

## 2.0

### INTRODUCTION

Independent laboratory validation of soil monitoring methods is required by the U.S. EPA OCSP 850.6100. The subject method is applicable for the quantitation of indoxacarb and metabolites in soil and sediment, as described in DuPont-41157.

The structure, CAS name, and CAS registry numbers for IN-MP819, IN-JT333, and indoxacarb can be found in Appendix 1. The method was validated on soil samples and sediment samples.

The samples (5 g) were weighed into 50-mL polypropylene centrifuge tubes and indoxacarb and its metabolites were extracted using three sequential extractions. An aliquot from each extract was transferred into a centrifuge tube and evaporated to 5-mL under nitrogen flow. An aliquot of the extracts was transferred to an autosampler vial and diluted with 0.01-M aqueous acetic acid. Indoxacarb and metabolites were separated from co-extracts by reversed-phase Liquid Chromatography (LC) and detected by positive ion Turbospray Ionization (TSI) Mass Spectrometry/Mass Spectrometry (MS/MS). All calibration standards were prepared in a combination of 0.1% acetic acid solution and extracted control solution.

The Limit of Quantitation (LOQ) was 1.0 ppb. The Limit of Detection (LOD) was estimated to be 0.5 ppb based on the least responsive analyte IN-MP819. During method validation, acceptable recoveries were generated for soil and sediment samples fortified at the LOQ and ten times the LOQ. Quantitative analysis is reported for the two ion transitions, the quantitative ion transition and a confirmatory ion transition.

The analytical method was performed without any significant modifications. The method was successfully validated for indoxacarb and two metabolites in soil and sediment. This independent laboratory validation study demonstrated that the analytical method DuPont-41157 is acceptable for the quantitation of indoxacarb and metabolites in soil and sediment, according to guidelines set forth by US EPA Ecological Effects Guidelines, OCSPP 850.6100 "Environmental Chemistry Methods and Associated Independent Laboratory Validation,".

### 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, SHIMADZU, LC-10ADVP.

Mass Spectrometer System, API 4000 triple quadrupole mass spectrometer using a Turbo Ion Spray and Analyst version 1.4.2 software.

Pipettors, 10-50  $\mu$ L (Gilson), 20-100  $\mu$ L (Gilson) 200-1000  $\mu$ L (Gilson), 200-1000 $\mu$ L (Globaltown Pipette).

Evaporator - N-Evap<sup>®</sup> Model 112 lab oratory sample evaporator/nitrogen manifold fitted with Teflon<sup>®</sup>-coated needles .

##### Chromatographic Supplies

HPLC Column: 3.0 mm i.d.  $\times$  50 mm, 2  $\mu$ m packing, Ace Excel 2 C18-AR analytical column, Part # EXL-109-0503U (Mac Mod, Chadds Ford, PA)

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

##### Extractor

Multi-Tube Vortexer, VWR – VX-2500. (VWR Scientific Co., Bridgeport, NJ) Ceramic Balls, 1/4 inch, Catalog No. 116540034 (MP Biomedicals, LLC)



### Labware

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 with stopper, Pyrex, 15-mL capacity, Cat. No. 21048-027 (VWR Scientific Co., Bridgeport, NJ)

## 3.2

### ***Reagents and Standards***

Acetic Acid - J. T. Baker, Batch No. 0000014114

Acetonitrile (ACN) – Pharmco-Aaper<sup>®</sup>, HPLC-grade acetonitrile, Batch No. W0097048.

Methanol – J. T. Baker, HPLC-grade methanol, Batch No. 0000090324.

Water – PASC LETS# 77 (Milli-Q water), calibration due: 01/2016.

Indoxacarb (DPX-KN128-223) reference substance (99.8% pure) used for sample analysis: Analytical standard grade reagent (DuPont Crop Protection, Global Technology Division, E. I. du Pont de Nemours and Company)

IN-JT333-023 reference substance (99.6% pure) used for sample analysis: Analytical standard grade reagent (DuPont Crop Protection, Global Technology Division, E. I. du Pont de Nemours and Company).

IN-MP819-004 reference substance (97% 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 were used in this method. All appropriate material safety data sheets have been followed and proper personal protective equipment was used.

