Method Validation Study for the Determination of Residues of (2,4-dichlorophenoxy)acetic acid and its Metabolites in Surface Water, Ground Water and Drinking Water

INTRODUCTION

Scope

This method is applicable for the quantitative determination of residues of (2,4-dichlorophenoxy)acetic acid (2,4-D), 2,4-dichlorophenol (2,4-DCP), 4-chlorophenol and 2,4-dichloroanisole (2,4-DCA) in surface water, ground water and drinking water. The method was validated over the concentration range of 0.10-5.0 µg/L with a validated limit of quantitation of 0.10 µg/L for each analyte. Common and chemical names, molecular formulas, and the nominal masses for the analyte and related compounds are given in Table 1.

This study was conducted to fulfill data requirements outlined in the U. S. EPA Residue Chemistry Test Guidelines, OPPTS 850.7100 (1), the European Commission Guidance Document on Residue Analytical Methods, SANCO/825/00 rev. 8.1 (2) and SANCO/3029/99 rev. 4 (3), and PMRA Residue Chemistry Guidelines as Regulatory Directive Dir 98-02 (4).

Method Principle

Residues of (2,4-dichlorophenoxy)acetic acid and its 2,4-dichlorophenol and 4-chlorophenol metabolites are prepared for analysis by adding 1.0 mL of 2 N hydrochloric acid to the sample aliquot. The samples are shaken for 30 minutes and then purified using an Oasis MCX solid-phase extraction (SPE) column. After elution from the SPE column with an acetonitrile:methanol (80:20) solution containing 0.1% acetic acid, the eluate is concentrated to approximately 500 µL and then diluted to 1.0 mL with a water solution containing 0.1% acetic acid. The resulting sample is analyzed for 2,4-D and 2,4-DCP by liquid chromatography with negative-ion APCI ionization tandem mass spectrometry (LC-MS/MS) and analyzed for 4-chlorophenol by liquid chromatography with negative-ion electrospray ionization tandem mass spectrometry (LC-MS/MS).

Residues of 2,4-dichloroanisole are prepared by adding 1.0 mL of 1 N hydrochloric acid to the sample aliquot and an appropriate volume of isooctane extraction solution. The samples are shaken for 30 minutes, centrifuged, and a portion of the top, organic layer is analyzed by electron impact gas chromatography with mass spectrometry (GC-MS).

Safety Precautions

Each analyst must be acquainted with the potential hazards of the equipment, reagents, products, solvents, and procedures used in this method before commencing laboratory work. Sources of information include: operation manuals, Material Safety Data Sheets, literature and other related date. Safety information should be obtained from the supplier. Disposal of waste materials, reagents, reactants, and solvents must be in compliance with applicable governmental requirements.

Acetone, acetonitrile, isooctane and methanol are flammable and should be used in well-ventilated areas away from ignition sources. Acetic acid and hydrochloric acid are corrosive and can cause severe burns. It is imperative that proper eye and personal protection equipment be worn when handling these reagents.

Test Substance/Analytical Standard

Analytical Standard	TSN Number	Percent Purity	Date of Certification	Reference
2,4-D	AGR275828	99.5%	21-Jan-2008	FAPC07-152704
2,4-DCP	AGR182992	>99%	21-Apr-2011	FAPC11-278475
2,4-DCA	TSN028154-0001	100%	28-Jan-2011	FAPC10-275580
4-chlorophenol	TSN100174	99.7%	20-Nov-2008	FAPC08-195891
2,4-D 2-EHE[a]	TSN027750-0024	99.3%	23-Nov-2010	DECO ML AL-2010-015106REV

[a] 2,4-dichlorophenoxy aceticacid 2-ethylhexyl ester; used to validate hydrolysis step of procedure

The Certificates of Analysis for the test substance can be found in Figure 1-Figures 5. The test substances may be obtained from the Test Substance Coordinator, Dow AgroSciences LLC, 9330 Zionsville Road, Building 304, Indianapolis, IN 46268-1054.

Equipment, Glassware, and Materials

Equipment, glassware, materials, reagents, and chemicals considered to be equivalent to those specified may be substituted with the understanding that their performance must be confirmed by appropriate tests. Common laboratory glassware and supplies are assumed to be readily available. Unless specified otherwise, class A volumetric glassware is used to prepare analytical standards, fortification solutions, and calibration standards.

Laboratory Equipment

Balance, analytical, Model AE100, Mettler-Toledo, Inc.

Balance, pan, Model BB2440, Mettler-Toledo, Inc.

Centrifuge, with rotor to accommodate 8-oz wide-mouth bottles, Model Legend XFR, Thermo International Equipment Company.

Pipet, positive-displacement, 100-1000 µL capacity, catalog number M1000, Gilson Inc.

Pipet, positive-displacement, 50-250 µL capacity, catalog number M250, Gilson Inc.

Pipet, positive-displacement, 3-25 µL capacity, catalog number M25, Gilson Inc.

Repeater, positive-displacement, 1-25 mL capacity, catalog number 022260201, Eppendorf.

Shaker, variable speed reciprocating with box carrier, Model 6000, Eberbach Corporation.

