INTRODUCTION

An analytical method for the determination of (2,4-dichlorophenoxy)acetic acid, 2,4-dichlorophenol, 4-chlorophenol and 2,4-dichloroanisole in drinking water, surface water and ground water, Dow AgroSciences Study Number 110504, "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" (Reference 6), was developed and validated at Dow AgroSciences LLC. The method was found to be suitable for the determination of residues of (2,4-dichlorophenoxy)acetic acid, 2,4-dichlorophenol, 4-chlorophenol and 2,4-dichloroanisole in drinking water, surface water and ground water over the concentration range of 0.1 to 5.0 µg/L. The validated limit of quantitation was confirmed to be 0.1 µg/L for all water types.

An independent laboratory validation of the analytical method was conducted on drinking water, surface water and ground water to satisfy the requirements of the European Commission Guidance Document on Residue Analytical Methods, SANCO/825/00 rev. 8.1 (Reference 1) and SANCO/3029/99 rev. 4 (Reference 2). The study was also conducted to satisfy the requirements of U.S. EPA Guideline OPPTS 860.1340(c)(6) (Reference 3), PR Notice 96-1 (Reference 4) and PR Notice 86-5 (Reference 5).

The independent laboratory, the Study Director, and the analyst chosen to conduct the ILV were unfamiliar with the method, both in its development and subsequent use in analyzing samples. The independent laboratory used all of its own equipment and supplies, so that there was no common link between Dow AgroSciences and the ILV analyst. Throughout the conduct of the study, any communications between Dow AgroSciences and the Study Director and/or the analyst were logged for inclusion in the report. No one from Dow AgroSciences 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

Preparation and Storage of Samples

The independent laboratory validation was carried out on three water specimens; surface water, ground water and drinking water. The drinking water was obtained from a "drinking water" tap at the Test Facility (CEMAS), the ground water was obtained from a well near Henley-on-Thames and the surface water was obtained from the River Loddon, Charvill, UK.

Specimen	CEMAS Sample Reference Number
Surface Water	CCON/037/001
Ground Water	CCON/038/001
Drinking Water	CCON/039/001

On receipt the specimens were stored at approximately 4 °C before and after analysis.

Characterisation of Samples

The water specimens were characterised at CEMAS, Study Number CEMS-5332. Details of the characterisation results are as follows:

Specimen	pH	Total Hardness mg/L as $CaCO_3$	Total Suspended Solids mg/L	Alkalinity mg/L as $CaCO_3$	Dissolved Organic Carbon
Surface Water	7.6	206	11.4	155.0	4.99
Ground Water	8.0	280	5.2	258.0	1.03
Drinking Water	7.8	263	5.2	196.0	2.73

Specimen	Electrical Conductivity µS/cm	Silt Content mg/L	Bicarbonate mg/L	Carbonate mg/L
Surface Water	645	<1.0	189.1	<0.1
Ground Water	600	<1.0	270.9	21.6
Drinking Water	779	15	219.6	9.6

pH of water specimens

The pH of the water samples was determined using CEMAS SOP CEM-3373 - Determination of the pH of Water, Soil and Sediment Samples in water and/or Salt Solutions (0.01 M Calcium Chloride, 0.1 M Potassium Chloride, 1.0 M Potassium Chloride).

The pH value reflects the relative number of hydrogen ions (H+) in solution. The more hydrogen ions present, compared to the hydroxyl ions (OH-), the more acidic the solution will be and the lower the pH value. A decrease in hydrogen ions and increase in hydroxyl ions will result in more alkaline or basic conditions.

The pH was determined, potentiometrically, using a glass combination electrode and a pH meter.

Hardness EDTA titration

Total Hardness by EDTA Titration in water was determined using CEMAS SOP CEM-3060 -Determination of Total Hardness by EDTA Titration in Water.

Water hardness is an expression for the sum of the calcium and magnesium cation concentrations in a water sample. The standard method of expressing water hardness is in mg/L calcium carbonate ($CaCO_3$) which has the formula weight of 100.1 g/mole.

Water hardness was determined using a complexometric titration method using a standard ethylenediaminetetraacetic acid (EDTA) solution. Due to steric hindrances EDTA will then complex with calcium and magnesium in a one-to-one molar ratio. Since EDTA and its hardness complexes are not colored, an additional chelating agent, eriochrome black T, was used to facilitate endpoint detection.

