ANALYTICAL METHOD FOR THE DETERMINATION OF DPX-MP062 [75% DPX-KN128 (INDOXACARB) AND 25% IN-KN127] AND METABOLITES IN-MS775, IN-JT333, IN-MP819, IN-JU873, AND IN-KG433 IN GROUND, SURFACE, AND DRINKING WATERS USING LC/MS/MS

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REASON FOR REVISION

Modifications to the original method were made to allow for the analysis of IN-MS775.

1.0 SUMMARY

The purpose of this study was to develop an analytical method for the detection, quantitative analysis, and confirmation of DPX-MP062 and metabolites IN-MS775, IN-JT333, IN-MP819, IN-JU873, and IN-KG433 in water. DPX-MP062 is a mixture of two optical isomers, 75% DPX-KN128 also known as indoxacarb and 25% IN-KN127. Indoxacarb is insecticidally active and IN-KN127 is not. This method was validated using ground, surface, and drinking water.

The samples (40-mL) were diluted with acetonitrile until the percent of acetonitrile was equal to 20% (40-mL sample and 10-mL acetonitrile). This was to assure the complete transfer of the analytes from the sample container to the SPE cartridge. The pH of each sample was adjusted by the addition of 40-µL of acetic acid. DPX-MP062, IN-MS775, IN-JT333, IN-MP819, IN-JU873, and IN-KG433 were extracted from the water samples by filtration through an Oasis HLB solid phase extraction (SPE) cartridge. The cartridges were washed with 10 mL of 70:30 water:acetonitrile followed by 5 mL of hexane. The analytes were eluted with 25 mL of acetonitrile. The extracts were evaporated under a flow of nitrogen to a volume of approximately 100-µL. One mL of acetonitrile was added to the extracts. The extracts were vortexed, sonicated, and then diluted to 2 mL using water. DPX-MP062, IN-MS775, IN-JT333, IN-MP819, IN-JU873, and IN-KG433 were separated from co-extracts by reversed-phase liquid chromatography (RPLC). DPX-MP062, IN-MS775, IN-JT333, IN-JU873, and IN-KG433 were detected by positive ion electrospray (ESI) mass spectrometry/mass spectrometry (MS/MS). IN-MP819 did not produce sufficient single using the electrospray interface. IN-MP819 was detected using the Atmospheric Pressure Chemical Ionization (APCI) interface and mass spectrometry/mass spectrometry (MS/MS). The isomers that make up DPX-MP062 (DPX-KN128 and IN-KN127) elute as one peak. The Limit of Quantitation (LOQ) was 0.050 µg/L (ppb) for all six analytes. The Limit of Detection (LOD) was estimated to be 0.02 µg/L (ppb).

2.0 INTRODUCTION

DPX-MP062 is a mixture of two optical isomers, 75% DPX-KN128 also known as indoxacarb and 25% IN-KN127. Indoxacarb is insecticidally active and IN-KN127 is not. The structure, CAS name, and various physical properties of DPX-KN128, IN-KN127, IN-MS775, IN-JT333, IN-MP819, IN-JU873, and IN-KG433 can be found in Appendix 1. The method was validated on ground, surface, and drinking water. An LC/MS/MS method (DuPont-7898) for the analysis of DPX-MP062, IN-JT333, and IN-KT413 with an LOQ of 0.05 µg/L (ppb) has been developed previously (Reference 1). The method described in DuPont-7898 will not detect or quantify IN-MS775, IN-MP819, IN-JU873, or IN-KG433 in water samples. A GC/MS method (DuPont-3328) for the analysis of DPX-KN128 and IN-KN127 (as one peak) with a LOQ of 0.05 µg/L (ppb) has been developed previously (Reference 2). The method described in DuPont-3328 will not detect or quantify IN-MS775, IN-JT333, IN-MP819, IN-JU873, or IN-KG433 in water samples.