Vacuum manifold, Model spe-12G, Mallinckrodt Baker, Inc.

Vortex mixer, Model G-560, Scientific Industries, Inc.

Chromatographic System

Column, analytical, Synergi Hydro-RP, 4.6 mm x 75 mm, 4.0-µm particle size, catalog number 00C-4375-E0, Phenomenex.

Column, Agilent J&W Scientific DB-5ms GC column, 30 m x 0.25 mm, catalog number 122-5533, Agilent Technologies.

Gas chromatograph, 6890 Series, Agilent Technologies.

Gas purifier, catalog number G1999-80410, Agilent Technologies.

Gas purifier, catalog number OT3-2, Agilent Technologies.

Injector, automatic, Model 7683, Agilent Technologies.

Inlet liner, splitless, double gooseneck, catalog number 5181-3315, Agilent Technologies.

Liquid chromatograph, Model 1290, Agilent Technologies.

Liquid chromatograph, Reliance, Spark Holland.

Mass spectrometer, Model QTRAP 5500, MDS/Sciex.

Mass spectrometer, Model API5000, MDS/Sciex.

Mass spectrometer, 5973 Mass Selective Detector, Agilent Technologies.

Mass spectrometer data system, Model Analyst 1.5.1, MDS/Sciex

Mass spectrometer data system, Model G1701CA, Agilent Technologies.

Glassware and Materials

Column, Oasis MCX, 60-mg sorbent, 3-mL reservoir, catalog number 186000254, Waters.

MicroSert, for autosampler vial, catalog number C4012-465, National Scientific Company.

Pipet tip, positive-displacement, 1000-µL capacity, catalog number CP1000, Gilson Inc.

Pipet tip, positive-displacement, 250-µL capacity, catalog number CP250, Gilson Inc.

Pipet tip, positive-displacement, 25-µL capacity, catalog number CP25, Gilson Inc.

Pipet tip, positive-displacement, 5 mL capacity, catalog number 022266403, Eppendorf.

Pipet tip, positive-displacement, 10 mL capacity, catalog number 022266501, Eppendorf.

Vial, autosampler, 2-mL, catalog number C4000-1W, National Scientific Company.

Vial, 3-dram, with PTFE-lined screw cap, catalog number 60940A-12, Kimble Glass Company.

Vial, 16x100 mm, catalog number 73770-16100, Kimble Glass Company.

Vial cap, for autosampler vial, catalog number C4000-55B, National Scientific Company.

Reagents

Acetic acid, glacial, ACS plus grade, catalog number A38S-500, Fisher Scientific.

Acetone, HPLC grade, catalog number 439126, Sigma Aldrich.

Acetonitrile, HPLC grade, catalog number 34998, Sigma Aldrich.

Helium, gas, 99.995% purity, BOC Gases, New Providence, NJ 07974

Hydrochloric acid, 2 N, certified concentration, catalog number RH170500, Fisher Scientific.

Hydrochloric acid, 1 N, certified concentration, catalog number SA48-1, Fisher Scientific.

Hydrochloric acid, 0.1 N, certified concentration, catalog number SA54-1, Fisher Scientific.

Isooctane, HPLC grade, catalog number 0296SK-1, Fisher Scientific.

Methanol, Chromasolv HPLC grade, catalog number 34860, Sigma Aldrich.

Nitrogen, refrigerated liquid, catalog number, LQNI, BOC Gases, New Providence, NJ 07974.

Water, Chromasolv HPLC grade, catalog number 270733, Sigma Aldrich.

Prepared Solutions

Acetonitrile:methanol (80:20) solution containing 0.1% acetic acid (v:v)

Add 3200 mL of acetonitrile to 800 mL methanol in an empty 4 L glass bottle. Pipet 4 mL acetic acid to the mixture. Cap and mix thoroughly.

water containing 0.1% acetic acid

Pipet 2.0 mL of acetic acid into a 2000-mL volumetric flask containing approximately 500 mL of HPLC water. Dilute to volume with HPLC water. Mix thoroughly.

50% acetonitrile:methanol (80:20) with 0.1% acetic acid and 50% water with 0.1% acetic acid (v:v)

Add 500 mL of acetonitrile:methanol (80:20) with 0.1% acetic acid to 500 mL of water with 0.1% acetic acid. Cap and mix thoroughly.

EXPERIMENTAL

Instrumental Conditions

Typical HPLC Operating Conditions (for determination of 2,4-D and 2,4-DCP)

Instrumentation:	Spark Holland Reliance LC system Applied Biosystems API5000 LC-MS/MS System MDS/Sciex Analyst 1.5.1 data system
Column:	Synergi Hydro-RP 4.6 x 75 mm, 4-µm
Injection Volume:	10 µL
Needle height:	5 mm
Autosampler wash:	700 µL acetonitrile:methanol (80:20) containing 0.1% acetic acid
Run Time:	Approximately 6.0 minutes
Mobile Phase:	A – Water containing 0.1% acetic acid B – Acetonitile:methanol (80:20) containing 0.1% acetic acid

