2.0 INTRODUCTION AND BACKGROUND

Analytical method, AU-287R0 was used for determination of propanil and 3,4-DCA in drinking and surface waters. Freshly fortified samples were analyzed using an LC/MS/MS with MRM quantitation *m/z 218.056→161.900* for propanil and *m/z 162.014→127.000* for 3,4 DCA. The confirmatory *m/z 218.056 →126.900* for propanil and *m/z 162.014→74.000* for 3,4 DCA were used.

3.0 SAFETY

The chemicals used in this study were treated as potential health hazards and exposure to these chemicals were minimized. The analyst is responsible for maintaining awareness of OSHA (Occupational Safety and Health Administration) regulations regarding the safe handling of the chemicals used in this method. A reference file of safety data sheets (SDS) should be available to all personnel involved in the chemical analyses, as well as GHS (Globally Harmonized System) SDS training if required.

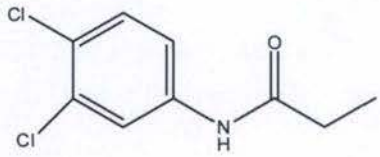
4.0 MATERIALS

4.1. Test and Reference Substance Identification

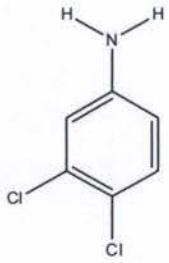
The characterization of Propanil and 3, 4-Dichloroaniline are summarized as follows:

Common Name:	Propanil
IUPAC Chemical Name:	N-(3, 4-dichlorophenyl) propanamide
CAS Registry No.:	709-98-8
Molecular Weight:	218.08 $g \cdot mol^{-1}$
Molecular Formula:	$C_9H_9Cl_2NO$
Batch No.:	SZBF166XV
Date Received:	May 3, 2017
Reassay Date:	May 3, 2018
Purity:	99.7%

Storage Condition:	Ambient
Source:	Sigma-Aldrich
JRFA No.:	SAS-130-J
Structure:	



Common Name:	**3,4-DCA**
IUPAC Chemical Name:	3,4-dichloroaniline
CAS Registry No.:	95-76-1
Molecular Weight:	162.02 $g \cdot mol^{-1}$
Molecular Formula:	$C_6H_5Cl_2N$
Batch No.:	13509KQV
Date Received:	January 1, 2016
Reassay Date:	September 1, 2018
Purity:	99.3%
Storage Condition:	Ambient
Source:	Sigma-Aldrich
JRFA No.:	SAS-130-M
Structure:	



4.2. Test Matrices

The matrices were selected to be representative of typical surface and drinking waters, and were collected as per guideline requirements. They were stored at 4°C. Information such as collection location and characteristics are included in the raw data. The test matrices are drinking water (Aquafina) and surface water from Skippack Creek in Collegeville, PA (coordinates 40.150077, -75.447406).

5.0 APPARATUS AND EQUIPMENT

5.1. Laboratory Glassware

Test tubes, glass, 20-mL

Vials and caps, autosampler, 2-mL, screw cap

Screw cap test tubes, 10, 15, and 20 mL
Graduated cylinders, 25, 50, 100 mL
Class A glassware (volumetric pipettes, flasks, graduated cylinders class A, etc.)

5.2. Laboratory Equipment

Pipettes, air-displacement, adjustable volume, Eppendorf
SNs: R10117C, 117127A, R32379C, 3105819, J201536C, R32413C

Balance, analytical, capable of weighing to the nearest 0.1 mg, Mettler Toledo AT 200, K51405

Syringe filters, polytetrafluoroethylene (PTFE), 0.45-µm, Agilent

5.3. Chromatographic System and Detector Options

Column: Phenomenex Luna® 3 µm C18(2) 100 Å ,100 x 2.0mm
(Part: 00D-4251-B0; Serial Number 680-335-15)

Liquid Chromatography System, Shimadzu UFLCXR
Mass Spectrometer, Sciex4000 API MS
Mass Spectrometer Data system, Analyst Software, version 1.6.3 or equivalent

The following instrument options were used during this study:

Analysis	Mass Spec Detector	HPLC System
Method Validation	SciEx 4000 API MS	Shimadzu UFLC XR
Extract Stability	SciEx 4000 API MS	Shimadzu UFLC XR

5.4. Reagents

Solvents and other reagents were LC/MS grade or better
Acetic Acid, Glacial, J.T. Baker, Avantor Performance Materials, Lot #9508-03, Q5289
Methanol, EMD Millipore Corporation, Lot # 57181, 57135, R080731
Acetonitrile, EMD Millipore Corporation, Lot # 56110
Water, EMD Millipore Corporation, Lot # 57156, 57200

5.5. Reagents and Materials to be Prepared

Mobile Phase A –0.1% acetic acid in water:

Transfer 1.00 mL of acetic acid to 1000-mL volumetric flask. Bring to volume with water.
Mobile Phase B – 0.1% acetic acid in MeOH:

Transfer 1.00 mL of acetic acid to 1000-mL volumetric flask. Bring to volume with methanol.

5.6. Stock Solutions

Propanil stock solution was prepared in LC-MS grade acetonitrile, whereas 3, 4-Dichloroaniline (3,4-DCA) stock standard solution was prepared in LC-MS grade methanol. The following is an example for preparing 100 mL of a 55,600 µg/L 3,4-DCA stock standard.

