INvariety of crops. The purpose of the current study is to develop a method for DPX-HGW86 and its environmentally significant metabolites in soil with a target limit of quantitation (LOQ) of 1.0 ppb. The method is intended to meet SANCO/825/00 rev.7 and EPA Guidance OPPTS. 850.7100 (Reference 1 and Reference 2).

The method consists of extraction with an acidic acetone solution followed by SPE cleanup with amine(NH2) and styrene-divinylbenzene(ENV) phases. The acidic acetone solution is the same extraction solvent system used in the aerobic soil metabolism study, DuPont-15775 (Reference 3). An additional extraction efficiency study, DuPont-17804 (Reference 4), was performed which demonstrated that the extraction procedure used in the current study was equivalent to the aerobic soil metabolism study.

The structures of DPX-HGW86 and its soil metabolites IN-K7H19, IN-JCZ38, IN-JSE76, IN-K5A77, IN-K5A78, IN-K5A79, IN-J9Z38, and IN-PLT97 can be found in Appendix 1.

## 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*

| EQUIPMENT DESCRIPTION | PRODUCT ID | SUPPLIER |
|---|---|---|
| Refrigerator | 6FAR | Marvel Industries, Inc. (Richmond, IN) |
| Analytical Balance | AG 104 Analytical Balance; PM600 top-loading balance | Mettler Instrument Corp. (Hightstown, NJ) |
| Analytical Evaporator | N-Evap® Model 111 with stainless steel luer fit needles with water bath | Organomation Assoc. (South Berlin, MA) |
| Sonication | 2200 Ultrasonic cleaner, 0.75-gal. capacity | Branson Ultrasonics Corp. (Danbury, CT) |
| Vortex Mixer | Vortex-2 Genie® | VWR, Inc. (West Chester, PA) |
| Filtration | Gelman Acrodisc® 13 CR, 0.2-µm PTFE 13-mm dia. membrane syringe filter, Cat. No. 4423 | VWR (Bridgeport, NJ) |
| Solid Phase Extraction | Visiprep DL™ SPE manifold, Cat. No. 5-7030M; | Supelco (Bellefonte, PA) |
| Solid Phase Extraction | Bond Elut™ NH2 SPE cartridge, 6 cc/0.5 gm, Cat. No. 12256045, Bond Elut™ ENV SPE cartridge, 6 cc/0.5 gm, Cat. No. 12255011; 60-mL plastic reservoirs, Cat. No. 12131012; union adapter for 6 mL columns, Cat. No. 12131001 | Varian, Inc. (Palo Alto, CA) |
| Wrist Action Shaker | Model 75 | Burrell Scientific (Pittsburg, PA) |
| Bench Top Centrifuge | Sorvall® Centrifuge, Model GLC-2B | Sorvall Instruments (Wilmington, DE) |
| Labware | Pyrex® Brand single metric scale graduated cylinders, 10-mL and 100-mL capacity, Cat. No. 24709-715 and 24709-748, respectively; Corning® 430291 (50 mL) disposable centrifuge tube 15-mL centrifuge tube, Cat. No. 352097 60-mL glass centrifuge tube Cat. No. 73785-50. | VWR (Bridgeport, NJ) |
| Labware | Biohit Proline Electronic pipettors, variable volume. 50-1000 µL, Cat. No. 53495-205; 0.1-5.0 mL, Cat. No. 53495-290. | VWR (Bridgeport, NJ) |
| Labware | 3-mL disposable syringe, Cat. No. BD309585 | Becton Dickinson (Franklin Lakes, NJ) |

