ANALYTICAL METHOD FOR THE DETERMINATION OF DPX-QGU42 AND METABOLITES IN-Q7D41, AND IN-RAB06 IN SOIL USING LC/MS/MS

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1.0 SUMMARY

The purpose of this study was to develop an analytical method for the detection, quantitative analysis, and confirmation of DPX-QGU42 and metabolites IN-Q7D41 and IN-RAB06 in soil.

DPX-QGU42, IN-Q7D41, and IN-RAB06 were extracted from soil samples using a solution of formic acid, water and acetonitrile. An aliquot of the extract was removed and diluted with 50:50 methanol: water. An aliquot of the diluted extract was transferred to an auto-sampler vial for analysis. DPX-QGU42, IN-Q7D41, and IN-RAB06 were separated from co-extracts by reversed phase liquid chromatography (LC). DPX-QGU42 and IN-Q7D41 were detected by positive ion electrospray mass spectrometry/mass spectrometry (MS/MS) and IN-RAB06 was detected by negative ion electrospray MS/MS. The Limit of Quantitation (LOQ) for each analyte was 1.0 µg/kg (ppb). The Limit of Detection (LOD) was estimated to be 0.3 µg/kg (ppb).

2.0 INTRODUCTION

The structure, CAS name, CAS registry number, and various physical properties of DPX-QGU42 and metabolites IN-Q7D41, and IN-RAB06 can be found in Appendix 1. The method was validated on soil from a site selected for a field soil dissipation study in Germany and France.

DPX-QGU42, IN-Q7D41, and IN-RAB06 were extracted from soil samples using a solution of formic acid, water, and acetonitrile. An aliquot of the extracts were

diluted and analyzed using reversed phase liquid chromatography (LC) and electrospray mass spectrometry/mass spectrometry (MS/MS). The Limit of Quantitation (LOQ) was 1.0 µg/kg (ppb). The Limit of Detection (LOD) was estimated to be 0.3 µg/kg (ppb).

Due to the selective nature of the LC/MS/MS method, a separate confirmation method was not necessary. Confirmation using LC/MS/MS of possible residues were based on the detection and relative ratios of two MS/MS ion fragments. Confirmation criteria and examples are discussed in this report.

3.0 MATERIALS

Equivalent equipment and materials may be substituted unless otherwise specified. Note any specification 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

Instrumentation

LC system, HP1200 with temperature controlled autosampler (Agilent Technologies, Wilmington, DE)

Mass Spectrometer System, API 5000 triple quadrupole mass spectrometer using a Turbo Ion Spray and Analyst version 1.4 software (Applied Biosystems/MDS Sciex, Foster City, CA)

VWR brand Vortex Geni 2 Mixer, Cat. No. 58815-178 (VWR Scientific Co., Bridgeport, NJ)

Biohit Proline Electronic Pipettors, Variable Volume with Tip Ejector, Vanguard, 5.0-100 µL Cat. No. 53495-200, 50-1000 µL Cat. No. 53495-205 and 0.10-5.0 mL Cat. No. 53495-290 (VWR Scientific Co., Bridgeport, NJ)

Chromatographic Supplies

HPLC Column: 2.0 mm i.d. × 15 cm, Phenomenex C8(2) analytical column with 3-µm diameter packing Part # 00F-4248-B0 (Phenomenex, Torrance, CA)

HPLC Vials, Target DP Amber Kit, T/S/T Septa, 100 PK, Part # 5182-0556 (Agilent Technologies, Wilmington, DE)

Low Flow Mixer Assembly, Part# 411-0050 (Analytical Scientific Instruments)

Labware

Pyrex Brand Single Metric Scale Graduated Cylinders, 10-mL and 100-mL capacity, Cat. No. 24709-715 and 24709-748, respectively (VWR Scientific Co., Bridgeport, NJ)

VWR brand Disposable Pasteur Pipettes, Borosilicate Glass, 9 in, Cat. No. 53283-914 equipped with 2 mL, 13 X 32 mm rubber bulbs, Cat. No. 56310-240 (VWR Scientific Co., Bridgeport, NJ)

Centrifuge tubes, Polystyrene 50-mL capacity, Cat. No. 21008-939 (VWR Scientific Co., Bridgeport, NJ)

Centrifuge tubes, Polystyrene 15-mL capacity, Cat. No. 21008-930 (VWR Scientific Co., Bridgeport, NJ)

Miscellaneous

6 Port Electrically Actuated Valve, Valco Instruments Co. Inc., PN 1384 (Alltech, Deerfield, IL)

Carbon Steel Balls, 1/4 inch, Catalog No. 00073254 (MSC Industrial Supply, Melville, NY)

Genogrinder: Spex SamplePrep Model number 2000.

