METHOD VALIDATION OF DPX-Q8U80 AND ITS METABOLITES IN SOIL

Lisa Swaim

1.0 SUMMARY

The purpose of this study was to validate the methodology developed and reported in ABC Laboratories, Inc. Method ABC 69683-M for the detection and quantitation of DPX-Q8U80 and its metabolites (IN-REG72, IN-VM862, IN-QEK31, IN-F4106, IN-A5760, and IN-RYC33) in soil. The method limit of quantitation (LOQ) was 1.0 ppb (µg/kg). The limit of detection (LOD) was estimated to be 0.3 ppb (µg/kg) in representative soil matrices. The method was validated at 1.0 and 10 ppb in soil matrices using an LC/MS/MS system operating with an electrospray interface (ESI) in positive and negative ion modes. This analytical method is suitable for enforcement/monitoring and data generation for regulatory studies. The method was validated on sandy soil (Florida soil, Reference 6) and loam soil (New Jersey soil, Reference 7).

A 7.5-g soil sample is extracted twice with 50:50 acetonitrile:2% formic acid solution by shaking on a Geno/grinder®. After adjusting to a known volume with water, a 7-mL aliquot of the extract is taken and diluted to approximately 22 mL with water. The diluted extract is then passed through an HLB SPE column. The analytes are retained on the HLB SPE column. DPX-Q8U80 and its metabolites are eluted from the HLB column with an acidic acetone solution. The extracts are evaporated and solvent exchanged to acetonitrile. Samples are then diluted to final volume with acidified water, filtered, and analyzed by LC/TIS-MS/MS.

The confirmatory analysis for the LC/TIS-MS/MS method was based on detection of secondary parent-to-daughter ion transitions monitored during the validation.

Before method validation, post-fortified samples were analyzed with controls for each soil matrix to determine if matrix effect, suppression or enhancement, influenced percent recovery of DPX-Q8U80 and its metabolites. The post-fortified samples, in this study, were extracts of control soil samples that were purified and prepared in the same manner as with the other fortified or treated samples, but fortified with the analytes prior to LC/TIS-MS/MS analysis.

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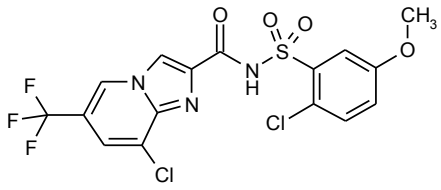
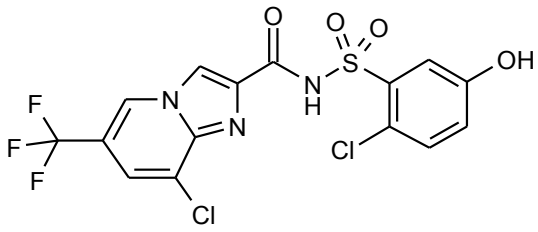
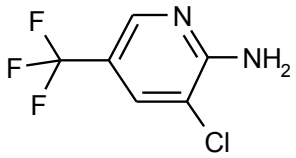
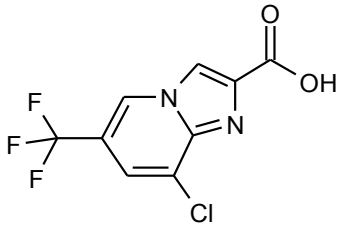
2.0 INTRODUCTION

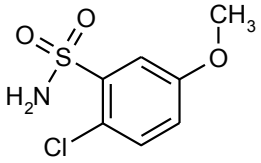
DPX-Q8U80 is an experimental crop protection product being developed for control of nematodes on a wide variety of crops. DPX-Q8U80 metabolizes in soil samples to IN-REG72, IN-VM862, IN-QEK31, IN-F4106, IN-A5760, and IN-RYC33. The chemical structures and pertinent information of the test substances are listed below.

