INDEPENDENT LABORATORY VALIDATION OF DUPONT-42574, "ANALYTICAL METHOD FOR THE DETERMINATION OF DPX-Q8U80 AND METABOLITES IN SURFACE, GROUND AND DRINKING WATER USING LC/MS/MS"

Yixiao Shen, Ph.D.

1.0 SUMMARY

The objective of this study was to conduct an independent laboratory validation of analytical method DuPont-42574 entitled, "Analytical Method for the Determination of DPX-Q8U80 and Metabolites in Surface, Ground and Drinking Water Using LC/MS/MS," as written (Reference 1). This study was designed to fulfill the requirements of the U.S. Environmental Protection Agency (EPA) Residue Chemistry Test Guidelines: OPPTS 860.1340. "Residue Analytical Method" (Reference 2) and the European Commission's Directorate General for Health and Consumer Protection. "Guidance Document on Residue Analytical Methods", SANCO/825/00 rev. 8.1, November 16, 2010 (Reference 3).

The method under evaluation has a stated limit of quantitation (LOQ) in surface water, ground water and drinking water of 0.10 µg/L (parts per billion, ppb). In this study, the method was validated at the LOQ and 10×LOQ in surface water, ground water and drinking water.

The independent laboratory validation was successful, and the method was used as written with no significant modifications—refer to section 3.6. A single scientist completed one sample set (one set consisting of two controls, five LOQ fortifications and five 10×LOQs in surface water, ground water, or drinking water) during the course of one workday (8 hours), with liquid chromatography–tandem mass spectrometry (LC-MS/MS) analysis performed unattended following the completion of sample preparation. [redacted]

[redacted]

2.0 INTRODUCTION

To satisfy US and EU regulatory independent laboratory validation (ILV) requirements, a residue analytical method must be validated at an independent laboratory prior to its submission to the appropriate regulatory authority. This study was conducted to fulfill those requirements.

The analytical method DuPont-42574 entitled, "Analytical Method for the Determination of DPX-Q8U80 and Metabolites in Surface, Ground and Drinking Water Using LC/MS/MS," is applicable for the quantitation of fluazaindolizine (DPX-Q8U80) and its metabolites IN-REG72, IN-F4106, IN-A5760, IN-VM862, IN-QEK31, and IN-RCY33 in surface water, ground water, and drinking water.

Fluazaindolizine and its metabolites were extracted from surface water, ground water, and drinking water that were fortified with the analytes at LOQ of 0.10 µg/L (ppb) and 10×LOQ of 1.0 µg/L (ppb). Two ion transitions were monitored for each analyte. All transitions of fluazaindolizine and its metabolites, IN-REG72, IN-F4106 and IN-A5760 were detected by negative ionization MS/MS. Both transitions of metabolites IN-VM862, IN-QEK31, and IN-RCY33 were detected by positive ionization MS/MS.

The analytical method was designed to achieve an LOQ of 0.10 µg/L (ppb) in surface water, ground water and drinking water. The independent validation thus evaluated recoveries of fluazaindolizine (DPX-Q8U80) and its metabolites, IN-REG72, IN-F4106, IN-A5760, IN-VM862, IN-QEK31 and IN-RCY33 in samples fortified at the 1×LOQ and 10×LOQ level. The method was used as written.

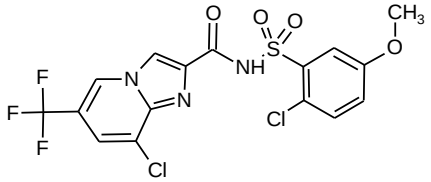
3.0 MATERIALS AND METHODS

3.1 Test Substances

The reference analytical standards (test substances) used for this study are listed below.

DuPont Code:	DPX-Q8U80 (Fluazaindolizine)

Chemical Structure:

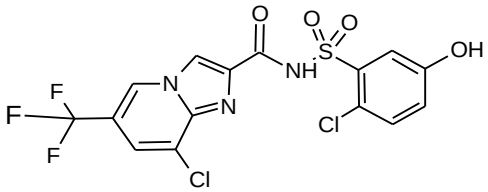


DPX-Q8U80

CAS Name:	8-Chloro-*N*-[(2-chloro-5-methoxyphenyl)sulfonyl]-6-(trifluoromethyl)-imidazo[1,2-*a*]pyridine-2-carboxamide
Molecular Weight:	468.2 g/mole
Source:	DuPont
CAS Number:	1254304-22-7
Batch/Lot Number:	FE114893-110
Purity:	99.6%
Receipt Date:	22 July 2016
Expiration Date:	28 July 2020
Storage:	Ambient