1. A mass of 0.0056 g of 3, 4-Dichloroaniline reference standard is weighed (adjusted for purity of 99.3%) and transferred to a 0.100 L class A volumetric flask.
2. Fill the volumetric flask halfway with methanol and agitate gently (sonicate if necessary) until standard is completely dissolved.
3. Dilute to volume with methanol and mix by inverting several times.
4. Calculate the exact concentration using the exact weight and purity, for example:

$$\left(\frac{0.0056\text{g}*0.993}{0.100\text{ L}}\right)*\left(\frac{10^6\mu\text{g}}{\text{g}}\right)= 55600\ \mu\text{g/L}$$

The following is an example for preparing 100 mL of a 125,000µg/L propanil stock standard.

1. A mass of 0.0125 g of propanil reference standard is weighed (adjusted for purity of 99.7%) and transferred to a 0.100 L class A volumetric flask.
2. Fill the volumetric flask halfway with methanol and agitate gently (sonicate if necessary) until standard is completely dissolved.
3. Dilute to volume with methanol and mix by inverting several times.
4. Calculate the exact concentration using the exact weight and purity, for example:

$$\left(\frac{0.0125\text{g}*0.997}{0.100\text{ L}}\right)*\left(\frac{10^6\mu\text{g}}{\text{g}}\right)= 125000\ \mu\text{g/L}$$

The table below lists the stock solutions prepared and used during this study.

Analyte ID	JRFA ID	Purity	Weight (mg)[1]	Final Volume (mL)	Concentration (µg/L)
Propanil	JRFA-444/75-1	99.7%	12.5	100	125000
3,4 DCA	JRFA-536/1-1	99.3%	5.56	100	55600

[1] Corrected for purity

5.7. Preparation of Fortification Solutions

Sample fortification solutions were prepared by serial dilution of the stock standard in drinking and surface waters. The table below lists the fortification solutions prepared and used during this study.

Analyte ID	Initial JRFA ID	Volume (mL)	Initial Concentration (ug/L)	Final Volume (mL)	Concentration (µg/L)	Final JRFA ID
Propanil	JRFA-444/75-1	0.800	125000	100	1000	JRFA-536/2-1[A]
3,4-DCA	JRFA-536/1-1	1.800	55600	100		
Mixed	JRFA-536/2-1[A]	10.0	1000	100	100	JRFA-536/3-1[A]
Propanil	JRFA-444/75-1	0.800	125000	100	1000	JRFA-536/2-8[B]
3,4-DCA	JRFA-536/1-1	1.800	55600	100		
Mixed	JRFA-536/2-8[B]	10.0	1000	100	100	JRFA-536/3-2[B]

[A]: prepared in drinking water. [B]: prepared in surface water

5.8. Preparation of Calibration Standards for LC-MS/MS

Calibration solutions suitable for LC-MS/MS analysis should be prepared in matrix (surface or drinking waters). At least five levels of external calibration standards should be prepared to develop calibration curves for calculation of sample residues. Typical dilution schemes used to prepare the LC-MS/MS calibration solutions are as follows:

Drinking Water Matrix Matched Calibration

Starting Concentration (µg/L)	Initial JRFA ID	Volume Used (mL)	Final Volume (mL)	Final Concentration (µg/L)	Final JRFA ID
100	JRFA-536/3-1	0.100	10.0	1.0	JRFA-536/3-3
1.0	JRFA-536/3-3	5.0	10.0	0.5	JRFA-536/3-4
0.5	JRFA-536/3-4	5.0	10.0	0.25	JRFA-536/3-5
0.25	JRFA-536/3-5	4.0	10.0	0.1	JRFA-536/3-6
0.1	JRFA-536/3-6	5.0	10.0	0.05	JRFA-536/3-7
0.05	JRFA-536/3-7	5.0	10.0	0.025	JRFA-536/3-8

Surface Water Matrix Matched Calibration

Starting Concentration (µg/L)	Initial JRFA ID	Volume Used (mL)	Final Volume (mL)	Final Concentration (µg/L)	Final JRFA ID
1000	JRFA-536/2-8	0.010	10.0	1.0	JRFA-536/2-9
1.0	JRFA-536/2-9	5.0	10.0	0.5	JRFA-536/2-10
0.5	JRFA-536/2-10	5.0	10.0	0.25	JRFA-536/2-11
0.25	JRFA-536/2-11	4.0	10.0	0.1	JRFA-536/2-12
0.1	JRFA-536/2-12	5.0	10.0	0.05	JRFA-536/2-13
0.05	JRFA-536/2-13	5.0	10.0	0.025	JRFA-536/2-14

5.9. Standard Solution Storage and Expiration

Propanil and 3, 4-Dichloroaniline stock and fortification solutions were stored in a refrigerator (~ 4° C) when not in use to prevent degradation and/or concentration of the standard. Standard solutions were allowed to equilibrate to room temperature prior to use.

An expiration date of 6 months for stock solutions, and 3 months for fortification standards, were followed as per JRFA SOPs.

Matrix matched calibration standards should be made fresh prior to analysis.