Flow Rate:	1000 µL/min		
Gradient:	Time, min:sec	Solvent A, %	Solvent B, %
	00:01	45	55
	06:01	45	55

Flow Diverter Program: 1) 0.0 to 1.5 min: flow to waste
2) 1.5 to 5.5 min: flow to source
3) 5.5 to end of the run: flow to waste

Typical Mass Spectrometry Operating Conditions (for determination of 2,4-D and 2,4-DCP)

Interface:	Heated Nebulizer
Polarity:	Negative
Scan Type:	MRM
Resolution:	Q1 – unit, Q3 – unit
Curtain Gas (CUR):	30 psi
Collision Gas (CAD):	4.0 (Medium)
Temperature (TEM):	450°C
Ion Source Gas 1 (GS1):	40 psi
Nebulizer Current:	-4
Acquisition Time	4.0 min
Dwell Time:	50 ms

Analytes:	Precursor Ion Q1	Product Ion Q3	Declustering Potential	Collision Energy	Cell Exit Potential
	m/z	*m/z*	V	V	V
2,4-D (219/161)	218.985	160.800	-40 V	-22 V	-17 V
2,4-D (221/163)	220.968	163.000	-40 V	-22 V	-15 V
2,4-DCP (161/125)	160.953	125.000	-40 V	-24 V	-15 V
2,4-DCP (163/127)	162.952	127.000	-40 V	-24 V	-11 V

Typical HPLC Operating Conditions (for determination of 4-chlorophenol)

Instrumentation:	Agilent 1290 Infinity LC system Applied Biosystems QTRAP 5500 LC-MS/MS System MDS/Sciex Analyst 1.5.1 data system
Column:	Synergi Hydro-RP 4.6 x 75 mm, 4-µm
Injection Volume:	10 µL
Flush time:	10 sec
Needle height:	5 mm
Needle wash:	Acetonitrile:methanol (80:20) containing 0.1% acetic acid – 10 seconds
Run Time:	Approximately 12.5 minutes
Mobile Phase:	A – Water containing 0.1% acetic acid B – Acetonitile:methanol (80:20) containing 0.1% acetic acid

Flow Rate: 1000 µL/min (approx 200 µL/min split to source)

Gradient:

Time, min:sec	Solvent A, %	Solvent B, %
00:01	80	20
04:50	0	100
07:50	0	100
08:00	80	20
12:50	80	20

Flow Diverter Program:
1) 0.0 to 1.5 min: flow to waste
2) 1.5 to 7.5 min: flow to source
3) 7.5 to end of the run: flow to waste

Typical Mass Spectrometry Operating Conditions (for determination of 4-chlorophenol)

Interface:	Electrospray
Polarity:	Negative
Scan Type:	MRM
Resolution:	Q1 – unit, Q3 – unit
Curtain Gas (CUR):	30 psi
Collision Gas (CAD):	Medium
Temperature (TEM):	500°C
Ion Source Gas 1 (GS1):	60 psi
Ion Source Gas 2 (GS2):	50 psi
Acquisition Time	6.0 min
IonSpray Voltage (IS):	-4500 volts
Dwell Time:	50 ms

Analytes:	Precursor Ion Q1	Product Ion Q3	Declustering Potential	Collision Energy	Cell Exit Potential
	m/z	*m/z*	V	V	V
4-CP (127/91)	126.920	90.948	-80 V	-24 V	-9 V
4-CP (129/91)	128.941	90.943	-75 V	-24 V	-17 V

Typical GC Operating Conditions (for the determination of 2,4-DCA)

Instrumentation:	Agilent Model 6890A gas chromatograph Agilent Model 7683 autoinjector Agilent Model 5973N mass spectrometer Agilent Model G1701CA data system
Column:	J & W fused silica capillary Durabond-5MS liquid phase 30 m x 0.25 mm i.d. 0.25-µm film thickness

Oven Method:	
Column	80 °C for 1.2 min 80 °C to 320 °C at 20 °C/min 320 °C for 2.0 min
Front inlet	50 °C for 0.1 min 50 °C to 280 °C at 1500 °C/min 280 °C for 5.0 min
Cryo	Use temp: 90 °C Timeout: 10 min Pressure: 67.8 kPa Purge flow: 50 ml/min Purge time: 1.2 min Total flow: 53.8 mL/min
Transfer Line	280 °C
Carrier Gas method:	Helium
Constant Flow	1.0 ml/min
Vacuum Compensation	On
Initial Head Pressure	~70 kPa
Linear Velocity	~36 cm/s
Injection Method:	Splitless
Injector Temperature	280 °C
Purge Flow	50 mL/min
Injection Volume	1 µL
Detector Mode:	Electron impact
Source Temperature	230 °C
Quad Temperature	150 °C
Calibration Program	Electron impact (low mass) autotune
Electron Multiplier	1705.9 volts
SIM Resolution	High
Dwell Time	50 msec

Ions Monitored:

2,4-DCA	*m/z* 176 (quantitation)
	m/z 178 (confirmation 1)
	m/z 161 (confirmation 2)

Full-scan and product-ion mass spectra for 2,4-dichlorophenoxyacetic acid and metabolites are shown in Figures 6-9.

