3.0 INTRODUCTION

Independent laboratory validation of water methods is required to fulfill the requirements under U.S. EPA OPPTS 850.7100, PR Notice 96-1.

The environmental analytical method for DPX-QGU42 and its metabolites in water as described in DuPont-32124, entitled "Analytical Method for the Determination of DPX-QGU42 and Metabolites in Water Using LC/MS/MS" (Reference 2) is applicable for the quantitation of DPX-QGU42 and its metabolites in water matrix.

DPX-QGU42, IN-E8S72, IN-Q7D41, IN-P3X26, IN-QPS10, IN-RAB06, and IN-RDT31 were recovered from fortified water samples using three liquid-liquid extractions. The combined organic extracts were concentrated under a stream of nitrogen and subsequently reconstituted with 0.90 mL acetonitrile. The extracts were diluted to a final volume of 3 mL using HPLC grade water. Aliquots of these extracts were then analyzed using reversed phase liquid chromatography (LC) coupled with electrospray ionization and mass spectrometry/mass spectrometry (MS/MS) detection. DPX-QGU42, IN-Q7D41, IN-P3X26, IN-QPS10 and IN-RDT31 were detected by positive ion electrospray MS/MS and IN-E8S72 and IN-RAB06 were detected by negative ion electrospray MS/MS.

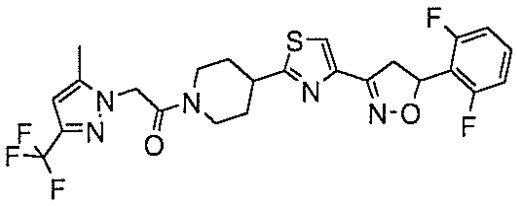
The analytical method was designed to achieve an LOQ of 0.10 µg/kg (ppb) for all analytes, and the LOD was estimated to be 0.3 µg/kg (ppb). The independent validation thus evaluated DPX-QGU42, IN-E8S72, IN-Q7D41, IN-P3X26, IN-QPS10, IN-RAB06 and IN-RDT31 recoveries on samples fortified at 1X and 10X the LOQ level. The method was used as written, with the exception of minor adjustments to the ion transitions monitored and a reduction in total extract volume from 15 mL to 14 mL. The Study Director received authorization for these changes prior to initiation of validation analyses.

4.0 MATERIALS AND METHODS

4.1 Test Substance

The reference analytical standards (test substances) used for this study were:

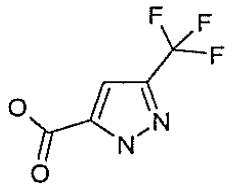
DuPont code: DPX-QGU42
Chemical Structure:



DPX-QGU42

Molecular weight: 539.52
Formula: $C_{24}H_{22}F_5N_5O_2S$
Source: E. I. du Pont de Nemours and Company
CAS Number: 1003318-67-9
Batch/Lot Number: E105317-115
Purity: 98.9%
Receipt date: 28 March, 2011
Expiration date: 18 June, 2012
Storage: Ambient temperature

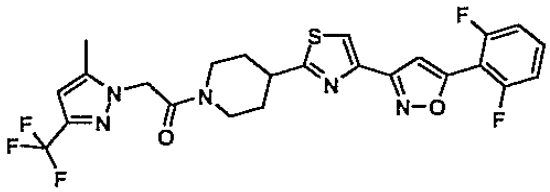
DuPont code: IN-E8S72
Chemical Structure:



IN-E8S72

Molecular weight: 180.09
Formula: $C_5H_3F_3N_2O_2$
Source: E. I. du Pont de Nemours and Company
Lot Number: GF1007175
Purity: 99.7%
Receipt date: 28 March, 2011
Expiration date: 12 October, 2013
Storage: Ambient temperature

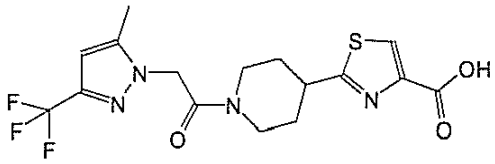
DuPont code: IN-Q7D41
Chemical Structure:



IN-Q7D41

Molecular weight: 537.51
Formula: $C_{24}H_{20}F_5N_5O_2S$
Source: E. I. du Pont de Nemours and Company
Lot Number: GF1001492
Purity: 91.5%
Receipt date: 28 March, 2011
Expiration date: 02 April, 2013
Storage: Ambient temperature