The samples (40-mL) were diluted with acetonitrile until the percent of acetonitrile was equal to 20% (40-mL sample and 10-mL acetonitrile). This was to assure the complete transfer of the analytes from the sample container to the SPE cartridge. The pH of each sample was adjusted by the addition of 40-µL of acetic acid. DPX-MP062, IN-MS775, IN-JT333, IN-MP819, IN-JU873, and IN-KG433 were

extracted from the water samples by filtration through an Oasis HLB (0.50 g) solid phase extraction (SPE) cartridge. The cartridges were washed with 10 mL of 70:30 water:acetonitrile followed by 5 mL of hexane. The analytes were eluted with 25 mL of acetonitrile. The extracts were evaporated under a flow of nitrogen to a volume of approximately 100-µL. One mL of acetonitrile was added to the extracts. The extracts were vortexed, sonicated, and then diluted to 2 mL using water. DPX-MP062, IN-MS775, IN-JT333 IN-MP819, IN-JU873, and IN-KG433 were separated from co-extracts by reversed-phase liquid chromatography. DPX-MP062, IN-MS775, IN-JT333, IN-JU873, and IN-KG433 were detected by positive ion electrospray (ESI) mass spectrometry/mass spectrometry (MS/MS). IN-MP819 did not produce sufficient single using the electrospray interface. IN-MP819 was detected using the Atmospheric Pressure Chemical Ionization (APCI) interface and mass spectrometry/mass spectrometry (MS/MS).

The LOQ for DPX-MP062 and metabolites, IN-MS775, IN-JT333 IN-MP819, IN-JU873, and IN-KG433 was 0.050 µg/L (ppb). The LOD was estimated to be 0.02 µg/L (ppb). During method validation, acceptable recoveries for water samples fortified at 1× and 10× the LOQ were generated.

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

HPLC system, HP1100 (Agilent, Wilmington, DE)

Mass Spectrometer System, Quattro II with APCI interface (Micromass Inc., Altrincham, UK)

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)

Evaporator - N-Evap® Model 111 laboratory sample evaporator/nitrogen manifold fitted with Teflon®-coated needles (Organomation Associates, South Berlin, MA). This unit is attached to a dry, clean nitrogen source.

Solid-Phase Extraction Equipment

Visiprep 12 port SPE vacuum manifold, PN 5-7030 (Supelco, Bellefonte, PA)

Solid-Phase Extraction Supplies

Oasis® HLB cartridge, 500 mg/12 cc, PN 186000116 (Waters, Milford, MA) - **Do not substitute**

Chromatographic Supplies

HPLC Column: 4.6 mm i.d. × 15 cm, Phenomenex® Luna C-18(2) analytical column with 3 µm diameter packing Part # 00F-4251-E0 (Phenomenex, Torrance, CA)

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

Labware

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

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

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

Centrifuge tubes, PYREX Brand Conical Centrifuge Tubes with Standard Taper Stopper, 40-mL capacity, Cat. No. 21021-993 (VWR Scientific Co., Bridgeport, NJ)

Miscellaneous

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

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)

Acetic Acid - Baker Analyzed® glacial acetic acid, #9524-00 (J. T. Baker, Inc. Danvers, MA).

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

Hexanes - EM Omni Solv®, #HX0296-1 (EM Science, Gibbstown, NJ)

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

DPX-MP062 reference substance (Dash 160, 99.4% pure) used for sample analysis: Analytical standard grade reagent (DuPont Crop Protection, Global Technology Division, E. I. du Pont de Nemours and Company)

IN-MS775 reference substance (Dash 2, 99.7% pure) used for sample analysis: Analytical standard grade reagent (DuPont Crop Protection, Global Technology Division, E. I. du Pont de Nemours and Company)

IN-JT333 reference substance (Dash 17, 97.5% pure) used for sample analysis: Analytical standard grade reagent (DuPont Crop Protection, Global Technology Division, E. I. du Pont de Nemours and Company)

IN-MP819 reference substance (Dash 1, 94.1% pure) used for sample analysis: Analytical standard grade reagent (DuPont Crop Protection, Global Technology Division, E. I. du Pont de Nemours and Company)

IN-JU873 reference substance (Dash 3, 96.3% pure) used for sample analysis: Analytical standard grade reagent (DuPont Crop Protection, Global Technology Division, E. I. du Pont de Nemours and Company)

IN-KG433 reference substance (Dash 1, 88.2% pure) used for sample analysis: Analytical standard grade reagent (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 (MSDS) 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

The quantitative analysis of DPX-MP062, IN-MS775, IN-JT333, IN-MP819, IN-JU873, and IN-KG433 using one analytical method required some special sample handling procedures as described below.

