

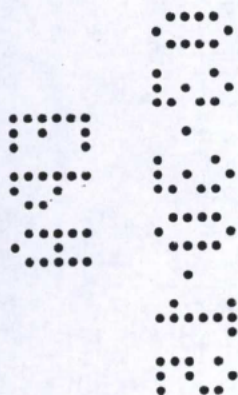
ANALYTICAL METHOD FOR THE DETERMINATION OF INDOXACARB AND METABOLITES IN SOIL AND SEDIMENT USING LC/MS/MS

Robert M. Henze and James J. Stry

1.0 SUMMARY

The purpose of this study was to develop an analytical method for the detection, quantitative analysis and confirmation of indoxacarb and metabolites IN-MP819 and IN-JT333 in soil and sediment.

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 then evaporated to 5.0-mL under nitrogen flow. An aliquot of the extracts were 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 solvent. 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 0.3 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

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

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 were 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 solvent.

The Limit of Quantitation (LOQ) was 1.0 ppb. The Limit of Detection (LOD) was estimated to be 0.3 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. Quantitative analysis is report for the two ion transition the quantitative ion transitions and a confirmatory ion transition.

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, HP1290 with temperature controlled autosampler (Agilent Technologies, Wilmington, DE)

Mass Spectrometer System, API 5000 triple quadrupole mass spectrometer using a Turbo Ion Spray and Analyst version 1.4 software (Applied Biosystems/MDS Sciex, Foster City, CA)

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 μ L Cat. No. 53495-200, 50-1000 μ L 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.

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

Genogrinder : Spex SamplePrep Model number 2000

Carbon Steel Balls, 1/4 inch, Catalog No. 00073254 (MSC Industrial Supply, Melville, NY)

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)

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.

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

Acetonitrile (ACN) - EM Omni Solv®, HPLC-grade acetonitrile, #AX0142-1 (EM Science, Gibbstown, NJ)

Methanol - EM Omni Solv®, HPLC-grade methanol, #MX0488-1 (EM Science, Gibbstown, NJ)

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

Indoxacarb (DPX-KN128) reference substance (Dash 89, 99.9% 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, 98.8% pure) used for sample analysis:
Analytical standard grade reagent (DuPont Crop Protection, Global Technology
Division, E. I. du Pont de Nemours and Company)

IN-MP819 reference substance (Dash 4, 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 are used in this method. All appropriate material safety data sheets 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 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 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 indoxacarb 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:

0.025% Aqueous Acetic Acid – Add 250 μ L of acetic acid to 1000 mL of HPLC-grade water, mix the resulting solution to homogeneity.

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

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

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

4.2.3 Preparation and Stability of Stock Standards

Use Class A volumetric flasks when preparing standard solutions.

Indoxacarb, IN-JT333 and IN-MP819 Stock Standards

Prepare standard stock solutions of Indoxacarb, IN-JT333 and IN-MP819 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 $\mu\text{g/mL}$.

4.2.4 Preparation and Stability of the Intermediate and Fortification Standards

Use Class A volumetric flasks when preparing standard solutions.

Prepare a 1.0- $\mu\text{g/mL}$ of Indoxacarb, IN-JT333 and IN-MP819 standard in acetonitrile by pipetting 1.00 mL of each of the 100.0- $\mu\text{g/mL}$ 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 2 weeks if stored in a refrigerator at approximately 4°C immediately after each use.

Prepare a 0.10- $\mu\text{g/mL}$ Indoxacarb, IN-JT333 and IN-MP819 standard in acetonitrile by pipetting 1.00 mL of the 1.00- $\mu\text{g/mL}$ standard into a 10-mL volumetric flask. Bring to volume using HPLC-grade acetonitrile and mix to homogeneity. The standard is stable for approximately 2 weeks if stored in a refrigerator at approximately 4°C immediately after each use.

Alternate or additional solutions may be prepared as needed.

4.2.5 Preparation and Stability of Calibration Standards

All calibration standards were prepared in control matrix. In order to exactly match the amount of control matrix in the calibration standard and the sample extracts a 1.5 mL aliquot of control extract was removed and diluted to 9.0-mL using 0.1% aqueous acetic acid.