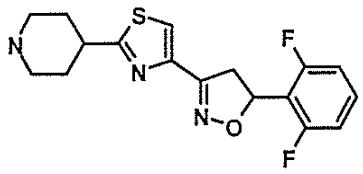
DuPont code: IN-P3X26
Chemical Structure:



IN-P3X26

Molecular weight: 402.40
Formula: $C_{16}H_{17}F_3N_4O_3S$
Source: E. I. du Pont de Nemours and Company
Lot Number: GF1011057
Purity: 93.8%
Receipt date: 28 March, 2011
Expiration date: 13 December, 2013
Storage: Ambient temperature

DuPont code: IN-QPS10
Chemical Structure:

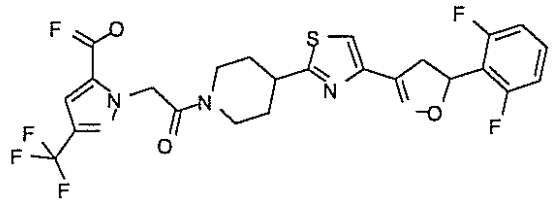


IN-QPS10

Molecular weight: 349.40
Formula: $C_{17}H_{17}F_2N_3OS$
Source: E. I. du Pont de Nemours and Company
Lot Number: GF1015939
Purity: 99.3%
Receipt date: 28 March, 2011
Expiration date: 13 August, 2013
Storage: Ambient temperature

DuPont code: IN-RAB06

Chemical Structure:

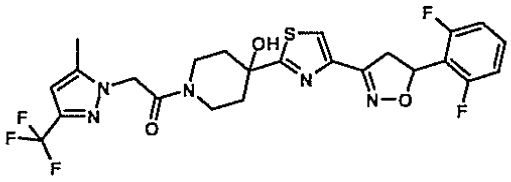


IN-RAB06

Molecular weight: 569.51
Formula: $C_{24}H_{20}F_5N_5O_4S$
Source: E. I. du Pont de Nemours and Company
Lot Number: GF912584
Purity: 97.3%
Receipt date: 28 March, 2010
Expiration date: 08 June, 2013
Storage: Ambient temperature

DuPont code: IN-RDT31

Chemical Structure:



IN-RDT31

Molecular weight: 555.53
Formula: $C_{24}H_{22}F_5N_5O_3S$
Source: E. I. du Pont de Nemours and Company
Lot Number: GF1000813
Purity: 94.0%
Receipt date: 28 March, 2011
Expiration date: 30 June, 2011
Revised Expiration Date: 18 May, 2014
Storage: Freezer ($\leq$ -10 ^{o}C)

Analytical standards and copies of characterization documentation for DPX-QGU42, IN-E8S72, IN-Q7D41, IN-P3X26, IN-QPS10, IN-RAB06, and IN-RDT31 were supplied by E. I. du Pont de Nemours and Company, DuPont Crop Protection, 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, Delaware.

4.2 Test Substance Disposition

A reserve sample of the test substance and records of sample disposition are maintained at Eurofins PSL. Any additional test substance is retained for at least 3 months following completion of the final report, unless otherwise specified by the Sponsor. After this time period all remaining test substance will be returned to the Sponsor or properly disposed.

4.3 Test System

In this study, the analytical method was validated in surface water, drinking water, and ground water matrices. Surface water was sampled from a pond located in East Brunswick, NJ 08816. Ground water was sampled from a residential well located in Monroe Township, NJ 08831, and drinking water was sampled from a water tap within the testing facility. GLP characterization was conducted for surface water only (Appendix 2). All samples were stored in a refrigerator at approximately -4°C and were shaken vigorously before subsampling.

Fortifications were made to 5.0 mL of water using 0.010-µg/mL and 0.10-µg/mL standard solutions.

The fortification and control samples were assigned unique identification by the laboratory, an alpha-numeric sample ID along with additional designations such as "control," and "LOQ" as appropriate.

4.4 Equipment

Equipment used was either the same as that specified in the analytical method or equivalent.

4.5 Reagents

Reagents used were either the same as that specified in the analytical method or equivalent grade of the quality.

4.6 Principles of the Analytical Method

The analyses in the study followed the analytical method for DPX-QGU42 and metabolites IN-E8S72, IN-Q7D41, IN-P3X26, IN-QPS10, IN-RAB06 and IN-RDT31 in water, as described in DuPont Study No. DuPont-32124 (Reference 2). The following is a summary of the method conducted at Product Safety Labs. The complete description of the method is reported in the original method (DuPont-32124).