6.0 METHOD SUMMARY

6.1. Analytical Method for the Determination of Propanil and 3, 4-DCA in Drinking and Surface Waters by LC-MS/MS Analysis

Samples should be prepared using an approved method for sample preparation for residue analysis. Water samples should be kept refrigerated.

1. Measure (10.00 – x) mL of water into 15 mL glass test tube, where "x" is the volume of fortification standard to be added. UTCs were not fortified (x = 0).
2. Fortify samples with the proper amount (if necessary).
3. Filter samples through 0.45 µm filter, if necessary. Dilute with untreated control (UTC) water, if necessary.

Instrumental analysis is accomplished using a LC-MS/MS system. Separation is achieved using a reversed phase column. The molecular ions formed in positive ion mode are fragmented by collision with neutral gas. The fragment ions generated are filtered, and one ion is selected for Quantitation and another product ion for confirmation.

6.2. Linearity

A series of standards were prepared and analyzed to empirically determine the linearity of the detector response (1/x weighting was used as an option). The calibration range extended beyond (by at least 20%) the lowest nominal concentration of the analyte in the relevant analytical solutions. Linearity was calculated as the correlation coefficient (r) resulting from a least squares equation that reflects the detector response as a function of the analyte concentrations.

6.3. Limits of Detection and Quantitation

The lower limit of Quantitation (LOQ) as per guideline for the method is 0.1 µg/L. The limit of detection (LOD) for the matrices is calculated from the data of the seven (7) LOQ recovery samples, as described in "Assigning Values to Non-detected/Non-quantified Pesticide Residues in Human Health Food Exposure Assessments, Item 6047, U.S. EPA, March 23, 2000." For this method, the LOD was determined to be 0.0175 µg/L and 0.0394 µg/L for propanil and 3,4-DCA respectively in drinking water. For surface water, the LOD was determined to be 0.0238 µg/L and 0.0374 µg/L for propanil and 3,4-DCA, respectively.

6.4. Validation of Confirmatory Techniques

Confirmation of the presence of the analytes were performed by using a primary quantitation

transition ion and one confirmatory transition ions MRM with same retention time for each.

Calibration curve and linearity values, recoveries for the fortified samples and precision data as well as results in blank samples were calculated for the quantitation and confirmatory ions and are reported in the appendices.

6.5. Propanil and 3, 4-DCA Stability

Propanil and 3,4-DCA fortification solutions were prepared and used on the day of analysis for surface water; drinking water fort solutions were prepared four days prior.

Calibration standard solutions were prepared fresh prior to instrument analysis.

6.6. Extract Stability

Sample extracts were analyzed after 7 (surface) and 10(drinking) days of storage at -20 ^{0}C. Stocks and fortification solutions in solvents stored at 4 ^{0}C were not analyzed as both analytes are known to be stable under these storage conditions.

7.0 CHROMATOGRAPHIC CONDITIONS

The following LC-MS/MS parameters were used to determine the concentration of Propanil and 3,4-DCA residues in water matrices. The parameters may be modified to achieve adequate chromatographic resolution and/or detector sensitivity. The actual parameters used are documented with each HPLC-MS/MS analysis sequence in the raw data.

HPLC System:	Shimadzu UFLC XR
MS Detector:	Sciex4000 API MS with Analyst™ software version 1.6.3
Mobile Phase A:	0.1% acetic acid in LC-MS grade water
Mobile Phase B:	0.1% acetic acid in LC-MS grade MeOH
Flow Rate:	500 µL/min
Column:	Luna 3µm 100 Å, 100 x 2.0 mm, SNo. 680-335-15
Column Oven Temp:	Ambient
Injection Vol.:	40 µL
Run Time:	12 minutes
Detector:	Sciex 4000 API MS
Retention Time:	Propanil: ~5.66 min
	3,4 DCA: ~5.23 min

Mobile Phase Composition (linear gradient changes):

A gradient elution, using an increased percentage of organic solvent (methanol) in the mobile phase, is used to resolve interferences and improve separation. See the specific gradient listed below:

Time (Min)	A% (0.1% Acetic Acid in water)	B% (0.1% Acetic Acid in MeOH)	Flow (µL/min)
0.00	80	20	500
2.00	80	20	500
5.00	10	90	500
9.00	10	90	500
10.00	80	20	500
12.00	80	20	500
12.01	80	20	500

Note: Retention times may differ depending upon the flow rate, column, and gradient used.

Acquisition Ions and Compound Dependent Parameters:

Analyte	Mass Transition (*m/z*)	Dwell (msec)	DP (V)	CE (V)	CXP (V)
Propanil (Quantitation)	*218.056→161.900*	150	86	21	10
Propanil (Confirmatory)	*218.056→126.900*	150	86	37	8
3,4-DCA (Quantitation)	*162.014→127.000*	150	81	29	8
3,4-DCA (Confirmatory)	*162.014→74.000*	150	81	69	14

Typical MS/MS Voltage Conditions Used:

Ionization Mode	ESI
Scan Type	MRM
Polarity	Positive
Resolution Q1	unit
Resolution Q3	unit
Curtain gas (N_2, psi)	20
GS1 (psi)	80
GS2 (psi)	60
CAD gas (N_2)	High
Ion Spray (V)	5500
Temperature (°C)	550
EP (V)	10

Initial and Final Q1 and Product Scans can be found in Appendix II.