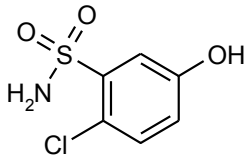
Structure	
DPX Number	DPX-Q8U80
Common Name	Not Available
CAS Chemical Name	8-Chloro-N-[(2-chloro-5-methoxyphenyl)sulfonyl]-6-(trifluoromethyl)-imidazo[1,2-a]pyridine-2-carboxamide
CAS Registration Number	1254304-22-7
Formula	$C_{16}H_{10}Cl_2F_3N_3O_4S$
Molecular Weight (g/mol)	468.2
pKa (25°C)	5.6 ± 0.07
Solubility (g/L), 20 ± 0.5 °C	Double distilled water (pH 6.40): 0.0561 ± 0.0036 Buffer Solutions: pH 4.0 = 0.0221 ± 0.0013 pH 7.0 = 2.1479 ± 0.0310 pH 9.0 = 2.8455 ± 0.0888 Acetonitrile: 35.05 Methanol: 3.47 Acetone: 99.76 Ethyl acetate: 27.62 1,2-dichloroethane: 19.29 o-Xylene: 1.247 n-Octanol: 2.00 n-Hexane: 0.002

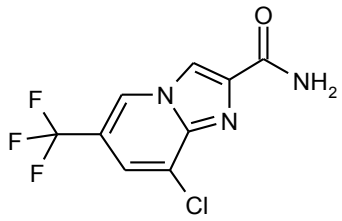
Structure	
DPX Number	IN-REG72
CAS Chemical Name	8-Chloro-N-[(2-chloro-5-hydroxyphenyl)sulfonyl]-6-(trifluoromethyl)imidazo[1,2-a]pyridine-2-carboxamide
CAS Registration Number	Not available
Formula	$C_{15}H_8Cl_2F_3N_3O_4S$
Molecular Weight	454.2

Structure	
DPX Number	IN-VM862
CAS Chemical Name	3-Chloro-5-(trifluoromethyl)-2-pyridinamine
CAS Registration Number	79456-26-1
Formula	$C_6H_4ClF_3N_2$
Molecular Weight	196.6

Structure	
DPX Number	IN-QEK31
CAS Chemical Name	8-Chloro-6-(trifluoromethyl)imidazo[1,2-a]pyridine-2-carboxylic acid
CAS Registration Number	353258-35-2
Formula	$C_9H_4ClF_3N_2O_2$
Molecular Weight	264.6

Structure	
DPX Number	IN-F4106
CAS Chemical Name	2-Chloro-5-methoxybenzenesulfonamide
CAS Registration Number	502187-53-3
Formula	$C_7H_8ClNO_3S$
Molecular Weight	221.7

Structure	
DPX Number	IN-A5760
CAS Chemical Name	2-Chloro-5-hydroxybenzenesulfonamide
CAS Registration Number	86093-06-3
Formula	$C_6H_6ClNO_3S$
Molecular Weight	207.6

Structure	
DPX Number	IN-RYC33
CAS Chemical Name	8-Chloro-6-(trifluoromethyl)imidazo[1,2-a]pyridine-2-carboxamide
CAS Registration Number	1228376-01-9
Formula	$C_9H_5ClF_3N_3O$
Molecular Weight	263.6

The analytical method (ABC 69683-M) for the determination of DPX-Q8U80, IN-REG72, IN-VM862, IN-QEK31, IN-F4106, IN-A5760, and IN-RYC33 in soil matrices, at a LOQ of approximately 1.0 ppb, was developed to satisfy the requirements of SANCO/825/00 rev. 8.1 and the US EPA OCSPP 850.6100 guidelines.

A 7.5-g soil sample is extracted twice with 50:50 acetonitrile:2% formic acid solution by shaking on a Geno/grinder®. After adjusting to a known volume with water, a

7-mL aliquot of the extract is taken and diluted to approximately 22 mL with water. The diluted extract is then passed through an HLB SPE column. The analytes are retained on the HLB SPE column. DPX-Q8U80 and its metabolites are eluted from the HLB column with an acidic acetone solution. The extracts are evaporated and then solvent exchanged to acetonitrile. Samples are then diluted to final volume with acidified water, filtered, and analyzed by LC/TIS-MS/MS.

The method was validated on two soil types (sandy and loam) from the United States, Florida (Reference 6) and New Jersey (Reference 7).

3.0 MATERIALS

Equivalent equipment and materials may be substituted unless otherwise specified; note any specifications in the following descriptions before making substitutions. Substitutions should only be made if equivalency/suitability has been verified with acceptable control and fortification recovery data.