**HPLC/MS System**

| | | |
|---|---|---|
| HPLC | HP1100: G1322A degasser, G1312A binary pump, G1311A quaternary pump; G1313A autosampler; G1316A column unit; G1314A variable wavelength detector | Agilent Technologies, Inc. (Palo Alto, CA) |
| Autosampler Vials | Target DP Amber Kit, T/S/T Septa, 100 PK, Cat. No. 5182-0556 | Agilent Technologies, Inc. (Palo Alto, CA) |
| HPLC Column | Luna® C8 (2); 2 mm × 150 mm, 5 µm particle size diameter 00F-4040-B0 | Phenomenex® (Torrance, CA) |
| Switching Valve | Valco 6-Port electrically actuated valve, Cat. No. 1384 | Valco Instruments, Inc. (Houston, TX) |
| Triple Quadrupole MS | API 5000 triple quadrupole mass spectrometer using an electrospray (ESI) and Analyst version 1.4 software | Applied Biosystems/MDS Sciex (Foster City, CA) |

## *3.2 Reagents and Standards*

### *3.2.1 Reagents*

The equivalency/suitability of substituted reagents should be verified.

| Reagents | Product Description | Product ID | Supplier |
|---|---|---|---|
| Formic Acid | GR, ACS, 98% | FX0440-11 | EM Science (Gibbstown, NJ) |
| Methanol | OmniSolv®, 4L | MX0488-1 | EM Science (Gibbstown, NJ) |
| Acetone | OmniSolv®, 4L | AX0116-1 | EM Science (Gibbstown, NJ) |
| Hexanes | OmniSolv®, 4L | HX0296-1 | EM Science (Gibbstown, NJ) |
| Acetonitrile | OmniSolv®, 4L | AX0142-1 | EM Science (Gibbstown, NJ) |
| Ammonium Hydoxide | GR (28-30%) | AX1303-13 | EM Science (Gibbstown, NJ) |
| Water | HLPC grade | NA | NA |
| Ammonium Formate | GR (96%) | M53-08 | J.T. Baker (Phillipsburg, NJ) |
| Methyl Sulfoxide | ACS Spectroscopic, 99.9% | 15,493-8 | Aldrich (Milwaukee, WI) |

### *3.2.2 Reference Analytical Standards*

Reference analytical standards of the following standards:

| Compound | Lot No. | Purity(%) |
|---|---|---|
| DPX-HGW86 | 209 | 98.4 |
| IN-K7H19 | 000 | 72.0 |
| IN-JCZ38 | 004 | 92.1 |
| IN-K5A77 | 001 | 95.3 |
| IN-J9Z38 | 002 | 96.4 |
| IN-K5A79 | 000 | 86.5 |
| IN-JSE76 | 001 | 89.9 |
| IN-PLT97 | 001 | 82.5 |
| IN-K5A78 | 001 | 94.9 |

The standards were synthesized at E. I. du Pont de Nemours and Company, DuPont Agricultural Products, Wilmington, DE. Characterization data are archived by DuPont Agricultural Products, E. I. du Pont de Nemours and Company, Wilmington, DE.

## *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 should be used.

# 4.0 METHODS

## *4.1 Principle of the Analytical Method*

A 10 g soil sample is extracted twice with 25 mL of a 90/10 mixture of acetone/1.0N formic acid solution by shaking at high speed on a wrist action shaker. A 10 mL aliquot is taken and diluted to 50 mL with water in the first step of the clean up procedure. The diluted extract is then passed through NH2 and ENV SPE columns. The NH2 removes co-extracts but does not retain DPX-HGW86 or its metabolites. The ENV SPE column retains the analytes. DPX-HGW86 and its metabolites are eluted from the ENV column with a basic acetonitrile solution. The samples are evaporated to dryness and brought up in mobile phase, filtered, and analyzed by LC/MS/MS.

## *4.2 Analytical Procedure*

### *4.2.1 Glassware & Equipment Cleaning Procedures*

The effectiveness of any cleaning procedure used should be demonstrated by preparation and analysis of reagent blanks. In general, all reusable glass- and plasticware should be washed in hot tap water with laboratory grade, non-phosphate detergent, rinsed several times with tap water, rinsed several times with deionized water, rinsed once with acetone, and allowed to fully dry before use. Care should be taken to avoid working with high levels of the analyte being monitored in the same laboratory where samples are being extracted and analyzed.

