INDEPENDENT LABORATORY VALIDATION OF ANALYTICAL METHOD FOR THE DETERMINATION OF INDOXACARB AND METABOLITES IN SOIL AND SEDIMENT USING LC/MS/MS

1.0 SUMMARY

The purpose of the study was to independently validate the analytical method for the determination of indoxacarb and two metabolites in soil and sediment. A validation trial of the analytical method by an independent laboratory is required before the method is submitted to the EPA. This study was designed to demonstrate the utility, ruggedness, efficiency, and any inherent weakness in the subject method as written.

The samples (5 g) were weighed into 50-mL polypropylene centrifuge tubes and indoxacarb and its metabolites were extracted using three sequential extractions. The first extraction used 10-mL of 80:20 acetonitrile: 0.025% aqueous acetic acid. The following extractions used 10-mL of 90:10 acetonitrile: 0.025% aqueous acetic acid and 10-mL of acetonitrile, respectively. A genogrinder bead mill was used to pulverize the samples during the extraction process. An aliquot of the extract was transferred into a centrifuge tube and was than evaporated to 5.0-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 (0.001 mg/kg) for all analytes. 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 samples fortified at the LOQ and ten times the LOQ.

2.0 INTRODUCTION

Independent laboratory validation of soil monitoring methods are required by the U.S. EPA OCSPP 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 µL (Gilson), 20-100 µL (Gilson) 200-1000 µL (Gilson), 200-1000µL (Globaltown Pipette).

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

Chromatographic Supplies

HPLC Column: 3.0 mm i.d. × 50 mm, 2 µm 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®, 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 µL 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 µL 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-mLvolumetric 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	pH_w	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 µL 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 µL aliquot of each extract into an auto-sampler vial and dilute with 700 µL 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. × 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	**528.1→ 203.0 AMU**	**60**	**53**	**15**
	528.1→ 150.1 AMU	60	35	15
IN-MP819	**470.2→ 238.1 AMU**	**60**	**18**	**15**
	470.2→ 206.0 AMU	60	29	15
IN-JT333	**470.2→ 267.1 AMU**	**60**	**15**	**15**
	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_{Avg}) was calculated as follows:

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

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

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

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

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:

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

4.4.2 *Example*

For a soil sample fortified with DPX-KN128 at 1.0 ppb [Date analyzed 21041216-ILV-soil, 1.0 ppb Fortification (LOQ 1 Soil)], the concentration found was calculated as follows:

Average Response Factor was calculated as follows:

$$RF_{Ave} = \frac{\begin{array}{c}(0.050\ ng/mL \div 4430) + (0.10\ ng/mL \div 9320) + \\ (0.25\ ng/mL \div 22600) + (0.50\ ng/mL \div 51900) + \\ (1.0\ ng/mL \div 109000) + (2.5\ ng/mL \div 204000) \\ + (5.0\ ng/mL \div 483000)\end{array}}{7}$$

(AC ≡ Area Counts)

$RF_{Avg} = 1.06419e^{-5}$ ng/mL/AC

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

$$ng/g\ Found = \frac{(9920\ \text{AC}) \times (1.06419\text{e}^{-5}\ ng/\text{mL/AC}) \times (1.0\ \text{mL}) \times (50)}{(5\ \text{grams})}$$

ng/g Found = 1.056

The percent recovery found was calculated as follows:

$$\%\ \text{Recovery} = \frac{(1.056\ ng/g\ )}{(1.0\ ng/g\ )} \times 100$$

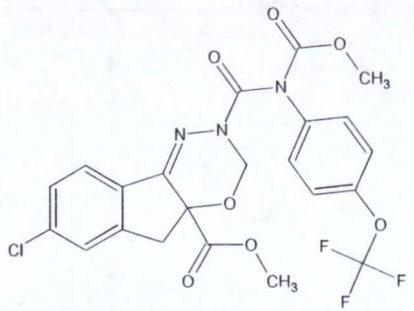
% Recovery = 106%

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

APPENDIX 1 STRUCTURE AND PROPERTIES OF INDOXACARB AND METABOLITE

1.Indoxacarb

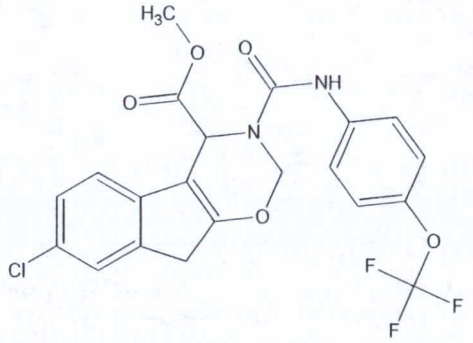
Structure:



	DPX Number DPX-KN128
Common Name	Indoxacarb
Chemical Abstracts Name	(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
CAS Number	173584-44-6
Molecular Formula	$C_{22}H_{17}ClF_3N_3O_7$
Molecular Mass	527.84 g/mol

2. IN-MP819

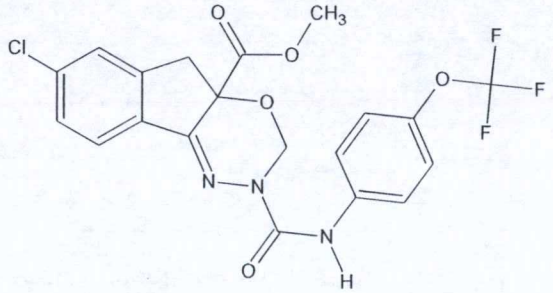
Structure:



DPX Number	IN-MP819
Chemical Abstracts Name	indeno[1,2-e][1,3,4]oxadiazine-1(2H)-carboxylic acid, 7-chloro-3,5-dihydro-2-[[[4-(trifluoromethoxy)-phenyl]amino] carbonyl]-, methyl ester
CAS Number	N/A
Molecular Formula	$C_{20}H_{15}ClF_3N_3O_5$
Molecular Mass	469.81 g/mol

3. IN-JT333

Structure:



DPX Number	IN-JT333
Chemical Abstracts Name	methyl 7-chloro-2,5-dihydro-2-[[[4-(trifluoromethoxy)phenyl]amino]carbonyl] indeno[1,2-e][1,3,4]oxadiazine-4a(3H)-carboxylate4a(3H)carboxylate
CAS Number	144171-39-1
Molecular Formula	$C_{20}H_{15}ClF_3N_3O_5$
Molecular Mass	469.81g/mol