A 5.0-mL water sample was measured into a 15 mL centrifuge tube and fortified with standard solution. To each sample, 0.10 mL formic acid, 1.5 g sodium chloride and 5.0 mL ethyl acetate were added. Samples were mixed on a vortex mixer for approximately 30 seconds and centrifuged at 3000rpm for 5 minutes. The organic layer from each sample was then transferred to a fresh 15 mL tube. The extraction was repeated twice, first using 5 mL acetonitrile, and then finally with 4 mL ethyl acetate. All three organic extracts were combined and evaporated to approximately 0.5 mL in a nitrogen evaporation system set to 30°C. Samples were reconstituted in 0.9 mL of acetonitrile, vortexed for approximately 30 seconds, and sonicated for 5 minutes. HPLC-grade water was added to each to a final volume of 3 mL. Samples were vortexed for an additional 30 seconds, and aliquots were transferred to autosampler vials for analysis by LC/MS/MS.

Method validation was accomplished by analyzing each of the seven analytes in the validation sets each consisting of 2 blank control samples, 5 replicate fortifications at LOQ and 5 replicate fortifications at 10xLOQ.

4.7 Modifications, Interpretations, and Critical Steps

The analytical method was run exactly as written with the following exceptions:

- The final extraction step was performed using 4 mL ethyl acetate rather than 5 mL as specified in the method.
- Extracts were evaporated to approximately 0.5 mL prior to dilution with acetonitrile and water.

- An injection volume of 0.030 mL was used instead of 0.010 mL for all samples in both positive and negative ion methods. The gradient table for the negative ion method was adjusted from the original method to the parameters listed in Section 4.8 below. As a result, the final run time changed from 15.0 minutes to 20.0 minutes.

Prior to the start of validation analyses, The Study Monitor received authorization from the Study Monitor to alter method conditions as needed. Adjustments were determined to have no significant effect on validation results.

No procedural step was identified as having a critical impact on analytical results.

4.8 Instrumentation

HPLC Method Conditions for the Analysis of DPX-QGU42, IN-Q7D41, IN-QPS10, IN-P3X26, and IN-RDT3:

System:	Applied Biosystems API4000; PerkinElmer LC Series 200
Column:	3.0 mm i.d. × 15 cm, 3.5 µm Agilent SB-phenyl
Column Temperature:	40°C
Autosampler Temperature:	4°C
Injection Volume:	30 µL
Flow Rate:	0.60 mL/minute
Conditions:	A: 0.05% Aqueous Formic Acid solution, 0.1% Methanol
	B: 0.01% Formic Acid in Methanol
	Flow in mL/minute
	Time(min) %A %B Flow 0.0 75 25 0.60 0.3 75 25 0.60 5.0 30 70 0.60 10.0 20 80 0.60 10.2 1.0 99 0.60 14.0 1.0 99 0.60 15.0 75 25 0.60 20.0 75 25 0.60
IN-QPS10 Retention Time:	5.24 minutes
IN-P3X26 Retention Time	6.87 minutes
IN-RDT31 Retention Time:	8.68 minutes
DPX-QGU42 Retention Time:	9.95 minutes
IN-Q7D41 Retention Time:	11.53 minutes
Total Run Time:	20.0 minutes

ANALYTES	IONS MONITORED	DECLUSTERING POTENTIAL (DP)	COLLISION ENERGY (CE)	EXIT POTENTIAL (CXP)
DPX-QGU42	**540.2→ 500.3 AMU**	**196**	**37**	**34**
	540.2→ 163.2 AMU	196	37	34
IN-QPS10	**349.8→ 81.9 AMU**	**21**	**41**	**10**
	349.8→ 209.8 AMU	21	35	54
IN-Q7D41	**538.1→ 498.0 AMU**	**196**	**33**	**20**
	538.→ 141.0 AMU	196	59	24
IN-P3X26	**403.09→ 362.9 AMU**	**51**	**29**	**34**
	403.09→ 384.9 AMU	51	29	26
IN-RDT31	**556.1→ 330.9 AMU**	**11**	**45**	**46**
	556.1→ 537.9 AMU	11	29	32

Time:	0.0 – 20.0 minutes		
Ion Mode:	Positive	CAD:	4
Turbo Spray Voltage:	4500 V	GS1:	40
Source Temperatures:	600 ^{0}C	GS2:	50
CUR:	30	Dwell	0.15 Seconds