3.1 Equipment

Balances

Mettler, Model XP205DR, for weighing solid standards (Columbus, OH)
Mettler, Model BB2440, for weighing soil samples (Columbus, OH)

Centrifuge

Beckman Coulter (Brea, CA)

Sample Collection

Volumetric glassware and pipettes, available from Fisher Scientific (Fairlawn, NJ)
Beakers and measuring cylinders, available from Fisher Scientific (Fairlawn, NJ)
Falcon™ 50-mL disposable centrifuge tubes, Cat. No. 14-959-49A, Fisher Scientific (Fairlawn, NJ)
Falcon™ 15-mL centrifuge tubes, Cat. No. 14-959-70C, Fisher Scientific (Fairlawn, NJ)

Extractor

SPEX Sample Prep 2010, aka Geno/grinder® (SPEX Sample Prep, LLC, Metuchen, NJ)

¼" steel balls (Ballistic Products, Hamel, MN)

Solid Phase Extraction Visiprep DL™

SPE vacuum manifold, available from Supelco (Bellefonte, PA)

Solid Phase Extraction Cartridges

Oasis HLB SPE cartridge, 6-mL/500-mg, Cat No. 186000115, Waters Corp. (Milford, MA)

Pipettes

Pasteur pipettes, disposable, available from Fisher Scientific (Fairlawn, NJ)
FisherBrand® Disposable 10 mL Pipettes, Cat. No. 13-678-27F, Fisher Scientific (Fairlawn, NJ)

Gilson Microman or Eppendorf type pipettes and tips, Gilson, Inc. (Middleton, WI)

Nitrogen Evaporator
N-Evap®, Organomation Assoc. (Berlin, MA)

Vortex Mixer
Fisher Scientific (Fairlawn, NJ)

Sonicator
Ultrasonic cleaner, available from Branson Ultrasonics Corp. (Danbury, CT)

Syringes
Disposable, 10 mL, luer-lok™ tip, Cat. No. 14-826-14, Fisher Scientific (Fairlawn, NJ)

Syringe Filters
0.45-µm PTFE, 17 mm diam., Cat. No. 44513-PV, Fisher Scientific (Fairlawn, NJ)

HPLC Vials
Snap-It Vials, C4011-6W or C4011-5, Fisher Scientific (Fairlawn, NJ)
Snap-It caps with Septa, Cat. No. C4011-54, Fisher Scientific (Fairlawn, NJ)

UPLC/MS/MS System:
Waters Acquity Column Manager
Waters Acquity Sample Manager
Waters Acquity Binary Solvent Manager
Waters Sample Organizer (Milford, MA)
Applied Biosystems/Sciex API 5000 MS/MS Detector, Turbo Ion Spray (TIS) and Analyst version 1.5 software; (Forest City, CA)
Waters Acquity UPLC Column, HSS T3, 50 x 2.1 mm, 1.8 µm particle size, Cat. No.186003538, Waters Corp. (Milford, MA)

3.2 Reagents and Standards

Equivalent reagents may be substituted for those listed below. To determine if impurities in substituted reagents interfere with analyses, a "reagent blank" should be prepared using appropriate amounts of the solvents, and using the chromatographic conditions established for the analysis of the samples to be tested prior to or during the method validation trial.

Acetone – HPLC grade (Cat. No. A949-4, Fisher Scientific, Fairlawn, NJ)

Acetonitrile - Optima grade (Cat. No. A996-4, Fisher Scientific, Fairlawn, NJ)

Formic Acid – p.a. 99% (Cat. No. AC27048-0025, Fisher Scientific, Fairlawn, NJ)
or GR ACS, 98% (Cat. No. FX0440-6, EMD Millipore, Darmstadt, Germany)

Methanol - Optima grade (Cat. No. A454-4, Fisher Scientific, Fairlawn, NJ)

Water –HPLC-grade, (Cat. No. W5-4, Fisher Scientific, Fairlawn, NJ)

Reference Standards

DuPont Crop Protection, Newark, DE:
DPX-Q8U80, IN-REG72, IN-QEK31, IN-F4106, IN-A5760, and IN-RYC33

Sigma-Aldrich Production GmbH:
IN-VM862

3.2.1 *Reference Analytical Standards*

ANALYTICAL STANDARD	LOT NO.	PURITY
DPX-Q8U80-046	FE114893-110	99.2%
IN-REG72-002	E117272-58	92.6%
IN-VM862-001	1401767	96.7%
IN-QEK31-006	IE114893-105	98.6%
IN-F4106-005	IE114893-098	97.9%
IN-A5760-003	EI77273-40	98.8%
IN-RYC33-001	E117272-48	99.3%

The reference analytical standards were provided by DuPont Crop Protection, Global Technology Division, E. I. du Pont de Nemours and Company.

