ABSTRACT

This report contains independent laboratory validation data for EAG Method No. 83640-M for the determination of 2,4-dichlorophenoxyacetic acid and its metabolites in soil and sediment. The method was independently validated over the concentration range of 0.0019 to 0.019 mg/kg for 2,4-D acid, and from 0.01 to 0.1 mg/kg for the metabolites 2,4-EHE, 2,4-D DMAS, 2,4-DCP, 2,4-DCA, 4-CP and 4-CPA in soil and sediment. During the independent laboratory validation of the method, the limit of quantification (LOQ) was confirmed to be 0.0019 mg/kg for 2,4-D acid and 0.01 mg/kg for each metabolite is soil and sediment.

The analytical procedure was demonstrated to be applicable for use in the determination of 2,4-D acid and its metabolites in soil and sediment matrices.

INTRODUCTION

An analytical method for the determination of 2,4-dichlorophenoxyacetic acid (2,4-D acid) and its metabolites in soil and sediment matrices, was developed and validated at EAG Laboratories, "Determination of 2,4-D Acid; 2,4-D 2-EHE; 2,4-D DMAS; 2,4-DCP; 2,4-DCA;

4-CP; and 4-CPA in Soil and/or Sediment Matrices" (Reference 1), as can be found in Appendix 1 of the Study Protocol 445G1444 (Report Appendix A). The method was found to be suitable for the determination of residues of 2,4-D acid in soil and sediment over the concentration range of 0.0019 mg/kg to 0.019 mg/kg. The method was suitable for the determination of the metabolites 2,4-dichlorophenoxyacetic acid 2-ethylhexyl ester (2,4-D EHE), 2,4-dichlorophenoxyacetic acid dimethylamine salt (2,4-D DMAS), 2,4-dichlorophenol (2,4-DCP), 2,4-dichloroanisole (2,4-DCA), 4-chlorophenol (4-CP) and 4-chlorophenoxyacetic acid (4-CPA) over the concentration range of 0.01 mg/kg to 0.1 mg/kg. The validated limit of quantitation was confirmed to be 0.0019 mg/kg for 2,4-D acid and 0.01 mg/kg for each metabolite.

An independent laboratory validation of the analytical method was conducted on soil and sediment. The study was conducted to satisfy the requirements of the United States Environmental Protection Agency (US EPA) Guideline OCSPP 850.6100 (Reference 2).

The independent laboratory, the Study Director and the analysts chosen to conduct the ILV were unfamiliar with the method, both in its development and its subsequent use in analyzing samples. The independent laboratory used all of its own equipment and supplies, so that there was no common link between EAG Laboratories and the ILV analysts. Throughout the conduct of the study, any communications between the Study Monitor and the Study Director were logged for inclusion in the report (Appendix B). No one from EAG Laboratories was allowed to visit the independent laboratory during the ILV trial to observe, offer help, or assist the chemists or technicians. These steps successfully maintained the integrity of the ILV study.

ANALYTICAL

Storage and Characterization of Control Samples

The control soil and sediment samples were provided by Dow AgroSciences, LLC. The soil and sediment selected were identified as the most difficult matrices in the original validation based on organic matter content and subsequent analytical precision and accuracy data obtained in the method validation. On arrival, the samples were placed in a freezer set to maintain a temperature of approximately -20°C where they were maintained at all time unless removed for analysis. Samples were received already ground and homogenized, so no additional processing at the Test Facility was necessary.

The control samples were characterized by Agvise Laboratories. Characterization analyses included texture (% sand/silt/clay), moisture content, pH, total nitrogen, total phosphorous, base saturation and microbial biomass. The control specimen certificates of analysis are found in Appendix C. The sample identified as M983 was the soil sample. The sediment sample was identified as M947.

Soil and Sediment Collection Sites

Soil M983:
53° 13' 9.4"N, 1° 51' 32.4"W
(British National Grid: SK09512 69147)
Grass field within Peak District National Park

Sediment M947:
Goose River, Grand Forks County, North Dakota
N47° 43, 779 W097137.312

Preparation of Stock Solutions, Calibration Standard Solutions and Fortification Solutions

The reference substance/analytical standard certificates of analysis can be found in Appendix D. Reagents used were of equivalent specifications as described in the analytical method.

The following reference substances/analytical standards were utilized during the independent laboratory method validation:

Chemical name:	2,4-dichlorophenoxyacetic acid
Common name:	2,4-D acid
CAS No.:	94-75-7
Lot number:	MORRIS/1710
Expiration date:	10/4/2018
Purity:	99.1%
Storage Conditions:	Ambient
Chemical name:	2,4-dichlorophenoxyacetic acid 2-ethylhexyl ester
Common name:	2,4-D EHE
CAS No.:	1928-43-4
Lot number:	YB1-100780-094
Expiration date:	10/20/2018
Purity:	99.3%
Storage Conditions:	Frozen
Chemical name:	(2,4-dichlorophenoxy)acetic acid dimethylamine salt
Common name:	2,4-D DMAS
CAS No.:	2008-39-1
Lot number:	V43-037861-8
Expiration date:	10/21/2017
Purity:	99.3%
Storage Conditions:	Refrigerated
Chemical name:	2,4-dichlorophenol
Common name:	2,4-DCP
CAS No.:	120-83-2
Lot number:	OCR 696-132-1
Expiration date:	10/7/2023
Purity:	100%
Storage Conditions:	Ambient
Chemical name:	4-chlorophenol
Common name:	4-CP
CAS No.:	106-48-9
Lot number:	MKBJ7452V
Expiration date:	10/7/2017
Purity:	100%
Storage Conditions:	Ambient
Chemical name:	4-chlorophenoxyacetic acid
Common name:	4-CPA
CAS No.:	122-88-3
Lot number:	089F002125
Expiration date:	10/13/2023
Purity:	100%
Storage Conditions:	Ambient

Standard stock solutions, calibration standard solutions and fortification solutions were prepared as described in the analytical method. Full details of these materials are included in the raw data package for the study along with details of the preparation of all analytical and

fortification standards prepared from the primary reference substances. The reference substances will be retained until expiration and then disposed of following relevant disposal SOP's with the approval of the Study Monitor or Sponsor.

Fortification of Recovery Samples

Soil and sediment sub-samples (10.0 g) were fortified as described below:

Analyte(s)	Replicates	Concentration (mg/kg)	Notes
2,4-D acid	1	0.00057	LOD
2,4-D EHE, 2,4-D DMAS, 2,4-DCP, 2,4-DCA	1	0.003	LOD
4-CP and 4-CPA	1	0.003	LOD (sediment only)
2,4-D acid	5	0.0019	LOQ
2,4-D 2-EHE, 2,4-D DMAS, 2,4-DCP, 2,4-DCA	5	0.01	LOQ
4-CP and 4-CPA	5	0.01	LOQ (sediment only)
2,4-D acid	5	0.019	10 x LOQ
2,4-D 2-EHE, 2,4-D DMAS, 2,4-DCP, 2,4-DCA	5	0.1	10 x LOQ
4-CP and 4-CPA	5	0.1	10 x LOQ (sediment only)
Untreated Controls	2	NA	
Reagent Blank	1	NA	

**LOD – Limit of detection
***LOQ – Limit of quantitation
NA – Not applicable

Method Principle

Soil and sediment were assayed according to EAG Method No. 83640-M.
Analytes 2,4-D acid, 2,4-D EHE, 2,4-D DMAS, 2,4-DCP and 2,4-DCA were extracted from soil; and analytes 2,4-D acid, 2,4-D EHE, 2,4-D DMAS, 2,4-DCP, 2,4-DCA, 4-CP and 4-CPA were extracted from sediment via vortexing, sonication and centrifugation in 5% acetic

acid in methanol; 50:50 5% acetic acid in methanol:5% acetic acid in water; and 5% acetic acid in water. The extracts were decanted, combined and brought to a known volume with water. The sample preparation scheme for each analyte was as follows:

Fraction A (2,4-DCA): An aliquot of the matrix extract was partitioned with iso-octane, the volume reduced under nitrogen, diluted with 0.2% peanut oil in iso-octane and submitted for GC-MSD analysis.