### *4.2.2 Preparation & Stability of Reagent Solutions*

The following procedures may be adjusted to prepare different volumes.

***1M Formic Acid***

Add 45 mL concentrated formic acid (98%) and dilute to 1.0 L with de-ionized water. The solution may be stored at room temperature and should be stable for 6 months.

***Extraction Solution****, 90% acetone/ 10% aqueous 1.0 M formic acid*

Add 100 mL aqueous 1.0 M formic acid to 900 mL of Acetone. The solution may be stored at room temperature and should be prepared at least monthly.

***1M Ammonium Hydroxide***

Add 7 mL of ammonium hydroxide (28-30%) to 93 mL of de-ionized water. The solution may be stored at room temperature and should be prepared every 6 months.

***0.02 M Formic Acid***

Add 2 mL of 1M formic acid and dilute to 100 mL with de-ionized water. The solution may be stored at room temperature and should be stable for 1 week.

***1 M Ammonium Formate***

Add 6.3 g of ammonium formate and dilute to 100 mL with de-ionized water. The solution may be stored at room temperature and should be stable for 6 months.

***0.1 M Ammonium Formate***

Add 10 mL 1M ammonium formate and dilute to 100 mL with de-ionized water. The solution may be stored at room temperature and should be stable for 1 month.

*4.2.3* *Stock Standard Preparation and Stability*

Individual stock solutions are required for each analyte. Use an analytical balance that provides a weight precision to three significant figures. To prepare a stock solution of 100 µg/mL, weigh approximately 10.0 mg (adjusted for purity) of the analyte in a tared 100-mL volumetric flask. Add approximately 50 mL of acetonitrile sonicate to dissolve. If analyte does not go into solution add DMSO in 10 mL increments sonicate after each addition. **IN-PLT97** may need up to 30 ml of DMSO. IN-JCZ38 and IN-K5A79 may need smaller amounts of DMSO. Once analyte dissolves, dilute to a total volume of 100 mL with acetonitrile. These solutions are stored in a refrigerator at approximately 4°C and are stable for at least six months. Stock standards use may be extended if supported by stability test data.

*4.2.4* *Fortification Standard Preparation and Stability*

The following standard preparation procedures are examples and may be adjusted to prepare different volumes.

Prepare fortification solutions from dilutions of the individual stock solutions. Prepare 1.0-µg/mL, 0.10-µg/mL, and 0.010-µg/mL fortification solutions for sample fortification at the 10 × LOQ and LOQ, respectively. Alternative concentrations may be prepared as needed for other fortification levels. Store fortification solutions at or below 4°C and replace monthly.

*1.0 µg/mL Fortification Solution*

Dilute 1.0 mL of the stock solution for each analyte into a common 100-mL volumetric flask and fill to line with acetonitrile, cap and mix well.

*0.10 µg/mL Fortification Solution*

Dilute 10.0 mL of the 1.0-µg/mL fortification standard into a 100-mL volumetric flask and fill to line with acetonitrile, cap and mix well.

*0.010 µg/mL Fortification Solution*

Dilute 10.0 mL of the 0.10-µg/mL fortification standard into a 100-mL volumetric flask and fill to line with acetonitrile, cap and mix well.

*4.2.5* *Calibration Standard Preparation and Stability*

LC Calibration standards are prepared from dilutions of fortification standards. Five or more calibration standards are recommended. The LOQ equivalent final concentration for the each analyte is 1.0 ng/mL. Keep calibration standards refrigerated and they should be stable for up to a week.