3.2 Reagents and Standards

Equivalent reagents may be substituted for those listed below. To determine if impurities in substituted reagents interfere with analyses, appropriate amounts of the solvents should be taken through the entire method using the chromatographic conditions specified in this report.

Acetonitrile (ACN) - EM Omni Solv®, HPLC-grade acetonitrile, #AX0142-1 (EM Science, Gibbstown, NJ)

Formic Acid - Guaranteed Reagent 98% minimum, #FX0440-5 (EM Science, Gibbstown, NJ)

Methanol - EM Omni Solv®, HPLC-grade methanol, #MX0488-1 (EM Science, Gibbstown, NJ)

Water - EM Omni Solv®, HPLC-grade water, #WX0004-1 (EM Science, Gibbstown, NJ)

DPX-QGU42-028, non-GLP characterized material used, prepared by DuPont Crop Protection, Global Technology Division, E. I. du Pont de Nemours and Company

IN-Q7D41, non-GLP characterized material used, prepared by DuPont Crop Protection, Global Technology Division, E. I. du Pont de Nemours and Company

IN-RAB06, non-GLP characterized material used, prepared by DuPont Crop Protection, Global Technology Division, E. I. du Pont de Nemours and Company

3.3 Safety and Health

No unusually hazardous materials are used in this method. All appropriate material safety data sheets should be read and followed, and proper personal protective equipment used. An MSDS sheet for the analytes is available from DuPont Crop Protection, Global Technology Division, E. I. du Pont de Nemours and Company.

4.0 METHOD

4.1 Principles of the Analytical Method

DPX-QGU42, IN-Q7D41, and IN-RAB06 were extracted from soil samples using a solution of water / acetonitrile. An aliquot of the extracts were diluted and analyzed using reversed phase liquid chromatography (LC) and electrospray mass spectrometry/mass spectrometry (MS/MS).

4.2 Analytical Procedure

4.2.1 Glassware and Equipment

Cleaning

Glassware should be scrubbed with a brush using a laboratory soap solution, rinsed two to five times with tap water, rinsed with distilled or deionized water and finally rinsed with acetone or another suitable solvent and allowed to air dry prior to each use.

4.2.2 Preparation of Solutions

The following solutions should be prepared monthly and stored at room temperature unless stated otherwise:

Mobile Phase A : 0.05 % aqueous formic acid solution - Add 500 µL of formic acid to 990 mL of water and 10 mL of methanol mix the resulting solution to homogeneity.

Mobile Phase B : 0.01 % formic acid solution - Add 100 µL of formic acid to 1000 mL of methanol and mix the resulting solution to homogeneity.

4.2.3 Preparation and Stability of Stock Standard

Use Class A volumetric flasks when preparing standard solutions.

Prepare standard stock solutions by accurately weighing 10 ± 0.01 mg of each analyte into individual 100-mL volumetric flask using an analytical balance. Record the accurate weight of the standard. Dissolve the standards in approximately 50 mL of HPLC-grade acetonitrile. After dissolving, bring the solution to a volume of 100 mL using HPLC-grade acetonitrile and invert the volumetric flask to mix the solution to homogeneity. The standard solutions are stable for approximately 3 months when stored in a freezer at approximately -20°C immediately after each use. The concentration of each analyte in solution is 100 µg/mL.

4.2.4 Preparation and Stability of Intermediate and Fortification Standards

Use Class A volumetric flasks when preparing standard solutions.

Prepare a 1.0-µg/mL DPX-QGU42, IN-Q7D41, and IN-RAB06 intermediate standard in acetonitrile by pipetting 1.00 mL of each 100.0-µg/mL stock standard into a 100-mL volumetric flask. Dilute the standard to approximately 50-mL with acetonitrile and add 1.0-mL of concentrated formic acid. Bring to volume using HPLC-grade acetonitrile and mix to homogeneity.