Note: The MS settings as provided above should be used as guidelines only. For optimal results, compound and source optimization should be performed by the analyst.

7.1. Statistics and Sample Calculations

Propanil and 3, 4-Dichloroanilinewere calculated in µg/L using a multi-point calibration procedure as follows:

1. Prepare standard solutions over a concentration range appropriate to the expected residues in the samples.
2. Make an injection of each standard solution and measure the areas under the peaks corresponding to Propanil and 3, 4-Dichloroaniline. Calibration standard solutions should be interspersed throughout the analysis, after four injections of sample solutions.

3. Calibration standards and samples were analyzed using HPLC/MS-MS. Calibration curves and residue values were calculated using Analyst 1.6.3 data handling software using linear regression (1/x weighting is recommended).

   The standards were fit to the linear equation y = mx + b

   Where: x is the concentration of sample in final extract
   m is the calibration line slope
   b is the calibration line intercept
   y is the peak area

4. The following equation can be rearranged and used to calculate residues:

$$Residue\ Found\ (^{\mu g}/_{L}) = \frac{Peak\ area - curve\ intercept}{curve\ slope}$$

7.1.1. Example Calculation I

1. Drinking water sample (propanil, quantitation ion) PR LOQ R4 (D) was analyzed.

   Sample volume = 0.0100 L

   Peak area for propanil in the quantitation transition was 78176.213002 counts

   Calibration curve generated in the run was y = 290513.0897*x + 49138.66254

$$x = \frac{y - b}{m}$$

$$x = \frac{78176.213002 - 49138.66254}{290513.0897}$$

$$x = 0.100\ ^{\mu g}/_{L}$$

2. Recovery Results

   The recovery factor, expressed as a percentage (Recovery %), is calculated using the following equation:

$$\%Recovery = \frac{Measured\ concentration\ (\mu g/L) - Control\ concentration\ (\mu g/L)}{Theoretical\ concentration\ (\mu g/L)} * 100\%$$

Where the measured and control concentrations are taken from instrument outputs for the sample and UTC, respectively, and the theoretical concentration is the known amount of analyte added to the sample, if applicable.

Note: For the analysis of 10xLOQ samples, samples were diluted 10 fold and the results obtained were multiplied by 10 for dilution factor.

Example:

Drinking water sample (propanil, quantitation ion) PR LOQ R4 (D) was analyzed.

As no residues of the analyte were found in the control, the recovery was calculated as:

$$\%Recovery = \frac{0.100\ \mu g/L - 0.00\ \mu g/L}{0.100\ \mu g/L} * 100\% = 100\%$$

7.1.2. Example Calculation II

1. Surface water sample (3,4-DCA, quantitation ion) PR LOQ R2 (B) was analyzed.

   Sample volume = 0.0100 L

   Peak area for 3,4-DCA in the quantitation transition was 33616.495732 counts

   Calibration curve generated in the run was y = 288750.0481*x + 5124.333886

$$x = \frac{y - b}{m}$$

$$x = \frac{33616.495732 - 5124.333886}{288750.0481}$$

$$x = 0.0987\ {}^{\mu g}/_{L}$$

2. Recovery Results

   The recovery factor, expressed as a percentage (Recovery %), is calculated using the following equation:

$$\%Recovery = \frac{Measured\ concentration\ (\mu g/L) - Control\ concentration\ (\mu g/L)}{Theoretical\ concentration\ (\mu g/L)} * 100\%$$

Where the measured and control concentrations are taken from instrument outputs for the sample and UTC, respectively, and the theoretical concentration is the known amount of analyte added to the sample, if applicable.

Note: For the analysis of 10xLOQ samples, samples were diluted 10 fold and the results obtained were multiplied by 10 for dilution factor.

Example:

Surface water sample (3,4-DCA, quantitation ion) PR LOQ R2 (B) was analyzed.

As no residues of the analyte were found in the control, the recovery was calculated as:

$$\%Recovery = \frac{0.0987\ \mu g/L - 0.00\ \mu g/L}{0.100\ \mu g/L} * 100\% = 98.7\%$$

7.1.3. Limit of Detection (LOD) Calculation

The LOD was calculated using the seven LOQ sample data and the following equation:

$$LOD = Stdev(LOQ\ R1{:}\ LOQ\ R7) * t_{0.99} * \frac{1\ \mu g}{1000\ ng}$$

The standard deviation is calculated using:

$$Stdev(LOQ\ R1: LOQ\ R7) = \sqrt{\frac{1}{n-1}\sum_{i=1}^{n}(x_i - \bar{x})^2}$$

Where: Steve is the sample standard deviation of the calculated concentrations of the seven LOQ samples; n is number of samples, and $\bar{x}$ is the average calculated concentration.

$t_{0.99}$ is the one-tailed t-statistic at the 99% confidence level for n-1 replicates and is equal to 3.143 for n=7 samples.

