobloch, Brian Graham	Email	Protocol comments received from Sergio Nanita. Emphasis placed on recommendations in Section 5.3 in regards to SPE purification and avoidance of contamination.	None	Prior to Trial 1
4/15/09	Melissa Gaubatz, Del Koch, David Rockwell	Email	Discussion of acceptance criteria, injection order and bracketing standards following transfer of Trial 1 results.	None	After Trial 1
4/20/09	Melissa Gaubatz, Del Koch, David Rockwell	Email	Discussion of accepting Trial 1 results based on chromatography and of re-injecting extracts for IN-QFH57. Trial 1 passes for DPX-KJM44, DPX-MAT28 and IN-LXT69.	None	After Trial 1
5/12/09	Melissa Gaubatz, Del Koch, David Rockwell, Brian Graham	Email	Summarize phone call from 5/8/09: Agreed to re-prepare calibration curve from 0.2 ng/mL to 7.0 ng/mL and experiment with injection order to determine how to proceed with IN-QFH57 analysis. Agreed to decrease injection volume by half.	None	After Trial 1
5/29/09	Melissa Gaubatz, Del Koch, David Rockwell	Email	Transfer results of new calibration curve.	None	After Trial 1
6/1/09	Melissa Gaubatz, Del Koch, David Rockwell	Email	Discussion of proposed injection sequences and if Trial 1 will be re-injected after 2 months of frozen storage.	None	After Trial 1

APPENDIX 2 COMMUNICATION LOG (CONTINUED)

DATE	PERSONS INVOLVED	METHOD	REASON FOR CONTACT	RESULTING CHANGES TO METHOD	TIMING
6/5/09	Melissa Gaubatz, Del Koch, David Rockwell, Sergio Nanita, John May, Kristin Milby	Email	Received direction to proceed to Trial 2 for IN-QFH57 only.	None	Prior to Trial 2
7/7/09	Melissa Gaubatz, Del Koch,	Email	Summarize phone call from 7/7/09: Agreed that preparation error in Batch 2, Trial 2 for IN-QFH57 invalidates the batch and Trial 2 will begin again with Batch 3.	None	Prior to Trial 2
8/13/09 to 8/14/09	Melissa Gaubatz, Del Koch, David Rockwell, Sergio Nanita	Email	Discussion of re-injecting IN-QFH57 extracts using a smaller injection volume following approval to use extracts stored frozen beyond the 5-week expiration.	None	After Trial 2
8/25/09	Melissa Gaubatz, Del Koch, David Rockwell	Phone	Review of Trial 2 results. One standard replicate had atypical results and it was agreed to exclude it from curve.	None	After Trial 2
9/23/09	Melissa Gaubatz, Del Koch, David Rockwell	Email	Transfer of Trial 2 results following exclusion of outlier standard. Approval received from Del Koch and Sergio Nanita to accept the 122% recovery from the LOQ-3 sample in Trial 2 of IN-QFH57. Trial 2 passes.	None	After Trial 2