Typical calibration curves for the determination of 2,4-D, 2,4-DCP, 4-chlorophenol and 2,4-DCA are shown in Figure 10, Figure 11, Figure 12 and Figure 13, respectively.

Representative spectra for a reagent blank and calibration standards used in the determination of 2,4-D and 2,4-DCP are shown in Figure 14 and Figure 15, respectively. Typical chromatograms for the determination of 2,4-D and 2,4-DCP in water are illustrated in Figures 16-18.

Representative spectra for a reagent blank and calibration standards used in the determination of 4-chlorophenol are shown in Figure 19. Typical chromatograms for the determination of 4-chlorophenol in water are illustrated in Figures 20-22.

Representative spectra for a reagent blank and calibration standards used in the determination of 2,4-DCA are shown in Figure 23 and Figure 24, respectively. Typical chromatograms for the determination of 2,4-DCA in water are illustrated in Figures 25-27.

Preparation of Standard Solutions

Preparation of (2,4-dichlorophenoxy)acetic acid Stock Solutions

Weigh 0.0500 g of (2,4-dichlorophenoxy)acetic acid, 2,4-dichlorophenol, and 4-chlorophenol analytical standards separately and quantitatively transfer to separate 50-mL volumetric flasks with methanol. Dilute to volume with methanol to obtain separate 1000-µg/mL stock solutions of each analyte.

Pipet 200 µL of each 1000-µg/mL solution from into a single 20-mL volumetric flask and dilute to volume with methanol to obtain a mixed solution containing 10.0-µg/mL of each analyte.

Pipet 200 µL of the 10.0-µg/mL solution into a 20-mL volumetric flask and dilute to volume with methanol to obtain a mixed solution containing 0.10-µg/mL of each analyte.

Weigh 0.0500 g of 2,4-dichloroanisole analytical standard and quantitatively transfer to 50-mL volumetric flask with methanol. Dilute to volume with methanol to obtain a 1000-µg/mL stock solution of 2,4-dichloroanisole.

Pipet 250 µL of the 1000-µg/mL solution into a 25-mL volumetric flask and dilute to volume with acetone to obtain a solution containing 10.0-µg/mL 2,4-dichloranisole.

Pipet 250 µL of the 10.0-µg/mL solution into a 25-mL volumetric flask and dilute to volume with acetone to obtain a solution containing 0.10-µg/mL of 2,4-dichloroanisole.

Preparation of Calibration Standard Solutions

Prepare calibration standards in separate 10-mL volumetric flasks by dispensing the appropriate amount of mixed (2,4-D, 2,4-DCP and 4-chlorophenol) spiking solution into the flasks. Dilute calibration standards to volume with a solution containing 50% acetonitrile:methanol (80:20) with 0.1% acetic acid and 50% water with 0.1% acetic acid.

Concentration of Stock Solution	Aliquot of Stock Solution	Final Solution Volume	Calibration Solution Final Conc.
µg/mL	µL	mL	ng/mL
0.10	120	10.0	1.2
0.10	250	10.0	2.5
0.10	400	10.0	4.0
0.10	1000	10.0	10
10	25.0	10.0	25
10	40.0	10.0	40
10	50.0	10.0	50

Prepare calibration standards in separate 10-mL volumetric flasks by dispensing the appropriate amount of 2,4-DCA spiking solution into the flasks. Dilute calibration standards to volume with isooctane.

Concentration of Stock Solution	Aliquot of Stock Solution	Final Solution Volume	Calibration Solution Final Conc.
µg/mL	µL	mL	ng/mL
0.10	120	10.0	1.2
0.10	250	10.0	2.5
0.10	400	10.0	4.0
0.10	1000	10.0	10
10	25.0	10.0	25
10	40.0	10.0	40
10	50.0	10.0	50

Sample Origin, Numbering, Preparation and Storage

Untreated control samples of the water sources were obtained from the Dow AgroSciences LLC Sample Management Group. All samples were tracked in the Dow AgroSciences LLC Regulatory Labs Information Management System (RLIMS) database. Unique sample numbers were assigned to the samples to track them during receipt, storage, and analysis. Complete source documentation is included in the study file.

Sample Group Number	Water Type	pH	Hardness (mg equiv. $CaCO_3$/L)	Total Suspended Solids (ppm)	Alkalinity (mg $CaCO_3$/L)	Total Organic Carbon (ppm)	Dissolved Organic Carbon (ppm)
001	(Pond) Surface Water	8.0	135	8	80	8.0	6.9
002	(Well) Ground Water	8.2	330	6	183	3.4	2.2
003	(Tap) Drinking Water	8.6	4	8	292	3.1	2.6

No sample preparation was required for the water samples prior to analysis. During the course of the study, the samples were stored in temperature-monitored refrigerators at approximately 4 °C, except when removed for analysis.