Fraction B (2,4-D EHE and 2,4-D DMAS): An aliquot of extract was hydrolyzed with 1N NaOH (to 2,4-D acid), acidified with 1:1 HCl;water, concentrated and cleaned up via HLB SPE, diluted with 0.1% formic acid (aq) and submitted for LC-MS/MS analysis.

Fraction C (2,4-D acid, 2,4-DCP, 4-CP and 4-CPA): An aliquot of extract was diluted with water, concentrated and cleaned up via HLB SPE, the volume reduced under nitrogen, diluted with 0.1% formic acid (aq) and submitted for LC-MS/MS analysis.

Extraction

1. Added 20 mL of 5% acetic acid in methanol to 10.0 g of soil or sediment in a 50 mL plastic centrifuge tube.
2. For recovery samples, an appropriate volume of the spiking solution was added prior to addition of the extraction solvent to obtain concentrations ranging from the LOD to 10 x the LOQ as described in the table below:

Description	Spiking Volume (mL)	Spiking Solution conc. (µg/mL)	Fortification Level (mg/kg)
Control	---	---	---
LOD	0.1	0.30 (0.057)	0.003 (0.00057)
LOQ	0.1	1.0 (0.19)	0.01 (0.0019)
10 x LOQ	0.1	10 (1.9)	0.1 (0.019)

Values in parentheses are for 2,4-D acid only.

3. The centrifuge tube was capped and vortex mixed for approximately 30 seconds.
4. Sample was sonicated for 20 minutes, assuring that the water bath was above the level of the slurry.

5. The sample was centrifuged at 2,000 rpm for 10 minutes. The supernatant extract was decanted into a 100 mL volumetric flask.
6. Added 20 mL of 50:50, 5% acetic acid in methanol:5% acetic acid in water to the sample.
7. Repeated the vortexing, sonication, centrifugation and decanting steps described above, combining the supernatant extract with the first extract in the 100 mL volumetric flasks.
8. Added 20 mL of 5% acetic acid in water to the samples.
9. Repeated the vortexing, sonication, centrifugation and decanting steps described above, combining the supernatant extracts with the previous extracts in the 100 mL volumetric flask. The flasks were stoppered and mixed. The extract was transferred to an amber glass bottle and stored in a refrigerator when not needed for analysis.

Fraction A (2,4-DCA)

1. Transferred a 20 mL aliquot of the sample extract (extraction step 9 above) to a glass culture tube.
2. Added 5 mL of iso-octane. The tube was capped and shaken by hand for about 30 seconds.
3. The sample was shaken in a horizontal position at high speed on a platform shaker for approximately 5 minutes.
4. The sample was centrifuged at 1,000 rpm for 2 minutes.
5. The top iso-octane layer was transferred to a 15 mL graduated plastic centrifuge tube.
6. Added 5 mL of iso-octane and repeated shaking and centrifugation. The top iso-octane layer was combined with the first extract in the plastic centrifuge tube.
7. The iso-octane was evaporated under a stream of nitrogen to $\leq$ 1 mL on an N-evap apparatus (heating block temperature of 40° C).
8. The volume was returned to 1 mL with iso-octane, then diluted to 2 mL with 0.2% peanut oil in iso-octane.
9. An aliquot of the sample solution was transferred to a glass vial containing a 500 µL glass insert and submitted for GC-MSD analysis.

Fraction B (2,4-D EHE and 2,4-D DMAS)

1. Transferred a 2.0 mL aliquot of the sample extract (extraction step 9 above) to a glass culture tube.
2. Added 10 mL of 1N NaOH to the tube and vortex mixed.
3. Incubated the sample in a water bath set at 40° C for 30 minutes.
4. Cooled the sample to room temperature and added 2.0 mL of 1:1 HCl:DI water. Vortex mixed and verified that pH was < 2.
5. Conditioned an HLB SPE cartridge with 3 mL of methanol followed by 3 mL of DI water.
6. Loaded the sample onto the SPE cartridge with a transfer pipet at a flow rate of 0.5-1 mL/minute. The eluent was discarded.
7. Washed the sample tube with 2.0 mL of DI water using the same transfer pipet, passing the water through the SPE cartridge. Discarded the eluent.
8. Washed the sample tube with 2.0 mL of acetonitrile using the same transfer pipet, passing the acetonitrile through the SPE cartridge. The eluent was collected in a 15 mL plastic centrifuge tube.
9. Strong vacuum was applied to recover the acetonitrile remaining on the SPE cartridge.
10. Diluted the sample to 10 mL with 0.1% formic acid (aq).
11. Transferred an aliquot of the sample to an HPLC autosampler vial for LC-MS/MS analysis.

Fraction C (2,4-D Acid; 2,4-DCP; 4-CP and 4-CPA)

1. Transferred a 2.0 mL aliquot of sample extract (extraction step 9 above) to a glass culture tube.
2. Added 12 mL of 0.1N HCl to the sample and vortex mixed.
3. Conditioned an HLB SPE cartridge with 3 mL of methanol followed by 3 mL of DI water.
4. Loaded the sample onto the SPE cartridge with a transfer pipet at a flow rate of 0.5-1 mL/minute. The eluent was discarded.
5. Washed the sample tube with 2.0 mL of DI water using the same transfer pipet, passing the water through the SPE cartridge. Discarded the eluent.

6. Washed the sample tube with 2.0 mL of acetonitrile using the same transfer pipet, passing the acetonitrile through the SPE cartridge. The eluent was collected in a 15 mL plastic centrifuge tube.
7. Strong vacuum was applied to recover the acetonitrile remaining on the SPE cartridge.
8. Reduced the sample volume to $\leq$ 1 mL on an N-evap set to approximately 40° C without allowing the sample to reach dryness.
9. Brought sample to 1 mL volume with acetonitrile and diluted to 5 mL final volume with 0.1% formic acid (aq).
10. Transferred an aliquot of the sample to an HPLC autosampler vial for LC-MS/MS analysis.

Analytical Instrumentation and Equipment

The instrumental conditions used during the ILV trial were optimized for the available instrumentation. Full instrumental conditions used are given below:

GC-MSD Parameters (2,4-DCA)

Instrumentation:	Agilent 7890 Gas Chromatograph	
	5975C XL MSD with triple-axis detection	
Column:	DB-5MS; 30 m x 0.25 mm x 0.25 µm	
Temperature Program:	Hold at 80° C for 3 min.	
	Ramp to 150° C at 10° C/min, hold 0 min.	
	Ramp to 310° C at 40° C/min. hold 5 min.	
Injector Temperature:	275° C	
Detector Temperature:	Source:	250° C
	Quad:	180° C
	Aux:	280° C
Carrier Gas:	Helium at 0.8 mL/min.	
Inlet:	Splitless	
Injection Volume:	2.0 µL	

Ions Monitored:

Analyte	Quantitation Ion	Confirmation Ion 1	Confirmation Ion 2	Dwell (msec)
2,4-DCA	178	163	161	50

LC-MS/MS Parameters (2,4-D; 2,4-DCP; and 4-CPA)

Instrumentation:	Agilent 1290 LC System API 6500 Q-Trap MS/MS Detector MDS/Sciex Analyst/MultiQuant Data system
Column Temperature:	40°C
Injection Volume:	25 µL
Column:	Phenomenex Synergi Hydro RP, 75 x 4.6 mm x 4 µm
Mobile Phase:	A: 5 mM ammonium acetate B: Methanol
Flow Rate:	1.0 mL/min

Gradient:

Time (min)	%A	%B
0.00	70	30
6.00	10	90
7.50	10	90
7.60	70	30
9.00	70	30

Typical Mass Spectrometry Operating Conditions

Ionization Mode:	APCI
Polarity:	Negative
Scan Type:	MRM
Resolution:	Q1-unit, Q3-unit
Curtain Gas (CUR):	25
Collision Gas (CAD):	High
Temperature (TEM):	500 °C

Ion Source Gas 1 (GS1): 30

Ion Source Gas 2 (GS2): 0

Entrance Potential (EP): -10

Collision Cell Exit Potential (CXP): -10

Analyte	Q1 (m/z)	Q3 (m/z)	Dwell (msec)	Declustering Potential	Collision Energy
2,4-D Acid	219	161	100	-15	-21
2,4-D Acid	221	163	100	-15	-21
2,4-DCP	161	125	200	-70	-23
2,4-DCP	163	127	200	-70	-23
4-CPA	185	127	100	-25	-19
4-CPA	187	129	100	-25	-19

2,4-D EHE and 2,4-D DMAS are detected as 2,4-D.