DPX-MP062, IN-MS775, IN-JT333, IN-MP819, IN-JU873, and IN-KG433 were extracted from the water samples by filtration through an Oasis HLB (0.50 g) solid phase extraction (SPE) cartridge. DPX-MP062 and IN-JT333 have a tendency to adhere to glass and plastic surfaces when in water. Therefore, the samples were diluted with acetonitrile (80:20 water:acetonitrile) prior to loading onto the SPE cartridge. In addition, it is good practice to measure out all washes and elution steps using the graduated 50-mL sample centrifuge tube. The use of extra sample handling accessories such as SPE solvent reservoirs and adapters should be avoided to minimize surface area. All samples or extracts should be loaded directly into the SPE cartridges. The pH of the samples was adjusted by the addition of 40 µL of acetic acid. When the pH of the samples was not adjusted low recoveries were observed for IN-KG433 and IN-JU874 for some water types. Following the load step, the cartridges were washed with 70:30 water:acetonitrile followed by hexane. The analytes were eluted in 25-mL of acetonitrile. The extracts were evaporated under a flow of nitrogen to a volume of approximately 100-µL. One mL of acetonitrile was added to the extracts. The extracts were vortexed, sonicated, and then diluted to 2 mL using water. DPX-MP062, IN-MS775, IN-JT333, IN-JU873, and IN-KG433 were detected by positive ion electrospray (ESI) mass spectrometry/mass spectrometry (MS/MS). IN-MP819 did not produce sufficient single in ESI it was detected by positive ion Atmospheric Pressure Chemical Ionization (APCI) 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.

Due to the tendency of DPX-MP062 and IN-JT333 to adhere to surfaces when in water, it is extremely important not to wash analyte contaminated glassware such as stock standard volumetric flasks in common wash areas. Contaminated glassware must be thoroughly rinsed with acetonitrile prior to following normal glassware cleaning procedures.

4.2.2 Preparation of Solutions

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

0.010 M aqueous acetic acid solution - Add 600 µL of acetic acid to 1000 mL of water and mix the resulting solution to homogeneity.

0.010 M aqueous formic acid solution - Add 460 µL of formic acid to 1000 mL of water and mix the resulting solution to homogeneity.

70:30 water:acetonitrile - Add 300 mL of HPLC grade acetonitrile to 700 mL of HPLC grade water. Mix the resulting solution to homogeneity. This solution may be prepared monthly.

50:50 water:acetonitrile - Add 500 mL of HPLC grade acetonitrile to 500 mL of HPLC grade water. Mix the resulting solution to homogeneity. This solution may be prepared monthly.

4.2.3 *Preparation and Stability of Stock Standards*

Use Class A volumetric flasks when preparing standard solutions.

DPX-MP062, IN-MS775, IN-JT333, IN-MP819, IN-JU873, and IN-KG433 Stock Standards

Prepare standard stock solutions of DPX-MP062, IN-MS775, IN-JT333, IN-MP819, IN-JU873, and IN-KG433 by accurately weighing 10.00 ± 0.1 mg of each analyte into individual 100-mL volumetric flasks 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 solutions to a volume of 100 mL using HPLC-grade acetonitrile and invert the volumetric flask to mix the solutions to homogeneity. These 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 Fortification Standards*

Use Class A volumetric flasks when preparing standard solutions.

DPX-MP062, IN-MS775, IN-JT333, IN-MP819, IN-JU873, and IN-KG433 Fortification Standards

Prepare a 1.0-µg/mL DPX-MP062, IN-MS775, IN-JT333, IN-MP819, IN-JU873, and IN-KG433 standard in acetonitrile by pipetting 1.00 mL of each of the 100.0-µg/mL stock standards into a 100-mL volumetric flask. Bring to volume using HPLC-grade acetonitrile and mix to homogeneity. The standard is stable for approximately 3 months if stored in a freezer at approximately -20°C immediately after each use.

Prepare a 0.10-µg/mL DPX-MP062, IN-MS775, IN-JT333, IN-MP819, IN-JU873, and IN-KG433 fortification standard in acetonitrile by pipetting 1.00 mL of the 1.00-µg/mL fortification standard into a 10-mL volumetric flask. Bring to volume using HPLC-grade acetonitrile and mix to homogeneity. The standard is stable for approximately 3 months if stored in a freezer at approximately -20°C immediately after each use.