Analysis Procedure

Sample Analysis of 2,4-D, 2,4-DCP and 4-chlorophenol by LC-MS/MS

1. Measure 40 ±0.4 ml portions of sample into 11 dram (45-mL) glass vials.

2. For preparing fortified samples, add an appropriate volume aliquot of the appropriate spiking solutions to encompass the necessary concentration range:

Concentration of Fortified Sample (µg/L)	Volume of Spiking Solution (µL)	Concentration of Spiking Solution (µg/mL)	Equivalent Concentration (ng/mL)
0.03	12	0.10 µg/mL	1.2
0.10	40	0.10 µg/mL	4.0
1.0	400	0.10 µg/mL	40
5.0	20	10 µg/mL	200

3. Add 1.0 mL of 2 N HCl to the sample vial.

4. Shake the sample for a minimum of 30 minutes on a reciprocating shaker at approximately 180 excursions/minute.

5. Clean up samples on the Oasis MCX SPE cartridge using the following procedure:

   a. Place an Oasis MCX SPE cartridge (60-mg, 3-mL) on a vacuum manifold box.

   b. Condition the SPE cartridge with 1 mL of methanol followed by <u>two</u> 1 mL aliquots of 0.1 N HCl, discarding the eluates. Apply full vacuum (approximately -25 inches Hg) for about 10 seconds between solvent additions.

   c. Transfer the entire acidified sample to the SPE cartridge. Pull the sample through the SPE cartridge at approximately 1 mL/min, using vacuum if necessary. Discard the eluate. Apply full vacuum to the cartridge for about 10 seconds after the sample load volume has eluted.

   d. Wash the SPE cartridge with 1 mL of 0.1 N HCl. Discard the eluate. Dry the cartridge under full vacuum for approximately 30 seconds.

e. Elute 2,4-D and its metabolites from the SPE cartridge with two 500µL aliquots of a acetonitrile:methanol (80:20) solution containing 0.1% acetic acid at a rate of approximately 1 mL/min, using vacuum if necessary. Collect the two aliquots in the same glass tube. Apply full vacuum (approximately 25 inches of Hg) for about 10 seconds between solvent additions.

6. Concentrate the sample to approximately 500 µL using an N-Evap evaporator set at 40 °C and a nitrogen flow rate of approximately 500 mL/min (Do NOT concentrate the 5.0 µg/L spiked samples).

7. For all samples, adjust the volume in the sample vial to 1.0 mL with approximately 500 µL of a water solution containing 0.1% acetic acid.

   For samples fortified with 5.0 µg/L, add 4.0 mL of water solution containing 0.1% acetic acid to the 1.0 mL of eluate to bring the sample to a total of 5.0 mL volume.

8. Cap the sample vial and vortex mix for 3-4 seconds.

9. Transfer a portion of the sample to an autosampler vial containing a limited insert.

10. Analyze the samples and calibration standards for determination of 2,4-D and 2,4-DCP by LC-MS/MS with negative-ion APCI tandem mass spectrometry and for determination of 4-chlorophenol by LC-MS/MS with negative-ion electrospray mass spectrometry. Determine the suitability of the chromatographic system using the following performance criteria:

    a. Standard curve linearity: Determine that the correlation coefficient equals or exceeds 0.995 for the least squares equation which describes the detector response as a function of standard curve concentration.

    b. Peak resolution: Visually determine that sufficient resolution has been achieved for the analyte and internal standard relative to background interferences.

    c. Appearance of chromatograms: Visually determine the chromatograms with respect to peak response, baseline noise, and background interference. Visually determine that a minimum signal-to-noise ratio of 10:1 has been attained for each analyte in the 4.0-ng/mL calibration standard.

11. Re-analyze the samples which contain concentrations of 2,4-D or any of its metabolites greater than 80% of the highest standard following the dilution in Step 7. A concentration range shall be covered from 30% of the LOQ to 20% above the highest sample concentration.

Sample Analysis of 2,4-DCA by GC-MS

1. Measure 40 ±0.4 mL portions of sample into 11 dram (45 mL) glass vials.

2. For preparing fortified samples, add an appropriate volume aliquot of the appropriate spiking solutions to encompass the necessary concentration range:

Concentration of Fortified Sample (µg/L)	Volume of Spiking Solution (µL)	Concentration of Spiking Solution (µg/mL)	Equivalent Concentration (ng/mL)
0.03	12	0.10 µg/mL	1.2
0.10	40	0.10 µg/mL	4.0
1.0	400	0.10 µg/mL	40
5.0	20	10 µg/mL	200

3. Add 1.0 mL of 1 N HCl to the sample vial.

4. For samples fortified at 5.0 µg/L, add 5.0 mL of isooctane extraction solution.

   For all other samples, add 1.0 mL of isooctane extraction solution to the sample and cap vial.

5. Shake the sample for a minimum of 30 minutes on a reciprocating shaker at approximately 180 excursions/minute.

6. Centrifuge the sample vial for 5 minutes at 2000 rpm.

7. Using a Pasteur pipet, transfer a portion of the top layer into a limited insert vial and cap.

8. Analyze the samples and calibration standards for determination of 2,4-DCA by electron-impact GC-MS. Determine the suitability of the chromatographic system using the following performance criteria:

   a. Standard curve linearity: Determine that the correlation coefficient equals or exceeds 0.995 for the least squares equation which describes the detector response as a function of standard curve concentration.

   b. Peak resolution: Visually determine that sufficient resolution has been achieved for the analyte and internal standard relative to background interferences.

   c. Appearance of chromatograms: Visually determine the chromatograms with respect to peak response, baseline noise, and background interference. Visually determine

that a minimum signal-to-noise ratio of 10:1 has been attained for each analyte in the 4.0-ng/mL calibration standard.