LC-MS/MS Parameters (4-CP)

Instrumentation: Agilent 1290 LC System
API 6500 Q-Trap MS/MS Detector
MDS/Sciex Analyst/MultiQuant Data system

Column Temperature: 40°C

Injection Volume: 20 µL

Column: Phenomenex Synergi Hydro RP,
75 x 4.6 mm x 4 µm

Mobile Phase: A: 5 mM ammonium acetate
B: Methanol

Flow Rate: 1.0 mL/min

Gradient:	Time (min)	%A	%B
	0.00	60	40
	3.00	10	90
	4.50	10	90
	4.60	60	40
	6.00	60	40

Typical Mass Spectrometry Operating Conditions

Ionization Mode:	Electrospray
Polarity:	Negative
Scan Type:	MRM
Resolution:	Q1-unit, Q3-unit
Curtain Gas (CUR):	15
Collision Gas (CAD):	High
IonSpray Voltage (IS):	-4500
Temperature (TEM):	600 °C
Ion Source Gas 1 (GS1):	70
Ion Source Gas 2 (GS2):	60
Entrance Potential (EP):	-10
Declustering Potential (DP):	-80
Collision Cell Exit Potential (CXP):	-10

Analyte	Q1 (m/z)	Q3 (m/z)	Dwell (msec)	Collision Energy
4-CP	127	91	100	-22
4-CP	127	35	100	-22

Calculation of Results

The concentration of each calibration standard in ng/mL was entered in the MultiQuant (LC-MS/MS) software or into Chemstation (GC-MSD) software in µg/mL. Linear regression calibration curves were generated by the respective software programs using 1/x weighting.

Microsoft Excel is used for subsequent concentration and recovery calculations using the formula:

$y = mx + b$

y: Peak Area m: Slope b: y-intercept x: Concentration

$x = (y - b) / m$

Example Calculations

Example: 2,4-D Acid in Soil at 0.0019 mg/kg (LOQ)

Laboratory ID: 1444-S001-S13

Set: V001-1

Transition: m/z 219 → 161

Peak Area = 21123

Regression equation: y = 164606 x + 9419.01096

Concentration (ng/mL) = (21123 – 9419.01096) / 164606 = 0.0711 ng/mL

Amount Found (mg/kg); GC-MSD =

$$\frac{\mu g/mL\ Found * Final\ volume\ (mL) * Extraction\ Volume\ (mL)}{Sample\ Weight\ (g) * Aliquot\ Volume\ (mL)}$$

Amount Found (mg/kg); LC-MS/MS =

$$\frac{ng/mL\ Found * Final\ volume\ (mL) * Extraction\ Volume\ (mL)}{Sample\ Weight\ (g) * Aliquot\ Volume\ (mL) * 1000}$$

Note: For 2,4-D EHE, multiply by the molecular weight conversion factor:

333.25 g/mol (2,4-D EHE) / 221.04 g/mol (2,4-D)

Where:

Final Volume (mL) = 2 mL for fraction A, 10 mL for fraction B and 5 mL for fraction C

Extraction Volume = 100 mL

Sample Weight = 10 g

Aliquot Volume = 20 mL for fraction A, 2 mL for fractions B and C

Example: 2,4-D Acid in Soil at 0.0019 mg/kg (LOQ)

Laboratory ID: 1444-S001-S13

Set: V001-1

Transition: m/z 219 → 161

ng/mL Found = 0.0711

Sample Weight: 9.987 g

Amount Found (mg/kg) = 0.0711 * 5 * 100 / 2 * 9.987 * 1000 = 0.0018

Fortification Level (mg/kg) =

$$\frac{\textit{Vol. of Fortification Solution (mL)} * \textit{Conc. of Fortification Solution (µg/mL)}}{\textit{Sample Volume (g)}}$$

Where:

Vol. of Fortification Solution = 0.100 mL

Conc. of Fortification Solution = 0.190 µg/mL

Sample Weight = 9.987 g

Fortification Level (mg/kg) = (0.100 * 0.190) / 9.987 = 0.00190

Recovery (%) =

$$\frac{\textit{mg/kg Found – mg/kg Found (Unfortified Control)} * 100}{\textit{Fortification Level (mg/kg)}}$$

Example: 2,4-D Acid in Soil at 0.0019 mg/kg (LOQ)

Laboratory ID: 1444-S001-S13

Set: V001-1

Transition: m/z 219 → 161

Amount Found (mg/kg): 0.0018

Amount Found Unfortified Control (mg/kg): 0.0002

Fortification Level (mg/kg): 0.0019

Recovery (%) = (0.0018 – 0.0002) / 0.0019 * 100 = 84

Statistical Treatment of Data

The mean recoveries for the fortified samples were calculated using the "AVERAGE" function of the Microsoft Excel spreadsheet computer program, which divides the sum of the selected cells by the number of determinations. The standard deviation of the recoveries for a

fortification level was calculated using the "STDEV" function of the same spreadsheet program, which sums the squares of the individual deviations from the mean, divides by the number of degrees of freedom, and extracts the square root of the quotient. Percent relative standard deviation, % RSD, was calculated by dividing the standard deviation by the mean, and then multiplying by 100.

Confirmation of Residue Identity

Confirmation was performed to demonstrate the selectivity of the primary method by monitoring one additional MRM transition (LC-MS/MS) or two additional SIM ions (GC-MSD) simultaneous to the primary detection. Untreated control matrix samples and samples fortified at the lowest level of quantitation for each analyte/matrix combination were provided to demonstrate the selectivity of the method.

Appendix 1. Analytical Method (without attachments)

EAG LABORATORIES	Industry Task Force II on 2,4-D Research Data c/o Dentons US LLP 1900 K Street NW Washington, D.C. 20006-1102	
Method No.: 83640-M	Version: 000	Supersedes: None
Effective Date: 06 January 2017		Page 1 of 196
Title: Determination of 2,4-D Acid; 2,4-D 2-EHE; 2,4-D DMAS; 2,4-DCP; 2,4-DCA; 4-CP; and 4-CPA in Soil and/or Sediment Matrices		

Prepared by/Date: Lisa Sudeen 06Jan17
ABC Management/Date: Clark D Chick 06 Jan 17
Revision History: Version 000: New method

EAG Laboratories
Method 83640-M

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1. PRINCIPLE

Analytes 2,4-D acid; 2,4-D 2-EHE; 2,4-D DMAS; 2,4-DCP; and 2,4-DCA are extracted from soil matrices; and analytes 2,4-D acid; 2,4-D 2-EHE; 2,4-D DMAS; 2,4-DCP; 2,4-DCA, 4-CP and 4-CPA are extracted from sediment via vortexing, sonication, and centrifugation in 5% acetic acid in methanol; 50:50, 5% acetic acid in methanol:5% acetic acid in water; and 5% acetic acid in water. The extracts are decanted, combined, and brought to a known volume with water. The sample preparation scheme for each analyte continues as follows:

- **FRACTION A** (2,4-DCA): An aliquot of extract is partitioned with iso-octane, the volume reduced under nitrogen blow down, diluted with 0.2% peanut oil in iso-octane, and submitted for GC-MSD analysis.
- **FRACTION B** (2,4-D 2-EHE and 2,4-D DMAS): An aliquot of extract is hydrolyzed with 1N NaOH (to 2,4-D acid), acidified with 1:1, HCl:water, concentrated and cleaned up via an HLB SPE, diluted with 0.1% formic acid (aq), and submitted for LC-MS/MS analysis.
- **FRACTION C** (2,4-D acid, 2,4-DCP, 4-CP, and 4-CPA): An aliquot of extract is diluted with water, concentrated and cleaned up via an HLB SPE, the volume reduced under nitrogen blow down, diluted with 0.1% formic acid (aq), and submitted for LC-MS/MS analysis.