Alternate or additional solutions may be prepared as needed.

4.2.5 *Preparation and Stability of Calibration Standards*

Prepare the DPX-MP062, IN-MS775, IN-JT333, IN-MP819, IN-JU873, and IN-KG433 calibration standards by pipetting volumes of the standard solutions shown in the following table into separate 10.0-mL volumetric flasks.

Desired Standard Concentration (ng/mL)	Volume of 1.00-µg/mL Multi-Analyte Standard Required (mL)	Volume of 0.10-µg/mL Multi-Analyte Standard Required (mL)
15	0.150	-
10	0.100	-
5.0	0.050	-
1.0	-	0.100
0.60	-	0.060

Dilute the standards to volume using 50:50 acetonitrile:water, cap, and mix thoroughly. These standard solutions should be prepared weekly and stored approximately 4°C. Each of the calibration standards was vortexed for 30 seconds prior to injection. Alternative or additional standards may be prepared as needed. If standards are to be stored more than 5 days, add 10 µL of acetic acid to each standard to reduce degradation.

4.2.6 *Source of Samples*

Water control samples were obtained from local water sources. All water sources are provided in the table below. Bottled water (drinking water) was purchased from a local grocery store.

Origin	Location
Lums Pond	Bear, Delaware
Brandywine River	Wilmington, Delaware
Kemblesville Well	Kemblesville, Pennsylvania
Bottled Spring	Vendor: ACME Source: Pine Valley Spring, New Ringgold, PA

All samples were refrigerated until use.

4.2.7 *Storage and Preparation of Samples*

Water samples should be stored at approximately 4°C. The water samples were shaken by hand prior to use to ensure homogeneity. No additional filtration or purification was performed prior to sample processing. Since DPX-MP062 and IN-JT333 have a tendency to adhere to plastic and glass surfaces when in water, the samples must be diluted with acetonitrile prior to transferring from one container to a second container. The samples should be diluted until the percent acetonitrile is equal to 20%. For example, a 100-mL water sample would require 25 mL of acetonitrile

(25/125 × 100 = 20%). If the quantity of water is unknown it may be determined from the mass of the sample. This can be accomplished by weighing an empty identical container and weighing the container containing the sample. The difference in grams will be equal to the weight of the sample. Since 1 g of water is equal to 1 mL of water, the volume in milliliters will be equal to the mass of the sample in grams. **If acetonitrile was added to the sample, the initial water volume and the amount of acetonitrile added must be clearly marked on the container. This is to assure proper sample processing and disposal.**

In the event water samples contain sediment, the sample must be centrifuged and the water decanted into a clean container prior to analysis.

4.2.8 *Sample Fortification Procedure*

All fortifications were made directly to the water following the measurement of the sample.

Fortified 40-mL samples were prepared using the 1.00 µg/mL and 0.10 µg/mL of the multi-analyte fortification standards.

Fortification Level (µg/L)	Volume of 1.00-µg/mL Multi-Analyte Standard Required (mL)	Volume of 0.10-µg/mL Multi-Analyte Standard Required (mL)
0.050	-	0.020
0.50	0.020	-

The total volume of standard added to the water should be less than 1.0 mL.

4.2.9 *Analyte Extraction and Purification Procedures*

1. Accurately measure 40.0 mL (± 1%) of water using a 50-mL centrifuge tube. Fortify sample if necessary. Cap and shake the samples vigorously. Add 40 µL of acetic acid followed by 10.0 mL (± 1%) of acetonitrile to the sample. Cap and shake the samples vigorously. If acetonitrile was added to the sample previously (Section 4.2.7), adjust the sample size and percent acetonitrile until it is equivalent to 40 mL of water diluted to 20% acetonitrile.
2. Attach a 12-cc, 0.5-g Oasis HLB cartridge to an SPE manifold. Condition the cartridge with 5 mL of methanol followed by 10 mL of HPLC grade water. Do not let the cartridge go to dryness.
3. Load the sample into the SPE. Using gravity flow, allow the sample to pass through the Oasis cartridges at a flow rate of 2-5 mL/min. Rinse[1] the centrifuge tube with 10 mL of 70:30 water:acetonitrile and load the rinse into the reservoir just before all of the sample passes through. Use vacuum to dry the cartridge for 5 minutes.