Total Suspended Solids

The total suspended solids in the water samples were determined using CEMAS SOP CEM-3448 -Determination of Total Suspended and Volatile Suspended Solids in Waters, which is a standard gravimetric procedure. Total suspended solids are described as those solids which are retained on a glass fibre filter and dried at 103-105°C. 500 mL of sample was filtered, under vacuum, onto a pre-weighed glass fibre filter (GF/F). The paper plus residue was dried for at least two hours and then reweighed. The weight of residue was expressed as mg/L total suspended solids.

Carbonate, Bicarbonate, Carbonate Hardness and Alkalinity

Alkalinity was determined using CEMAS SOP CEM-3384 - Determination of Alkalinity of Water – Carbonate, Bicarbonate and Carbonate Hardness.

Alkalinity is the measure of a water sample's ability to neutralize hydrogen ions (its acid-neutralizing ability). Alkalinity may be caused by dissolved strong bases such as sodium hydroxide or potassium hydroxide (and other hydroxide-containing compounds), and it may, also, be caused by dissolved carbonates, bicarbonates, borates, and phosphates. The measured alkalinity is the total of all of these species found in a water sample. For the sake of simplicity, it is expressed in terms of mg $CaCO_3$/L although many species other than dissolved calcium carbonate may actually contribute to the alkalinity. Total Alkalinity is referred to as Carbonate Hardness.

The carbonate concentration was determined by titration with hydrochloric acid using phenolpthalein as an indicator and the bicarbonate hardness level was determined by further titration with the same acid using bromophenol blue as the indicator.

Silt Content

The silt content of water was measured using CEMAS Standard Operating Procedure CEM-3385 Determination of particle size distribution in Water, Fractionation/sedimentation Method.

The sand fraction was removed from the water specimen by sieving. The silt and clay particles after suspension were sampled with a pipette at different sedimentation times and were then determined gravimetrically. The method is dependent on the fact that the sedimentation rate of particles in water is proportional to their size and temperature of the water (Stokes Law).

Dissolved and Total Organic Carbon

The dissolved organic carbon and the total organic carbon of the water samples were determined using CEMAS SOP CEM-3396 - Determination of the Total and Dissolved Organic Carbon, Inorganic Carbon and Carbon in Water. The dissolved organic carbon (DOC) is a measure of the organic material, contained in a water sample that is soluble and/or colloidal, that can pass through a 0.45um filter.

A Sievers Model 5310C TOC Analyser (GE Analytical Instruments) was used to measure the concentration of Dissolved Organic Carbon (DOC), Inorganic Carbon (DIC), and Carbon (DTC) in the water samples. The analyser principle is based on the oxidation of organic compounds to form carbon dioxide using UV radiation and a chemical oxidising agent (ammonium persulphate). Carbon dioxide is measured using a sensitive, selective membrane-based conductometric detection technique. For each DOC measurement, the concentration of inorganic carbon species (carbonates, bicarbonates and carbon dioxide) is determined and, after oxidation of the organic compounds, the total carbon (DTC) content of the sample is measured. The concentration of the organic compounds (DOC) was calculated from the difference between the concentrations of the dissolved carbon (DC) and dissolved inorganic carbon (DIC).

Preparation of Solutions and Standards

Reagents used were of equivalent specifications as described in the analytical method.
The following analytical test substances/analytical standards were utilized during the independent laboratory method validation:

Test Substance/ Analytical Standard:	(2,4-Dichlorophenoxy) acetic acid
Supplier:	Sponsor
Reference Number:	AGR275828
Batch/Lot no:	MORRIS/1710
Purity:	99.5%
Expiry date:	19 October 2016
Storage:	Ambient

Test Substance/ Analytical Standard:	2,4-Dichlorophenol
Supplier:	Sponsor
Reference Number:	AGR182992
Batch/Lot no:	OCR 696-132-1
Purity:	99%
Expiry date:	07 October 2015
Storage:	Ambient

Test Substance/ Analytical Standard:	4-Chlorophenol
Supplier:	Sponsor
Reference Number:	TSN100174
Batch/Lot no:	HZ 03530CX
Purity:	99.7%
Expiry date:	03 November 2012
Storage:	Ambient

Test Substance/ Analytical Standard:	2,4-Dichloroanisole
Supplier:	Sponsor
Reference Number:	TSN028154-0001
Batch/Lot no:	S67935
Purity:	100%
Expiry date:	17 October 2012
Storage:	Coldroom

Standard solutions and calibration standard 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 items. The test/reference items and specimens will be retained until expiry and then disposed of.