Calibration standards can be prepared by pipetting the following volumes directly into a LC vial according to the following table (alternative or additional standards may be prepared as needed). Prepare weekly.

| Initial Standard (µg/mL) | Volume of Initial Standard (mL) | Volume of Methanol (mL) | Volume of 0.02 M Formic Acid (mL) | Final Concentration (ng/mL) |
|---|---|---|---|---|
| 0.10 | 0.200 | 0.300 | 0.50 | 20.0 |
| 0.10 | 0.100 | 0.400 | 0.50 | 10.0 |
| 0.10 | 0.050 | 0.450 | 0.50 | 5.0 |
| 0.010 | 0.200 | 0.300 | 0.50 | 2.0 |
| 0.010 | 0.100 | 0.400 | 0.50 | 1.0 |
| 0.010 | 0.050 | 0.450 | 0.50 | 0.50 |

*4.2.6* *Source (& Characterization) of Samples*

Five different soils were chosen for analysis covering a range of soil types and locations. Soils were obtained from DuPont Discovery Soil Bank at the Stine-Haskell Research Center. The classification and characteristics of each soil is listed below:

| Soil Type Lot No. (Location) | Classification | Sand (%) | Silt (%) | Clay (%) | pH | OM (%) |
|---|---|---|---|---|---|---|
| Drummer-2004-073 (Rochelle, IL) | Silty Clay Loam | 19.2 | 48.0 | 32.8 | 5.4 | 6.2 |
| Hildalgo-2005-025 (Madera, CA) | Sandy Loam | 50 | 24 | 26 | 8.0 | 1.3 |
| Myakka-2004-16 (Bradenton, FL) | Sand | 94 | 2 | 4 | 5.6 | 1.0 |
| Nambsheim-2005-23 (Nambsheim, France) | Sandy Loam | 52 | 38 | 10 | 7.3 | 1.8 |
| Sassafras-2004-72 (Chesapeake Farms, MD) | Sandy Loam | 55 | 37 | 8 | 5.2 | 2.0 |

*4.2.7* *Storage & Preparation of Samples*

In order to obtain a homogeneous representative sample the entire soil sample should be homogenized using a Hobart processor (or equivalent). Dry ice should be added to keep sample frozen during processing.

*4.2.8* *Sample Fortification Procedure*

All fortifications were made directly to the soil in the centrifuge tubes after weighing the sample. 10-gram samples were fortified with the 1.0-µg/mL multi-analyte fortification standard solution or the 0.10-µg/mL multi-analyte fortification standard solution.

| Sample Identification | Amount (g) | Fortification Solution | | Fortification (ng/g) |
|---|---|---|---|---|
| | | µg/mL | mL | |
| LOQ Fort | 10.0±0.05 | 0.10 | 0.10 | 1.0 |
| 10×LOQ Fort | 10.0±0.05 | 1.0 | 0.10 | 10.0 |

*4.2.9* *Analyte Extraction Procedure*

1. Extract 10-gram soil sample in 50-mL centrifuge tube with 25 mL of the soil extraction solution on a wrist action shaker at high speed for 15 minutes (90% Acetone and 10% 1.0 M Formic Acid).
2. Centrifuge sample for 5 minutes at approximately 3000 RPM in a bench top centrifuge. Decant supernadent into a 50 mL centrifuge tube.
3. Repeat Steps 1 and 2 on a shaker for and combine extracts in a 50-mL centrifuge tube (bring volume to total of 50 mL with extraction solution, mix).

*4.2.10* *Analyte Purification Procedure*

1. To a 10-mL aliquot of the extract from Step 3 of the extraction procedure, add 40 mL of water and mix thoroughly.
2. Condition a (6-cc/500-mg) NH2 Cartridge with 5 mL of methanol followed by 5 mL of water. Attach a 60-mL reservoir to the NH2 Cartridge; add 5 mL of water to it also. Add the sample before reservoir drains completely.
3. Collect eluent in a 60-mL centrifuge tube. (Use vacuum if needed to achieve a flow of 1-2 drops per second). After the sample has gone through the NH2 cartridge, wash with 8 mL of 10% acetonitrile in water. The sample is now ready for the ENV SPE clean up.
4. Condition a (6-cc/500-mg) ENV cartridge for clean-up using 5 mL of methanol followed by 5 mL of acidic water (100µL of 1 M Formic acid in 100 mL water). Attach a 60 mL reservoir; and add an additional 5mL of acidic water.