The sensitivity of the method is as follows:

2,4-D

Matrix	Sample Size (g)	LOQ (ppm)	LOD (ppm)
Soil	10.00	0.0019	0.00057
Sediment	10.00	0.0019	0.00057

2,4-D 2-EHE; 2,4-D DMAS; 2,4-DCP; 2,4-DCP; 4-CP; 4-CPA

Matrix	Sample Size (g)	LOQ (ppm)	LOD (ppm)
Soil	10.00	0.010	0.003
Sediment	10.00	0.010	0.003

2. EQUIVALENCE STATEMENT

During the conduct of this analysis, comparable apparatus, solvents, glassware, and techniques may be substituted for those described in this method, except where specifically stated otherwise. In the event a substituted piece of equipment or technique is used, its use will be documented in the study records.

3. APPARATUS

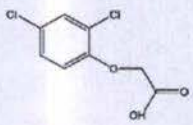
Balances:	Analytical balance capable of weighing to ± 0.1 mg Top-loading balance capable of weighing to ± 0.01 g
Bottles:	4-dram amber glass (Fisher Cat. No. 03-339-23D) with PTFE-lined screw caps (Fisher Cat. No. 02-993-42a) 4-ounce amber glass bottles (Fisher Cat. No. 02-911-904) with PTFE-lined screw caps (Fisher Cat. No. 02-993-396)
Centrifuge:	Thermo Scientific Legend XTR, bench-top
Centrifuge tubes:	Polypropylene Falcon Tubes, conical bottom, screw cap, graduated, 15-mL (Fisher Cat. No. 14-959-70C)
Columns:	HP-5MS 30 x 0.25 mm, 0.25 µm (GC-MSD) Phenomenex Synergi Hydro-RP 75 x 4.6 mm, 4µm (LC-MS/MS)
Culture tubes:	Glass, 25 × 150 mm (Fisher Cat. No. 14-933D) Glass, 16 x 125 mm (Fisher Cat. No. 14-959-35A), with PTFE-lined screw caps (Fisher Cat. No. 02-993-413)
Gas chromatograph:	Agilent 7890 GC with 5975C inert XL EI/CI MSD and Gerstel multi-purpose sampler
Gas chromatograph data system:	ChemStation G1701EA E.02.02
Glassware:	Various
Graduated mixing cylinders:	Glass, Various
Graduated cylinders:	Glass, Various
LC Vials:	Glass, 2-mL (Fisher Cat. No. 03-395C) Snap Caps (Fisher Cat. No. 03-396S) Glass, 500-µL insert (Fisher Cat. No. 03-375-3B)
Liquid chromatograph:	Shimadzu System Controller CBM-20A, Shimadzu Degasser DGU-20A3R, Shimadzu Pump LC30AD, Shimadzu Column Heater CTO-20AC, Shimadzu Autosampler SIL-30ACMP
Mass spectrometer:	AB Sciex API 6500 Q Trap
Mass spectrometer data system:	Model Analyst 1.6.2, AB Sciex

N-Evap:	Organomation Assoc., Inc. Model 112
pH paper:	pH 0-6 (Fisher Cat. No. 88-811)
Pipettes:	Glass, disposable, 25-mL serological, multipacks (Fisher Cat. No. 13-664-18D) Glass, disposable, Pasteur type (Fisher Cat. No. 13-678-20B)
Pipettors:	Set of adjustable positive-displacement pipettors, checked for accuracy and precision, and capable of delivering volumes ranging from 10-1000 µL, with disposable polypropylene tips
Shaker:	Platform Shaker
Solid-phase extraction (SPE):	Oasis HLB cartridges (60 mg, 3 mL) (Waters Corp., Milford, MA, Part No. WAT094226) SPE vacuum manifold apparatus such as Supelco Visiprep or equivalent, with waste trap and vacuum pump
Sonicator:	Branson Model 8800
Thermometer:	Digital, Traceable (Fisher Cat. No. 14-648-12)
Volumetric flasks:	Glass, Class A, various sizes
Vortexer:	Thermo Scientific Model M16715 (Fisher Cat. No. 12-815-50)

4. REFERENCE SUBSTANCES

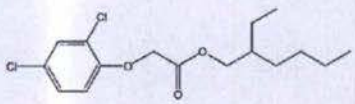
2,4-D Acid

Common Name: 2,4-D acid
Synonym:
Chemical Name: 2,4-dichlorophenoxy acetic acid
Molecular Mass: 221.04 g/mol
CAS Number: 94-75-7



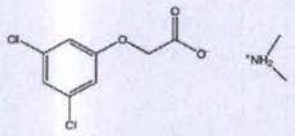
2,4-D 2-EHE

Common Name: 2,4-D 2-EHE (also known as 2,4-D EHE)
Synonym:
Chemical Name: 2,4-dichlorophenoxyacetic acid 2-ethylhexyl ester
Molecular Mass: 333.25 g/mol
CAS Number: 1928-43-4



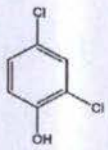
2,4-D DMAS

Common Name: 2,4-D dimethylamine salt
Synonym:
Chemical Name: (2,4-dichlorophenoxy)acetic acid dimethylamine salt
Molecular Mass: 266.12 g/mol
CAS Number: 2008-39-1



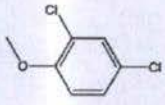
2,4-D DCP

Common Name: 2,4-DCP
Synonym:
Chemical Name: 2,4-dichlorophenol
Molecular Mass: 163.00 g/mol
CAS Number: 120-83-2



2,4-DCA

Common Name: 2,4-DCA
Synonym:
Chemical Name: 2,4-dichloroanisole
Molecular Mass: 177.03 g/mol
CAS Number: 553-82-2

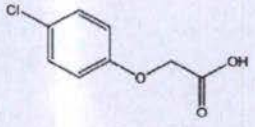


4-CP

Common Name: 4-CP
Synonym:
Chemical Name: 4-chlorophenol
Molecular Mass: 128.56 g/mol
CAS Number: 106-48-9

4-CPA

Common Name: 4-CPA
Synonym:
Chemical Name: 4-chlorophenoxyacetic acid
Molecular Mass: 186.59 g/mol
CAS Number: 122-88-3