9. Re-analyze the samples which contain concentrations of 2,4-DCA greater than 80% of the highest standard following the dilution in Step 4. A concentration range shall be covered from 30% of the LOQ to 20% above the highest sample concentration.

Calculations

Inject the series of calibration standards using the conditions listed in the instrument section and determine the peak areas for each analyte as indicated below.

(2,4-dichlorophenoxy)acetic acid	*m/z* Q1/Q3 219/161 (quantitation) *m/z* Q1/Q3 221/163 (confirmation)
2,4-dichlorophenol	*m/z* Q1/Q3 161/125 (quantitation) *m/z* Q1/Q3 163/127 (confirmation)
4-chlorophenol	*m/z* Q1/Q3 127/91 (quantitation) *m/z* Q1/Q3 129/91 (confirmation)
2,4-dichloroanisole	*m/z* 176 (quantitation) *m/z* 178 (confirmation 1) *m/z* 161 (confirmation 2)

Prepare a standard curve by plotting the analyte concentration on the abscissa (x-axis) and the respective peak area on the ordinate (y-axis). Using regression analysis, determine the equation for the curve with respect to the abscissa. Determination of net concentration and calculation of percent recovery for each analyte is done similarly; therefore, only a single example calculation will be presented below.

For example, using linear regression with 1/x weighting, sample, 110504-001-0001A22 + 0.10 µg/L, from analytical set 110504 S07:

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

$$X = \left(\frac{\text{Analyte peak area - intercept}}{\text{slope}}\right)$$

$$\text{2,4-D (ng/mL)} = \left(\frac{\text{2,4-D peak area - intercept}}{\text{slope}}\right)$$

$$\begin{matrix}\text{2,4 - D} \\ \text{(gross ng/mL)}\end{matrix} = \left(\frac{49100 - 564.849}{11689.7}\right)$$

Calculation of Percent Recovery

Determine the gross concentration in each recovery sample by substituting the quantitative peak area obtained into the above equation and solving for the concentration.

$$\begin{matrix}\text{2,4 - D} \\ \text{(gross ng/mL)}\end{matrix} = \left(\frac{\text{2,4 - D peak area - intercept}}{\text{slope}}\right)$$

$$\begin{matrix}\text{2,4 - D} \\ \text{(gross ng/mL)}\end{matrix} = \left(\frac{49100 - 564.849}{11689.7}\right)$$

$$\begin{matrix}\text{2,4 - D} \\ \text{(gross)}\end{matrix} = 4.152 \text{ ng/mL}$$

Convert the concentration (ng/mL) of the analyte found in the prepared extract to the concentration (µg/L) of the analyte found in the original sample as follows:

$$\begin{matrix}\text{2,4 - D} \\ \text{(gross µg/L)}\end{matrix} = \begin{matrix}\text{2,4 - D} \\ \text{(gross ng/mL)}\end{matrix} \times (\text{MF} \times \text{DF} \times \text{UC})^{A}$$

[A] MF = method factor; DF = dilution factor; UC = unit conversion

$$\text{Where MF} = \frac{\text{Final Volume} \times \text{Extraction Solution Volume}}{\text{Aliquot Factor} \times \text{Nominal Weight}}$$

$$\underset{(\text{gross } \mu g/L)}{2,4\text{-D}} = \underset{(\text{gross ng/mL})}{2,4\text{-D}} \times \left(0.025 \times 1 \times \frac{1.0\,\mu g/L}{1.0\,\text{ng/mL}} \right)$$

$$\underset{(\text{gross } \mu g/L)}{2,4\text{-D}} = 4.152\ \text{ng/mL} \times 0.025\ \frac{\text{mL} \times \mu g}{\text{L} \times \text{ng}}$$

$$\underset{(\text{gross})}{2,4\text{-D}} = 0.1038\ \mu g/L$$

Determine the net concentration in each recovery sample by subtracting any contribution found at the retention time of the analyte in the untreated control sample from that of the gross analyte concentration found in the recovery samples.

$$\underset{(\text{net } \mu g/L)}{2,4\text{-D}} = \underset{(\text{gross } \mu g/L)}{2,4\text{-D}} - \underset{(\text{control } \mu g/L)}{2,4\text{-D}}$$

$$\underset{(\text{net } \mu g/L)}{2,4\text{-D}} = 0.1038\,\mu g/L - 0.0000\,\mu g/L$$

$$\underset{(\text{net})}{2,4\text{-D}} = 0.1038\ \mu g/L$$

Determine the percent recovery by dividing the net concentration of each recovery sample by the theoretical concentration added.

$$\text{Recovery} = \frac{\text{Concentration Found}}{\text{Concentration Added}} \times 100\%$$

$$\text{Recovery} = \frac{0.1038\,\mu g/L}{0.1000\,\mu g/L} \times 100\%$$

$$\text{Recovery} = 104\%$$

Confirmation of Residue Identity

The method is selective for the determination of (2,4-dichlorophenoxy)acetic acid and its metabolites by virtue of the chromatographic separation and selective detection system used. To demonstrate further confirmation for 2,4-D, 2,4-DCP and 4-chlorophenol, an additional MS/MS ion transition can be monitored, and for 2,4-DCA, the resulting peak area ratio from selected-ion monitoring of three ions during GC-MS analysis can be calculated.