5. Pass the sample from Step 3 through the ENV cartridge using gravity flow. After the sample is through the column, discard the 60-mL reservoir and wash the ENV cartridge with 5 mL of water. Apply vacuum for approximately 20-30 seconds to remove any excess solution. Analytes are retained on the ENV cartridge.
6. Elute cartridges into a 60 mL glass centrifugetube with 3 x 5 mL of basic acetonitrile (2 mL of 1 M ammonium hydroxide in 100 mL of acetonitrile).
7. Evaporate to dryness on an N-Evap at approximately 40°C. Add 1.0 mL of methanol for 2 minutes. Homogenize the sample for 20 seconds using a vortex mixer, and then sonicate.
8. Add 1.0 mL of aqueous mobile phase. Homogenize the sample for 20 seconds using a vortex mixer, and then sonicate for 2 minutes. Instrument sensitivity may vary, the final volume may be adjusted to so that a signal to noise ratio of approximately 10 is achieved for the least sensitive analyte.
9. Filter sample with a 3-mL syringe and a -, 0.2-µm PTFE filter into an HPLC vial. Samples are ready for LC/MS/MS analysis.

## *4.3 Instrumentation*

### *4.3.1 Analysis of DPX-HGW86 and Metabolites*

This method uses a gradient-elution, reversed-phase LC analysis on a C-8 column. The column choice reflects experimental results indicating optimum chromatographic separation from co-extractants. Alternative chromatographic conditions can be used provided the analytical method is validated and acceptable recoveries are obtained.

### *4.3.2 HPLC Operating Conditions*

The LC is operating at a flow rate of 0.25 mL/min. To accommodate the low flow rate the solvent mixing chamber (Agilent part no. G1312-87330) is replaced with two in-line solvent filters (Upchurch Part No.A-314) connected using a short piece of 0.005 inch inner diameter tubing (this serves as the low flow solvent mixer). Use 0.005 inch id stainless steel or PEEK tubing to make all connections from the pump outlet to the autosampler to the column oven to the MS detector (keep all tubing lengths as short as possible for all connections).

| | |
|---|---|
| System: | Agilent HP1100 HPLC |
| Column: | 2.0 mm i.d. × 15 cm, Phenomenex C-8(2) analytical column with 5-µm diameter packing. |
| Column Temperature: | 40°C |
| Injection Volume: | 5.0 µL |
| Autosampler Temperature: | 4°C |
| Flow Rate: | 0.25 mL/min |
| Conditions: | Time %A %B Flow A: Aqueous.<br>0.0 50 50 0.25 100 µL of 1M Formic Acid<br>15.0 13 87 0.25 100 µL of 0.1 mM NH4 formate<br>15.1 1 99 0.25<br>19.0 1 99 0.25 B: Methanol<br>19.1 50 50 0.25 100 µL of 1M Formic Acid<br>30.0 50 50 0.25 |
| Approximate Retention Times | (minutes) |
| IN-K7H19 | 3.4 |
| IN-JCZ38 | 4.1 |
| DPX-HGW86 | 6.0 |
| IN-K5A77 | 6.6 |
| IN-J9Z38 | 9.9 |
| IN-K5A79 | 11.6 |
| IN-JSE76 | 12.6 |
| IN-PLT97 | 13.7 |
| IN-K5A78 | 14.4 |
| Total Run Time: | 30.0 minutes |