3.3 Safety and Health

Each analyst must be acquainted with the potential hazards of the reagents, products and solvents used in this method before commencing laboratory work. All appropriate safety data sheets should be read and followed, and proper personal protective equipment should be used.

4.0 METHODS

4.1 Principle of the Analytical Method

A 7.5-g soil sample is extracted twice with 50:50 acetonitrile:2% formic acid solution by shaking on a Geno/grinder®. After adjusting to a known volume with water, a 7-mL aliquot of the extract is taken and diluted to approximately 22 mL with water. The diluted extract is then passed through an HLB SPE column. The analytes are retained on the HLB SPE column. DPX-Q8U80 and its metabolites are eluted from the HLB column with an acidic acetone solution. The extracts are evaporated and then solvent exchanged to acetonitrile. Samples are then diluted to final volume with acidified water, filtered, and analyzed by LC/TIS-MS/MS.

The confirmatory method for the LC/TIS-MS/MS method was based on detection of a second MS/MS parent-to-daughter ion transition monitored during the validation for each analyte.

Before method validation, post-fortified samples were analyzed with controls for each soil matrix to determine if matrix effect, suppression or enhancement, influenced percent recovery of DPX-Q8U80 and its metabolites. The post-fortified samples, in this study, were extracts of control soil samples that were purified and prepared in the

same manner as the other fortified samples, but fortified with the analytes at the LOQ level just prior to LC/TIS-MS/MS analysis.

4.2 Analytical Procedure

4.2.1 Glassware and Equipment Cleaning Procedures

Due to the potential for contamination resulting from the low detection limit, disposable equipment was used for sample preparation when possible. If glassware is used, care should be taken to minimize the potential for contamination due to insufficient cleaning of the glassware.

4.2.2 Preparation and Stability of Reagent Solutions

2% Formic Acid in Water
Add 20 mL of concentrated formic acid to 980 mL of HPLC-grade water, and mix well. The solution may be stored at room temperature and should be stable for 3 months.

0.1% Formic Acid in Water
Add 0.50 mL of concentrated formic acid to a 500-mL volumetric flask partially filled with HPLC water, and bring to volume with HPLC water; mix well. The solution may be stored at room temperature and should be stable for 6 months.

0.1% Formic Acid in Acetone
Add 0.5 mL of concentrated formic acid to a 500-mL volumetric flask partially filled with acetone, and bring to volume with acetone; mix well. The solution may be stored at room temperature and should be stable for 3 months.

50:50 Acetonitrile:2% Formic Acid (aq) (Extraction Solution)
Combine equal portions of acetonitrile with 2% formic acid (aq); mix well. The solution may be stored at room temperature and should be stable for 6 months.

20% Acetonitrile:Water (SPE Rinse)
Add 60 mL of acetonitrile to 240 mL of HPLC water; mix well. The solution may be stored at room temperature and should be stable for 3 months.

2:8 Acetonitrile:0.1% Formic Acid in Water
Combine 20 mL of acetonitrile with 80 mL of 0.1% formic acid in water; mix well. The solution may be stored at room temperature and should be stable for 3 months.

0.1% Formic Acid in Water (Mobile Phase A)
Measure 20 L HPLC-grade water into a carboy. Add 20.0 mL formic acid (99+% purity). Mix well. The solution may be stored at room temperature and should be stable for 3 months.

0.1% Formic Acid in Methanol (Mobile Phase B)
Measure 20 L methanol into a carboy. Add 20.0 mL formic acid (99+% purity). Mix well. The solution may be stored at room temperature and should be stable for 12 months.

1:1:1 Acetonitrile:Methanol:Water (Needle Rinse Solution)
Add 4000 mL of acetonitrile, methanol, and HPLC-grade water. Mix well. The solution may be stored at room temperature and should be stable for 12 months.