[1] All rinse steps require vortexing prior loading on the column.

4. Rinse the centrifuge tube with 5 mL of hexane and load it on the cartridge. Vacuum will be required to start the flow of hexane through the SPE cartridge. Use vacuum to dry the cartridge for 5 minutes. Discard the eluate.
5. Elution of the analyte. Rinse the sample centrifuge tube with 25 mL of acetonitrile and load the acetonitrile onto the cartridge, vacuum may be required to start the flow but should be turned off once the flow has started. Once the acetonitrile has passed through the cartridge, use a small amount of vacuum to empty the remaining liquid in the cartridge into a centrifuge tube. Collect the acetonitrile eluate in a 40-mL glass centrifuge tube.
6. Evaporate the extract to approximately 100 µL using a flow of nitrogen in an N-Evap at 35°C. Add 1 mL of acetonitrile, vortex the extract for 30 seconds and sonicate it for 5 minutes. Using a pipette, dilute the extract to 2-mL using water. Vortex the centrifuge tube and transfer an aliquot of the extract using a disposable pipette into an HPLC vial. Analyze the solution by LC/MS/MS as described in the following section.

Extracts will be stable for approximately 72 hours if stored at 4°C. If extracts are to be stored more than 72 hours, add 10 µL of acetic acid to each extract to reduce degradation.

4.3 Instrumentation for the Method

4.3.1 Chromatography

Reversed-phase chromatography was used to separate DPX-MP062 and metabolites from co-extracts. A Phenomenex® Luna C-18 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. The conditions used for the analysis of DPX-MP062, IN-MS775, IN-JT333, IN-JU873, and IN-KG433 are provided below.

System:	Agilent HP1100 HPLC			
Column:	4.6 mm i.d. × 150 mm, C18 Luna Phenomenex®			
Column Temperature:	30°C			
Sample Temperature:	4°C			
Injection Volume:	0.100 mL			
Conditions:	A: 0.010 M aqueous Acetic Acid B: Acetonitrile			
	Time	%A	%B	Flow (mL/Min.)
	0.0	70	30	1.00
	0.5	70	30	1.00
	4.0	35	65	1.00
	12.0	5	95	1.00
	15.0	5	95	1.00
	15.1	70	30	1.00
DPX-MP062 Retention Time:	10.7 minutes			
IN-MS775 Retention Time:	12.1 minutes			
IN- JT333 Retention Time:	10.8 minutes			
IN-JU873 Retention Time:	9.5 minutes			
IN-KG433 Retention Time:	8.2 minutes			
Total Run Time:	22 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
0.00-5.0	Waste
5.00-15.0	MS source
15.0-End	Waste

Since the electrospray interface is optimal at low flow rates, the HPLC flow is split post-column such that only 100 µL/min actually passes through the interface (approximately 10:1 split), the remainder going to a waste container.

The conditions used for the APcI analysis of IN-MP819 is provided below.

System:	Agilent HP1100 HPLC			
Column:	4.6 mm i.d. × 150 mm, C18 Luna Phenomenex®			
Column Temperature:	30°C			
Sample Temperature:	4°C			
Injection Volume:	0.100 mL			
Conditions:	A: 0.010 M aqueous Formic Acid B: Acetonitrile			
	Time	%A	%B	Flow (mL/Min.)
	0.0	50	50	0.80
	0.5	50	50	0.80
	9.0	2	98	0.80
	12	2	98	0.80
	12.5	50	50	0.80
IN-MP819 Retention Time:	10.3 minutes			
Total Run Time:	16 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
0.00-9.0	Waste
9.0-11.0	MS source
11.0-End	Waste

Since APCI LC/MS systems perform optimally at high flow rates, the column eluate was introduced into the source at 0.8 mL/minute.

