INTRODUCTION

US EPA requires petitioners to furnish results of a successful confirmatory trial of a method by an independent laboratory to ensure its suitability as an enforcement method. This report details the results of the confirmatory trial of Independent Laboratory Validation of Analytical Method, DuPont-14819 entitled, "Analytical Method for the Determination of DPX-E2Y45, IN-EQW78, IN-ECD73, IN-F6L99, and IN-GAZ70 in Soil". The study was carried out according to Study Protocol P0001221, included as the appendix.

The first trial of Exygen Protocol P0001221 for the exhaustive extraction of DPX-E2Y45, IN-EQW78, IN-ECD73, and IN-GAZ70 from soil was not acceptable for DPX-E2Y45 and IN-ECD73. The validation set consisted of one reagent blank, two control samples not fortified with the analytes, five control samples fortified at the Limit of Quantitation (LOQ, 0.5 µg/kg), and five control samples fortified at 10x the LOQ (5 µg/kg).

The second trial of Exygen Protocol P0001221 for the exhaustive extraction of DPX-E2Y45, IN-EQW78, IN-ECD73, and IN-GAZ70 from soil was acceptable for DPX-E2Y45 and IN-ECD73. The validation set consisted of one reagent blank, two control samples not fortified with the analytes, five control samples fortified at the Limit of Quantitation (LOQ, 0.5 µg/kg), and five control samples fortified at 10x the LOQ (5 µg/kg).

The first trial of Exygen Protocol P0001221 for the conventional extraction of IN-F6L99 from soil was acceptable. The validation set consisted of one reagent blank, two control samples not fortified with the analyte, five control samples fortified at the Limit of Quantitation (LOQ, 0.5 µg/kg), and five control samples fortified at 10x the LOQ (5 µg/kg).

The study was initiated on December 07, 2004 when the Study Director signed Exygen Protocol P0001221. The experimental start date was December 20, 2004 and the experimental termination date was January 26, 2005.

TEST SYSTEM

Exygen acquired control soil locally from the property of Paul Connolly in Centre Hall, PA located approximately 10 miles east of Exygen's facility in State College, PA. The soil was assigned Exygen sample ID L0003978-0001, Exygen homogenized the soil with dry ice in a Hobart chopper and allowed the sample to sublime in a freezer overnight before use. The prepared sample was placed in a freezer at a temperature of -20 ± 5°C for storage. The sample was removed from the freezer for extraction, and immediately returned to a freezer after use. Sample log-in

information can be found in the raw data package associated with this study. Storage records will be kept at Exygen Research.

TEST AND REFERENCE MATERIALS

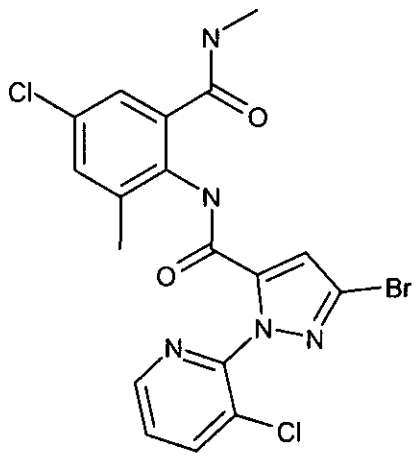
The test substances for soil (DPX-E2Y45) and metabolites (IN-EQW78, IN-ECD73, IN-F6L99, and IN-GAZ70) were supplied by DuPont. Characterization data for each test substance will be maintained by DuPont Crop Protection. The Exygen ExyLIMS numbers, lots, purities, expiration dates, structures, and CAS registry numbers for these test substances are listed below.