STANDARD CONCENTRATION (NG/ML)	STANDARD USED	VOLUME OF STANDARD ADDED (μ L)	VOLUME OF CONTROL EXTRACT ADDED (μ L)
5.0	0.1 μ g/mL	50	950
2.5	0.1 μ g/mL	25	975
1.0	0.1 μ g/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 should be freshly prepared with each sample set and stored 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
Drummer	Silt Loam	18	23	59	6.1	3.9	L1058-130
Sassafras	Sandy Loam	8	64	28	5.4	1.7	2004-006
Goose River	Clay Loam	29	49	22	8.1	3.2	568 Goose River

4.2.7 *Storage and Preparation of Samples*

Soil samples should be 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 the 5.0-g samples. Fortified samples were prepared using a 0.10- μ g/mL indoxacarb, IN-JT333 and IN-MP819 fortification standard.

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

4.2.9 Analyte Extraction and Purification Procedures

1. Accurately measure 5.0-g ($\pm 1\%$) of soil or sediment into a 50-mL plastic centrifuge tubes. Fortify samples if necessary and allow the fortification to dry in a fume hood for approximately 15-minutes. Add two 1/4" steel 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 genogrinder and homogenize for 3 minutes at a rate of approximately 1200 strokes per minute.
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 a 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 μ L of concentrated acetic acid to the extracts and place the extracts on a 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 μ L aliquot of each extract into an auto-sampler vial and dilute with 700 μ L of 0.01 M aqueous acetic acid. Mix the extracts using a vortex mixer and analyze using LC/MS/MS.

Extracts will be stable for approximately 72 hours if stored at 4°C. **If poor recoveries occur due to LC/MS matrix effects or if co-eluting peaks are observed, an optional extract clean-up procedure is summarized in Appendix 4.**

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. Alternative chromatographic conditions can be used, provided the analytical method is validated and provides acceptable recoveries as defined by regulatory method guidelines.

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

SYSTEM:	Agilent 1290 HPLC			
COLUMN:	3.0 mm i.d. × 50 mm, Ace Excel 2 C18-AR			
COLUMN TEMPERATURE:	40°C			
SAMPLE TEMPERATURE	20°C			
INJECTION VOLUME:	0.020 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:	2.95 minutes			
IN-MP819 RETENTION TIME:	3.02 minutes			
IN-JT333 RETENTION TIME:	3.27 minutes			
TOTAL RUN TIME:	11.0 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
0.00-1.0	Waste
1.0-6.0	MS source
6.0-End	Waste

4.3.2 *LC/MS/MS Analysis*

The quantitative analysis of indoxacarb and metabolites was performed using an API 5000 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 1 ANALYTES	IONS MONITORED	DECLUSTERING POTENTIAL (DP)	COLLISION ENERGY (CE)	EXIT POTENTIAL (CXP)
Indoxacarb	528.1 → 203.0 AMU	81	55	14
	528.1→ 150.1 AMU	81	35	20
IN-MP819	470.2 → 238.1 AMU	111	21	40
	470.2→ 205.9 AMU	111	21	40
IN-JT333	470.2 → 267.1 AMU	111	19	16
	470.2→ 207.1 AMU	111	31	24
Time:	0-11 minutes			
Ion Mode:	Positive			
Turbopray Voltage:	5500 V			
Source Temperatures:	600 C			
CUR:	30			
CAD:	4			
GS1:	40			
GS2:	50			
Dwell	0.15 Seconds			

A complete list of the experimental parameters is given in Appendix 2. 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 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 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:

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

Corrected Area = (Area in the standard – Area on the control)

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

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

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:

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

4.4.2 Example

For a Sassafras sample fortified with DPX-KN128 at 1.0 ppb [Date analyzed 29-April-14, 1.0 ppb Fortification (LOQ 1 Soil)], the concentration found was calculated as follows:

Average Response Factor was calculated as follows:

$$RF_{Ave} = \frac{(0.050 \text{ ng/mL} \div 7240) + (0.10 \text{ ng/mL} \div 15400) + (0.25 \text{ ng/mL} \div 29800) + (0.50 \text{ ng/mL} \div 70300) + (1.0 \text{ ng/mL} \div 154000) + (2.5 \text{ ng/mL} \div 314000)}{6}$$