Fortification of Recovery Samples

The control specimens were fortified as described below with (2,4-dichlorophenoxy)acetic acid, 2,4-dichlorophenol, 4-chlorophenol and 2,4-dichloroanisole:

Matrix	Untreated Control Specimen Reps	Replicates at Fortification Level (LOD)*	Replicates at Fortification Level (LOQ)**	Replicates at Fortification Level	Replicates at Fortification Level
Drinking Water	2	1 at 0.03 µg/L	5 at 0.1 µg/L	5 at 1.0 µg/L	5 at 5.0 µg/L
Groundwater	2	1 at 0.03 µg/L	5 at 0.1 µg/L	5 at1.0 µg/L	5 at 5.0 µg/L
Surface Water	2	1 at 0.03 µg/L	5 at 0.1 µg/L	5 at 1.0 µg/L	5 at 5.0 µg/L

*LOD – Limit of determination
**LOQ – Limit of quantitation

Forty-mL aliquots of the control specimen were measured into individual glass vials. Each sample was fortified as per the table above. One sample was fortified to achieve the fortification level 0.03 µg/L (LOD), five samples were fortified to achieve the fortification level of 0.1 µg/L(LOQ), five samples were fortified to achieve the fortification level of 1.0 µg/L and five samples were fortified to achieve the upper fortification level of 5.0 µg/L for drinking water, surface water and ground water. The fortification solution was injected directly into the matrix.

Sample Extraction, Purification and Analysis

Specimens were assayed according to the analytical method Dow Study Number 110504, "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" (reference 6).

Residues of (2,4-dichlorophenoxy)acetic acid, 2,4-dichlorophenol and 4-chlorophenol were extracted from the water sample matrices by acidifying the sample using a hydrochloric acid

solution and then the entire sample was purified using a reverse-phase polymeric solid-phase extraction (SPE) column.

After elution from the SPE column, the eluate was concentrated under a stream of nitrogen until approximately 500 µL remained. The final sample was adjusted to 1.0 mL using a water solution containing 0.1% acetic acid. The sample was analyzed for the determination of (2,4-dichlorophenoxy)acetic acid and 2,4-dichlorophenol by liquid chromatography with negative-ion APCI tandem mass spectrometry, and for determination of 4-chlorophenol by liquid chromatography with negative-ion electrospray ionization (ESI) tandem mass spectrometry (LC-MS/MS).

Residues of 2,4-dichloroanisole were extracted from the water sample matrices by acidifying the sample using a hydrochloric acid solution and then partitioning it against isooctane. The sample was analyzed by GC/MS electron impact selected ion monitoring.

Full extraction details:

Sample Analysis of (2,4-dichlorophenoxy)acetic acid, 2,4-dichlorophenol and 4-chlorophenol.

1. Forty ±0.4 mL portions of each matrix were measured into a 45-mL glass vial.
2. For preparing fortified samples, appropriate aliquots of the appropriate spiking solutions were added 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. One-mL of 2 N HCl was added to each glass vial.
4. Each sample was shaken for a minimum of 30 minutes on a reciprocating shaker at approximately 180 excursions/minute.

5. The clean up of samples on the Strata-X polymeric sorbent reversed-phase SPE cartridge used the following procedure:

   a. An Oasis polymeric sorbent reversed-phase SPE cartridge (60-mg, 3-mL) was placed on a vacuum manifold box.

   b. The SPE cartridge was conditioned with 1-mL of methanol followed by <u>two</u> 1-mL of 0.1 N HCl discarding the eluates. Full vacuum applied (approximately -25 inches Hg) for about 10 seconds between solvent additions.

   c. The entire acidified samples (from Step 4) were each consecutively transferred to the SPE cartridge. The samples were pulled through the SPE cartridge at approximately 1 mL/min, using vacuum if necessary. The eluate was discarded. Full vacuum was applied to the cartridge for about 10 seconds between solvent additions and after the sample had eluted.

   d. SPE cartridge was washed with 1 mL of 0.1 N HCl . The eluate was discarded. Cartridges were dried under full vacuum for about 30 seconds.

   e. The 2,4-D, 2,4 DCP and 4-CP was eluted from the SPE cartridge at a rate of approximately 1 mL/min (using vacuum if necessary) with <u>two</u> 500-µL aliquots of an acetonitrile/methanol (80:20) solution containing 0.1% acetic acid. The two eluates were collected in the same glass tube. Full vacuum (approximately -25 inches of Hg) was applied for about 10 seconds between solvent additions and after the final elution.