## 4.0

### **METHOD**

### 4.1

#### ***Principles of the Analytical Method***

The samples (5 g) were weighed into 50-mL polypropylene centrifuge tubes and indoxacarb and its metabolites were extracted using three sequential extractions. An aliquot from each extract was transferred into a centrifuge tube and evaporated to approximately 5-mL under nitrogen flow and diluted to 5.0-mL with acetonitrile. A 0.30-mL aliquot of the extract was diluted to 1.0-mL with 0.01-M aqueous acetic acid in an autosampler vial. Indoxacarb and metabolites were separated from co-extracts by reversed-phase Liquid Chromatography (LC) and detected by positive ion Turbospray Ionization Mass Spectrometry/Mass



Spectrometry (MS/MS).

## 4.2 *Analytical Procedure*

### 4.2.1 *Glassware and Equipment*

#### *Cleaning*

Glassware was scrubbed with a brush using a laboratory soap solution, rinsed three times with tap water, rinsed with Milli-Q water, and finally rinsed with methanol and allowed to air dry prior to each use.

Due to the tendency of indoxacarb and IN-JT333 to adhere to surfaces when in water, The stock standard volumetric flasks and other intermediate standard solution flasks were rinsed with acetonitrile prior to following normal glassware cleaning procedures.

### 4.2.2 *Preparation of Solutions*

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

**0.025% Aqueous Acetic Acid** – Added 250  $\mu\text{L}$  of acetic acid to 1000 mL of HPLC-grade water, and mixed the resulting solution to homogeneity.

**80:20 Acetonitrile: 0.025% Aqueous Acetic Acid** - Combined 800 mL of acetonitrile and 200 mL of 0.025% aqueous acetic acid, and mixed the resulting solution to homogeneity.

**90:10 Acetonitrile: 0.025% Aqueous Acetic Acid** - Combined 900 mL of acetonitrile and 100 mL of 0.025% aqueous acetic acid, and mixed the resulting solution to homogeneity.

**0.010 M aqueous acetic acid solution** - Added 600  $\mu\text{L}$  of acetic acid to 1000 mL of water and mixed the resulting solution to homogeneity.

### 4.2.3 *Preparation and Stability of Stock Standards*

*Class A volumetric flasks were used when preparing standard solutions.*

#### Indoxacarb, IN-JT333, and IN-MP819 Stock Standards

Prepared standard stock solutions of Indoxacarb, IN-JT333, and IN-MP819 by accurately weighing 10.31 (99.8%), 10.76 (97.0%) and 10.10 (99.6%) mg respectively into 100 mL volumetric flasks, and add 103.0, 104.4 and 100.6 mL of acetonitrile to each flask respectively. The procedure was to weigh 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. Using the appropriate pipette, additional amounts of HPLC-grade acetonitrile were added to each weighing to obtain an exact



concentration of 100 µg/mL. These standard solutions were stored in a freezer at approximately -20°C, and used within three months.

#### 4.2.4 Preparation and Stability of the Intermediate and Fortification

Standards Use Class A volumetric flasks when preparing standard solutions.

Prepared a 1.0-µg/mL standard in acetonitrile of Indoxacarb, IN-JT333, and IN-MP819 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 to be used within 2 weeks when stored in a refrigerator at approximately 4°C.

Prepare a 0.10-µg/mL standard in acetonitrile Indoxacarb, IN-JT333, and IN-MP819 by pipetting 1.00 mL of the 1.00-µg/mL standard into a 10-mL volumetric flask. Bring to volume using HPLC-grade acetonitrile and mix to homogeneity. The standard is to be used within 2 weeks when stored in a refrigerator at approximately 4°C.

#### 4.2.5 Preparation and Stability of Calibration Standards

All calibration standards were prepared in control matrix. In the preparation, 1.5 mL aliquot of control extract was removed and diluted to 9.0-mL using 0.1% aqueous acetic acid, and was used in the following calibration standard preparation table.

