INDEPENDENT LABORATORY VALIDATION OF DUPONT-37404, "METHOD VALIDATION OF DPX-Q8U80 AND ITS METABOLITES IN SOIL"

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1.0 SUMMARY

The objective of this study was to conduct an independent laboratory validation of the analytical method DuPont-37404 entitled, "Method Validation of DPX-Q8U80 and Its Metabolites in Soil," 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) of 1.0 µg/kg (parts per billion, ppb) in Drummer, Tama, and Sassafras soil. In this study, the method was validated at the LOQ and 10×LOQ in Drummer, Tama, and Sassafras soil.

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×LOQ fortifications in Drummer, Tama, or Sassafras soil) during the course of one workday (8 hours), with liquid chromatography–tandem mass spectrometry (HPLC-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-37404 entitled, "Method Validation of DPX-Q8U80 and Its Metabolites in Soil" is applicable for the quantitation of fluazaindolizine (DPX-Q8U80) and its metabolites IN-REG72, IN-F4106, IN-A5760, IN-VM862, IN-QEK31, and IN-RYC33 in three types of soil (Drummer, Tama, and Sassafras).

Fluazaindolizine and its metabolites were extracted from three types of soil (Drummer, Tama, and Sassafras) that were fortified with the analytes at the LOQ of 1.0 µg/kg (ppb) and 10×LOQ of 10 µg/kg (ppb). Two ion transitions were monitored for each analyte. Both 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-RYC33 were detected by positive ionization MS/MS.

The analytical method was designed to achieve an LOQ of 1.0 µg/kg (ppb) in three types of soil (Drummer, Tama and Sassafras). 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-RYC33 in samples fortified at the LOQ and 10×LOQ level. The method was used as written.

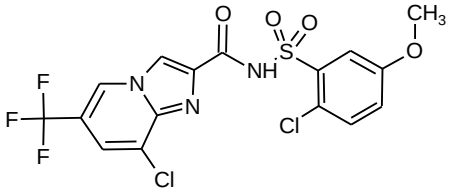
3.0 MATERIALS AND METHODS

3.1 Test Substances

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

DuPont Code:	DPX-Q8U80 (Fluazaindolizine)

Chemical Structure:

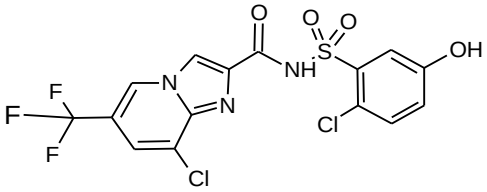


DPX-Q8U80

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:

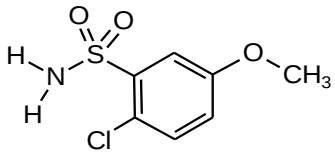


IN-REG72

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:

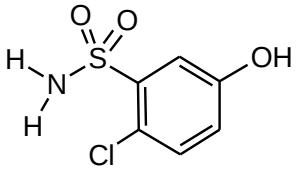


IN-F4106

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:

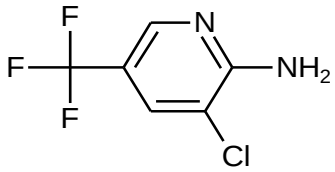


IN-A5760

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:

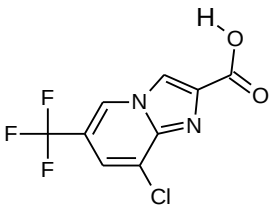


IN-VM862

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:

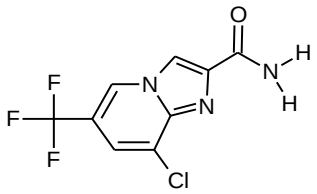


IN-QEK31

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-RYC33

CAS Name:	8-Chloro-6-(trifluoromethyl)imidazo[1,2-*a*]pyridine-2-carboxamide
Molecular Weight:	263.6 g/mole
Source:	DuPont
CAS Number:	122.18376-01-9
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-RYC33 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 seven compounds listed above are included in **Appendix 1**.