1:1:2 Acetonitrile:Methanol:Water (Needle Rinse Solution)
Add 2000 mL of acetonitrile, methanol, and 4000 mL of HPLC-grade water. Mix well. The solution may be stored at room temperature and should be stable for 12 months.

Note: *The expiration dates of the above listed solvents and reagents may be extended if their suitability has been verified with acceptable control and fortification recovery data.*

4.2.3 *Stock and Intermediate Standards Preparation and Stability*

Individual stock solutions were prepared for each analyte. To a 100-mL volumetric flask, 10.0 mg (adjusted for purity and recorded to the nearest 0.01 mg) of analytical standard were quantitatively transferred with acetonitrile. The solution was sonicated, allowed to come to room temperature, and brought to volume with acetonitrile to make stock standard solutions of approximately 100 µg/mL.

The stock standard solutions are stable for at least six months when stored capped under frozen conditions.

4.2.4 *Fortification Standard Preparation and Stability*

A 5.0-µg/mL mixed fortification solution in acetonitrile was prepared by adding 0.50 mL each of the 100-µg/mL individual DPX-Q8U80, IN-REG72, IN-VM862, IN-QEK31, IN-F4106, IN-A5760, and IN-RYC33 stock standards to a 10-mL volumetric flask. The solution was diluted to the mark with acetonitrile and mixed to homogeneity.

A 0.50-µg/mL mixed fortification standard in acetonitrile was prepared by adding 1.0 mL of the 5.0-µg/mL mixed fortification solution of DPX-Q8U80, IN-REG72, IN-VM862, IN-QEK31, IN-F4106, IN-A5760, and IN-RYC33 to a 10-mL volumetric flask. The solution was diluted to the mark with acetonitrile and mixed to homogeneity.

These solutions are stable for at least six months when stored under frozen conditions.

4.2.5 *Calibration Standard Preparation and Stability*

Calibration standards in 2:8 acetonitrile:0.1% formic acid in water ranging from 0.10 to 5.0 ng/mL were prepared from the fortification mixed standards. All calibration standards were stored in a freezer after preparation. The table below describes how standards were prepared for the validation work presented in this report:

Initial Standard (µg/mL)	Volume of Initial Standard (mL)	Volume of Acetonitrile (mL)	Volume of 0.1% Formic Acid in Water (mL)	Final Concentration (ng/mL)
0.50	1.0	1.0	8.0	50[a]

[a]Intermediate calibration standard (not injected as part of the calibration curve)

Initial Standard (ng/mL)	Volume of Initial Standard (mL)	Volume of 2:8 Acetonitrile:0.1% Formic Acid in Water (mL)	Final Concentration (ng/mL)
50	1.00	9.00	5.0
50	0.50	9.50	2.5
50	0.30	9.70	1.5
50	0.15	9.85	0.75
5.0	0.80	9.20	0.40
5.0	0.40	9.60	0.20
5.0	0.20	9.80	0.10

The calibration standards were stable for at least a week when stored capped in a freezer.

4.2.6 *Source of Samples*

The method was validated on two soil types (sandy and loam) from the United States, Florida (Reference 6) and New Jersey (Reference 7).

4.2.7 *Storage and Preparation of Samples*

Soil samples were received frozen and stored in a freezer set at a temperature below 0°C prior to preparation for analysis. Samples were previously homogenized (References 6 and 7).

4.2.8 *Sample Fortification*

Fifteen microliters of the 0.50- and 5.0-µg/mL mixed (DPX-Q8U80, IN-REG72, IN-VM862, IN-QEK31, IN-F4106, IN-A5760, and IN-RYC33) fortification solutions were fortified directly to 7.5-g soil samples for 1.0- and 10-ppb (µg/kg) fortification levels, respectively.

Sample Identification	Amount (g)	Fortification Solution		Fortification (ppb, µg/kg)
		µg/mL	mL	
LOQ Fort	7.5 ± 0.05	0.50	0.015	1.0
10×LOQ Fort	7.5 ± 0.05	5.0	0.015	10

4.2.9 *Analyte Extraction Procedure*

The following procedures were used to validate the soil methodology.