***Triple Quadrupole MS Operating Conditions***

| | |
|---|---|
| Splitter: | None ( all flow from column goes to source) |
| Interface: | Turbospray |
| Mode: | MRM |
| Resolution: | Unit |
| ESI Source Voltage: | 4.5 kV (positive) |
| APCI Source Temperature: | 550 °C |
| Divert Valve: | 0.0–3.3 min to waste |
| | 3.3–16.0 min to source |
| | 16.0–30.0 min to waste |

| AB Sciex API-4000 Acquisition Parameters (ESI interface, MRM mode) | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Period (min) | Analyte | Polarity (+/-) | Q1 (m/z) | Q3 (m/z) | Dwell (msecs) | CUR (psi) | GS1 (psi) | GS2 (psi) | TEM (°C) | ihe | IS (V) | CAD (psi) | DP (V) | EP (V) | CE (V) | CXP (V) |
| 2.0-7.7 | IN-K7H19 | + | 479.00 | 286.00 | 200.00 | 10.00 | 60.00 | 60.00 | 400.00 | on | 4500 | 8.00 | 150.00 | 10.00 | 25.00 | 15.00 |
| | | | 479.00 | 462.00 | | | | | | | | | | | | |
| | IN-JCZ38 | | 493.00 | 286.00 | | | | | | | | | | | 25.00 | |
| | | | 493.00 | 444.00 | | | | | | | | | | | | |
| | DPX-HGW86 | | 475.00 | 286.00 | | | | | | | | | | | 30.00 | |
| | | | 475.00 | 444.00 | | | | | | | | | | | | |
| | IN-K5A77 | | 475.00 | 188.00 | | | | | | | | | | | 50.00 | |
| | | | 475.00 | 299.00 | | | | | | | | | | | | |
| 7.7-15.0 | IN-J9Z38 | + | 457.00 | 188.00 | 200.00 | 10.00 | 60.00 | 60.00 | 400.00 | on | 4500 | 8.00 | 150.00 | 10.00 | 45.00 | 15.00 |
| | | | 457.00 | 299.00 | | | | | | | | | | | | |
| | IN-K5A79 | | 480.00 | 286.00 | | | | | | | | | | | 25.00 | |
| | | | 480.00 | 463.00 | | | | | | | | | | | | |
| | IN-JSE76 | | 494.00 | 286.00 | | | | | | | | | | | 30.00 | |
| | | | 494.00 | 463.00 | | | | | | | | | | | | |
| | IN-PLT97 | | 462.00 | 317.00 | | | | | | | | | | | 51.00 | |
| | | | 462.00 | 426.00 | | | | | | | | | | | 35.00 | |
| | | | 462.00 | 286.00 | | | | | | | | | | | 39.00 | |
| | IN-K5A78 | | 476.00 | 188.00 | | | | | | | | | | | 45.00 | |
| | | | 476.00 | 299.00 | | | | | | | | | | | | |

Mass Spectra shown in Appendix 2 provided the data for the transitions described in the above table. Each transition described above was used to quantitate analyte levels in samples as well as for confirmation purposes.

### *4.3.3 Calibration Procedure and Sample Analysis*

A chromatographic standard should be analyzed prior to the start of analyses to establish that the instrument is working properly. 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 it may be necessary to add some ion channels other than those used for development of this method 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. A control will be used to demonstrate that baseline interference is less then signal-to-noise 3:1. Begin each sample set by injecting a minimum of 2 calibration standards. The first injection of a calibration standard should always be disregarded.

## *4.4 Calculations*

### *4.4.1 Methods*

The response factor, RF, for each analytical standard is the ratio of the analyte concentration to the analyte peak area.

$$\text{RFstd} = \frac{\text{Concentration of analyte (µg/mL)}}{\text{Analyte peak area}}$$

The average response factor, $RF_{ave}$, calculated from each standard level analyzed in an analytical set containing control, fortified, or treated samples was used to calculate the concentration of DPX-HGW86 and its metabolites in these samples.

$$RF_{ave} = \frac{(RFstd1 + RFstd2 + RFstd3 + .....RFstdn)}{\text{Total Number of Standards Injected}}$$