5. SOLVENTS, REAGENTS, AND SOLUTIONS

5.1. Solvents

5.1.1. Methanol (MeOH), HPLC Grade, for LC-MS/MS (Cat. No. AH230-4, Burdick & Jackson, Seelze, Germany)

5.1.2. Methanol (MeOH), Optima Grade (Cat. No. A454-4, Fisher Scientific, Fair Lawn, NJ)

5.1.3. Iso-octane, HPLC Grade (Cat. No. O296-4, Fisher Scientific, Fair Lawn, NJ)

5.1.4. Water, HPLC Grade (Cat. No. W5-4, Fisher Scientific, Fair Lawn, NJ)

5.1.5. Acetone, HPLC Grade (Cat. No. A949-4, Fisher Scientific, Fair Lawn, NJ)

5.1.6. Acetonitrile (ACN), Optima Grade (Cat. No. A996-4, Fisher Scientific, Fair Lawn, NJ)

5.2. Reagents

5.2.1. Ammonium Acetate, HPLC Grade (Cat. No. A639-500, Fisher Scientific, Fair Lawn, NJ)

5.2.2. Glacial Acetic Acid (Cat. No. A38SI-212, Fisher Scientific, Fair Lawn, NJ)

5.2.3. Formic Acid, 99+%, Optima Grade (Cat. No. A117-50, Fisher Scientific, Fair Lawn, NJ)

5.2.4. Peanut Oil (Cat. No. AC41685, Fisher Scientific, Fair Lawn, NJ)

5.2.5. Sodium Hydroxide, Pellets, Reagent Grade (Cat. No. S318-500, Fisher Scientific, Fair Lawn, NJ)

5.2.6. Hydrochloric Acid, Concentrated (Cat. No. A144-212, Fisher Scientific, Fair Lawn, NJ)

5.3. Solutions

5.3.1. 0.1% Formic Acid (*aq*)
Add 2 L of water to a 2-L bottle and add 2.0 mL of formic acid. Cap and shake to mix. The solution may be stored at room temperature for up to 3 months. Adjust volumes accordingly for different quantities.

5.3.2. 20:80, ACN:0.1% Formic Acid (*aq*)
Add 320 mL of 0.1% formic acid (*aq*) to a 500-mL glass jar. Add 80 mL ACN to the jar. Cap and shake to mix. The solution may be stored at room temperature for up to 3 months. Adjust volumes accordingly for different quantities.

5.3.3. 1:1 HCl:Water
Add 100 mL of water to a 200-mL glass jar. Slowly add 100 mL of concentrated HCl to the jar. Cap and shake to mix. The solution may be stored at room temperature for up to 12 months. Adjust volumes accordingly for different quantities.

5.3.4. 0.1N HCl
Add 8.3 mL of concentrated HCl to a 1-L graduated mixing cylinder containing ~800 mL of water. Bring to volume with water. Cap and shake to mix. The solution may be stored at room temperature for up to 12 months. Adjust volumes accordingly for different quantities.

5.3.5. 1N Sodium Hydroxide
Weigh 40 g of sodium hydroxide and add to a 1-L graduated cylinder containing ~800 mL of water. Allow pellets to dissolve and bring to a final volume of 1000 mL with water. Transfer the solution to a 1-L plastic bottle and hand shake well to mix. The solution may be stored at room temperature for up to 12 months. Adjust volumes accordingly for different quantities.

5.3.6. 0.2% Peanut Oil in Iso-octane
Add 500 mL of iso-octane to a 500-mL glass jar. Add 1 mL of peanut oil to the jar. Cap and shake to mix. The solution may be stored at room temperature for up to 12 months. Adjust volumes accordingly for different quantities.

5.3.7. 0.1% Peanut Oil in Iso-octane
Add 100 mL of 0.2% peanut oil in iso-octane to a 500-mL glass jar. Add 100 mL of iso-octane to the jar. Cap and shake to mix. The solution may be stored at room temperature for up to 12 months. Adjust volumes accordingly for different quantities.

5.3.8. 5% Acetic Acid in Methanol (First Extraction Solution)
Add approximately 400 mL of methanol to a 1-L mixing cylinder. Add 50 mL of acetic acid to the 1-L mixing cylinder containing the methanol. Bring to a 1-L volume with methanol. Cap and shake to mix. Transfer the solution to an empty 1-L bottle. The solution may be stored at room temperature for up to 12 months. Adjust volumes accordingly for different quantities.

5.3.9. 50:50, 5% Acetic Acid in Methanol:5% Acetic Acid in Water (Second Extraction Solution)
Add ~400 mL of water to one 1-L mixing cylinder and ~400 mL of methanol to another 1-L mixing cylinder. Add 50 mL of acetic acid to each of the 1-L mixing

cylinders, containing water and the other containing methanol. Bring each to a 1-L volume, the one containing methanol with methanol and the one containing water with water. Cap and shake to mix. Combine the solutions from both 1-L mixing cylinders in an empty 2-L bottle. The solution may be stored at room temperature for up to 12 months. Adjust volumes accordingly for different quantities.

5.3.10. 5% Acetic Acid in Water (Third Extraction Solution)
Add approximately 400 mL of water to a 1-L mixing cylinder. Add 50 mL of acetic acid to the 1-L mixing cylinder. Bring to a 1-L volume with water. Cap and shake to mix. Transfer the solution to an empty 1-L bottle. The solution may be stored at room temperature for up to 3 months. Adjust volumes accordingly for different quantities.

5.3.11. 1.0M Ammonium Acetate (*aq*)
Measure approximately 50 mL of water into a 100-mL mixing cylinder. Measure and add 7.71 g of ammonium acetate to the same mixing cylinder, and bring to volume with water. Cap and shake to mix. The solution may be stored at room temperature for up to 3 months. Adjust volumes accordingly for different quantities.

5.3.12. 5mM Ammonium Acetate (*aq*)
Pipette approximately 20 mL of 1.0M ammonium acetate into a 4-L bottle of water. Cap and shake to mix. The solution may be stored at room temperature for up to 3 months. Adjust volumes accordingly for different quantities.

5.3.13. 1:1:1, Acetonitrile:Methanol:Water
Add approximately 4 L each of acetonitrile, methanol, and water to a carboy. Cap and shake to mix. The solution may be stored at room temperature for up to 12 months. Adjust volumes accordingly for different quantities.

5.3.14. 1:1:2, Acetonitrile:Methanol:Water
Add approximately 4 L each of acetonitrile, and methanol, and 8 L of water to a carboy. Cap and shake to mix. The solution may be stored at room temperature for up to 12 months. Adjust volumes accordingly for different quantities.

6. STANDARD PREPARATION

6.1. Stock Standard Solutions

6.1.1. 1000 µg/mL: Weigh approximately 10 mg of each analyte (corrected for purity) into individual 10-mL volumetric flasks. 2,4-D DMAS is weighed out as 2,4-D acid equivalent using a conversion factor of 221.04 g/mol (molecular mass of 2,4-D) / 266.12 g/mol (molecular mass of 2,4-D DMAS). Dissolve in ACN (brief sonication may be needed) and dilute to volume with ACN. Cap and vortex mix.

6.1.2. Store all stock standards at ~2-8 °C (refrigerator) in 4-dram amber glass bottles with PTFE-lined caps.

6.2. Fortification Solutions

The following dilution scheme is a suggestion only and may be scaled up or modified as needed.

2,4-D Acid; 2,4-DCP; 2,4-DCA; 4-CP; and 4-CPA

6.2.1. 100 µg/mL (19 µg/mL for 2,4-D): Pipette 1.00 mL (0.190 mL of 2,4-D) of each (2,4-DCP; 2,4-DCA; 4-CP, and 4-CPA) of the 1000-µg/mL stock standards prepared in Section 6.1.1 into a 4-dram amber bottle containing 5.81 mL of HPLC acetone. Cap and vortex mix.

6.2.2. 10 µg/mL (1.9 µg/mL for 2,4-D): Pipette 1.00 mL of the 100-µg/mL mixed standard solution prepared in Section 6.2.1 into a 4-dram amber bottle containing 9.0 mL of HPLC acetone. Cap and vortex mix.