1. Weigh 7.5 (± 0.05) g of soil sample into a 50-mL centrifuge tube. Fortify, if necessary. Let the sample sit under the fume hood for 15-20 minutes to evaporate the fortification solvent.
2. Add two steel balls (~¼ inch) to the centrifuge tube.
3. Add 20 mL of extraction solution [50:50 acetonitrile:2% formic acid (aq)] to the soil sample. Cap the tube and vortex for 10-15 seconds to mix the solution thoroughly with the soil.
4. Place the sample on a Geno/grinder® set to 1100 shakes per minute. Shake the sample for 3 minutes.
5. Centrifuge the sample for 10 minutes at ~3000 rpm.
6. Decant the extract into a clean 50-mL centrifuge tube.
7. Add 15 mL of extraction solution to the soil pellet. Cap the tube and vortex mix the soil sample until the pellet on the bottom breaks up.
8. Place the sample on a Geno/grinder® set to 1100 shakes per minute. Shake the sample for 3 minutes.
9. Centrifuge the sample for 10 minutes at ~3000 rpm.
10. Decant the extract to the same 50-mL centrifuge tube as in Step 6.
11. Adjust the volume of the extract to 35 mL with HPLC water. Cap the tube and vortex the extract.
12. Transfer a 7-mL aliquot of the sample extract to a clean 50-mL centrifuge tube. Adjust the volume of the extract to approximately 22 mL using HPLC water and mix well.
13. Attach an Oasis HLB (6 mL/500 mg) SPE column to an SPE manifold. Using gravity, condition the SPE with one column volume of acetonitrile followed by one column volume of 0.1% formic acid in water.
14. Using gravity, pass the soil extract through the SPE column. Rinse the sample tube with 5 mL of 20% acetonitrile in water solution. Using gravity, pass this rinse through the SPE tube. After all of the rinse has passed through the SPE, dry the cartridge with full vacuum for at least one minute.
15. Place a clean 15-mL centrifuge tube under the SPE column. Using gravity, elute the analytes using two column volumes (~6 mL each) of 0.1% formic acid in acetone. (A small amount of vacuum may be used to start the flow.) After all of the solution has passed through the SPE, use full vacuum for 10-15 seconds to remove any remaining solution from the SPE.
16. Using an N-evap® with a moderate nitrogen flow and water bath set at 35 ± 5°C, evaporate the eluate until approximately 2 mL is left in the tube. **Do not let the sample to go to dryness!**

17. Add 2 mL of acetonitrile to the sample tube and mix the sample. Using an N-evap® with a moderate nitrogen flow and water bath set at 35 ± 5°C, evaporate the eluate until approximately 1 mL is left in the tube. **Do not let the sample go to dryness!** Adjust the sample volume to 2 mL with acetonitrile.
18. Bring the sample to a 10-mL final volume using 0.1% formic acid in water. Cap and vortex the sample tube. Sonicate the sample for approximately 2 minutes. Vortex the sample again.
19. Filter the sample through a 0.45-µm PTFE syringe filter. Vial and submit for instrumental analysis. Any dilutions should be prepared using 2:8 acetonitrile: 0.1% formic acid in water.
20. Any remaining extracts or final volume may be stored frozen at approximately -20°C for up to one week.

4.3 Instrumentation

4.3.1 Description and Operating Conditions

Method validation data in this study were generated using a Waters Acquity UPLC system coupled to an Applied Biosystems API5000 (a triple quadrupole MS) with a turbo ion spray interface (TIS). The operating conditions are described below.

LC Conditions:

Injection Volume: 20 µL
Column: Acuity HSS T3 1.8 µm, 50mm × 2.1mm
Column Temperature: 40°C
Solvent A: 0.1% Formic acid in Water
Solvent B: 0.1% Formic acid in Methanol

TIME	FLOW RATE (ML/MIN)	%A	%B
0.00	0.700	95.0	5.0
4.00	0.700	10.0	90.0
5.00	0.700	10.0	90.0
5.01	0.700	95.0	5.0
6.00	0.700	95.0	5.0

Approximate Analyte Retention Times:
DPX-Q8U80 = 3.5 min
IN-REG72 = 3.3 min
IN-VM862 = 2.8 min
IN-QEK31 = 2.4 min
IN-F4106 = 1.7 min
IN-A5760 = 1.2 min
IN-RYC33 = 2.5 min

Triple Quadropole MS Conditions:

Interface:	Turbo Ion Spray (TIS)
Mode:	MRM
Resolution Q1:	Unit
Resolution Q3:	Unit
TIS Source:	Negative (DPX-Q8U80, IN-REG72, IN-F4106, and IN-A5760) Positive (IN-VM862, IN-QEK31, and IN-RYC33)

AB SCIEX API-5000 ACQUISITION PARAMETERS (TIS INTERFACE, MRM MODE)															
ANALYTE	Q1 (*M/Z*)	Q3 (*M/Z*)	TYPE	DWELL (MSEC)	CUR (PSI)	GS1 (PSI)	GS2 (PSI)	TEM (°C)	IHE	IS (V)	CAD (PSI)	DP (V)	EP (V)	CE (V)	CXP (V)
DPX-Q8U80	466.1	157.1	Quant	50	25	70	30	500	On	-4500	8	-180	-10	-40	-10
	466.1	142.0	Conf											-60	-15
IN-REG72	452.0	123.2	Quant	50	25	70	30	500	On	-4500	8	-130	-10	-40	-10
	452.0	143.0	Conf											-35	-7
IN-VM862	197.0	141.2	Conf	50	25	70	30	500	On	4500	8	135	10	38	16
	197.0	64.1	Conf											66	10
	197.0	69.0	Conf											80	12
	197.0	75.0	Quant											92	11
IN-QEK31	265.0	157.0	Quant	50	25	70	30	500	On	4500	8	65	10	60	26
	265.0	219.0	Conf											40	15
IN-F4106	220.0	78.0	Quant	50	25	70	30	500	On	-4500	8	-80	-10	-40	-11
	220.0	156.1	Conf											-20	
IN-A5760	206.0	122.1	Quant	50	25	70	30	500	On	-4500	8	-100	-10	-21	-20
	206.0	142.0	Conf												
IN-RYC33	264.0	157.2	Quant	50	25	70	30	500	On	4500	8	100	10	65	10
	264.0	219.3	Conf											45	

4.3.2 *Calibration Procedures*

Use standard mass spectrometer tuning and calibration techniques. If confidence in the mass calibration needs to be established (modern mass spectrometers under digital control generally do not need frequent mass calibration, especially for quantitative modes), use vendor recommended calibrating solution. Optimization tuning of the MS system should be accomplished by infusion of the test analytes. This method used external standards, prepared as described in Section 4.2.5.

Instrument calibration was based on the average response factor (ARF; defined here as analyte concentration/peak area response) obtained for external calibration standards and was evaluated using the Excel® functions AVERAGE, STDEV, and RSD. Two ion transitions were monitored and used for quantitative and confirmatory calculations for each analyte (as shown in Section 4.3.2); instrument calibration was

performed using the most responsive ion transition for each compound. Acceptance criteria for valid quantitation are: (1) a %RSD ≤20% for the calibration standard response factors, and (2) an r^2 value >0.98 for linear regression analysis of the calibration standards.

The calibrated range of instrument response was 0.10 to 5.0 ng/mL. With the 0.10-ng/mL standard, it is necessary that the "limiting analyte" IN-VM862 (least MS responder) shows a signal-to-noise ratio for each of the two transition ions monitored to be ≥ 3. Typically, 7 calibration standards were interspersed with sample extracts for quantitative LC/MS/MS analysis.

4.3.3 *Sample Analysis*

Prior to sample analysis, the system was equilibrated with standard and sample injections. Calibration standard analyses should precede the first sample analysis and follow the last sample analysis. Calibration standard runs were intermixed with the test samples and were analyzed before and after every 1–4 samples in each analytical set.

4.4 Calculations

4.4.1 *Methods*

DPX-Q8U80, IN-REG72, IN-VM862, IN-QEK31, IN-F4106, IN-A5760, and IN-RYC33 residue recoveries for fortified samples are reported to the nearest whole number percentage (%).

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

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

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

$$RF_{avg} = \frac{(RF\,std\,1 + RF\,std\,2 + RF\,std\,3 + \ldots\ldots RF\,std\,N)}{\text{Total Number of Standards Injected}}$$

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

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

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

The percent recovery found was calculated as follows:

$$\%\,\text{Recovery} = \frac{(\text{ppb found} - \text{ppb found in control sample})}{(\text{Fortification level, ppb})} \text{ x } 100\%$$

[redacted]