4.3.2 *LC/MS/MS Analysis*

The quantitative analysis of DPX-MP062 and metabolites was performed using a Micromass Quattro II LC/MS/MS system. The system parameters were adjusted while a solution of each analyte was infused directly into the ion source. The solution composition was 50% acetonitrile/50% 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. Extensive in-source fragmentation was observed for IN-MP819 in the APcI source. As a result, fragments generated in the source were used for quantitative analysis. A summary of the experimental conditions is provided in the following table:

Micromass Quattro LC ESI-LC/MS/MS Mass Spectrometer Conditions

Analytes	Ions Monitored	Cone Voltage	Collision Energy	Dwell (Seconds)
DPX-MP062	528.0→ 292.9 ± 0.5 AMU	32V	14V	0.30
	528.0→ 217.8 ± 0.5 AMU	32V	22V	0.30
	528.0→ 202.9 ± 0.5 AMU	32V	40V	0.30
IN-MS775	411.9→ 208.8 ± 0.5 AMU	40V	14V	0.30
	411.9→ 190.8 ± 0.5 AMU	40V	22V	0.30
IN-JT333	470.0→ 149.8 ± 0.5 AMU	25V	48V	0.30
	470.0→ 266.9 ± 0.5 AMU	**25V**	**12V**	**0.30**
	470.0→ 206.9 ± 0.5 AMU	25V	24V	0.30
IN-JU873	458.0→ 149.0 ± 0.5 AMU	42V	45V	0.30
	458.0→ 204.8 ± 0.5 AMU	**42V**	**22V**	**0.30**
	458.0→ 254.8 ± 0.5 AMU	42V	15V	0.30
IN-KG433	516.0→ 220.9 ± 0.5 AMU	35V	30V	0.30
	516.0→ 280.8 ± 0.5 AMU	**35V**	**18V**	**0.30**
Ion Mode:	Positive			
Capillary Voltage:	3.50 KV			
Detector Voltage:	750 V			
Source Temperature:	80°C			
Collision Gas Pressure:	1.3e-3 to 2.6e-3mBar			
Drying Gas Flow:	350 L/h			

Micromass Quattro LC APcI-LC/MS/MS Mass Spectrometer Conditions

Analytes	Ions Monitored	Cone Voltage	Collision Energy	Dwell (Seconds)
IN-MP819	238.5→ 194.2 ± 0.5 AMU	23V	11V	0.20
	238.5→ 131.2 ± 0.5 AMU	**23V**	**19V**	**0.20**
Ion Mode:	Positive			
Corona Voltage:	3.25 KV			
Detector Voltage:	750 V			
Probe Temperature:	600°C			
Source Temperature:	150°C			
Collision Gas Pressure:	1.6e-3 to 2.6e-3mBar			
APCI Sheath Gas Flow:	125 L/h			
Drying Gas Flow:	350 L/h			

A complete list of the experimental parameters is given in Appendix 4. Typical LC/MS and LC/MS/MS full scan spectra are shown in Figure 1 and Figure 2, respectively.

The instrument was operated in MS/MS-(MRM) positive ion mode for quantitative analysis. Peak area was used for quantitation. Quantitation of DPX-MP062 and IN-MS775 was performed using the TIC. **Quantitation of IN-JT333, IN-JU873,**

IN-KG433 and IN-MP819 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 0.60-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 at least 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

In order to calculate recovery data more accurately, the average response factor for the three standards closest in response to the fortification analyzed was used. The standards selected must include a minimum of one standard above and one standard below.

4.4.1 *Method*

Average Response Factor (RFAvg) was calculated as follows:

$$RF_{Ave} = \frac{(Conc.\,A \div Area\,A) + (Conc.\,B \div Area\,B) + (Conc.\,C \div Area\,C)}{3}$$

ppb found was calculated as follows:

$$ppb\ Found = \frac{(Peak\ Area) \times (RF_{Ave}) \times (Final\ Volume)}{(Sample\ Volume)}$$

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:

$$\%\ Recovery = \frac{(ppb\ Found)}{(Fortification\ level)} \times 100$$

4.4.2 *Example*

For a Lums Pond water sample fortified with IN-KG433 at 0.05 µg/L (0.05 ppb) [Date Extracted 4-Feb-2003, H-74], the concentration found was calculated as follows:

Average Response Factor was calculated as follows:

$$RF_{Ave} = \frac{(0.60\ ng/mL \div 384\ AC) + (1.0\ ng/mL \div 548\ AC) + (5.0\ ng/mL \div 2759\ AC)}{3}$$