6. The eluate was concentrated to approximately 500 µL under a stream of nitrogen using a N-vap evaporator set at 40°C and a nitrogen flow rate of approximately 500 mL/min. (The water samples fortified at 5.0 µg/L were not concentrated).

7. The final sample was adjusted to 1.0 mL by adding approximately 500 µL of a water solution containing 0.1% acetic acid. 4.0 mL of the water solution containing 0.1% acetic acid was added to the samples fortified at 5.0 µg/L to bring them to a total of 5 mL volume.

8. Sample vials were capped with a PTFE-lined cap and vortex mixed for 1-2 seconds.

9. A portion of sample was transferred to 2-mL autosampler vials using a limited insert and the vials were capped.

10. The sample was analyzed for the determination of (2,4-dichlorophenoxy)acetic acid and 2,4-dichlorophenol by liquid chromatography with negative-ion APCI tandem mass spectrometry, and for determination of 4-chlorophenol by liquid chromatography with negative-ion electrospray ionization (ESI) tandem mass spectrometry (LC-MS/MS).

Sample Analysis of 2,4-Dichloroanisole

1. 40 ±0.4 mL portions of each matrix were measured into a 45-mL glass vial.

2. For preparing fortified samples, appropriate aliquots of the appropriate spiking solutions were added 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. One-mL of 1 N HCl was added to each glass vial.

4. One-mL of isooctane extraction solution was added to each glass vial and sample vials were capped.

   Five-mL of isooctane extraction solution were added to the samples fortified at 5.0 µg/L.

5. Each sample was shaken for a minimum of 30 minutes on a reciprocating shaker at approximately 180 excursions/minute.

6. Sample vials were centrifuged for 5 minutes at 2000 rpm.

7. Using a Pasteur pipet, a portion of the top layer was transferred into a limited insert vial and capped.

8. The samples were analysed by GC/MS electron impact selected ion monitoring.

Analytical Instrumentation and Equipment

The instrumental conditions used during the ILV trial were as described in the analytical method. The instrumental conditions used are given below.

Typical HPLC Operating Conditions (For the analysis of 2,4-D and 2,4-DCP by LC-MS/MS)

Instrumentation:	Symbiosis Pharma
Column:	Synergi Hydro-RP (75x4.6 mm id)
Column Temperature:	Ambient
Injection Volume:	10 µL
Injection Wash	1) methanol containing 0.5% NH_4OH
Run Time:	6.0 minutes
Mobile Phase:	A – Water with 0.1% acetic acid B – Acetonitrile:methanol (80:20) with 0.1% acetic acid
Flow Rate:	1.0 mL/min.

Isocratic:

Time, min	A, %	B, %
0:00	45	55
6:00	45	55

Flow Diverter Program:

1) 0.0→1.5 min: flow to waste
2) 1.5→4 min: flow to source
3) 4→end of run: flow to waste

Typical Mass Spectrometry Operating Conditions (For the analysis of 2,4-D and 2,4-DCP by LC-MS/MS)

Instrumentation:	MDS SCIEX API 5000 LC-MS/MS System
	MDS SCIEX Analyst 1.5.1 data system
Interface:	Heated Nebulizer
Polarity:	Negative
Scan Type:	MRM
Resolution:	Q1 – unit, Q3 – unit
Curtain Gas (CUR):	30 psi
Collision Gas (CAD):	4.0 (High)
Temperature (TEM):	450°C
Ion Source Gas 1	30 psi
Pre-acquisition Delay:	1.5 min
Acquisition Time	4.5 min
Dwell Time:	50 ms
EP	-10

Analytes:	Precursor Ion Q1	Product Ion Q3	Declustering Potential	Collision Energy	Cell Exit Potential
2,4-D - quantitation	218.985	160.800	-40 V	-22 V	-17 V
2,4-D - confirmation	220.968	163.0	-40 V	-22 V	-15 V
2,4-DCP - quantitation	160.953	125.00	-40 V	-24 V	-15 V
2,4-DCP - confirmation	162.952	127.00	-40 V	-24 V	-11 V

Typical HPLC Operating Conditions (For the analysis 4-CP by LC-MS/MS)