Example: Propanil in Drinking water was analyzed.

$$LOD = Stdev(LOQ\ R1: LOQ\ R7) * 3.143$$

$$LOD = 0.0056 * 3.143 = 0.0175 \frac{\mu g}{L}$$

Analytical Method – (continued)

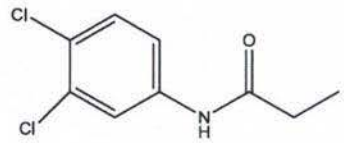
1.0 INTRODUCTION

1.1. Scope and Chemical Structures

Analytical Method AU-287R0 was developed for analysis of propanil and 3,4-dichloroaniline (3,4-DCA) in surface and drinking waters. The method was developed using LC-MS/MS for detection. The limit of quantitation (LOQ) of the method has been established at 0.1 µg/L for both analytes, with a concentration range of 0.1 (LOQ) µg/L to 1.0 (10x LOQ) µg/L. This method satisfies US EPA guidelines OCSPP (formerly OPPTS) 860.1340, SANCO/3029/99 rev. 4 and SANCO/825/00 rev. 8.1.

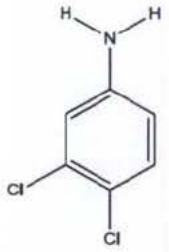
The chemical structures of propanil and 3,4-DCA are summarized as follows:

Common Name:	Propanil
Chemical Name (IUPAC):	N-(3, 4-dichlorophenyl) propanamide
CAS Registry No.:	709-98-8
Molecular Formula:	$C_9H_9Cl_2NO$
Molecular Weight:	218.08 $g \cdot mol^{-1}$
Batch No.:	SZBF166XV
Reassay Date:	May 3, 2018
Purity:	99.7%
Storage Condition:	Ambient
Source:	Sigma-Aldrich
JRFA No.	SAS-130-J
Structure:	



Common Name:	3,4-DCA
Chemical Name (IUPAC):	3,4-dichloroaniline
CAS Registry No.:	95-76-1
Molecular Formula:	$C_6H_5Cl_2N$
Molecular Weight:	162.02 $g \cdot mol^{-1}$
Batch No.:	13509KQV
Reassay Date:	September 1, 2018
Purity:	99.3%
Storage Condition:	Ambient
Source:	Sigma-Aldrich
JRFA No.	SAS-130-M
Structure:	

Analytical Method – (continued)



Analytical Method – (continued)

2.0 MATERIALS AND APPARATUS

2.1. Apparatus

The recommended equipment and apparatus are listed in Appendix I. Equipment with equivalent performance specifications may be substituted.

2.2. Reagents

All solvents and other reagents are to be of high purity, e.g., glass distilled/HPLC grade solvents and analytical grade reagents. Water must be deionized prior to use or purchased HPLC grade water utilized. Particular care must be taken to avoid contamination of the reagents used. Reagents of comparable purity may be substituted as long as acceptable performance is demonstrated. A list of reagents used in this method along with details of preparation of solutions is included in Appendix II.

2.3. Preparation of Analytical Standard Solutions

It is recommended that the following precautions should be taken when weighing the analytical materials.

1. Ensure good ventilation.
2. Wear gloves, protective eyewear and lab coat.
3. Prevent inhalation and contact with mouth.
4. Wash any contaminated areas.

2.3.1. Stock Solutions

Propanil stock solution was prepared in LC-MS grade acetonitrile, whereas 3, 4-Dichloroaniline (3,4-DCA) stock standard solution was prepared in LCMS grade methanol. The following is an example for preparing 100 mL of a 55,600 µg/L 3,4-DCA stock standard.

1. A mass of 0.0056 g of 3, 4-Dichloroaniline reference standard is weighed (adjusted for purity of 99.3%) and transferred to a 0.100 L class A volumetric flask.
2. Fill the volumetric flask halfway with methanol and agitate gently (sonicate if necessary) until standard is completely dissolved.
3. Dilute to volume with methanol and mix by inverting several times.
4. Calculate the exact concentration using the exact weight and purity, for example:

$$\left(\frac{0.0056\text{g}*0.993}{0.100\text{ L}}\right)*\left(\frac{10^6\mu\text{g}}{\text{g}}\right) = 55600\ \mu\text{g/L}$$

The following is an example for preparing 100 mL of a 125,000 µg/L propanil stock standard.

1. A mass of 0.0125 g of propanil reference standard is weighed (adjusted for purity of 99.7%) and transferred to a 0.100 L class A volumetric flask.
2. Fill the volumetric flask halfway with methanol and agitate gently (sonicate if necessary)

Analytical Method – (continued)

until standard is completely dissolved.

3. Dilute to volume with methanol and mix by inverting several times.
4. Calculate the exact concentration using the exact weight and purity, for example:

$$\left(\frac{0.0125\text{g}*0.997}{0.100\text{ L}}\right)*\left(\frac{10^6\mu\text{g}}{\text{g}}\right) = 125000\ \mu\text{g/L}$$

The table below lists the stock solutions prepared and used during this study.

Analyte ID	JRFA ID	Purity	Weight (mg) [1]	Final Volume (mL)	Concentration (µg/L)
Propanil	JRFA-444/75-1	99.7%	12.5	100	125000
3,4 DCA	JRFA-536/1-1	99.3%	5.56	100	55600

[1] Corrected for purity

2.3.2. Preparation of Fortification Solutions

Sample fortification solutions were prepared by serial dilution of the stock standard in drinking and surface waters. The table below lists the fort solutions prepared and used during this study.