Prepare a 0.10-µg/mL DPX-QGU42, IN-Q7D41, and IN-RAB06 standard in acetonitrile by pipetting 1.00 mL of the 1.0-µg/mL standard into a 10-mL volumetric flask. Dilute the standard to approximately 5-mL with acetonitrile and add 0.10-mL of concentrated formic acid. Bring to volume using HPLC-grade acetonitrile and mix to homogeneity.

Prepare a 0.010-µg/mL DPX-QGU42, IN-Q7D41, and IN-RAB06 standard in acetonitrile by pipetting 1.00 mL of the 0.10-µg/mL standard into a 10-mL volumetric flask. Dilute the standard to approximately 5-mL with acetonitrile and add 0.10-mL of concentrated formic acid. Bring to volume using HPLC-grade acetonitrile and mix to homogeneity.

Prepare a 0.0010-µg/mL DPX-QGU42, IN-Q7D41, and IN-RAB06 standard in acetonitrile by pipetting 1.00 mL of the 0.010-µg/mL standard into a 10-mL volumetric flask. Dilute the standard to approximately 5-mL with acetonitrile and add 0.10-mL of concentrated formic acid. Bring to volume using HPLC-grade acetonitrile and mix to homogeneity.

Prepare a 0.00010-µg/mL DPX-QGU42, IN-Q7D41, and IN-RAB06 standard in acetonitrile by pipetting 1.00 mL of the 0.0010-µg/mL standard into a 10-mL volumetric flask. Dilute the standard to approximately 5-mL with acetonitrile and add 0.10-mL of concentrated formic acid. Bring to volume using HPLC-grade acetonitrile and mix to homogeneity.

Alternate or additional solutions may be prepared as needed. All standard solutions prepared in acetonitrile or methanol are stable for approximately 3 months if stored in a freezer at approximately -20°C immediately after each use.

4.2.5 *Preparation and Stability of Calibration Standards*

Prepare the calibration standards as showed in the table below. (Alternative or additional standards may be prepared as needed):

Standard Used (µg/mL)	Volume Pippetted (µL)	Volume of acetonitrile added (µL)	Volume of Water added (µL)	Final Concentration (ng/mL)
0.001	150	350	500	0.15
0.001	100	400	500	0.10
0.001	50	450	500	0.05
0.001	20	480	500	0.02
0.0001	100	400	500	0.01
0.0001	40	460	500	0.004

These standard solutions should be freshly prepared with each sample set and stored approximately 4°C prior to use. Each of the calibration standards was vortex mixed for 30 seconds prior to filling the auto-sampler vials.

4.2.6 *Source of Samples*

Soil control samples were obtained from a field test site located in Goch in Germany and Nambsheim in France. The soil characteristics are shown in the following table:

SOIL NAME	COUNTRY	TYPE	% CLAY	% SAND	% SILT	pH_w	OM (%)	NOTEBOOK
Nambsheim	France	Clay Loam	27.6	22.0	50.4	7.9	2.5	2006-088
Goch	Germany	Silt Loam	8.4	29.2	62.4	6.3	2.4	2004-042A

4.2.7 *Storage and Preparation of Samples*

Soil samples should be stored frozen at approximately -20°C until use. The soil core was divided into segments based on depth. For method development purposes only the 0-5 cm cores were selected. Both cores were mixed by hand prior to analysis.

4.2.8 *Sample Fortification Procedure*

All fortifications were made directly to the 5.0-g soil sample after weighing the sample. Fortified samples were prepared using a 0.10-µg/mL standard solution.

FORTIFICATION LEVEL (µG/KG)	VOLUME OF STANDARD (ML)
1.00	0.050
10.0	0.500

4.2.9 *Analyte Extraction and Purification Procedures*

1. Accurately measure 5.0-g (± 1%) of soil into a 50-mL plastic centrifuge tubes. Fortify samples if necessary and allow the fortification to dry in a fume hood for approximately 15-minutes. Cap and shake the samples vigorously.
2. Add 2-mL of water to each sample and let the sample soak for approximately 5-minutes. Add three 1/4" steel balls, 15-mL of acetonitrile and 0.5-mL of formic acid to each sample.
3. Place samples on a genogrinder and homogenize for 2 minutes at a rate of approximately 1200 strokes per minute.
4. Centrifuge the samples for 5 minutes to drive the particulates to the bottom of the tube at a rate of approximately 3000 RPM.
5. Transfer the supernatants into a clean 50-mL centrifuge tubes. Repeat steps 2-4 using 15-mL of acetonitrile and 0.5-mL of formic acid. Do not add water for the second extraction. Combining the two extracts into the same 50-mL centrifuge tube.