HPLC Method Conditions for the Analysis ofIN-E8S72 and IN-RAB06:

System:	Applied Biosystems API4000; PerkinElmer LC Series 200
Column:	3.0 mm i.d. × 15 cm, 3.5 µm Agilent SB-phenyl
Column Temperature:	40°C
Autosampler Temperature:	4°C
Injection Volume:	0.030 mL
Flow Rate:	0.60 mL/minute
Conditions:	A: 0.05% Aqueous Formic Acid solution, 0.1% Methanol
	B: 0.01% Formic Acid in Methanol
	Flow in mL/minute
	Time %A %B Flow 0.0 75 25 0.60 0.3 75 25 0.60 4.0 40 60 0.60 5.0 30 70 0.60 10.0 20 80 0.60 10.2 1.0 99 0.60 14.0 1.0 99 0.60 15.0 75 25 0.60 20.0 75 25 0.60
IN-E8S72 Retention Time:	4.95 minutes
IN-RAB06 Retention Time:	10.29 minutes
Total Run Time:	20.0 minutes

ANALYTES	IONS MONITORED	DECLUSTERING POTENTIAL (DP)	COLLISION ENERGY (CE)	EXIT POTENTIAL (CXP)
IN-E8S72	**178.77→ 65.01 AMU**	**-25**	**-26**	**-11**
	178.77→ 134.85 AMU	-25	-16	-13
IN-RAB06	**567.9→ 524.7 AMU**	**-115**	**-20**	**-33**
	567.9→ 134.9 AMU	-115	-54	-15
Time:	0 - 15 minutes			
Ion Mode:	Negative	CAD:	10	
Turbo Spray Voltage:	-4500 V	GS1:	70	
Source Temperatures:	600 ^{0}C	GS2:	70	
CUR:	15	Dwell	0.15 Seconds	

Peak detection was accomplished using LC-MS/MS with Turbo Ion Spray ionization in the positive mode on a triple quadrupole instrument. The acquisition method was adjusted to maximize the response of the fragment ions detected. Ion transitions are included in the method tables above.

For quantitative analysis, the instrument was operated in the MS/MS positive ion mode for analytes DPX-QGU42 IN-Q7D41, IN-P3X26, IN-QPS10, and IN-RDT31 and in the negative ion mode for analytes IN-E8S72 and IN-RAB06. The ion chromatograms were integrated and the peak area used for quantitation.

For each analytical run, a seven-point standard curve was prepared by injecting constant volumes of standard solutions of each analyte of interest at a frequency of at least one standard injection for every three sample injections. Constant volume injections were used for sample extracts as well. The relative ratio of the fragment ions was evaluated to confirm the presence of an analyte in an unknown sample.

4.9 Calculations

Residues of DPX-QGU42 and its metabolites IN-E8S72, IN-Q7D41, IN-P3X26, IN-QPS10, IN-RAB06 and IN-RDT31 were quantitated using calibration standards. A calibration curve for each analyte was generated by plotting the detector's response in peak area versus the concentration (ng/mL) of standard injected. The data collection system derived an equation for the fit of the standard curve and this equation was used to calculate intercept and slope of the linear regression curve.

The calibration curve was obtained by direct injection of 15 µL of standard solutions of all analytes into LC-MS/MS in the range of 0.05 ng/mL to 5.0 ng/mL. In a given injection run, the same injection volume was used for all samples and standards.

Peak integration and quantitation were performed using Applied Biosystems' Analyst software version 1.4.2. Detected analyte concentrations and percent recoveries were also determined by this software. Additional calculations including statistical operations were computed for each set of samples by Microsoft's Excel® spreadsheet. Equations used for quantitation are shown below:

For the calculation of percent recoveries, the following formula was used:

$$\text{Rec} = \frac{C_{end}}{\left(\frac{R_{fortified} \times V_i}{V_f}\right)} \times 100$$

Where:

Rec: Percent recovery

C_{End}: Final analyte concentration derived from calibration curve in ng/mL (ppb)

V_f: Final reconstituted volume: 3 mL

V_i: Volume of initial water sample: 5 mL

Example: S-1-1, DPX-QGU42, Surface Water, Fortified @ 0.10 ppb (Figure 9):

C_{End} = 0.157 ppb

$R_{fortified}$ = 0.10 ppb

$$Rec = \frac{0.157 ppb}{\left(\frac{0.10 ppb \times 5 mL}{3 mL}\right)} \times 100$$

Rec = 94%