1. Prepare samples for analysis by following the steps of the Analysis Procedure section.

2. Transfer a portion of the sample to a 2-mL autosampler vial and firmly seal the vial with a cap.

3. Analyze the calibration standards interspersed with the samples as described in the Instrumental Conditions section. Determine the peak areas for each analyte as indicated below.

(2,4-dichlorophenoxy)acetic acid	*m/z* Q1/Q3 219/161 (quantitation) *m/z* Q1/Q3 221/163 (confirmation)
2,4-dichlorophenol	*m/z* Q1/Q3 161/125 (quantitation) *m/z* Q1/Q3 163/127 (confirmation)
4-chlorophenol	*m/z* Q1/Q3 127/91 (quantitation) *m/z* Q1/Q3 129/91 (confirmation)
2,4-dichloroanisole	*m/z* 176 (quantitation) *m/z* 178 (confirmation 1) *m/z* 161 (confirmation 2)

4. For each standard, calculate the confirmation ratio. Use the average confirmation ratio of all standards in the analytical set to confirm the presence of the analyte in the water samples.

For example, using the data for 2,4-D from the 4.0-ng/mL standard in Figure 15:

$$\text{Confirmation Ratio} = \frac{\text{peak area of confirmation ion transition}}{\text{peak area of quantitation ion transition}}$$

$$\text{Confirmation Ratio} = \frac{\text{Confirmation peak area at } m/z \text{ 221/163}}{\text{Quantitation peak area at } m/z \text{ 219/161}}$$

$$\text{Confirmation Ratio} = \frac{35332}{49968}$$

$$\text{Confirmation Ratio} = 0.7071$$

Confirmation ratio deviation is calculated as a percent difference relative to the average confirmation ratio found for the standards.

For example, using the data for 2,4-dichlorophenoxyacetic acid from sample 110504-001-0001A22 + 0.10 µg/L in Figure 16 and the average confirmation ratio found for the standards for analytical set 110504 S07:

$$\text{Confirmation Ratio Difference} = \frac{\text{Confirmation Ratio - Avg. Conf. Ratio}}{\text{Avg. Conf. Ratio}} \times 100\%$$

Where Avg. Conf. Ratio = Average Confirmation Ratio of the Standards

$$\text{Confirmation Ratio Difference} = \frac{0.7520 - 0.7275}{0.7275} \times 100\%$$

$$\text{Confirmation Ratio Difference} = 0.0337 \times 100\%$$

$$\text{Confirmation Ratio Difference} = 3.36\%$$

Confirmation of the presence of the analyte is indicated when the retention time of the samples matches that of the standards and the confirmation ratio is in the range of ±20% of the average found for the standards.

Validation of Hydrolysis

For method validation, the procedure was designed to measure residues of 2,4-D EHE (2-ethylhexyl ester) as 2,4-D (free acid). An additional step is required to base hydrolyze 2,4-D esters in order to quantitate residues as 2,4-D acid. The completion of this hydrolysis was tested to demonstrate complete conversion to 2,4-D (free acid) during sample analysis. Representative water matrix samples were spiked in duplicate with 0.10 µg/L a.e. of a 2,4-D EHE spiking solution and analyzed in parallel with duplicate water samples spiked with 0.10 µg/L 2,4-D (acid). Samples were analyzed according to the Analysis Procedure (for determination of 2,4-D, 2,4-DCP and 4-chlorophenol) with an additional hydrolysis step (addition of 1.0 mL of 2 N sodium hydroxide and a 20 minute shake, prior to the addition of 1.0 mL of 2 N HCl – Step 3) and then evaluated, monitoring the amount of 2,4-D (acid) present, following LC-MS/MS analysis.

The results are detailed in the raw study file and are summarized in Table 41. Results from the determination of the hydrolysis step demonstrate an average conversion from 2,4-D EHE (2-ethylhxyl ester) to 2,4-D (free acid) of 77%, for the quantitation transition, in the analysis of water samples using this procedure.

Calculated Limits of Quantitation and Detection

The limits of detection (LOD) and quantitation (LOQ) were proposed at the initiation of the study at 0.03 µg/L and 0.10 µg/L, respectively. Following established guidelines (6), the LOQ and LOD for the determination of residues of 2,4-D and its metabolites in surface water, ground water and drinking water were calculated using the standard deviation derived from the 0.10-µg/L recovery results. The LOQ was calculated as ten times the standard deviation (10*s*), and the LOD was calculated as three times the standard deviation (3*s*) of the 0.10 µg/L recovery results. The results are summarized in Table 42. The results showed that the calculated LOD and LOQ were either lower than 0.03 µg/L and 0.10 µg/L, or not significantly higher than 0.03 µg/L and 0.10 µg/L, respectively. The results supported the limits of detection and quantitation established for the study.