6. Repeat steps 2-4 using 15-mL of acetonitrile and 0.5-mL of formic acid a third time. Do not add water for the third extraction. Combining the three extracts and adjust the volume of the extracts from each sample to 50-mL using acetonitrile. Mix the extract using a vortex mixer for approximately 30 seconds.
7. Pipette 1.0-mL of each extract into a clean 14-mL centrifuge tubes and dilute to 12-mL using 50:50 acetonitrile / water. Mix the extract using a vortex mixer for approximately 30 seconds. Transfer an aliquot of each extract into an auto-sampler vial for LC/MS/MS analysis.

Extracts will be stable for approximately 48 hours if stored at 20°C.

4.3 Instrumentation for the Method

4.3.1 Chromatography

Reversed-phase chromatography was used to separate DPX-QGU42 and metabolites from co-extracts. A Phenomenex C8(2) column was selected. The column choice reflected experimental results indicating preferred separation from co-extractants. Alternative chromatographic conditions can be used, provided the analytical method is validated and provides acceptable recoveries as defined by regulatory method guidelines.

For this method the HPLC is operating at a flow rate of 0.45 mL/min. To accommodate the low flow rate the solvent mixing chamber (Agilent part no. G1312-87330) is replaced with a low flow mixer assembly from Analytical Scientific Instruments (ASI part no. 411-0050). This reduces the volume of the mixing chamber from 450 to 50 microliters.

SYSTEM:	Agilent 1200 HPLC			
COLUMN:	2.0 mm i.d. × 15 cm, 3.5 μm Phenomenex Aqua C8(2)			
COLUMN TEMPERATURE:	45°C			
SAMPLE TEMPERATURE	20°C			
INJECTION VOLUME:	0.050 mL			
FLOW RATE:	0.450 mL/min			
CONDITIONS:	A: 0.05 % aqueous Formic Acid B: 0.01 % Formic Acid in Methanol			
	Time	%A	%B	Flow (mL/Min.)
	0.0	40	60	0.45
	6.5	35	65	0.45
	9.0	35	65	0.45
	10.0	1	99	0.45
	12.0	1	99	0.45
	13.0	40	60	0.45
	18.0	40	60	0.45
DPX-QGU42 RETENTION TIME:	5.1 minutes			
IN-RAB06 RETENTION TIME:	6.8 minutes			
IN-Q7D41 RETENTION TIME:	8.7 minutes			
TOTAL RUN TIME:	18.0 minutes			

A six-port electronically activated switching valve was used to direct the flow to waste prior to and following the elution of the compounds of interest. The use of this valve reduces source contamination and enables additional samples to be analyzed prior to source cleaning. The valve switching times are given in the following table.

TIME (MINUTES)	COLUMN ELUATE FLOW
0.00-3.0	Waste
3.0-10.0	MS source
11.0-End	Waste

4.3.2 *LC/MS/MS Analysis*

The quantitative analysis of DPX-QGU42 and metabolites was performed using an Applied Biosystem API 5000 LC/MS/MS system. Quantitative analysis was based on the integration of a single ion transition. The system parameters were adjusted while a solution of each analyte was infused directly into the ion source. The solution composition was 85% methanol/15% water, so that it would approximate the composition of the mobile phase at the retention time of the analyte. The solution concentration was approximately 2 μg/mL. A summary of the experimental conditions is provided in the following table:

Period 1Analytes	Ions Monitored	Declustering Potential (DP)	Collision Energy (CE)	Exit Potential (CXP)
DPX-QGU42	**540.1→ 499.9 AMU**	196	37	34
	540.1→ 522.0 AMU	196	37	34
Time:	0-5.4 minutes			
Ion Mode:	Positive			
Turbo spray Voltage:	5500 V			
Source Temperatures:	600 C			
CUR:	30			
CAD:	4			
GS1:	40			
GS2:	50			
Dwell	0.30 Seconds			