(AC ≡ Area Counts)

$RF_{Avg} = 1.73319e^{-3}$ ng/mL/AC

ppb found was calculated as follows:

$$ppb\ Found = \frac{(501\ AC) \times (1.73319e-3\ ng/mL/AC) \times (2.0\ mL)}{(40\ mL)} \times \frac{1\ \mu g/L}{1\ ng/mL}$$

ppb Found = 0.0434164 µg/L

(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:

$$\%\ Recovery = \frac{(0.0434164\ \mu g/L)}{(0.050\ \mu g/L)} \times 100$$

% Recovery = 87%

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

An LOD was estimated for each analyte based on signal-to-noise. An LOD value should be estimated by each lab using this method. The LOD is estimated to be approximately 0.02 µg/L for DPX-MP062 and metabolites. The LOD is defined as the concentration of IN-MP062, the least responsive analyte at which analyte peaks are approximately three times the chromatographic baseline noise observed near the retention time or approximately 1/3 the concentration of the LOQ. Variation in the LOD was observed. Instrument response may fluctuate between routine instrument maintenance.

5.2 Time Required for Analysis

Typically ten to twelve samples can be prepared during the course of an eight-hour day. LC/MS/MS analyses were run unattended overnight.

5.3 Modifications or Special Precautions

Care must be taken when transferring the sample to a new container. DPX-MP062 and IN-JT333 have a tendency to adhere to glass and plastic when in the presence of water. The sample must be measured and diluted with acetonitrile until the sample composition is approximately 20% acetonitrile.

Low recoveries for IN-KG433 and IN-JU873 were observed when the pH of the samples was not adjusted prior to loading the samples on the Oasis SPE. We believe the cause of the low recoveries to be poor stability of the analytes at pHs above 6.5.

5.4 Method Ruggedness

5.4.1 Stability and Ruggedness Testing

The stability of the analyte has been stated in the respective sections of this report. The stability of reagents used in this method has also been stated.

5.4.2 Specificity/Potential Interference

Due to the selective nature of the detection of this method (two individual ion transitions monitored), interference peaks were not observed at the retention time of the analytes. As a result of the selective detection used, interference testing is not necessary for this method.

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.

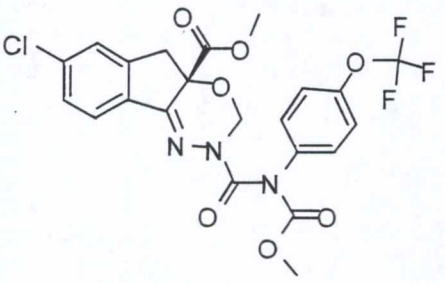
If an instrument is used that is not capable of monitoring two ion transitions for one or more of the analytes at the method low standard (0.6 ng/mL) and a detected residue must be confirmed, the samples should be re-injected at a greater injection volume. Therefore, allowing for sufficient signal to be collected and the confirmation criteria assessed. The data collected from the re-injection will be used to calculate confirmation criteria **only**. Due to phenomena such as matrix effects, the collected data **cannot** be used to calculate recovery or magnitude of residue data unless rigorous validation data at that injection volume has been collected.

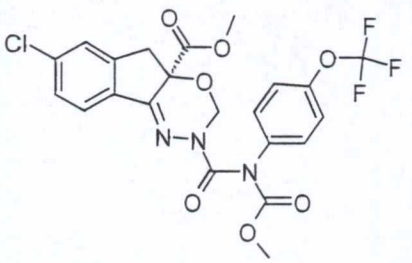
6.2 Confirmation Criteria

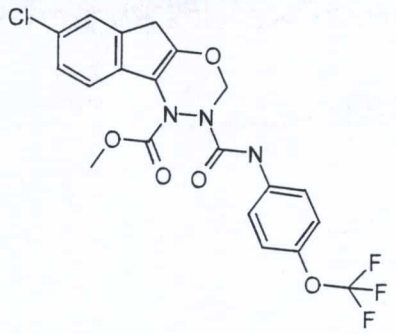
In order for a sample set to be valid, the relative standard deviation of the ion ratios calculated from the calibration standards analyzed must be less than 20%. For the confirmation of possible DPX-MP062 and metabolite residues in a water sample, the ion ratio must fall within ±30% of the average ratio for all calibration standards for a specific sample set.