Name or Code: DPX-E2Y45

ExyLIMS ID: SP0004950, Lot: 100, Purity: 99.2%, Expiration Date: 02/27/06

CAS No.: 500008-45-7

Molecular Weight: 483.1511

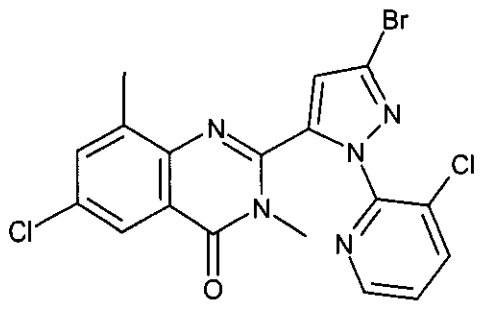


Name or Code: IN-EQW78

ExyLIMS ID: SP0004952, Lot: 004, Purity: 99.8%, Expiration Date: 01/14/07

CAS No.: N/A

Molecular Weight: 465.1358

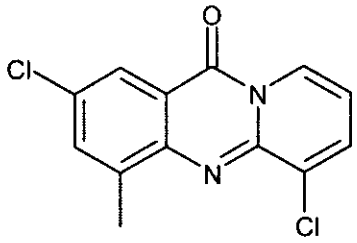


Name or Code: IN-ECD73

ExyLIMS ID: SP0004951, Lot: 003, Purity: 99.8%, Expiration Date: 02/11/06

CAS No.: N/A

Molecular Weight: 279.1248

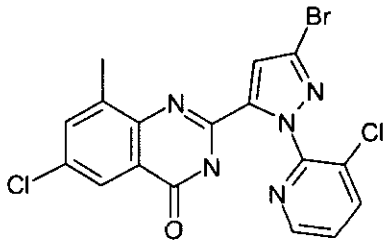


Name or Code: IN-GAZ70

ExyLIMS ID: SP0004953, Lot: 001, Purity: 96%, Expiration Date: 07/07/06

CAS No.: N/A

Molecular Weight: 450.1010

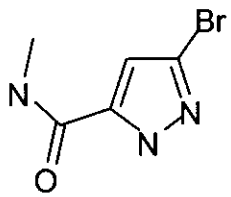


Name or Code: IN-F6L99

ExyLIMS ID: SP0005062, Lot: 003, Purity: 97.7%, Expiration Date: 03/17/06

CAS No.: N/A

Molecular Weight: 204.0263



Stock standard solutions were prepared on December 20 and 22, 2004 as well as January 17 and 19, 2005. Fortification solutions were prepared on December 20, 21, and 22, 2004 as well as January 4, 7, 17, and 19, 2005 as specified in the analytical method portion of the protocol. Calibration solutions were prepared on December 21, 2004 as well as January 4, 7, 17, and 19, 2005 by making appropriate dilutions to the fortification solutions. All stock and fortification standard solutions were stored in amber vials in a refrigerator at a temperature of 2-8°C when not in use. Documentation of standard preparation can be found in the raw data associated with this report.

DESCRIPTION OF ANALYTICAL METHOD

Reference: DuPont-14819 entitled, "Analytical Method for the Determination of DPX-E2Y45, IN-EQW78, IN-ECD73, IN-F6L99, and IN-GAZ70 in Soil", Inveresk Analytical Method No.: 0335B, "Determination of DPX-E2Y45, IN-EQW78, IN-ECD73, and IN-GAZ70 in Soil" – **Exhaustive Extraction Procedure** *(Reference 1).*

DPX-E2Y45 and the metabolites were extracted from a 10-g sample of soil by the addition of 25 mL of a 0.2% formic acid extraction solution and shaking vigorously by hand. Samples were placed on flat bed shaker for 15 minutes. Samples were centrifuged at ca 3000 r.p.m. for 10 minutes and the supernatant was decanted into a 250 mL graduated mixing cylinder with stopper. Twenty-five milliters of 1% hydrochloric acid extraction solution was added to the samples and they were shaken vigorously by hand. Samples were placed on flat bed shaker for 15 minutes. Samples were placed in a water bath set to ca 60°C for 30 minutes. Samples were removed from water bath and the caps were slowly opened to release any pressure. Samples were centrifuged at ca 3000 r.p.m. for 10 minutes and the supernatant was decanted into the same 250 mL graduated mixing cylinder with stopper. Fifty milliters of 5% hydrochloric acid extraction solution was added to the samples and they were shaken vigorously by hand. Samples were placed on flat bed shaker for 5 minutes. Samples were placed in a water bath set at ca 60°C for 1 hour. Samples were removed from water bath and the caps were slowly opened to release any pressure. Samples were placed on flat bed shaker for 10 minutes. Samples were centrifuged at 3000 r.p.m. for 10 minutes and the supernatant was decanted into the same 250 mL graduated mixing cylinder. The volume of each extract was adjusted to 100 mL with 1% hydrochloric acid extraction solution and the extract was mixed thoroughly. A 35-mL aliquot was pipetted into a 250-mL graduated mixing cylinder with stopper, made up to 115 mL with HPLC grade water and mixed thoroughly.