6.2.3. 1.0 µg/mL (0.19 µg/mL for 2,4-D): Pipette 1.00 mL of the 10-µg/mL mixed standard solution prepared in Section 6.2.2 into a 4-dram amber bottle containing 9.0 mL of HPLC acetone. Cap and vortex mix.

6.2.4. 0.30 µg/mL (0.057 µg/mL for 2,4-D): Pipette 0.300 mL of the 10-µg/mL mixed standard solution prepared in Section 6.2.2 into a 4-dram amber bottle containing 9.7 mL of HPLC acetone. Cap and vortex mix.

2,4-D EHE

6.2.5. 100 µg/mL: Pipette 1.00 mL of the 1000-µg/mL 2,4-D EHE stock standard prepared in Section 6.1.1 into a 4-dram amber bottle containing 9.0 mL of HPLC acetone. Cap and mix well (vortex).

6.2.6. 10 µg/mL: Pipette 1.00 mL of the 100-µg/mL standard prepared in Section 6.2.5 into a 4-dram amber bottle containing 9.0 mL of HPLC acetone. Cap and vortex mix.

6.2.7. 1.0 µg/mL: Pipette 1.00 mL of the 10-µg/mL standard solution prepared in Section 6.2.6 into a 4-dram amber bottle containing 9.0 mL of HPLC acetone. Cap and vortex mix.

6.2.8. 0.30 µg/mL: Pipette 0.300 mL of the 10-µg/mL standard solution prepared in Section 6.2.6 into a 4-dram amber bottle containing 9.7 mL of HPLC acetone. Cap and vortex mix.

2,4-D DMAS

6.2.9. 100 µg/mL: Pipette 1.00 mL of the 1000-µg/mL 2,4-D DMAS stock standard prepared in Section 6.1.1 into a 4-dram amber bottle containing 9.0 mL of HPLC acetone. Cap and vortex mix.

6.2.10. 10 µg/mL: Pipette 1.00 mL of the 100-µg/mL standard prepared in Section 6.2.9 into a 4-dram amber bottle containing 9.0 mL of HPLC acetone. Cap and vortex mix.

6.2.11. 1.0 µg/mL: Pipette 1.00 mL of the 10-µg/mL standard solution prepared in Section 6.2.10 into a 4-dram amber bottle containing 9.0 mL of HPLC acetone. Cap and vortex mix.

6.2.12. 0.30 µg/mL: Pipette 0.300 mL of the 10-µg/mL standard solution prepared in Section 6.2.10 into a 4-dram amber bottle containing 9.7 mL of HPLC acetone. Cap and vortex mix.

6.2.13. Store all fortification standards at ~2-8 °C (refrigerator) in 4-dram amber glass bottles with PTFE-lined caps.

6.3. GC Calibration Standards (2,4-DCA, Fraction A Analysis)

The following table presents a suggested dilution scheme for the preparation of GC calibration standards. Each calibration standard contains the indicated concentration of 2,4-DCA. The 10- and 1-µg/mL standards are used only for the preparation of other standards, not for analysis. Any dilution scheme generating at least 5 non-zero calibration points spanning the needed range of concentrations may be used. Use calibrated pipettes to measure 10 mL of 0.1% peanut oil in iso-octane into 4-dram amber glass bottles. Remove from the standard bottle the indicated aliquot volumes and use the same calibrated pipette to add the corresponding amount of standard solution. Store calibration solutions at ~2-8 °C (refrigerator).

Parent Solution Concentration (µg/mL)	Aliquot Volume (mL)	Dilution Volume (mL)	Final Concentration (µg/mL)
1000	0.100	10.0	10 intermediate
10.0	1.00	10.0	1.0 intermediate
1.00	1.00	10.0	0.100
1.00	0.500	10.0	0.0500
1.00	0.250	10.0	0.0250
1.00	0.100	10.0	0.0100
0.100	0.500	10.0	0.00500
0.100	0.300	10.0	0.00300

6.4. HPLC Calibration Standards (2,4-D; 2,4-D EHE; 2,4-D DMAS; 2,4-DCP; 4-CP; and 4-CPA, Fractions B and C Analysis)

The following table presents a suggested dilution scheme for the preparation of HPLC calibration standards. Each calibration standard contains the indicated concentration of 2,4-D Acid; 2,4-D EHE; 2,4-D DMAS; 2,4-DCP; 4-CP; and 4-CPA. The 100- and 10-ng/mL standards are used only for the preparation of other standards, not for analysis. Any dilution scheme generating at least 5 non-zero calibration points spanning the needed range of concentrations may be used. Use calibrated pipettes to measure 10 mL of 20:80 ACN:0.1% formic acid (*aq*) into 4-dram amber bottles. Remove from the standard bottle the indicated aliquot volumes and use the same calibrated pipette to add the corresponding amount of standard solution. Store calibration solutions at ~2-8 °C (refrigerator) in 4-dram amber glass bottles.

Parent Solution Concentration (ng/mL)[a]	Aliquot Volume (mL)	Dilution Volume (mL)	Final Concentration (ng/mL)[a]
10 µg/mL (1.9 µg/mL)	0.100	10.0	100 (19) intermediate
100 (19)	1.00	10.0	10 (1.9) intermediate
100 (19)	0.600	10.0	6.0 (1.14)
100 (19)	0.250	10.0	2.5 (0.475)
10 (1.9)	1.00	10.0	1.0 (0.19)
10 (1.9)	0.400	10.0	0.40 (0.076)
1.0 (0.19)	1.20	10.0	0.12 (0.0228)

[a] Values in parentheses are for 2,4-D only.

7. SAMPLE PREPARATION

7.1. Fortification

7.1.1. This method is for processed (finely ground, homogeneous) samples.

7.1.2. Weigh 10 ± 0.05-g samples into 25 x 150 mm glass culture tubes.

7.1.3. Use a calibrated pipette to apply fortification solutions to the samples as appropriate. The following table indicates the fortification scheme analyzed during the method validation.

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Method 83640-M

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Matrix	Sample Type	Fortifying Compound	Fortification Level[a] (ppm)	Fortification Solution		No. of Samples
				Concentration[a] (ng/mL)	Amount Added (mL)	
Soil	Reagent Blank	N/A	0.0	N/A	N/A	1
	Control	none	0.0	N/A	N/A	1
	Fortified control	2,4-D; 2,4-DCP; 2,4-DCA	LOD: 0.003 (0.00057)	0.30 (0.057)	0.100	1
		2,4-D EHE	LOD: 0.003	0.30	0.100	1
		2,4-D DMAS	LOD: 0.003	0.30	0.100	1
		2,4-D; 2,4-DCP; 2,4-DCA	LOQ: 0.01 (0.0019)	1.0 (0.19)	0.100	5
		2,4-D EHE	LOQ: 0.01	1.0	0.100	5
		2,4-D DMAS	LOQ: 0.01	1.0	0.100	5
		2,4-D; 2,4-DCP; 2,4-DCA	10×LOQ: 0.10 (0.019)	10 (1.9)	0.100	5
		2,4-D EHE	10×LOQ: 0.10	10	0.100	5
		2,4-D DMAS	10×LOQ: 0.10	10	0.100	5
		2,4-D; 2,4-DCP; 2,4-DCA	100×LOQ: 1.0 (0.19)	100 (19)	0.100	5
		2,4-D EHE	100×LOQ: 1.0	100	0.100	5
		2,4-D DMAS	100×LOQ: 1.0	100	0.100	5
Sediment	Reagent Blank	N/A	0.0	N/A	N/A	1
	Control	none	0.0	N/A	N/A	1
	Fortified control	2,4-D; 2,4-DCP; 2,4-DCA; 4-CP; 4-CPA	LOD: 0.003 (0.00057)	0.30 (0.057)	0.100	1
		2,4-D EHE	LOD: 0.003	0.30	0.100	1
		2,4-D DMAS	LOD: 0.003	0.30	0.100	1
		2,4-D; 2,4-DCP; 2,4-DCA; 4-CP; 4-CPA	LOQ: 0.01 (0.0019)	1.0 (0.19)	0.100	5
		2,4-D EHE	LOQ: 0.01	1.0	0.100	5
		2,4-D DMAS	LOQ: 0.01	1.0	0.100	5
		2,4-D; 2,4-DCP; 2,4-DCA; 4-CP; 4-CPA	10×LOQ: 0.10 (0.019)	10 (1.9)	0.100	5
		2,4-D EHE	10×LOQ: 0.10	10	0.100	5
		2,4-D DMAS	10×LOQ: 0.10	10	0.100	5
		2,4-D; 2,4-DCP; 2,4-DCA; 4-CP; 4-CPA	100×LOQ: 1.0 (0.19)	100 (19)	0.100	5
		2,4-D EHE	100×LOQ: 1.0	100	0.100	5
		2,4-D DMAS	100×LOQ: 1.0	100	0.100	5

[a] Values in parentheses are for 2,4-D only.