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

ng/g analyte Found =

$$\frac{[Peak\ Area(sample - control)\ x\ RFave]\ \ x\ \ [Final\ Vol.(mL)\ x\ mL\ solv\ \ x\ \ Dil\ Fctr]}{Sample\ Wt.(g)\ \ x\ \ Aliquot\ Taken\ (mL)}$$

Where:

| | | |
|---|---|---|
| Total Extract Volume (mL solv) | = | 50 mL |
| Final Extract Volume (Final Vol.) | = | 2 mL |
| Aliquot Taken | = | 10 mL |
| Sample Weight | = | 10 ± 0.10 grams |

The percent recovery found was calculated as follows:

$$\%\ Recovery = \frac{(ng/g\ Found)}{(Fortification\ level, ng/g)}\ x\ 100\%$$

*4.4.2* *Example*

The calculation below shows the concentration of DPX-HGW86 from a sample of Drummer soil fortified at 1.0 ng/g, (LOQ 1) in the first validation set. See chromatogram in Figure 1 and Residue Summary Sheet in Appendix 3 for values to substitute into calculations:

$$RFstd = \frac{0.5\,(ng/ml)}{53300} = 9.381E-6$$

$$RF_{ave} = \frac{(9.381E-6) + (9.009E-6) + (9.302E-6) + (8.432E-6) + (8.403E-6)}{5}$$

$RF_{ave}$ = 8.905E-6

Peak Area HGW86 Fortified Sample: = 106000

Peak Area HGW86 Control Sample: = 0

mL Solvent: = 50 mL

mL Aliquot 1: = 10.0 mL

Final Volume: = 2 mL

HPLC Dil Factor: = 1

Sample Weight: = 10.0 grams

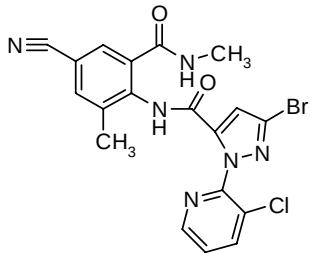
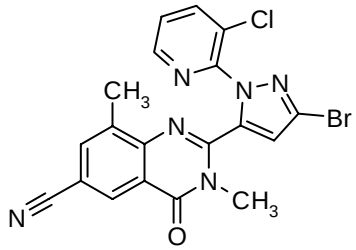
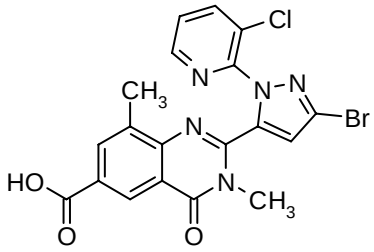
$$\text{ng/g HGW86 Found} = \frac{(106000 - 0)\ x\ 8.905E-6)\ \text{x}\ (2.00\ \text{mL x}\ 50.0\ \text{mL x}\ 1)}{10.0\ \text{mL} \times 10.0\text{g}} =$$

0.944 ppb

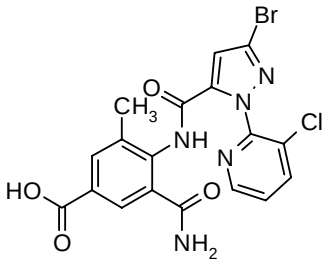
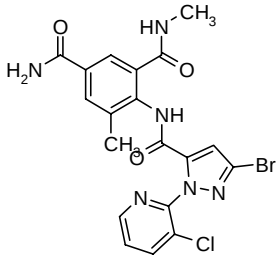
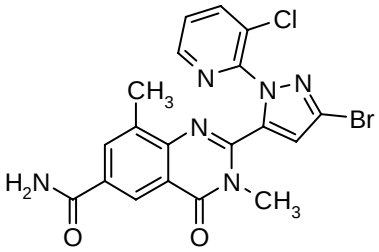
$$\%\ \text{Recovery} = \frac{0.944\ \text{ng/g}}{1.00\ \text{ng/g}}\ \text{x}\ 100\% = 94\%$$

All individual recoveries reported can be calculated using this set of equations and the Data Summary Sheets in Appendix 3.