Instrumentation	Agilent 1200 HPLC system Applied Biosystems QTRAP 5500 LC-MS/MS System MDS/Sciex Analyst 1.5.1 data system
Column:	Zorbax SB-C8 4.6 x 75 mm, 3.5-µm
Injection	10 µL
Speed:	Normal
Needle height:	5 mm
Autosampler wash:	methanol/water (50:50) containing 0.1% acetic acid
Run Time:	Approximately 12.5 minutes
Mobile Phase:	A – Water containing 0.10% acetic acid B – Acetonitile/Methanol (80:20) containing 0.10% acetic acid
Flow Rate:	1000 µL/min

Gradient:

Time,	Solvent A,	Solvent
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 run: flow to waste

Typical Mass Spectrometry Operating Conditions (For the analysis 4-CP by LC-MS/MS)

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
Pre-acquisition Delay:	0.0 min
Acquisition Time	6.0 min
IonSpray Voltage (IS):	-4500 volts
Dwell Time:	100 ms
EP	-10

Analytes:	Precursor Ion Q1	Product Ion Q3	Declustering Potential	Collision Energy	Cell Exit Potential
4-CP - quantitation	126.920	90.948	-80 V	-24 V	-9 V
4-CP - confirmation	128.941	90.943	-75 V	-24 V	-17 V

Typical GC Operating Conditions (For the analysis of 2,4-DCA by GC/MS)

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. 1.0-µm film thickness
Liner:	Agilent Liner 1.8 mm ID PTV M – p/n: 5183-2037
Oven Method: Column	80 °C for 1.2 min 80 °C to 320 °C at 20 °C/min 320 °C for 0.85 min
Transfer Line	280 °C
Carrier Gas method:	Helium
Constant Flow Vacuum Compensation Initial Head Pressure Linear Velocity	1.0 ml/min On ~70 kPa ~36 cm/s
Injection Method:	Splitless
Injector Temperature	50 °C for 0.1 min Ramps:

Rate	Final Temperature	Final time
1500	280 °C	5.00

Cryo use Temperature	90 °C
Cryo timeout	15.00min
Pressure	9.34 psi
Purge flow	50 mL/min
Purge time	1.20 min
Total flow	54 mL/min
Septum Purge	On
Injection Volume	1 µL
Detector Mode:	Electron Impact with selected ion monitoring (EI-mode)
Source Temperature	230 °C
Quad Temperature	150 °C
Calibration Program	autotune
Electron Multiplier	1900 volts (~200 volts above autotune)
SIM Resolution	High
Dwell Time	50 msecIons
Monitored:	
2,4-DCA	*m/z* 176 (quantitation) *m/z* 178 (confirmation 1) *m/z* 161 (confirmation 2)

Calculation of Results

For each analytical batch, a range of calibration standards was injected over the range 1.2 ng/mL to 50 ng/mL. A calibration curve was prepared by plotting the quantitation peak area obtained versus analyte concentration.

Example

(2,4-dichlorophenoxy)acetic acid recovery at 0.1 ng/mL

ASR number = 0060/12/07

Peak area (2,4-dichlorophenoxy)acetic acid = 185707

Slope of calibration curve (forced through origin) = 40148.7609

(2,4-dichlorophenoxy)acetic acid concentration in final extract = $\frac{185707}{40148.76}$ = 4.62547 ng/mL

Sample concentration = 40 mL/mL

Dilution factor = 1

(2,4-dichlorophenoxy)acetic acid residue = $\frac{4.462547. \times 1}{40}$ = 0.11564 ng/mL

Mean residue in control sample = 0.0098 ng/ml, (used below for background subtraction)

$$\text{Recovery} = \frac{0.11563 - 0.0098}{0.1} \times 100 = 106\%$$

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 of one matrix type 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 (n-1), 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

The LC-MS/MS methods are highly selective for the determination of (2,4-dichlorophenoxy)acetic acid, 2,4-dichlorophenol and 4-chlorophenol by virtue of the chromatographic separation and selective detection system used. To demonstrate further confirmation, a second MS/MS ion transition was monitored for each analyte. Calculations of %Recovery and %RSD were carried out on the confirmatory ions data (Tables 1,2,4,5,7 and 8).

The GC/MS method is highly selective for the determination of 2,4-dichloroanisole by virtue of the chromatographic separation and selective detection system used. To demonstrate further confirmation, two additional ions were monitored. Calculations of %Recovery and %RSD were carried out on both of the confirmatory ions data (Tables 3, 6 and 9).