3.2 Test System

In this study, the analytical method was validated using samples of Drummer, Tama, and Sassafras soils supplied by DuPont Crop Protection, E. I. du Pont de Nemours and Company (Newark, Delaware). Fortifications of the samples in soil were made using 7.5 ± 0.05 g of blank soil. 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 a Waters Acquity UPLC system. An AB SCIEX Triple Quad 5500 was used instead of an

API 5000 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-37404 method for DPX-Q8U80 and its 6 metabolites. The following is a summary of the method used to conduct the independent laboratory validation at Alliance Pharma.

Sample Preparation

Soil sample (7.5 ± 0.05 g) was weighted into a 50-mL centrifuge tube and fortified at either the LOQ (1.0 µg/kg) or 10×LOQ (10 µg/kg) if necessary. The sample was placed under the fume hood for 15-20 minutes to evaporate the fortification solvent. Two steel balls (~¼ inch) and 20 mL of extraction solution [50:50 acetonitrile: 2% formic acid (aq)] were added to the centrifuge tube. The sample tube was capped and vortexed for 10-15 seconds to mix the solution thoroughly with the soil. The sample was placed on a Geno/Grinder® at 1100 shakes per minute for 3 minutes and centrifuged for 10 minutes at ~3000 rpm. The extract was then decanted into a clean 50-mL centrifuge tube. Another aliquot of 15 mL extraction solution was added to the soil pellet. The soil sample was vortex mixed until the pellet on the bottom was broken up. The sample tube was placed on a Geno/Grinder® at 1100 shakes per minute for 3 minutes and centrifuged for 10 minutes at ~3000 rpm again. The extract was decanted into to the same 50-mL centrifuge tube. The final volume of the extract was adjusted to 35 mL with HPLC-grade water. The extract was capped and vortexed.

An aliquot (7 mL) of sample extract was transferred to a clean 50-mL centrifuge tube. The volume of the extract was adjusted to approximately 22 mL using HPLC-grade water and mixed well. An Oasis HLB (6 mL/500 mg) SPE (solid phase extraction) column was attached to an SPE manifold and conditioned with one column volume of acetonitrile and one column volume of 0.1% formic acid in water by gravity. The soil extract passed through the SPE column using gravity. The sample tube was rinsed with 5 mL of 20% acetonitrile in water solution and the rinse passed through the SPE tube by gravity. After all of the rinse has passed through the SPE, the cartridge was dried with full vacuum setting for at least 1 minute. A clean 15-mL centrifuge tube was placed under the SPE column. The analytes were eluted using two column volumes (~6 mL each) of 0.1% formic acid in acetone (a small amount of vacuum was used to start the flow). After all the solution had passed through the SPE, full vacuum was used for 10-15 seconds to remove any remaining solution from the SPE. The eluate was evaporated with a moderate nitrogen flow in a water bath at 35 ± 5ºC until approximately 2 mL was left in the tube.

Two milliliters of methanol were added to the sample tube and mixed with the sample. The sample was evaporated with a moderate nitrogen flow in a water bath at 25 ± 5ºC until approximately 1 mL was left in the tube. The sample volume was adjusted to 2

mL with methanol. The final volume of 10 mL was adjusted with 0.1% formic acid in water. The sample tube was capped, vortexed, and sonicated for approximately 2 minutes. After vortexing again, the sample was filtered through a 0.45-µm PTFE syringe filter and transferred to an HPLC vial for analysis.

For Drummer, Tama, and Sassafras samples, the analytes fluazaindolizine (DPX-Q8U80), IN-REG72, IN-F4106, and IN-A5760 were detected using HPLC-MS/MS in negative ionization mode, whereas IN-VM862, IN-QEK31, and IN-RYC33 were detected using HPLC-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*) 466.1→157.1 and 466.1→142.0; IN-REG72 using *m/z* 452.0→123.2 and 452.0→143.0; IN-F4106 using *m/z* 220.0→78.0 and 220.0→156.1; IN-A5760 using *m/z* 206.0→122.1 and 206.0→142.0; IN-VM862 using *m/z* 197.0→75.0 and 197.0→69.0; IN-QEK31 using *m/z* 265.0→157.0 and 265.0→219.0; and IN-RYC33 using *m/z* 264.0→157.2 and 264.0→219.3. The confirmatory method was based on the recovery of secondary MS/MS ion transitions.