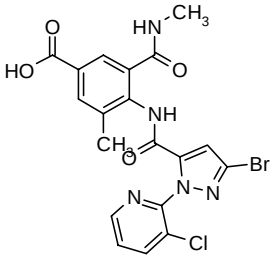
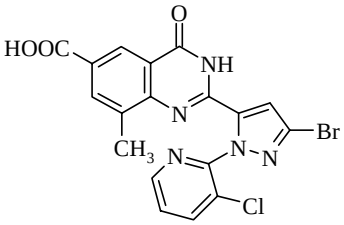
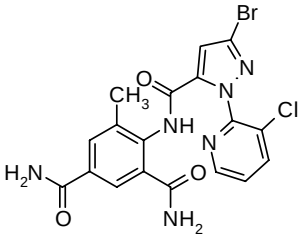
## APPENDIX 1 STRUCTURES OF DPX-HGW86 AND METABOLITES

| Structure | Compound Name |
|---|---|
|  <br>DPX-HGW86<br>MW 473.72 | 3-Bromo-*N*-[4-cyano-2-methyl-6-(methylcarbamoyl)phenyl]-1-(3-chloropyridin-2-yl)-1*H*-pyrazole-5-carboxamide |
|  <br>IN-J9Z38<br>MW 455.70 | 2-[3-bromo-1-(3-chloropyridin-2-yl)-1*H*-pyrazol-5-yl]-3,8-dimethyl-4-oxo-3,4-dihydroquinazoline-6-carbonitrile |
|  <br>IN-K5A78<br>MW 474.70 | 2-[3-bromo-1-(3-chloropyridin-2-yl)-1*H*-pyrazol-5-yl]-3,8-dimethyl-4-oxo-3,4-dihydroquinazoline-6-carboxylic acid |

## APPENDIX 1 STRUCTURES OF DPX-HGW86 AND METABOLITES (CONTINUED)

| Structure | Compound Name |
|---|---|
|  <br>IN-K5A79<br>MW 478.69 | 4-({[3-bromo-1-(3-chloropyridin-2-yl)-1*H*-pyrazol-5-yl]carbonyl}amino)-3-carbamoyl-5-methylbenzoic acid |
|  <br>IN-JCZ38<br>MW 491.73 | 4-({[3-bromo-1-(3-chloropyridin-2-yl)-1*H*-pyrazol-5-yl]carbonyl}amino)-N3,5-dimethylisophthalamide |
|  <br>IN-K5A77<br>MW 473.72 (same as HGW86) | 2-[3-bromo-1-(3-chloropyridin-2-yl)-1*H*-pyrazol-5-yl]-3,8-dimethyl-4-oxo-3,4-dihydroquinazoline-6-carboxamide |

## APPENDIX 1 STRUCTURES OF DPX-HGW86 AND METABOLITES (CONTINUED)

| Structure | Compound Name |
|---|---|
|  <br>IN-JSE76<br>MW 492.72 | 4-({[3-bromo-1-(3-chloropyridin-2-yl)-1*H*-pyrazol-5-yl]carbonyl}amino)-3-methyl-5-(methylcarbamoyl)benzoic acid |
|  <br>IN-PLT97<br>MW 460.68 | 2-[3-bromo-1-(3-chloropyridin-2-yl)-1*H*-pyrazol-5-yl]-8-methyl-4-oxo-3,4-dihydroquinazoline-6-carboxylic acid |
|  <br>IN-K7H19<br>MW 477.70 | 4-({[3-bromo-1-(3-chloropyridin-2-yl)-1*H*-pyrazol-5-yl]carbonyl}amino)-5-methylisophthalamide |