Analytical Method for use in ILV:

7. PREPARATION OF STANDARDS

The preparation of these standard solutions may be achieved by the use of alternative dilutions if necessary and alternative concentrations may be used as appropriate to the analysis.

7.1 Preparation of Spiking Solutions

7.1.1. 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.

7.1.2. Pipet 250 µL of each 1000-µg/mL solution from Section 7.1.1 into separate 25-mL volumetric flask and dilute to volume with methanol to obtain a solution containing 10.0-µg/mL of each analyte.

7.1.3 Pipet 250 µL of each 10.0-µg/mL solution from Section 7.1.2 into separate 25-mL volumetric flask and dilute to volume with methanol to obtain a solution containing 0.10-µg/mL of each analyte.

7.1.4 Weigh 0.0500 g of 2,4-dichloroanisole analytical standard and quantitatively transfer to 50-mL volumetric flasks with acetone. Dilute to volume with acetone to obtain a 1000-µg/mL stock solutions of each 2,4-dichloroanisole.

7.1.5. Pipet 250 µL of the 1000-µg/mL solution from Section 7.1.4 into a 25-mL volumetric flask and dilute to volume with acetone to obtain a solution containing 10.0-µg/mL.

7.1.6 Pipet 250 µL of each 10.0-µg/mL solution from Section 7.1.5 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.

7.2. Preparation of Calibration Standard Solutions

7.2.1. Prepare calibration standards in separate 10 mL volumetric flasks by dispensing the appropriate amount of 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

7.2.2. 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

8. Typical Instrumental Operating Conditions

8.1 Typical HPLC Operating Conditions (For the analysis of 2,4-D and 2,4-DCP by LC/MS/MS)

Instrumentation:	Symbiosis Pharma
Column:	Synergi Hydro-RP (75x4.6 mm id)
Column Temperature:	Ambient
Injection Volume:	10 µL
Injection Wash Program	1) 2 x 700 µL methanol containing 0.5% NH_4OH 2) 3 x 700 µL water
Run Time:	6.0 minutes
Mobile Phase:	A –Acetonitrile:methanol (80:20) with 0.1% acetic acid B –Water with 0.1% acetic acid
Flow Rate:	1.0 mL/min. Flow diverted for first 1.5 minutes

Isocratic:

Time, min	A, %	B, %
0:00	55	45
6:00	55	45

Flow Diverter Program:

1) 0.0→1.5 min: flow to waste
2) 1.5→4 min: flow to source
3) 4→end of run: flow to waste

8.2 Typical Mass Spectrometry Operating Conditions

Instrumentation:	MDS SCIEX API 5000 LC/MS/MS System
	MDS SCIEX Analyst 1.5.1 data system
Interface:	Heated Nebulizer
Polarity:	Negative
Scan Type:	MRM
Resolution:	Q1 – unit, Q3 – unit
Curtain Gas (CUR):	30 psi
Collision Gas (CAD):	4.0 (High)
Temperature (TEM):	450°C
Ion Source Gas 1 (GS1):	30 psi
Pre-acquisition Delay:	1.5 min
Acquisition Time	4.5 min
Dwell Time:	50 ms

Analytes:	Precursor Ion Q1	Product Ion Q3	Declustering Potential	Collision Energy	Cell Exit Potential
2,4-D - quantitation	218.985	160.800	-40 V	-22 V	-17 V
2,4-D - confirmation	220.968	163.0	-40 V	-22 V	-15 V
2,4-DCP - quantitation	160.953	125.00	-40 V	-24 V	-15 V
2,4-DCP - confirmation	162.952	127.00	-40 V	-24 V	-11 V

8.3. Typical HPLC Operating Conditions (For the analysis 4-CP by LC/MS/MS)

Instrumentation:	Agilent 1290 HPLC system Applied Biosystems QTRAP 5500 LC/MS/MS System MDS/Sciex Analyst 1.5.1 data system
Column:	Zorbax SB-C8 4.6 x 75 mm, 4-µm
Injection Volume:	10 µL
Speed:	Normal
Needle height:	5 mm
Autosampler wash:	700 µL methanol/water (50:50) containing 0.1% acetic acid
Run Time:	Approximately 12.5 minutes
Mobile Phase:	A – Water containing 0.10% acetic acid B – Acetonitile/Methanol (80:20) containing 0.10% 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 run: flow to waste