7.2. Extraction

7.2.1. Add 20 mL of First Extraction Solution (5% acetic acid in methanol) to each sample. Cap the sample tube.

7.2.2. Vortex mix the sample on high speed for approximately 30 seconds.

7.2.3. Sonicate the sample for approximately 20 minutes, assuring water is at or above the level of the sample slurry.

7.2.4. Centrifuge sample at approximately 2000 rpm for approximately 10 minutes and decant supernatant into a 100-mL graduated mixing cylinder.

7.2.5. Add 20 mL of Second Extraction Solution (50:50, 5% acetic acid in methanol:5% acetic acid in water) to the sample. Cap the sample tube.

7.2.6. Repeat Steps 7.2.2 through 7.2.4, combining the supernatants in the same 100-mL mixing cylinder.

7.2.7. Add 20 mL of Third Extraction Solution (5% acetic acid in water) to the sample. Cap the sample tube.

7.2.8. Repeat Steps 7.2.2 through 7.2.4, combining the supernatants in the same 100-mL mixing cylinder.

7.2.9. Bring to 100 mL final volume with water. Cap and mix well by inversion. Transfer the extracts into 4-oz amber glass bottles for refrigerator storage.

Fraction A (2,4-DCA)

7.2.10. Transfer a 20-mL aliquot of sample from Step 7.2.9 to a 25 x 150 mm glass culture tube.

7.2.11. Add 5 mL of iso-octane to the sample and cap the sample. Shake the sample by hand for approximately 30 seconds.

7.2.12. Shake the sample in a horizontal position at high speed on a platform shaker for approximately 5 minutes.

7.2.13. Centrifuge at approximately 1000 rpm for approximately 2 minutes.

7.2.14. Transfer the iso-octane (top) layer to a 15-mL graduated polypropylene centrifuge tube.

7.2.15. Repeat Steps 7.2.11 through 7.2.14, combining the iso-octane layer in the centrifuge tube.

7.2.16. Reduce the volume of the iso-octane to ≤1 mL on an N-evap set to approximately 40 °C. (Do not allow to go to dryness.)

7.2.17. Return to 1-mL volume with iso-octane and dilute to 2 mL with 0.2% peanut oil in iso-octane. Further dilute the sample with 0.1% peanut oil in iso-octane if necessary to bring the sample concentration within the standard curve range.

7.2.18. Vial in 2-mL clear glass injection vial containing a 500-µL glass insert and submit for GC-MSD analysis.

Fraction B (2,4-D EHE and 2,4-DMAS)

7.2.10. Transfer a 2-mL aliquot of sample from Step 7.2.9 to a 25 x 150 mm glass culture tube.

7.2.11. Add 10 mL of 1N NaOH to the sample. Cap and vortex to mix.

7.2.12. Incubate for approximately 30 minutes in a water bath set at approximately 40 °C.

7.2.13. Cool sample to room temperature and add 2 mL of 1:1 HCl:water. Vortex mix and check pH: add 1:1 HCl:water as needed to achieve pH <2.

HLB SPE Cleanup

7.2.14. For each sample, place an HLB SPE cartridge onto a vacuum manifold. Using gravity, condition the SPE cartridge with one column volume (~3 mL) of methanol followed by one column volume (~3 mL) of water. Do not allow the cartridge to go dry.

7.2.15. Load the sample onto the SPE cartridge using a transfer pipette, passing the sample through the cartridge at a flow rate of 0.5-1 mL/min (discard the eluent).

7.2.16. Wash the sample tube with 2 mL of water, using the same transfer pipette, passing the water through the cartridge (discard the eluent).

7.2.17. Wash the sample tube with 2 mL of ACN, using the same transfer pipette, passing ACN through the cartridge. Collect the eluent in a 15-mL graduated polypropylene centrifuge tube.

7.2.18. Apply strong vacuum to recover any remaining eluent.

7.2.19. Dilute to 10 mL with 0.1% formic acid. Further dilute the sample with 20:80, ACN:0.1% formic acid if necessary to bring the sample concentration within the standard curve range.

7.2.20. Vial in 2-mL clear glass injection vial and submit for LC-MS/MS analysis.

Fraction C (2,4-D Acid; 2,4-DCP; 4-CP; and 4-CPA)

7.2.10. Transfer a 2-mL aliquot of sample from Step 7.2.9 to a 16 x 125 mm glass culture tube.

7.2.11. Add 12 mL of 0.1N HCl to the sample. Cap and vortex to mix the sample.

HLB SPE Cleanup

7.2.12. For each sample, place an HLB SPE cartridge onto a vacuum manifold. Using gravity, condition the SPE cartridge with one column volume (~3 mL) of methanol followed by one column volume (~3 mL) of water.

7.2.13. Load the sample onto the SPE cartridge using a transfer pipette, passing the sample through the cartridge at a flow rate of 0.5-1 mL/min (discard the eluent).

7.2.14. Wash the sample tube with 2 mL of water, using the same transfer pipette, passing water through the cartridge (discard the eluent).

7.2.15. Wash sample tubes with 2 mL of ACN, using the same transfer pipette, passing ACN through the cartridge. Collect eluent in a 15-mL graduated polypropylene centrifuge tube.

7.2.16. Apply strong vacuum to recover any remaining eluent.

7.2.17. Reduce the volume of the sample to ≤1 mL on an N-evap set to approximately 40 °C. (Do not allow to go to dryness.)

7.2.18. Return to 1-mL volume with ACN and dilute to 5 mL with 0.1% formic acid. Further dilute the sample with 20:80, ACN:0.1% formic acid if necessary to bring the sample concentration within the standard curve range.

7.2.19. Vial in a 2-mL clear glass injection vial and submit for LC-MS/MS analysis.

8. ANALYSIS

Note: The following instrument parameters have been optimized for soil and sediment. Modifications to these instrument parameters may be necessary for other matrices or instruments. Any equipment and settings that yield acceptable recoveries may be used. Record the exact conditions used for a particular run in the data package for that run.