Analyte ID	Initial JRFA ID	Volume (mL)	Initial Concentration (ug/L)	Final Volume (mL)	Concentration (µg/L)	Final JRFA ID
Propanil	JRFA-444/75-1	0.800	125000	100	1000	JRFA-536/2-1[A]
3,4-DCA	JRFA-536/1-1	1.800	556000	100		
Mixed	JRFA-536/2-1[A]	10.0	1000	100	100	JRFA-536/3-1[A]
Propanil	JRFA-444/75-1	0.800	125000	100	1000	JRFA-536/2-8[B]
3,4-DCA	JRFA-536/1-1	1.800	556000	100		
Mixed	JRFA-536/2-8[B]	10.0	1000	100	100	JRFA-536/3-2[B]

[A]: prepared in drinking water. [B]: prepared in surface water

2.3.3. Preparation of Calibration Standards for LC-MS/MS

Calibration solutions suitable for LC-MS/MS analysis should be prepared in matrix (surface or drinking waters). At least five levels of external calibration standards should be prepared to develop calibration curves for calculation of sample residues. Typical dilution schemes used to prepare the LC-MS/MS calibration solutions are as follows:

Analytical Method – (continued)

Drinking Water Matrix Matched Calibration

Starting Concentration (µg/L)	Initial JRFA ID	Volume Used (mL)	Final Volume (mL)	Final Concentration (µg/L)	Final JRFA ID
100	JRFA-536/3-1	0.010	10.0	1.0	JRFA-536/3-3
1.0	JRFA-536/3-3	5.0	10.0	0.5	JRFA-536/3-4
0.5	JRFA-536/3-4	5.0	10.0	0.25	JRFA-536/3-5
0.25	JRFA-536/3-5	4.0	10.0	0.1	JRFA-536/3-6
0.1	JRFA-536/3-6	5.0	10.0	0.05	JRFA-536/3-7
0.05	JRFA-536/3-7	5.0	10.0	0.025	JRFA-536/3-8

Surface Water Matrix Matched Calibration

Starting Concentration (µg/L)	Initial JRFA ID	Volume Used (mL)	Final Volume (mL)	Final Concentration (µg/L)	Final JRFA ID
1000	JRFA-536/2-8	0.100	10.0	1.0	JRFA-536/2-9
1.0	JRFA-536/2-9	5.0	10.0	0.5	JRFA-536/2-10
0.5	JRFA-536/2-10	5.0	10.0	0.25	JRFA-536/2-11
0.25	JRFA-536/2-11	4.0	10.0	0.1	JRFA-536/2-12
0.1	JRFA-536/2-12	5.0	10.0	0.05	JRFA-536/2-13
0.05	JRFA-536/2-13	5.0	10.0	0.025	JRFA-536/2-14

2.3.4. Standard Solution Storage and Expiration

Propanil and 3, 4-Dichloroaniline stock and fortification solutions were stored in a refrigerator (~ 4° C) when not in use to prevent degradation and/or concentration of the standard. Standard solutions were allowed to equilibrate to room temperature prior to use.

An expiration date of 6 months for stock solutions, and 3 months for fortification standards, were followed as per JRFA SOPs.

Matrix matched calibration standards should be made fresh prior to analysis.

2.4. Safety Precautions and Hazards

All caution should be exercised when handling pure material or concentrated stock solutions. Avoid skin contact and inhalation. See Safety Data Sheet (SDS) documentation accompanying standard shipment. All personnel should be familiar with all solvents and equipment precautions and hazards prior to use.

3.0 ANALYTICAL PROCEDURE

3.1. Sample Preparation

Samples should be prepared using an approved method for sample preparation for residue analysis. Water samples should be kept refrigerated.

Analytical Method – (continued)

1. Measure (10.00 – x) mL of water into 15 mL glass test tube, where "x" is the volume of fortification standard to be added. UTCs were not fortified (x = 0).
2. Fortify samples the proper amount (if necessary).
3. Filter samples through 0.45 µm filter, if necessary. Dilute with untreated control (UTC) water, if necessary.

3.2. Time Required for Analysis

The methodology is normally performed with a batch of 15 samples. In average, one chemist can complete the analysis of one batch of 15 samples including instrument analysis and data processing in a period of 8 working hours.

3.3. Method Stopping Points

No stop point is considered necessary.

3.4. Modifications and Potential Problems

Samples should be analyzed within a week after extraction. An expiration date of 6 months is recommended for propanil and 3,4-DCA stock standard solutions, and 3 months for fortification solutions as described in JRFA SOPs. Calibration standard solutions should be made fresh prior to analysis.

4.0 FINAL DETERMINATION

The method has been developed for use on a SCIEX 4000 API MS and Shimadzu HPLC system. The following instrumentation and conditions can be used as a general guidance. Other instrumentation, column and mobile phases can also be used, though optimization may be required to achieve the desired separation and sensitivity.