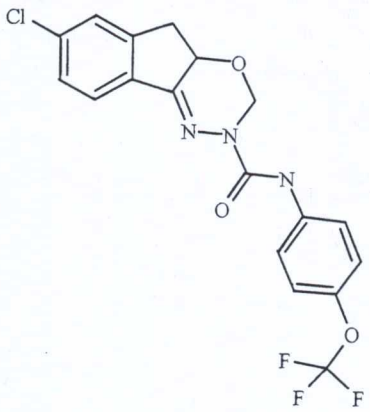
If the ion ratio is outside the ±30% range, the signal was most likely generated from a compound that is unrelated to DPX-MP062. The unknown compound also has the same ion by LC/MS and a similar fragmentation pattern. In addition to meeting the defined ion ratio criteria, the elution time of the compound of interest must fall within 2% of the elution time of the standards analyzed for that sample set.

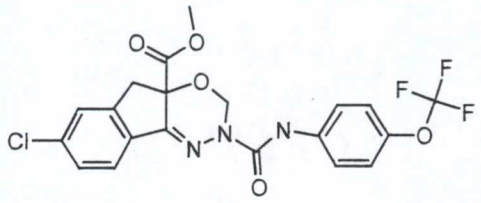
APPENDIX 1 STRUCTURE AND PROPERTIES OF INDOXACARB AND METABOLITES

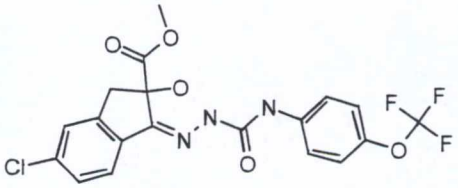
Common Name	Indoxacarb
Structure	
DPX Number	DPX-KN128
CAS Chemical Name	(S)-methyl 7-chloro-2,5-dihydro-2-[[(methoxycarbonyl)[4-(trifluoromethoxy)phenyl]amino]carbonyl]= indeno[1,2-*e*][1,3,4]oxadiazine-4a(3*H*)-carboxylate
CAS Number	173584-44-6
Formula	$C_{22}H_{17}ClF_3N_3O_7$
Molecular Weight	527.8409
Monoisotopic Weight	527.0707

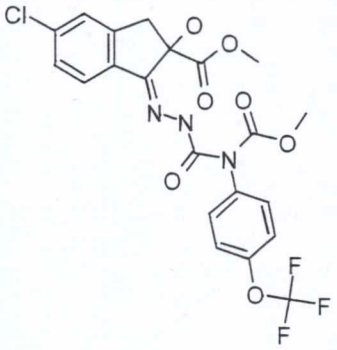
Common Name	None
Structure	
DPX Number	DPX-KN127
CAS Chemical Name	(R)-methyl 7-chloro-2,5-dihydro-2-[[(methoxycarbonyl)[4-(trifluoromethoxy)phenyl]amino]carbonyl]= indeno[1,2-*e*][1,3,4]oxadiazine-4a(3*H*)-carboxylate
Formula	$C_{22}H_{17}ClF_3N_3O_7$
Molecular Weight	527.8409
Monoisotopic Weight	527.0707

Common Name	None
Structure	
DPX Number	IN-MP819
Formula	$C_{20}H_{15}ClF_3N_3O_5$
Molecular Weight	469.8042
Monoisotopic Weight	469.0652

Common Name	None
Structure	
DPX Number	IN-MS775
Formula	$C_{18}H_{13}ClF_3N_3O_3$
Molecular Weight	411.7675
Monoisotopic Weight	411.0598

Common Name	None
Structure	
DPX Number	IN-JT333
Formula	$C_{20}H_{15}ClF_3N_3O_5$
Molecular Weight	469.8042
Monoisotopic Weight	469.0652

Common Name	None
Structure	
DPX Number	IN-JU873
Formula	$C_{19}H_{15}ClF_3N_3O_5$
Molecular Weight	457.7932
Monoisotopic Weight	457.0652

Common Name	None
Structure	
DPX Number	IN-KG433
Formula	$C_{21}H_{17}ClF_3N_3O_7$
Molecular Weight	515.8299
Monoisotopic Weight	515.0707