STANDARD CONCENTRATION (NG/ML)	STANDARD USED	VOLUME OF STANDARD ADDED (µL)	VOLUME OF CONTROL EXTRACT ADDED (µL)
5.0	0.1 µg/mL	50	950
2.5	0.1 µg/mL	25	975
1.0	0.1 µg/mL	10	990
0.50	5.0 ng/mL	100	900
0.25	2.5 ng/mL	100	900
0.10	1.0 ng/mL	100	900
0.050	0.50 ng/mL	100	900

These standard solutions were freshly prepared with each sample set and stored at approximately 4°C prior to use. Each of the calibration standards were mixed using a vortex mixer for 30 seconds prior to being placed on the autosampler tray. Alternative or additional standards may be prepared as needed

#### 4.2.6 Source of Samples

Soil and sediment control samples were obtained from a field test site located in the

USA. The soil and sediment characteristics are shown in the following table.

NAME	TYPE	% CLAY	% SAND	% SILT	PHW	OM (%)	REFERENCE
88 NJ 01 Nascna Soil	Loam	25	28	47	6.9	2.0	Agvise 13-311
Goose River Sediment	Clay Loam	33	28	39	7.8	6.0	Agvise 14-2475

#### 4.2.7 Storage and Preparation of Samples

Soil samples were stored frozen at approximately -20°C until use. The samples were mixed by hand prior to analysis.

#### 4.2.8 Sample Fortification Procedure

All fortifications were made directly to 5g ±0.1 samples. Fortified samples were prepared using a 0.10-µg/mL indoxacarb, IN-JT333, and IN-MP819 fortification standard.

FORTIFICATION LEVEL (µG/KG, PPB)	VOLUME OF 0.10-µG/ML MULTI-ANALYTE STANDARD (ML)
1.00	0.050
10.0	0.50

#### 4.2.9 Analyte Extraction and Purification Procedures

1. Accurately weigh 5.0-g (± 1%) of soil or sediment into 50-mL plastic centrifuge tubes. Fortify samples except reagent and controls and allow the fortification to dry in a fume hood for approximately 15-minutes. Add two 1/4" ceramic balls to each sample cap and shake the samples vigorously.
2. Add 10-mL of 80:20 acetonitrile: 0.025% aqueous acetic acid to each sample and let the sample soak for approximately 5-minutes.
3. Place samples on a Multi-Tube Vortexer and homogenized for 3 minutes at a high speed.
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 clean 50-mL centrifuge tubes. Extract the samples a second time using 10-mL of 90:10 acetonitrile: 0.025% aqueous acetic acid. Combine the two extracts into the same 50-mL centrifuge tube.



6. Extract the samples a third time using 10-mL of acetonitrile. Combine the three extracts and adjust the volume of the extracts from each sample to 30-mL using acetonitrile. Mix the extract using a vortex mixer for approximately 30 seconds.
7. Pipette 10-mL of each extract into a centrifuge tube. Add 10  $\mu$ L of concentrated acetic acid to the extracts and place the extracts on an N-EVAP nitrogen evaporator set to 40°C. Evaporate the extracts using a nitrogen flow to a volume of approximately 5-mL.
8. Adjust the volume of the extracts to 5.0-mL with acetonitrile. Place the extracts in a sonicator for approximately 5-minutes and mix the extracts using a vortex mixer for approximately 30 seconds. Transfer a 300  $\mu$ L aliquot of each extract into an auto-sampler vial and dilute with 700  $\mu$ L of 0.01 M aqueous acetic acid. Mix the extracts using a vortex mixer and analyze using LC/MS/MS.

The extracts are stable for approximately 72 hours if stored at 4°C.

### 4.3

#### *Instrumentation for the Method*

#### 4.3.1

##### Chromatography

Reversed-phase chromatography was used to separate indoxacarb and metabolites from co-extracts. The column choice reflected experimental results indicating preferred separation from co-extractants.