A Waters Oasis HLB cartridge (1 g/20 cc) was conditioned with 5 mL of methanol followed by 20 mL HPLC grade water. A SAX cartridge (1 g/6 mL) was conditioned with 15 mL of SAX conditioning solution. An adapter was used to stack the SAX cartridge above the Water Oasis HLB cartridge and a sample reservoir was placed above the SAX cartridge to simplify loading the extract. Forty milliters of the 115 mL extract was measured using a graduated cylinder and was passed through the

joined cartridges at a flow rate of approximately 4-5 mL/min. The eluate was discarded. The graduated cylinder was rinsed with 2 x 10 mL of SAX conditioning solution and passed through the cartridges. This eluate was also discarded. A vacuum was applied for ca 30 seconds once all extracts and rinses had passed through cartridges to ensure that the SAX cartridges were completely dry. The SAX cartridge was removed. The vacuum was continued for a another 5 minutes to ensure that the Water Oasis HLB cartridges were completely dry. DPX-E2Y45, IN-EQW78, IN-ECD73, and IN-GAZ70 were eluted by the addition of 20 mL acetonitrile followed by 25 mL ethyl acetate. A vacuum was required to start the flow, but was turned off once the flow had started. The eluate was collected in a glass tube. The extract was evaporated to dryness using a flow of nitrogen at approximately 50°C and reconstituted as follows: Add 0.5 mL acetonitrile, ultrasonicate for 5 minutes, and add 0.5 mL 0.01 M aqueous formic acid, ultrasonicate for another 5 minutes, vortex for ca 30 seconds, and remove a portion for LC-MS/MS analysis. The analytes were separated from co-extracts by reversed-phase liquid chromatography (LC) and detected by mass spectrometry/mass spectrometry (MS/MS).

Reference: DuPont-14819 entitled, "Analytical Method for the Determination of DPX-E2Y45, IN-EQW78, IN-ECD73, IN-F6L99, and IN-GAZ70 in Soil", Inveresk Analytical Method No.: 0335A, "Determination of DPX-E2Y45, IN-EQW78, IN-ECD73, and IN-F6L99 in Soil", – **Conventional Extraction Procedure** (Reference 2).

IN-F6L99 was extracted from a 10-g sample of soil by the addition of 10 mL of HPLC grade water and shaking vigorously by hand. The sample was placed in a refrigerator for ca 18 hours at ca +4°C. The sample was removed from the refrigerator and 50 mL of 0.2% formic acid extraction solvent was added. The sample was placed on a flat bed shaker for ca 60 minutes. The sample was then centrifuged at ca 3000 r.p.m. for 10 minutes. The supernatant was decanted into a 250-mL graduated mixing cylinder with stopper. Another 50 mL of 0.2% formic acid extraction solvent was added. The sample was placed on flat bed shaker for ca 60 minutes. The sample was then centrifuged at ca 3000 r.p.m. for 10 minutes. The supernatant was decanted into the same 250-mL graduated mixing cylinder. The volume of each extract was adjusted to 120 mL with 0.2% formic acid extraction solvent and mixed thoroughly. A 40-mL aliquot was pipetted into a 50-mL centrifuge tube. A 10-mL aliquot was removed from the 40-mL extract and evaporated to approximately 0.1 mL under a stream of nitrogen at ca 35°C. The extract was diluted to 10 mL using HPLC grade water and vortexed.

A Waters Oasis HLB cartridge (1 g/20 cc) was conditioned with 5 mL of methanol followed by 20-mL HPLC grade water. Ten milliters of the extract was loaded onto the cartridge. The eluate was discarded. The tube was rinsed with 2 x 5 mL of HPLC grade water and passed through the cartridge. The cartridge was then dried under vacuum for 5 minutes. IN-F6L99 was eluted by the addition of 30 mL of acetonitrile. A vacuum was required to start the flow but was turned off once the flow had started.