In actual residue samples, numerical results should be reported as less than the LOQ for residues that are equal to or above the LOD but less than the validated LOQ, indicating that the results are being reported at a lower confidence level. For residues less than the LOD, results should be reported as not detected.

Standardization of SPE Elution Profile

There is a possibility that variation in the Oasis MCX SPE columns may influence the elution profile of 2,4-D and its metabolites. If it is necessary to obtain an elution profile for each lot of SPE columns used to ensure optimum recovery and clean-up efficiency, the following procedure can be used:

1. For profiling with sample matrix present, weigh 40 ± 0.4 mL of untreated control samples into individual 11 dram glass vials with caps. (Note: Drinking water was used for profiling with matrix present during method development.)

2. Fortify samples with 40 µL of the 0.10 µg/mL mixed spiking solution (containing 2,4-D, 2,4-DCP and 4-chlorophenol).

3. Add 1.0 mL of 2 N HCl to the sample vial.

4. Shake the sample for a minimum of 30 minutes on a reciprocating shaker at approximately 180 excursions/minute.

5. Clean up samples on the Oasis MCX SPE cartridge using the following procedure:

    a. Place an Oasis MCX SPE cartridge (60-mg, 3-mL) on a vacuum manifold box.

    b. Condition the SPE cartridge with 1 mL of methanol followed by two 1 mL aliquots of 0.1 N HCl, discarding the eluates. Apply full vacuum (approximately -25 inches Hg) for about 10 seconds between solvent additions.

c. Transfer the entire acidified sample to the SPE cartridge. Pull the sample through the SPE cartridge at approximately 1 mL/min, using vacuum if necessary. Discard the eluate. Apply full vacuum to the cartridge for about 10 seconds after the sample load volume has eluted.

d. Wash the SPE cartridge with 1 mL of 0.1 N HCl. Discard the eluate. Dry the cartridge under full vacuum for approximately 30 seconds.

e. Elute 2,4-D and its metabolites from the SPE cartridge with six 500µL aliquots of a acetonitrile:methanol (80:20) solution containing 0.1% acetic acid at a rate of approximately 1 mL/min, using vacuum if necessary. Collect the six eluates in separate glass tubes. Apply full vacuum (approximately 25 inches of Hg) for about 10 seconds between solvent additions.

6. Add 500 µL of a water solution containing 0.1% acetic acid.

7. Cap the sample vial and vortex mix for 3-4 seconds.

8. Transfer a portion of the sample to an autosampler vial containing a limited insert.

9. Analyze the samples and calibration standards for determination of 2.4-D and 2,4-DCP by LC-MS/MS with negative-ion APCI tandem mass spectrometry and for determination of 4-chlorophenol by LC-MS/MS with negative-ion electrospray mass spectrometry as described in the Instrumental Conditions section.

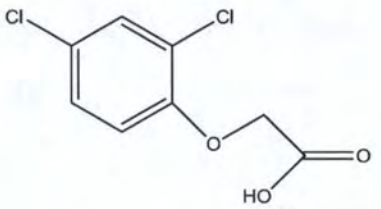
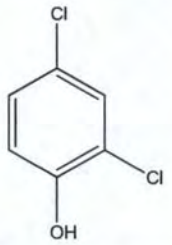
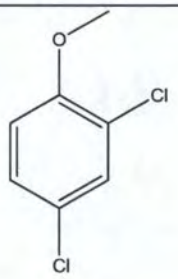
10. Calculate the percent recovery as described in the Calculations section.

A typical elution profile is illustrated in Figure 28. If the elution profile differs from that shown, adjust the volume of the acetonitrile:methanol (80:20) solution containing 0.1% acetic acid to be collected in Step 5.e. of the Analysis Procedure section.

Supplemental Notes

1. The instrumental conditions may be modified to obtain optimal chromatographic separation and sensitivity.

Table 1. Identity and Structures of (2,4-dichlorophenoxy)acetic acid and Related Compounds

Identifying information	Chemical Structure
(2,4-dichlorophenoxy)acetic acid (2,4-D) IUPAC name: (2,4-dichlorophenoxy)acetic acid Chemical Formula: $C_8H_6Cl_2O_3$ Molecular Weight: 221.04 Nominal Mass: 220 CAS Number: 94-75-7	
2,4-dichlorophenol (2,4-DCP) IUPAC name: 2,4-dichlorophenol Chemical Formula: $C_6H_4Cl_2O$ Molecular Weight: 163.0 Nominal Mass: 162 CAS Number: 120-83-2	
2,4-dichloroanisole (2,4-DCA) IUPAC name: 2,4-dichloroanisole Chemical Formula: $C_7H_6Cl_2O$ Molecular Weight: 177.03 Nominal Mass: 176 CAS Number: 553-82-2	
4-chlorophenol IUPAC name: 4-chlorophenol Chemical Formula: C_6H_5ClO Molecular Weight: 128.56 Nominal Mass: 128 CAS Number: 106-48-9