**Conditions used for the analysis of indoxacarb, IN-MP819 and IN-JT333:**

SYSTEM:	Shimadzu LC-10ADVP HPLC			
COLUMN:	3.0 mm i.d. x 50 mm, Ace Excel 2 C18-AR			
COLUMN TEMPERATURE:	40°C			
SAMPLE TEMPERATURE	20°C			
INJECTION VOLUME:	0.050 mL			
FLOW RATE:	0.800 mL/min			
CONDITIONS:	A: 0.01 M aqueous acetic acid			
	B: acetonitrile			
	Time	% A	% B	Flow (mL/min.)
	0.0	50	50	0.80
	1.0	50	50	0.80
	6.0	1	99	0.80
	8.0	1	99	0.80
8.1	50	50	0.80	
10.0	50	50	0.80	
INDOXACARB RETENTION TIME:	3.26 minutes			
IN-MP819 RETENTION TIME:	3.30 minutes			
IN-JT333 RETENTION TIME:	3.62 minutes			
TOTAL RUN TIME:	10.01 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
0.00-0.99	Waste
1.0-8.0	MS source
8.0-End	Waste

## 4.3.2

LC/MS/MS Analysis

The quantitative analysis of indoxacarb and metabolites was performed using an API 4000 LC/MS/MS system. The system parameters were adjusted while a solution of each analyte was infused directly into the TSI 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. A summary of the experimental conditions is provided in the following table.



PERIOD ANALYTES	IONS MONITORED	DECLUSTERING POTENTIAL (DP)	COLLISION ENERGY (CE)	EXIT POTENTIAL (CXP)
Indoxacarb	<b>528.1→ 203.0 AMU</b>	<b>60</b>	<b>53</b>	<b>15</b>
	528.1→ 150.1 AMU	60	35	15
IN-MP819	<b>470.2→ 238.1 AMU</b>	<b>60</b>	<b>18</b>	<b>15</b>
	470.2→ 206.0 AMU	60	29	15
IN-JT333	<b>470.2→ 267.1 AMU</b>	<b>60</b>	<b>15</b>	<b>15</b>
	470.2→ 207.1 AMU	60	31	15
Time:	0 – 10.01 minutes			
Ion Mode:	Positive			
Turbopray Voltage:	5500 V			
Source Temperatures:	450 C			
CUR:	15			
CAD:	6			
GS1:	60			
GS2:	65			
Dwell	0.06 Seconds (528.1→ 203.0 AMU, 528.1→ 150.1 AMU, 470.2→ 238.1 AMU, 470.2→ 267.1 AMU, 470.2 → 207.1 AMU) 0.10 Seconds (470.2 → 267.1 AMU)			

A complete list of the experimental parameters is given in Appendix 3. 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 was performed using the ion transition displayed in bold face print.** The second ion transition was used for confirmation.

#### 4.3.3

##### Calibration Procedure and Sample Analysis

A 0.050-ng/mL chromatographic standard was tested prior to the start of analyses to establish that the instrument is working properly and to insure that a signal-to-noise ratio of at least approximately 5-10 to 1 is attained. The instrument must be tuned or cleaned prior to sample analysis and the operating parameters optimized. Each ion channel used for sample analysis/quantitation has been checked to insure it is free of interference. Two controls were used to demonstrate that baseline interference is less than signal-to-noise 3:1. Begin each sample set by injecting a few solvent samples and a 0.05 ng/mL standard to insure that the instrument is at acceptable status

#### 4.4 **Calculations**

In order to more accurately calculate recovery data 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 ( $RF_{Ave}$ ) was calculated as follows:

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

$$\text{Corrected Area} = (\text{Area in the standard} - \text{Area in the control})$$

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

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

*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:

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



**5.1**      ***Method Validation Results***

The method was successfully validated on the first attempt for soil and sediment at the LOQ concentration level (1.0 ppb) and 10x LOQ (10 ppb) concentration level for all the analytes without the optional of clean-up procedure.

**5.1.1**      ***Detector Response***

Standard calibration solutions used for quantitative analysis ranged from 0.05 to 2.5 ng/mL. The instrument response during selected sample sets is provided in Appendix 2. Typical LC/MS/MS chromatograms for standards analyzed during method validation are provided in Figure 4. For all sample sets analyzed during method validation, the relative standard deviation of the response factors of the standards used was less than 20%. Control samples fortified at the LOQ and 10XLOQ were successfully extracted, cleaned-up, and analyzed using this method.

**5.1.2**      ***Unfortified Samples***

Interference peaks in unfortified sample chromatograms were less than the LOD at the retention time for indoxacarb and metabolites.