DuPont Code:	IN-REG72

Chemical Structure:

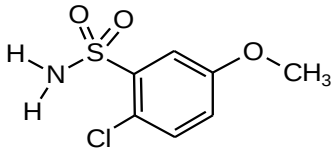


IN-REG72

CAS Name:	8-Chloro-*N*-[(2-chloro-5-hydroxyphenyl) sulfonyl]-6-(trifluoromethyl)imidazo[1,2-*a*]pyridine-2-carboxamide
Molecular Weight:	454.2 g/mole
Source:	DuPont
CAS Number:	Not available
Batch/Lot Number:	103533-736RD
Purity:	100.0%
Receipt Date:	22 July 2016
Expiration Date:	17 September 2018
Storage:	Ambient

DuPont Code: IN-F4106

Chemical Structure:

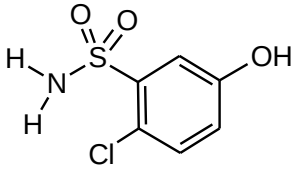


IN-F4106

CAS Name:	2-Chloro-5-methoxybenzenesulfonamide
Molecular Weight:	221.7 g/mole
Source:	DuPont
CAS Number:	502187-53-3
Batch/Lot Number:	IE114893-098
Purity:	97.9%
Receipt Date:	22 July 2016
Expiration Date:	27 February 2020
Storage:	Ambient

DuPont Code: IN-A5760

Chemical Structure:

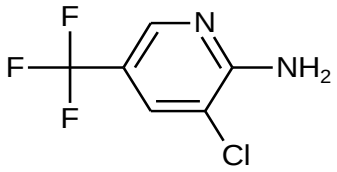


IN-A5760

CAS Name:	2-Chloro-5-hydroxybenzenesulfonamide
Molecular weight:	207.6 g/mole
Source:	DuPont
CAS Number:	86093-06-3
Batch/Lot Number:	E118883-19
Purity:	99.3%
Receipt Date:	22 July 2016
Expiration Date:	22 June 2022
Storage:	Ambient

DuPont Code: IN-VM862

Chemical Structure:

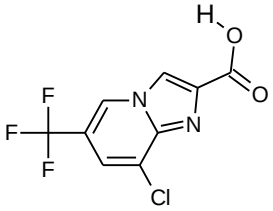


IN-VM862

CAS Name:	3-Chloro-5-(trifluoromethyl)-2-pyridinamine
Molecular Weight:	196.6 g/mole
Source:	DuPont
CAS Number:	79456-26-1
Batch/Lot Number:	R20K
Purity:	100.0%
Receipt Date:	22 July 2016
Expiration Date:	07 May 2017
Storage:	Ambient

DuPont Code: IN-QEK31

Chemical Structure:

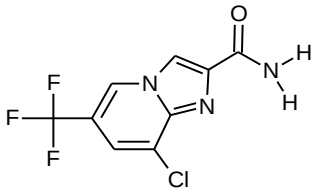


IN-QEK31

CAS Name:	8-Chloro-6-(trifluoromethyl)imidazo[1,2-*a*]pyridine-2-carboxylic acid
Molecular Weight:	264.6 g/mole
Source:	DuPont
CAS Number:	353258-35-2
Batch/Lot Number:	IE114893-105
Purity:	98.6%
Receipt Date:	22 July 2016
Expiration Date:	07 April 2020
Storage:	Ambient

DuPont Code:	IN-RYC33

Chemical Structure:



IN-RCY33

CAS Name:	8-Chloro-6-(trifluoromethyl)imidazo[1,2-*a*]pyridine-2-carboxamide
Molecular Weight:	263.6 g/mole
Source:	DuPont
CAS Number:	1228376-01-9
Batch/Lot Number:	E117272-48
Purity:	99.3%
Receipt Date:	22 July 2016
Expiration Date:	15 July 2020
Storage:	Ambient

Fluazaindolizine (DPX-Q8U80) and its metabolites IN-REG72, IN-VM862, IN-F4106, IN-A5760, IN-VM862, IN-QEK31, and IN-RCY33 were supplied by E. I. du Pont de Nemours and Company, DuPont Crop Protection, Newark, Delaware. Information pertaining to the characterization and stability of these test substances is archived by DuPont Crop Protection, E. I. du Pont de Nemours and Company, Newark, Delaware. Certificates of analysis for the above compounds are included in **Appendix 1**.