GC-MSD Parameters (2,4-DCA)

Instrumentation:	Agilent 7890 Gas Chromatograph
Detector:	5975C inert XL EI/CI MSD with triple-axis detection
Injector:	Gerstel Multi-Purpose Sampler
Column:	HP-5MS 30 x 0.25 mm, 0.25 µm
Oven Temperature:	Hold at 80 °C for 3 min, then 80 to 150 °C at 10 °C/min, then 150 to 310 °C at 40 °C/min, then hold 5 min
Injector Temperature:	275 °C
Detector Temperature:	NCI Source: 230 °C Quad: 150 °C Thermal Aux: 280 °C
Carrier Gas:	Helium
Carrier Gas Flow Rate:	0.8 mL/min
Head Pressure:	30 psi 1 min, 6.84 psi initial
Inlet Liner:	Gooseneck splitless liner packed with CarboFrit, 4 mm i.d.
Injector Purge Delay:	1.5 min
Septum Purge:	3.0 mL/min
Injection Volume:	2.0 µL
Wash #1:	Acetone
Wash #2:	Iso-octane

Analyte	Quantitation Ion	Qualifier Ion 1	Qualifier Ion 2	Dwell time (msec)
2,4-DCA	178	161	163	100

Typical Retention Time: 2,4-DCA = ~9.6 min

LC-MS/MS Parameters (2,4-D; 2,4-DCP; and 4-CPA)

HPLC System:	Shimadzu
MS/MS System:	AB Sciex API 6500 Q Trap
Column:	Phenomenex Synergi Hydro-RP 75 x 4.6 mm, 4µm
Column Temperature:	40 °C
Injection Volume:	25 µL
Wash #1:	1:1:1 ACN:MeOH:H_2O
Wash #2:	1:1:2 ACN:MeOH:H_2O
Mobile Phase A:	5mM ammonium acetate (aq)
Mobile Phase B:	Methanol

Gradient:			
Time (min)	Flow (mL/min)	% A	% B
0.00	1.0	70	30
6.00	1.0	10	90
7.50	1.0	10	90
7.60	1.0	70	30
9.00	1.0	Stop	

Interface: APCI
Ionization Mode: Negative
Ion Source Parameters:
Curtain Gas: 20
Source Temperature: 500
GS1 (Nebulizing Gas): 30
GS2 (Auxiliary Gas): 0
Entrance Potential: -10
Collision Gas Pressure: High
Collision Cell Exit Potential: -10

Analyte	Q1 *m/z*	Q3 *m/z*	Dwell time (msec)	Declustering Potential	Collision Energy
2,4-D Acid	219	161	100	-15	-21
2,4-D Acid a	221	163	100	-15	-21
2,4-DCP	161	125	200	-70	-23
2,4-DCP a	163	127	200	-70	-23
4-CPA	185	127	100	-25	-19
4-CPA a	187	129	100	-25	-19

Typical Retention Times: 2,4-D = ~2.9 min
2,4-DCP = ~5.2 min
4-CPA = ~2.0 min

Note: 2,4-D EHE and; 2,4-D DMAS are detected as 2,4-D.

LC-MS/MS Parameters (4-CP)

HPLC System: Shimadzu
MS/MS System: AB Sciex API 6500 Q Trap
Column: Phenomenex Synergi Hydro-RP 75 x 4.6 mm, 4µm
Column Temperature: 40 °C
Injection Volume: 35 µL
Wash #1: 1:1:1 ACN:MeOH:H_2O
Wash #2: 1:1:2 ACN:MeOH:H_2O
Mobile Phase A: 5mM ammonium acetate (aq)
Mobile Phase B: Methanol

Gradient:			
Time (min)	Flow (mL/min)	% A	% B
0.00	1.0	60	40
3.00	1.0	10	90
4.50	1.0	10	90
4.60	1.0	60	40
6.00	1.0	Stop	

Interface: Electrospray
Ionization Mode: Negative
Ion Source Parameters:
Curtain Gas: 15
Spray Voltage: -4500
Source Temperature: 600
GS1 (Nebulizing Gas): 70
GS2 (Auxiliary Gas): 60
Entrance Potential: -10
Collision Gas Pressure: High
Declustering Potential: -80
Collision Cell Exit Potential: -10

Analyte	Q1 *m/z*	Q3 *m/z*	Dwell time (msec)	Collision Energy
4-CP	127	91	100	-22
4-CP a	127	35	100	-36
4-CP b	129	91	100	-22
4-CP c	129	37	100	-36

Typical Retention Times: 4-CP – ~2.7 min

9. CALCULATIONS

9.2. The concentration of each calibration standard in ng/mL is input into the Analyst software (LC-MS/MS) or into the ChemStation (GC-MSD) software in µg/mL when the run is set up. Area counts from the GC-MSD ChemStation software are transferred into an Excel spreadsheet to generate the regression curve.

9.3. Both software programs use regression curves (usually linear with 1/x weighting) to convert the observed chromatographic peak areas to sample analyte concentrations in ng/mL or µg/mL. Correlation coefficient (r) should be >0.990.

All standards injected and their corresponding peak responses were entered into the program to create the standard curve. Weighting (1/x) was used.

The equation used for the least squares fit is:

$$Y = \text{slope} \times X - \text{intercept}$$

Y = detector response (peak area) for each analyte

X = analyte concentration in the sample in ng/mL or µg/mL

$$X = \frac{Y - \text{intercept}}{\text{slope}} = \text{ng/mL or } \mu\text{g/mL}$$

9.4. For each terminal analyte, convert the analyte concentration found in the sample to analyte concentration in the raw commodity sample using the following calculation:

GC-MSD:

$$\textit{sample ppm} = \frac{\mu g/mL\ Found \times Final\ Volume\ (mL) \times Extract\ Volume\ (mL) \times DF}{Sample\ Wt\ (g) \times Aliquot\ Volume\ (mL)}$$

LC-MS/MS:

$$\textit{sample ppm} = \frac{ng/mL\ Found \times Final\ Volume\ (mL) \times Extract\ Volume\ (mL) \times DF}{Sample\ Wt\ (g) \times Aliquot\ Volume\ (mL) \times Conversion\ Factor}$$

Note: For 2,4-D EHE, multiply by the molecular mass conversion factor [333.25 g/mol (2,4-D EHE) / 221.04 g/mol (2,4-D) 1.50765]

where:

µg/mL Found	=	analyte concentration found in the GC-MSD sample
ng/mL Found	=	analyte concentration found in the LC-MS/MS sample
Final Volume (mL)	=	total volume of the LC-MS/MS or GC-MSD sample (2 mL for Fraction A; 10 mL for Fraction B; 5 mL for Fraction C)
Extract Volume (mL)	=	volume of total extract (100 mL)
Sample Wt (g)	=	amount of sample weighed for analysis (10 g)
Aliquot Volume (mL)		volume of extract taken for cleanup and analysis (20 mL for Fraction A; 2 mL for Fraction B; 2 mL for Fraction C)
DF	=	Dilution Factor

Conversion Factor = 1000 (ppb to ppm)

Procedural recovery data from fortified samples are calculated via the following equation:

$$Percentage\ Recovery = \frac{ppm\ found - ppm\ found\ in\ control}{ppm\ added} \times 100$$

10. REFERENCES

1. B.A. Sorenson, P. (1994). *Determination of 2,4-Dichlorophenoxyacetic Acid 2-Ethylhexyl Ester (2,4-D 2-EHE), 2,4-Dichlorophenoxyacetic Acid Dimethylamine Salt (2,4-D DMAS) as its 2,4-Dichlorophenoxyacetic Acid (2,4-D) Equivalent, 2,4-D, 2,4-Dichlorophenol (2,4-DCP), 2,4-Dichloroanisole (2,4-DCA), 4-Chlorophenol (4-CP), and 4-Chlorophenoxyacetic Acid (4-CPA) in Soil Sediment Samples by Gas Chromatography with Mass Selective Detection. Method Number QMAM94006 (1994).* Industry Task Force II on 2,4-D Research Data.

2. N.L. Steed and J.S.C. Chang, P. (1994). *Determination of 2,4-Dichlorophenoxyacetic Acid 2-Ethylhexyl Ester, 2,4-Dichlorophenoxyacetic Acid Dimethylamine Salt, as its 2,4-D Acid Equivalent, 2,4-Dichlorophenoxyacetic Acid, 2,4-Dichlorophenol, and 2,4-Dichloroanisole in Soil by Gas Chromatography/Mass Selective Detection. Method Number RAM 8862-93-001 (1994).* Battelle Health Division, Residue and Product Chemistry.