4.1. Instrument Description

HPLC System:	Shimadzu UFLC XR
Detector:	Sciex 4000 API MS with Analyst™ software version 1.6.3

4.2. Chromatography Conditions for Propanil/3,4-DCA Analysis

Mobile Phase A:	0.1% acetic acid in LC-MS H_2O
Mobile Phase B:	0.1% acetic acid in LC-MS MeOH
Flow Rate:	500 µL/min
Column:	Phenomenex Luna® 3 µm C18(2) 100 Å ,100 x 2.0mm
Column Oven Temp:	Ambient
Injection Vol.:	40 µL
Run Time:	12 minutes
Detector:	Sciex 4000 API MS
Retention Time:	Propanil: ~5.66 min

Analytical Method – (continued)

3,4-DCA:~5.23 min

Mobile Phase Composition (linear gradient changes):

A gradient elution, using an increased percentage of organic solvent (methanol) in the mobile phase, is used to resolve interferences and improve separation. See the specific gradient listed below:

Time (Min)	A% (0.1% Acetic Acid in water)	B% (0.1% Acetic Acid in MeOH)	Flow (µL/min)
0.00	80	20	500
2.00	80	20	500
5.00	10	90	500
9.00	10	90	500
10.00	80	20	500
12.00	80	20	500
12.01	80	20	500

Note: Retention times may differ depending upon the flow rate, column, and gradient used.

Acquisition Ions and Compound Dependent Parameters:

Analyte	Mass Transition (*m/z*)	Dwell (msec)	DP (V)	CE (V)	CXP (V)
Propanil (Quantitation)	*218.056→161.900*	150	86	21	10
Propanil (Confirmatory)	*218.056→126.900*	150	86	37	8
3,4-DCA (Quantitation)	*162.014→127.000*	150	81	29	8
3,4-DCA (Confirmatory)	*162.014→74.000*	150	81	69	14

Typical MS/MS Voltage Conditions Used:

Ionization Mode	ESI
Scan Type	MRM
Polarity	Positive
Resolution Q1	unit
Resolution Q3	unit
Curtain gas (N_2, psi)	20
GS1 (psi)	80
GS2 (psi)	60
CAD gas (N_2)	High
Ion Spray (V)	5500
Temperature (°C)	550
EP (V)	10

Initial and Final Q1 and Product Scans can be found in Appendix VI: .

Note: The MS settings as provided above should be used as guidelines only. For optimal results, compound and source optimization should be performed by the analyst.

Analytical Method – (continued)

5.0 CALCULATION OF RESULTS

5.1. Multi Point Calibration Procedure

Propanil and 3,4-DCA residues may be calculated in µg/L using a multi-point calibration procedure as follows.

1. Prepare standard solutions over a concentration range appropriate to the expected residues in the samples. An appropriate number of different concentrations within this range should be prepared (at least four).
2. Make an injection of each sample solution and measure the areas of the peaks corresponding to Propanil or 3,4-DCA. Calibration standard solutions should be interspersed throughout the analysis, after approximately five injections of sample solutions.
3. Calibration standards and samples were analyzed using HPLC/MS-MS. Calibration curves and residue values were calculated using Analyst 1.6.3 data handling software using linear regression (1/x weighting is recommended).

   The standards were fit to the linear equation y = mx + b

   Where: x is the concentration of sample in final extract

   m is the calibration line slope
   b is the calibration line intercept
   y is the peak area
4. The following equation can be rearranged and used to calculate residues:

$$Residue\ Found\ (^{\mu g}/_{L}) = \frac{Peak\ area - curve\ intercept}{curve\ slope}$$

5.2. Example Calculation I

1. Drinking water sample (propanil, quantitation ion) PR LOQ R4 (D) was analyzed.

   Sample volume = 0.0100 L
   Peak area for propanil in the quantitation transition was 78176.213002 counts
   Calibration curve generated in the run was y = 290513.0897*x + 49138.66254

$$x = \frac{y - b}{m}$$

$$x = \frac{78176.213002 - 49138.66254}{290513.0897}$$

$$x = 0.100\ ^{\mu g}/_{L}$$

2. Recovery Results

Analytical Method – (continued)

The recovery factor, expressed as a percentage (Recovery %), is calculated using the following equation:

$$\%Recovery = \frac{Measured\ concentration\ (\mu g/L) - Control\ concentration\ (\mu g/L)}{Theoretical\ concentration\ (\mu g/L)} * 100\%$$

Where the measured and control concentrations are taken from instrument outputs for the sample and UTC, respectively, and the theoretical concentration is the known amount of analyte added to the sample, if applicable.

Example:

Drinking water sample (propanil, quantitation ion) PR LOQ R4 (D) was analyzed.

As no residues of the analyte were found in the control, the recovery was calculated as:

$$\%Recovery = \frac{0.100\ \mu g/L - 0.00\ \mu g/L}{0.100\ \mu g/L} * 100\% = 100\%$$

5.3. Example Calculation II

1. Surface water sample (3,4-DCA, quantitation ion) PR LOQ R2 (B) was analyzed.

   Sample volume = 0.0100 L
   Peak area for 3,4-DCA in the quantitation transition was 33616.495732 counts
   Calibration curve generated in the run was y = 288750.0481*x + 5124.333886

$$x = \frac{y - b}{m}$$

$$x = \frac{33616.495732 - 5124.333886}{288750.0481}$$

$$x = 0.0987\ {}^{\mu g}/_{L}$$

2. Recovery Results

   The recovery factor, expressed as a percentage (Recovery %), is calculated using the following equation:

$$\%Recovery = \frac{Measured\ concentration\ (\mu g/L) - Control\ concentration\ (\mu g/L)}{Theoretical\ concentration\ (\mu g/L)} * 100\%$$

Where the measured and control concentrations are taken from instrument outputs for the sample and UTC, respectively, and the theoretical concentration is the known amount of analyte added to the sample, if applicable.