3.2 Test System

In this study, the analytical method was validated using samples of drinking water and ground water supplied by DuPont Crop Protection, E. I. du Pont de Nemours and Company, Newark, Delaware. The sample of surface water was collected in Downingtown, Pennsylvania, on 16 May 2016. The characterization reports of the drinking water (Tap water was used as drinking water), surface water and ground water (Well water was used as ground water) are included in **Appendix 2 to Appendix 4**. Fortifications of the samples in water were made using 5.0 ±0.10 mL of blank water. The samples were each assigned a unique identifier by the laboratory, namely, an alpha-numeric sample ID along with an additional designation such as "Control" or "LOQ", as appropriate.

3.3 Equipment

The equipment used was either the same as that specified in the analytical method or the equivalent. A Shimadzu Nexera X2 UHPLC system was used instead of an Agilent 1290 HPLC system. An AB SCIEX Triple Quad 5500 was used instead of an API QTRAP 6500 system. These equipment substitutions were demonstrated to be equivalent to those specified in the method.

3.4 Reagents

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

3.5 Principles of the Analytical Method

The analyses in this study followed DuPont-42574 method for fluazaindolizine and its 6 metabolites. The following is a summary of the method used to conduct the independent laboratory validation at Alliance Pharma.

Sample Preparation

Sample aliquots of 5.0 (±0.10) mL of surface water, ground water, and drinking water were fortified at either LOQ (0.10 ppb) or 10×LOQ (1.0 ppb), if necessary. The samples were capped and vortex-mixed for 10 seconds. The fortified samples were left uncapped for 10 minutes to allow the carrier to evaporate. The water samples were centrifuged at room temperature for 10 minutes at 3500 rpm. In an amber LC vial, 900 µL of each sample was diluted to 1 mL with HPLC-grade methanol. The samples were caped and vortex-mixed for 10 seconds and then analyzed using LC/ESI-MS/MS (liquid chromatography/electrospray ionization–tandem mass spectrometry).

For surface water, ground water, and drinking water samples, the analytes fluazaindolizine (DPX-Q8U80), IN-REG72, IN-F4106, and IN-A5760 were detected using LC-MS/MS in negative ionization mode, whereas IN-VM862, IN-QEK31 and IN-RCY33 were detected using LC-MS/MS in positive ionization mode. Two parent-to-daughter ion transitions of each analyte were monitored as follows: fluazaindolizine (DPX-Q8U80) using mass-to-charge ratios (*m/z*) 466→157 and 466→142, IN-REG72 using *m/z* 452→123 and 452→244, IN-F4106 using *m/z* 220→156 and 220→141, IN-A5760 using *m/z* 206→122 and 206→142, IN-VM862 using *m/z* 197→141 and 197→114, IN-QEK31 using *m/z* 265→219 and 265→184, and IN-RCY33 using *m/z* 264→157 and 264→184. The confirmatory method was based on the recovery of secondary MS/MS ion transitions.

Method validation on each type of water sample was accomplished by analyzing the analytes in validation sets consisting of 2 blank control samples, 5 replicate samples fortified at the LOQ, and 5 replicate samples fortified at 10×LOQ.

3.6 Modifications, Interpretations, and Critical Steps

The analytical method was followed exactly as written with only a few exceptions. A Shimadzu Nexera X2 UHPLC system was used instead of an Agilent HPLC.

An AB SCIEX Triple Quad 5500 was used instead of an API QTRAP 6500 system. Separate HPLC injections were made in positive and negative ionization modes instead of using the positive/negative switch mode. These substitutions were demonstrated to be equivalent those specified in the method and thus did not impact the analytical results.