(AC \equiv Area Counts)

$$RF_{Avg} = 7.22609e^{-6} \text{ ng/mL/AC}$$

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

$$\text{ng/g Found} = \frac{(17500 - 2980 \text{ AC}) \times (7.22609e^{-6} \text{ ng/mL/AC}) \times (1.0 \text{ mL}) \times (50)}{(5 \text{ grams})}$$

$$\text{ng/g Found} = 1.049$$

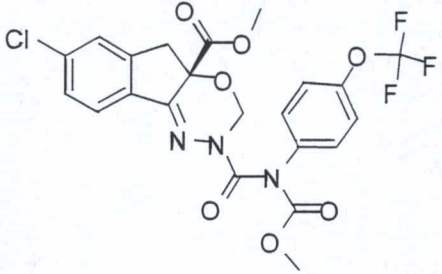
The percent recovery found was calculated as follows:

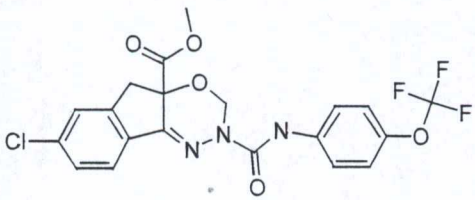
$$\% \text{ Recovery} = \frac{(1.049 \text{ ng/g})}{(1.0 \text{ ng/g})} \times 100$$

$$\% \text{ Recovery} = 105\%$$

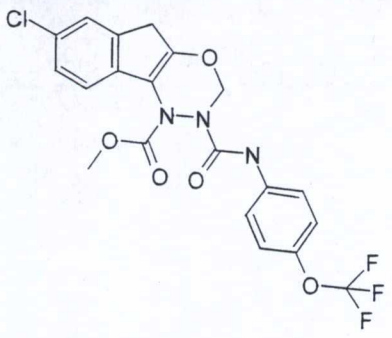
(percent recoveries are rounded to the nearest whole number in Table 1, without rounding the concentration or ppb found)

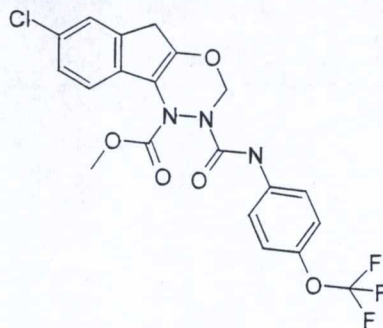
APPENDIX 1 STRUCTURE AND PROPERTIES OF INDOXACARB AND METABOLITES

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(3H)-carboxylate
CAS Number	173584-44-6
Formula	C ₂₂ H ₁₇ ClF ₃ N ₃ O ₇
Molecular Weight	527.8409
Monoisotopic Weight	527.0707

Common Name	None
Structure	
DPX Number	IN-JT333
Formula	C ₂₀ H ₁₅ ClF ₃ N ₃ O ₅
Molecular Weight	469.8042
Monoisotopic Weight	469.0652

Common Name	None
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Structure	
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DPX Number	IN-MP819
Formula	C ₂₀ H ₁₅ ClF ₃ N ₃ O ₅
Molecular Weight	469.8042
Monoisotopic Weight	469.0652

APPENDIX 4 OPTIONAL SAMPLE CLEAN-UP PROCEDURE

Analyte Extraction and Purification Procedures

1. Accurately measure 5.0-g ($\pm 1\%$) of soil or sediment into a 50-mL plastic centrifuge tubes. Fortify samples if necessary and allow the fortification to dry in a fume hood for approximately 15-minutes. Add two 1/4" steel 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 genogrinder and homogenize for 3 minutes at a rate of approximately 1200 strokes per minute.
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 a 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 a 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. Add 0.25 grams of bulk Bondesal SAX material (PN 12213042, 40 uM, 100 grams, Agilent, Wilmington, DE, Do not substitute). Shake by hand for approximately 30 seconds. Centrifuge extracts for 10-minutes at 3000 rpms.
9. 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.

Extracts will be stable for approximately 72 hours if stored at 4°C.