Example:

Surface water sample (3,4-DCA, quantitation ion) PR LOQ R2 (B) was analyzed.

As no residues of the analyte were found in the control, the recovery was calculated as:

Analytical Method – (continued)

$$\%Recovery = \frac{0.0987\ \mu g/L - 0.00\ \mu g/L}{0.100\ \mu g/L} * 100\% = 98.7\%$$

5.4. Limit of Detection (LOD) Calculation

The LOD was calculated using the seven LOQ sample data and the following equation:

$$LOD = Stdev(LOQ\ R1{:}\ LOQ\ R7) * t_{0.99}$$

The standard deviation is calculated using:

$$Stdev(LOQ\ R1{:}\ LOQ\ R7) = \sqrt{\frac{1}{n-1}\sum_{i=1}^{n}(x_i - \bar{x})^2}$$

Where: Stdev is the sample standard deviation of the calculated concentrations of the seven LOQ samples; n is number of samples, and $\bar{x}$ is the average calculated concentration

$t_{0.99}$ is the one-tailed t-statistic at the 99% confidence level for n-1 replicates and is equal to 3.143 for n=7 samples.

Example: Propanil in Drinking water was analyzed.

$$LOD = Stdev(LOQ\ R1{:}\ LOQ\ R7) * 3.143$$

$$LOD = 0.0056 * 3.143 = 0.0175\ \frac{\mu g}{L}$$

6.0 UNTREATED CONTROL AND RECOVERY SAMPLES

If untreated control samples are available, untreated control samples should be analyzed for each set of samples analyzed to verify that samples are free from analyte contamination. A minimum of one control should be analyzed with each batch of samples.

A total of two recovery samples, which are untreated samples accurately fortified with a known amount of propanil and 3,4-DCA, should also be analyzed in each analytical set. The recovery levels should be run at the LOQ and a higher level to encompass the treated sample results.

7.0 SPECIFICITY

7.1. Labware Interference

All reusable glassware is suggested to be detergent washed in hot water and then rinsed with deionized water and acetone prior to use.

7.2. Reagent and Solvent Interference

None.

Analytical Method – (continued)

11.0 REFERENCES

1. USEPA, "Residue Chemistry Test Guidelines OPPTS 860.1340 Residue Analytical Method". OPPTS, EPA 712–C–96–174, August 1996.
2. European Commission, "Residues: Guidance for generating and reporting methods of analysis in support of pre-registration data requirements for Annex II (part A, Section 4) and Annex III (part A, Section 5) of Directive 91/414". SANCO/3029/99 rev.4, 11 July 2000.
3. European Commission, "Guidance document on pesticide residue analytical methods". SANCO/825/00 rev. 8.1, 16 November 2010.
4. T. Hayama and M. Takada. 2008. "Simple and rapid method for the determination of ethylenebisdithiocarbamate fungicides in fruits and vegetables using liquid chromatography with tandem mass spectrometry". Anal Bioanal Chem 392: 969-976.
5. Office of Pesticide Programs, 2000. "Assigning Values to Non-detected/Non-quantified Pesticide Residues in Human Health Food Exposure Assessments". Item 6047, U.S. EPA, March 23, 2000.

Analytical Method – (continued)

12.0 APPENDICES

12.1. Appendix I: Apparatus with Recommended Suppliers

A. HPLC/MS-MS System

1. Shimadzu UFLC XR, Shimadzu, Kyoto, Japan or equivalent.
2. Sciex 4000 API mass spectrometer with Analyst™ software version 1.6.3., SCIEX Framingham, MA or equivalent.

B. Column: Phenomenex Luna® 3 µm C18(2) 100 Å ,100 x 2.0mm

C. Eppendorf adjustable pipettes, assorted sizes

D. Balance, top-loading, Mettler or equivalent

E. Analytical Balance, for standard prep

F. Sonicator, Fisher Scientific

G. Vortex, Fisher Scientific

H. Glassware

Class A volumetric flasks, assorted sizes

Graduated cylinders, various sizes

Beakers, various sizes

12.2. Appendix II: Reagents

A. Solvents and Reagents.

1. LC/MS grade solvents or better should be utilized. Other brands and grades of solvents may be substituted as long as they do not produce interferences with the chromatography.
   a. Acetonitrile, EMD Millipore Corporation, Billerica, MA
   b. Water, EMD Millipore Corporation, Billerica, MA
   c. Methanol, EMD Millipore Corporation, Billerica, MA
   d. Acetic Acid, Glacial, JT Baker, Center Valley, PA
2. Working Solutions
   a. Mobile phase A: 0.1% acetic acid in water: 1.00 mL of acetic acid to 999 mL with water
   b. Mobile phase B: 0.1% acetic acid in methanol: dilute 1 mL of formic acid to 999 mL with MeOH.