Period 2Analyte	Ions Monitored	Declustering Potential (DP)	Collision Energy (CE)	Dwell (Seconds)
IN-RAB06	**568.0→ 523.9 AMU**	-70	-22	-25
	568.0→ 409.8 AMU	-70	-22	-25
Time:	5.4 – 6.5 minutes			
Ion Mode:	Negative			
Turbo spray Voltage:	-4500 V			
Source Temperatures:	600 C			
CUR:	30			
CAD:	4			
GS1:	40			
GS2:	50			
Dwell	0.30 Seconds			

Period 3Analytes	Ions Monitored	Declustering Potential (DP)	Collision Energy (CE)	Exit Potential (CXP)
IN-Q7D41	**538.1→ 498.0 AMU**	196	33	20
	538.1→ 141.0 AMU	196	59	24
Time:	6.5-14 minutes			
Ion Mode:	Positive			
Turbo spray Voltage:	5500 V			
Source Temperatures:	600 C			
CUR:	30			
CAD:	4			
GS1:	40			
GS2:	50			
Dwell	0.30 Seconds			

A complete list of the experimental parameters is given in Appendix 4. A typical LC/MS and LC/MS/MS full scan spectrum of each analyte is shown in Figure 1 and Figure 2, respectively.

The instrument was operated in MS/MS-(MRM) positive and negative ion modes for quantitative analysis. Peak area was used for quantitation. **Quantitation was performed using the ion transition displayed in bold face print.** The relative ratio of the fragment ions was evaluated to confirm the presence of an analyte in an unknown sample.

4.3.3 *Calibration Procedure and Sample Analysis*

A 0.004-ng/mL chromatographic standard should be analyzed prior to the start of analyses to establish that the instrument is working properly. If a signal-to-noise ratio of approximately 5-10 to 1 is not attained, the instrument must be tuned or cleaned prior to sample analysis. Operating parameters must be tailored to the particular instrument used, especially if it is to be an alternate vendor's instrument, and should be checked daily. Note that some ion channels other than those used for development of this method may need to be added or eliminated when utilizing this method on other instrumentation. Each ion channel used for sample analysis/quantitation must be checked to insure it is free of interference. The control will be used to demonstrate that baseline interference is less than signal-to-noise 3:1. Begin each sample set by injecting a minimum of 2 calibration standards. The first injection should always be disregarded.

4.4 Calculations

4.4.1 *Methods*

Average Response Factor (RF_{Avg}) was calculated as follows:

$$RF_{Ave} = \frac{(\text{Conc. A} \div \text{A Corrected Area A}) + (\text{Conc. B} \div \text{Corrected Area B}) + (\text{Conc. C} \div \text{Corrected Area C}) + (\text{Conc. D} \div \text{Corrected Area D})}{\text{Total Number of Standards Injected}}$$

Corrected Area = (Area in the standard – Area on the control)

ng/g (ppb) found was calculated as follows:

$$\text{ng/g Found} = \frac{(\text{Peak Area}) \times (RF_{Ave}) \times (\text{Final Volume}) \times (\text{Aliquot Factor})}{(\text{grams of Sample})}$$

In the event a peak was detected in the control, a corrected peak area was used to calculate ppb found for 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:

$$\text{\% Recovery} = \frac{(\text{ng/g Found})}{(\text{ng/g Fortified})} \text{ x } 100$$

4.4.2 *Example*

For a soil sample fortified with DPX-QGU42 at 1.0 ppb) [Date analyzed 13-May-09, 1.0 ppb Fortification (a)], the concentration found was calculated as follows:

Average Response Factor was calculated as follows:

$$RF_{Ave} = \frac{(0.0040 ng/mL \div 2780) + (0.010 ng/mL \div 7280) + (0.020 ng/mL \div 10200) + (0.050 ng/mL \div 23500) + (0.100 ng/mL \div 47300) + (0.150 ng/mL \div 69000)}{6}$$

(AC ≡ Area Counts)

$RF_{Avg} = 1.86483^{-6}$ ng/mL/AC

ng/g (ppb) found was calculated as follows:

$$\text{ng/g Found} = \frac{(3980\ AC) \times (1.86483e-6\ ng/mL/AC) \times (12.0\ mL) \times (50)}{(5\ grams)}$$

ng/g Found = 0.891

(ppb values are reported to two significant figures in Table 1 of this report. Rounding was performed using the Microsoft Excel version 7.0 for Windows 95 rounding function)