3.7 Instrumentation

HPLC Conditions (for Analyses of DPX-Q8U80 and Its Metabolites IN-REG72, IN-F4106, IN-A5760, IN-VM862, IN-QEK31, and IN-RCY33)

System:	Shimadzu LC-30AD / Sil-30ACMP Autosampler			
Column:	Zorbax Eclipse Plus Phenyl-Hexyl, RPHD 1.8 µm, 4.6 x 50 mm			
Column Temperature:	50°C			
Injection Volume:	20 µL			
Autosampler Temperature:	10°C			
Mobile Phases:	**A**: HPLC-grade water **B**: 0.01% Formic acid in methanol			
Conditions:	Time (min)	%A	%B	Flow Rate (mL/min)
	0.0	90	10	0.3
	0.1	90	10	0.3
	3.5	1.0	99	0.3
	5.5	1.0	99	0.3
	5.6	90	10	0.3
	8.0	90	10	0.3

Analyte	**Retention Time (minutes)**
DPX-Q8U80	~4.7
IN-REG72	~4.5
IN-F4106	~3.4
IN-A5760	~2.5
IN-VM862	~4.1
IN-QEK31	~3.9
IN-RCY33	~4.1

The detection method utilized was LC/ESI-MS/MS employing atmospheric pressure electrospray ionization interface in both positive and negative modes on a triple quadrupole instrument. The acquisition method was adjusted to maximize the response of the ion transitions detected. The ion transitions for fluazaindolizine and its metabolites are shown in the table below.

MS System:		AB SCIEX API 5500						
Compounds	**Parent Ion (*m/z*)**	**Product Ion (*m/z*)**		**Dwell Time (ms)**	**DP**	**EP**	**CE**	**CXP**
DPX-Q8U80	466	Q[1]	157	50	-80	-10	-35	-13
		C[2]	142				-44	-13
IN-REG72	452	Q[1]	123	50	-80	-10	-37	-11
		C[2]	244				-36	-11
IN-F4106	220	Q[1]	156	50	-80	-10	-19	-18
		C[2]	141				-25	-18
IN-A5760	206	Q[1]	122	50	-80	-10	-21	-15
		C[2]	142				-22	-15
IN-VM862	197	Q[1]	141	50	80	10	35	18
		C[2]	114				45	18
IN-QEK31	265	Q[1]	219	50	80	10	39	23
		C[2]	184				47	23
IN-RCY33	264	Q[1]	157	50	80	10	58	15
		C[2]	184				50	15

Mode	Positive	Negative
Turbo Spray Voltage :	5500 V	−4500 V
Source Temperatures:	400°C	400°C
CUR:	30	30
CAD:	10	10
GS1:	65	60
GS2:	30	30

[1] Quantitation
[2] Confirmation

The HPLC system was operated in the MS/MS (multiple reaction monitoring [MRM]) negative ionization mode for quantitative analysis of fluazaindolizine (DPX-Q8U80), IN-REG72, IN-F4106, and IN-A5760. And, the HPLC system was operated in the MS/MS positive ionization mode for the quantitative analysis of IN-VM862, IN-QEK31, and IN-RCY33. The ion chromatograms were integrated, and the peak areas were used for quantitation.

For each analytical run, a 5-point standard curve was prepared by injecting constant volumes of mixed standard solutions composed of each analyte of interest. Constant volume injections were also used for sample extracts.

3.8 Calculations

Average response factors (Rf_{avg} values) were used for quantitation and were calculated as follows:

$$Rf_{avg}=[(\text{Conc A} \div \text{Area A}) + (\text{Conc B} \div \text{Area B}) + (\text{Conc C} \div \text{Area C}) + (\text{Conc D} \div \text{Area D}) + (\text{Conc E} \div \text{Area E})])/\text{Total number of standards injected}$$

Dilution factor was calculated as follows:

$$\text{HPLC Dilution Factor}= \frac{\text{Total volume in HPLC Vial}}{\text{Volume of sample in HPLC Vial}}$$

Residues found (expressed in ng/mL, µg/L, or ppm) were calculated as follows:

$$= \frac{(\text{Peak Area}) \times (Rf_{avg}) \times (\text{Extract Volume}) \times (\text{HPLC Dilution Factor})}{(\text{Sample Weight})}$$

where,

Rf_{avg} = Average response factor

NOTE.—In the event a peak was detected in the control, a corrected peak area was used to calculate residues (ppm) found in freshly fortified samples. The corrected peak area is the area of the fortified sample minus the area of the control sample.

Percent recoveries (reported to the nearest whole number) from fortified samples were calculated as follows:

$$\% \text{ Recovery} = \frac{\text{Residue found (ng/mL , µg/L, or ppm)}}{\text{Actual Fortification Level (ng/mL , µg/L, or ppm)}} \times 100$$

[redacted]