The eluate was collected in a glass tube. The extract was evaporated to dryness using a flow of nitrogen at approximately 50°C and reconstituted as follows: Add 0.2 mL acetonitrile, ultrasonicate for 5 minutes, and add 1.8 mL 0.01 M aqueous formic acid, ultrasonicate for a further 5 minutes, vortex for ca 30 seconds, and remove a portion for LC-MS/MS analysis. The analyte was separated from co-extracts by reversed-phase liquid chromatography (LC) and detected by mass spectrometry/mass spectrometry (MS/MS).

The following reagents/materials/instrumentation were used in the study.

1. Acetonitrile, EMD, HPLC Grade
2. Methanol, EMD, HPLC Grade
3. Water, EMD, HPLC Grade
4. Ethyl Acetate, JT Baker, HPLC Grade
5. Formic Acid, EMD, 98%
6. Dimethylsulfoxide, EMD, 99.99%
7. Hydrochloric acid, JT Baker, 36.5-38%
8. Waters Oasis HLB, 1g/20cc
9. Varian Mega BondElute SAX, 1g/6mL
10. National Scientific Autosampler vials, Amber
11. Pyrex 50-mL capacity centrifuge tubes
12. Corning disposable pasteur pipets
13. DuPont Sorvall RC 5B Centrifuge
14. Beckman Coulter Avanti J-E Centrifuge
15. Beckman Allegra 6R Centrifuge
16. Organomation Associates, Inc N-EVAP 112
17. VWR Aquasonic Model 250D Sonicator
18. VWR Vortex Genie 2 Vortexer
19. Water Bath, Stovall Life Science, Inc., Hybridization water bath
20. Eberbach Corporation Flat Bed Shaker
21. Analytical Balance, Mettler-Toledo, Inc. Microbalance
22. Top-loader Balance, Mettler-Toledo, Inc.
23. Rainin digital autopipettes 10-100 µL and 100-1000 µL

The following are the analytical conditions that were employed for the exhaustive extraction procedure.

Instrument:	AB SCIEX API 4000 LCMSMS Turbo V Atmospheric Pressure Chemical Ionization Source
Computer:	Dell Precision 360
Software:	PE Sciex Analyst 1.4
HPLC Equipment:	Hewlett Packard, HP Series 1100 HP Quat Pump HP Vacuum Degasser HP Autosampler HP Column Oven

HPLC Column: Phenomenex Luna C18(2) 3 µm, 150 mm x 4.6 mm
Guard Column: Phenomenex C18(ODS),
4.0 mm x 3.0 mm

Column Temperature: 40°C

Mobile Phase (A): 0.01 M Formic Acid in Water
Mobile Phase (B): Methanol

Time (min)	Flow Rate (µL/min)	% A	% B
0.0	1000	40	60
0.5	1000	40	60
2.0	1000	20	80
5.0	1000	2	98
8.0	1000	2	98
8.5	1000	40	60
11.5	1000	40	60

Total run time = 11.5 min

Injected Volume: 25 µL

Ions monitored:

Analyte	Ionization Mode	Target Ion	Confirmatory Ion
DPX-E2Y45	APCI, Positive	283.9 → 177.1	484.0 → 453.0
IN-EQW78	APCI, Positive	465.7 → 188.0	465.7 → 186.2
IN-ECD73	APCI, Positive	278.9 → 244.1	278.9 → 209.1
IN-GAZ70	APCI, Positive	452.0 → 416.1	449.9 → 414.0

The conventional extraction procedure used the same analytical conditions as the exhaustive extraction procedure; however, the gradient was slightly different and the monitored ions differed as shown below.

Time (min)	Flow Rate (µL/min)	% A	% B
0.0	1000	90	10
0.5	1000	90	10
5.5	1000	20	80
5.8	1000	10	90
8.8	1000	10	90
9.0	1000	90	10
11.0	1000	90	10

Total run time = 11 min

Ions monitored:

Analyte	Ionization Mode	Target Ion	Confirmatory Ion
IN-F6L99	APCI, Positive	204.0 → 173.0	204.0 → 66.2

EXPERIMENTAL DESIGN

Step 1

The Study Director will read the subject method completely and ensure all necessary equipment and reagents are available. Substitutions of equipment and reagents that are permitted are indicated by the phrase "or equivalent" after the listed item in the method. No other substitutions are permitted.