8.4 Typical Mass Spectrometry Operating Conditions

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
Pre-acquisition Delay:	0.0 min
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
4-CP – quantitation	126.920	90.948	-80 V	-24 V	-9 V
4-CP - confirmation	128.941	90.943	-75 V	-24 V	-17 V

8.5 Typical GC Operating Conditions (For the analysis of 2,4-DCA by GC/MS)

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
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 Delay	1.25 min
Splitter Flow	50 mL/min
Septum Purge	On
Injection Volume	1 µL

Detector Mode:	Negative-ion chemical ionization
Source Temperature	230 °C
Quad Temperature	150 °C
Reagent Gas	Methane
Flow Setting	44%
Pressure	2.2×10^{-4} torr
Calibration Program	Negative-ion chemical ionization autotune
Electron Multiplier	1900 volts (~200 volts above autotune)
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)

9. DETERMINATION OF RECOVERY OF (2,4-DICHLOROPHENOXY)ACETIC ACID AND ITS METABOLITES IN SURFACE WATER, GROUND WATER AND DRINKING WATER

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

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

9.1.2. 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

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

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

9.1.5. Clean up samples on the Oasis polymeric sorbent reversed phase SPE cartridge using the following procedure:

a. Place an Oasis polymeric sorbent reversed phase 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 (from Step 9.2.4) 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 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 eluates in the same glass tube. Apply full vacuum (approximately -25 inches of Hg) for about 10 seconds between solvent additions.

9.1.6 Concentrate the sample to approximately 500 µL using an N-vap 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).

9.1.7 Adjust the volume in the sample vial to 1.0 mL with approximately 500 µL of a water solution containing 0.1% acetic acid.

Add 4 mL of the water solution containing 0.1% acetic acid to the 5.0 µg/L spiked samples to bring them to a total of 5 mL volume.

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

9.1.9. Transfer a portion of the sample to an autosampler vial using a limited insert.

9.1.10. 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.

9.2. Sample Analysis of 2,4-DCA by GC/MS

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

9.2.2. 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

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

9.2.4. Add 1.0 mL of isooctane extraction solution to the sample and cap vial.

Add 5.0 mL of isooctane extraction solution to the 5.0 µg/L fortified samples.

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

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

9.2.7. Using a Pasteur pipet, transfer a portion of the top layer into a limited insert vial and cap. (NOTE: Aliquot to be injected should be void of aqueous solution.)

9.2.8. Analyze the samples and calibration standards by GC/MS.

CEMAS

Amendment No 1

Study Number: **CEMS-5324** **DAS Study Number:** **110821**

Study Title: **INDEPENDENT LABORATORY VALIDATION OF AN ANALYTICAL METHOD FOR THE DETERMINATION OF (2,4-DICHLOROPHENOXY)ACETIC ACID, 2,4-DICHLOROPHENOL, 4-CHLOROPHENOL AND 2,4-DICHLOROANISOLE IN WATER**

Reference: **Page Number:** 14 **Section Number:** Appendix 1

Amendment: GCMS Operating Conditions were incorrect. New Operating Conditions are detailed on pages 3 and 4.

Reason: An error in the analytical method was made.

Impact: No adverse impact.

Signature Study Director
M Garcia Alix **Date:** 11 January 2012

Signature Management
A Whittle **Date:** 11 January 2012

Signature Sponsor
D Shackelford **Date:** 11-Jan-2012

QA Authorisation
M Griffiths **Date:** 11 January 2012

Signature
Sponsor
Distribution:

Original	Study Director
Copies	Study monitor
	CEMAS QA

8.5 Typical GC Operating Conditions (For the analysis of 2,4-DCA by GC/MS)

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
Liner:	Agilent Liner 1.8 mm ID PTV M – p/n: 5183-2037
Oven Method: Column	80 °C for 1.2 min 80 °C to 320 °C at 20 °C/min 320 °C for 2.0 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	50 °C for 0.1 min Ramps:

Rate	Final Temperature	Final time
1500	280 °C	5.00

Cryo use Temperature	90 °C
Cryo timeout	15.00min
Pressure	9.34 psi
Purge flow	50 mL/min
Purge time	1.20 min
Total flow	54 mL/min
Septum Purge	On
Injection Volume	1 µL

Detector Mode:	Electron-ion (EI-mode)
Source Temperature	230 °C
Quad Temperature	150 °C
Reagent Gas	Helium
Calibration Program	autotune
Electron Multiplier	1900 volts (~200 volts above autotune)
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)