The percent recovery found was calculated as follows:

$$\%\ \text{Recovery} = \frac{(0.891\ ng/g)}{(1.00\ ng/g)} \text{ x } 100$$

% Recovery = 89.1%

(percent recoveries are rounded to the nearest whole number in Table 1, without rounding the concentration or ppb found)

5.3 Modifications or Special Precautions

Due to the poor solubility of DPX-QGU42 and IN-RAB06 in aqueous solutions it is important to prepare the calibration standards in 50:50 acetonitrile: water. Also to prevent the standards from dropping out of solution during the analysis the auto-sampler tray should be maintained at 20°C.

The column selected for the analysis generated very high pressure when operated with a flow rate of 0.45 ml/minute. In order to operate the column at this flow rate the column oven should be heated to 45°C prior to turning on the instrument pump. When operated at room temperature the system may exceed the maximum allowed pressure.

6.0 CONFIRMATION OF DETECTED RESIDUES

6.1 Method

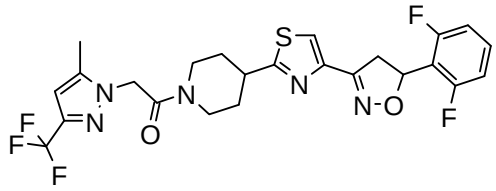
The confirmation method is based on evaluating the ion ratios collected during method validation. During the quantitative analysis of possible residues, two ion transitions were monitored. The ion ratio from the transitions monitored was used to establish criteria against which possibly detected residues are compared. The ratio of the ion intensity (area) of (A→B/A→C) was used to positively confirm the identity of an unknown compound. Since the ions detected originate by collision-induced fragmentation in an MS/MS system, the absolute intensity is dependent on gas cell pressure, gas cell size, storage time, system geometry, and other instrument specific parameters. Therefore, the ratio is expected to vary from day to day and when

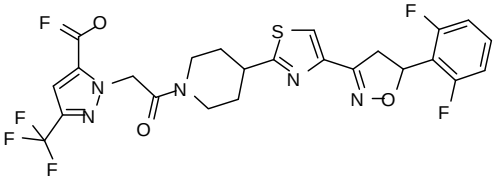
different vendor's instrumentation is used. For every sample set, the ion ratio data must be calculated based on the calibration standards and compared to actual sample data.

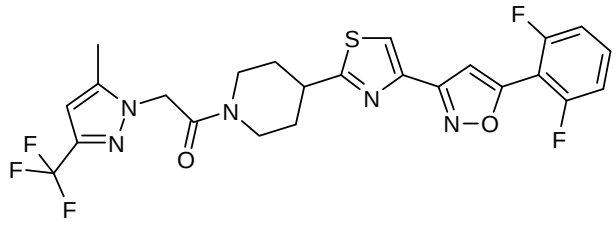
6.2 Confirmation Criteria

In order for a sample set to be valid, the relative standard deviation of the ion ratios calculated from the calibration standards analyzed must be less than 20%. For the confirmation of possible DPX-QGU42 and metabolite residues in a soil sample, the ion ratio must fall within ±30% of the average ratio for all calibration standards for a specific sample set. If the ion ratio is outside the ±30% range, the signal was most likely generated from a compound that is unrelated to DPX-QGU42. The unknown compound also has the same ion by LC/MS and a similar fragmentation pattern. In addition to meeting the defined ion ratio criteria, the elution time of the compound of interest must fall within 2% of the elution time of the standards analyzed for that sample set.

APPENDIX 1 STRUCTURE AND PROPERTIES OF DPX-QGU42 AND METABOLITES

Common Name	DPX-QGU42
Structure	
DPX Number	DPX-QGU42
Formula	$C_{24}H_{22}F_5N_5O_2S$
Molecular Weight	539.53
Monoisotopic Weight	539.14

Structure	
DPX Number	IN-RAB06
Formula	$C_{24}H_{20}F_5N_5O_4S$
Molecular Weight	569.51
Monoisotopic Weight	569.12

Common Name	None
Structure	
DPX Number	IN-Q7D41
Formula	$C_{24}H_{22}F_5N_5O_2S$
Molecular Weight	537.51
Monoisotopic Weight	537.13