Method validation in each type of soil 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 a Waters Acquity UPLC system. An AB SCIEX Triple Quad 5500 was used instead of an API 5000 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-RYC33)

System:	Shimadzu LC-30AD / Sil-30ACMP Autosampler			
Column:	HSS T3, 50 x 2.1 mm, 1.8 µm particle size			
Column Temperature:	40°C			
Injection Volume:	35 µL			
Autosampler Temperature:	10°C			
Mobile Phases:	**A**: 0.1% Formic acid in water **B**: 0.1% Formic acid in methanol			
Conditions:	Time (min)	%A	%B	Flow Rate (mL/min)
	0.00	95	5	0.7
	4.00	10	90	0.7
	5.00	10	90	0.7
	5.01	95	5	0.7
	6.00	95	5	0.7
Analyte	**Retention Time (minutes)**			
DPX-Q8U80	~3.7			
IN-REG72	~3.5			
IN-F4106	~2.0			
IN-A5760	~1.4			
IN-VM862	~3.0			
IN-QEK31	~2.7			
IN-RYC33	~2.8			

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	466.1	Q[1]	157.1	50	-80	-10	-30	-15
		C[2]	142.0				-40	-15
IN-REG72	452.0	Q[1]	123.2	50	-80	-10	-30	-15
		C[2]	143.0				-25	-15
IN-F4106	220.0	Q[1]	78.0	50	-80	-10	-40	-13
		C[2]	156.1				-22	-13
IN-A5760	206.0	Q[1]	122.1	50	-80	-10	-21	-15
		C[2]	142.0				-21	-15
IN-VM862	197.0	Q[1]	75.0	50	80	9	89	12
		C[2]	69.0				88	14
IN-QEK31	265.0	Q[1]	157.0	50	80	9	57	15
		C[2]	219.0				38	20
IN-RYC33	264.0	Q[1]	157.2	50	80	9	40	10
		C[2]	219.3				30	10

Mode	Positive	Negative
Turbo Spray Voltage :	4500 V	−4500 V
Source Temperatures:	500°C	650°C
CUR:	25	25
CAD:	10	10
GS1:	85	70
GS2:	70	30

[1] Quantitation
[2] Confirmation

The HPLC system was operated in the MS/MS (multiple reaction monitoring) 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-RYC33. The ion chromatograms were integrated, and the peak areas were used for quantitation.

For each analytical run, a 7-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

The standard response factor, RF_{std}, for each analytical standard is the ratio of the analyte concentration to the analyte peak area.

$$RF_{std} = \frac{\text{analyte of concentration(ng/mL)}}{\text{Analyte peak area}}$$

The average response factor, RF_{avg}, calculated using each standard level analyzed in an analytical set containing control and fortified, samples was used to calculate the concentration of DPX-Q8U80 and its metabolites in these samples.

$$RF_{avg} = \frac{(RFstd1 + RFstd2 + RFstd3 + \ldots\ldots RFstdN)}{\text{Total number of standards injected}}$$

The concentration (ppb) of analyte found in each sample was calculated as follows:

$$\text{ppb analyte Found} = \frac{(\text{ng/mL Found}) \times (\text{Aliquot Factor}) \times (\text{Final Vol. mL}) \times (DF)}{(\text{Sample Weight g})}$$

where:

ng/mL Found = Peak Area × RF_{avg}
Extract Volume = 35 mL
Aliquot Taken = 7 mL
Aliquot Factor = Extract Volume / Aliquot Taken = 5
Final Vol. (mL) = 10 mL
Sample Weight (g) = 7.5 g
Dilution Factor (DF) =1

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.

The percent recovery found was calculated as follows:

$$\%\ \text{Recovery} = \frac{(\text{ppb found} - \text{ppb found in control sample})}{\text{Fortification Level (ppb)}} \times 100\%$$

[redacted]