Step 2

After reading the subject method, the Study Director will document areas that are not clear or require special interpretation to implement. The Study Director will write an interpretation of each area and how that interpretation may affect the results obtained with the method. The description will be submitted as part of the final report, whether or not the subject method is confirmed satisfactorily in the study. At this point in the study, the Study Director may contact the Study Monitor for clarification(s), but this communication must be documented in the study records.

Step 3

The independent laboratory will establish the relationship between the instrument response and the concentration of analyte in order to assess the linearity of the system. Following the tuning of the instrument, a range of standards should be analyzed to establish the working range for the instrumentation used. The standard range should be adjusted such that the signal to noise of the lowest standard used is approximately 10:1 for the least responsive analyte. The final extracts will be diluted such that the LOQ fortification is approximately 150-200% of the concentration of the low standard.

A standard curve should be constructed with at least five standards covering the range from approximately 10:1 signal to noise for the least responsive analyte to at least 120% of the anticipated highest injected concentration. The independent lab should also verify that the matrix control samples are free of interference and matrix effects at the retention time of the analyte. This should be accomplished by analysis of a soil control sample and examination of the regions of analyte retention. Response greater than 30% of the proposed LOQ constitutes an interference, and the Study Monitor should be contacted. A control sample should then be fortified at the appropriate concentration for an LOQ sample after being processed through the entire method. The recovery of a post-fortified sample should be calculated using a standard curve or bracketing average response factor. A recovery greater than 120% or less than 70%

of the proposed LOQ may constitute a matrix effect and the Study Monitor should be contacted.

Step 4

Analyze method validation sample set for DPX-E2Y45, IN-EQW78, IN-ECD73, and IN-GAZ70 in soil. Analyze an additional validation set for IN-F6L99 in soil. Results from the test should be similar to those published in the subject method (DuPont-14819). Fortification will be made to the matrix just before extraction at the levels defined above. This is the typical route of administration for validation studies. The fortification solution containing a standard solution will be applied to control samples via a Hamilton syringe, or equivalent to maintain consistency and accuracy.

Step 5

After completion of the first validation set, the Study Director (or designate) will contact the Study Monitor to provide a summary of the results and discuss the next step of the study. This communication will be documented. If results for all samples in the test are within 70-120% of fortification concentrations, then the analytical phase is complete. If the result of one or more of the individual fortification samples falls outside 70-120% of the fortification concentration, the Study Monitor will be contacted for discussion of method implications and future experiments. A second validation set of soil may be analyzed. A maximum of three (3) validation sets may be analyzed to show the subject method is valid.

Communication between the Study Director and the Study Monitor should occur after each validation set is analyzed, and must be fully documented in the study records. Under no circumstances should personnel familiar with the method visit the independent laboratory to observe or to offer help.

If all three validation sets fail, the Study Director will inform the Study Monitor and will proceed to write the final report. In the report an explanation of what failed in each trial (i.e., low recoveries, high recoveries) should be included; however, no speculation should be included in this discussion.

METHOD OBSERVATIONS

A. Problems Encountered

Stability issues with DPX-E2Y45 were observed during the validation. These were overcome by the addition of concentrated formic acid to the stock and fortification standard solutions.

B. Critical Steps

None observed.

C. Matrix or Solvent Effects

No matrix or solvent effects were noted.

RECOMMENDED CHANGES TO METHOD

In order to analyze the tested compounds in the subject matrix on a Sciex API 4000 LC/MS/MS, the following changes to the method are suggested:

1. Section 5.4.2 and 5.4.5. - For DPX-E2Y45, add approximately 50 µL of concentrated formic acid prior to adjusting the volume to 100 mL with acetonitrile.
2. Section 5.4.3 and 5.4.6. - For the multi-analyte fortification solutions add approximately 50 µL of concentrated formic acid prior to adjusting the volume to 100 mL with acetonitrile, and approximately 5 µL prior to adjusting the volume to 10 mL.
3. Section 8.2.7. - Elute analytes by addition of 20 mL of acetonitrile followed by 30 mL of ethyl acetate.