5.1.4

Limit of Quantitation and Limit of Detection

The LOQ was determined to be 1.0 ppb (1.0 µg/kg) for indoxacarb and metabolites. This quantitation limit is defined as the lowest fortification level evaluated at which acceptable average recoveries (70 - 120%, % RSD < 20%) were achieved. This quantitation limit also reflects the fortification level at which analyte peaks were consistently generated at a level approximately 10 - 20 times the signal at the retention time of each analyte in an untreated control sample for the lowest responding analyte.

An LOD was estimated for each analyte based on the signal-to-noise ratio. The LOD value should be estimated by each lab using this method. An LOD of approximately 0.5 ppb for indoxacarb and its metabolites was established at PASC using this method. The LOD is defined as the concentration of IN-MP819, the least responsive analyte, at which analyte peaks are approximately three times the chromatographic baseline noise observed near the retention time.

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***

Carryover may be observed for some autosamplers. To minimize the chance of carryover, when possible use a needle wash and insert blank samples following the injection of the high concentration standards.

**5.4**      ***Extraction Efficiency***

The extraction used closely matches the extraction used in the aerobic soil metabolism study (Reference 1).

**5.5**      ***Method Ruggedness***

**5.5.1**    ***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.

**5.5.2**    ***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***

Two LC/MS/MS ion transitions were monitored for each analyte during method validation. A detected soil residue may be confirmed by evaluating the recovery results for the second ion transition.

## 7.0

### CONCLUSIONS

This method for determination of residues of indoxacarb and metabolites in soil and sediment samples meets the U.S. EPA Ecological Effects Test Guidelines: OCSPP 850.6100: Environmental Chemistry Methods and Associated Independent Laboratory Validation and the European Commission, Directorate General Health and Consumer Protection. "Guidance Document on Residue Analytical Methods", SANCO/825/00 rev. 8.1, November 16, 2010.

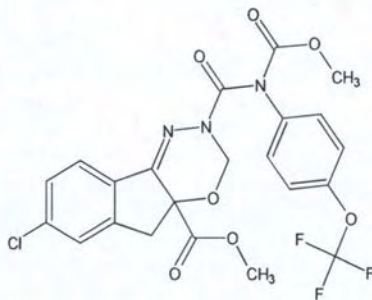
The LC/MS/MS method is free of interference above the LOD at the retention time of indoxacarb and metabolites in unfortified samples. The method generated acceptable recoveries over the concentration levels tested in soil and sediment.



## APPENDIX 1 STRUCTURE AND PROPERTIES OF INDOXACARB AND METABOLITE

### 1. Indoxacarb

Structure:



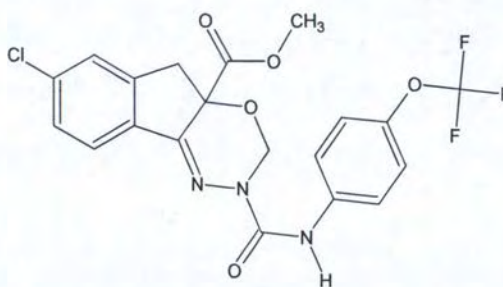
Common Name  
Chemical Abstracts Name

CAS Number  
Molecular Formula  
Molecular Mass

DPX Number DPX-KN128  
Indoxacarb  
(S)-methyl 7-chloro-2,5-dihydro-2-[[[(methoxycarbonyl)[4-(trifluoromethoxy)phenyl]amino]carbonyl]indeno  
[1,2-e][1,3,4]oxadiazine-4a(3H)-carboxylate  
173584-44-6  
 $C_{22}H_{17}ClF_3N_3O_7$   
527.84 g/mol

### 3. IN-JT333

Structure:



DPX Number  
Chemical Abstracts Name

IN-JT333  
methyl 7-chloro-2,5-dihydro-2-[[[4-(trifluoromethoxy)phenyl]amino]carbonyl]indeno[1,2-e][1,3,4]oxadiazine-4a(3H)-carboxylate

CAS Number  
Molecular Formula  
Molecular Mass

144171-39-1  
 $C_{20}H_{15}ClF_3N_3O_5$   
469.81 g/mol