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Chlorothalonil
**Chlorothalonil - Independent Laboratory Validation (ILV)
of Analytical Method (GRM005.08A) for the Residue
Determination of Chlorothalonil in Soil by LC-MS/MS**
Final Report Amendment 2

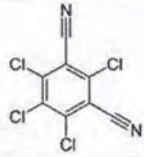
DATA REQUIREMENT(S): EPA OCSPP 850.6100 (2012)
EC SANCO/3029/99 rev 4 (2000)
EC SANCO/825/00 rev 8.1 (2010)



3.0 MATERIALS AND METHODS

3.1 Test/Reference Substance

The test/reference substances were obtained from Syngenta Crop Protection, LLC. The following test/reference substances were used:

Compound Structure	
Syngenta Code:	R44686
Common Name:	Chlorothalonil
CAS Name:	2,4,5,6-tetrachloro-isophthalonitrille
Molecular Weight:	265.9
Storage Conditions:	< 30°C
Purity:	99.6 ± 0.3%
Expiration Date:	01/2015

Characterization data for the test/reference standard are maintained by the Sponsor, Syngenta Crop Protection, LLC. The Certificate of Analysis is included in Appendix 3.

The test/reference substance (analytical standard) used in this study was procured from the Sponsor and stored as directed on "Analytical Standards Chain of Custody" documents. All solutions made from the reference substances (analytical standards) were stored according to the method.

3.2 Test System

The soil samples evaluated in this study were clay loam soil and sandy loam soil, as summarized in the following table. These matrices were chosen because they were representative of the matrices the method was designed for. Control samples used in this study were provided by the Sponsor. Control soil samples were characterized by AGVISE Laboratories of Northwood, North Dakota, reported to Syngenta, and archived under Syngenta Study TK0002309. GLP characterization results are presented in Table 1 and summarized below:

Sample ID	pH	Calcium (ppm)	Magnesium (ppm)	Hardness CaCO ₃ (mg/L)	TDS (ppm)	TSS (ppm)	DOC (ppm)
Clay Loam Soil	N/A	2442	250	N/A	N/A	N/A	N/A
Sandy Loam Soil	N/A	2099	431	N/A	N/A	N/A	N/A

The samples were received at PASC on Aug. 29, 2014, and were stored in a freezer at a nominal temperature of -20°C.

These control samples were checked for contamination prior to use in this ILV study by employing the same extraction and detection method as described in Syngenta Method GRM005.08A. They were found to be free of contamination.

3.3 Equipment and Reagents

3.3.1 Equipment

Equipment	Description	Supplier
General glassware	General glassware	VWR
Polypropylene centrifuge tube	15 mL capacity	Biologix Research Company
Nalgene bottle	250 mL	Thermo Scientific
Crimp cap autosampler vials and caps	2 mL capacity	Agela Technologies
SPE cartridges	Bond Elut C18 500 mg, 6 mL	Agilent
LC-MS/MS system	Triple Quad 6500	AB Sciex
HPLC with autosampler	Acquity UPLC	Waters
HPLC column	Agilent SB-Aq 1.8 um x 50 mm x 4.6 mm	Agilent

3.3.2 Reagents

Reagent	Description	Supplier
Milli-Q Water	Millipore Milli-Q Gradient A10 purified water generator	Milli-Q Water, LETS#77, Calibration Due: 01/2015
Acetonitrile	HPLC grade	Pharmco Product, Inc.
Methanol	HPLC grade	J. T. Baker, Avantor Performance Material, Inc.
Acetone	HPLC grade	BDH
Formic Acid	A.C.S grade	Sigma
Sulfuric Acid	A.C.S grade	Sigma
Ammonium Acetate	A.C.S grade	Sigma

3.3.3 Preparation of Reagents

Reagent Solution	Preparation
0.1 mM ammonium acetate in Milli-Q water	7.708 mg of ammonium acetate 1000 mL of Milli-Q water
0.1% Formic acid	1 mL of formic acid 999 mL of Milli-Q water
20/80 (v/v) methanol/0.1 % formic acid in Milli-Q water	200 mL of methanol 800 mL of 0.1 % formic acid in Milli-Q water
Acidic acetone	1,540 mL of acetone 60 mL of 50% H ₂ SO ₄ in Milli-Q water.
50/50 (v/v) methanol/Milli-Q water	500 mL of methanol 500 mL of Milli-Q Water
40/60 (v/v) methanol/0.1% formic acid in Milli-Q water	400 mL of methanol 600 mL of 0.1 % formic acid in Milli-Q water

3.4 Preparation of Standard Solutions

3.4.1 Stock Standard

A stock standard solution of chlorothalonil was made by weighing 10.082 mg of chlorothalonil into a 100 mL "Class A" volumetric flask and diluting to the mark with methanol. The final concentration of chlorothalonil in the stock standard solution was 100.042 µg/mL.

3.4.2 Fortification Standard

The first level fortification standard solution (containing chlorothalonil at a concentration of 1.0042 µg/mL) was prepared by adding 1.0 mL of chlorothalonil stock standard to a 10 mL volumetric flask and diluting to the mark with methanol. The ID of the resulting solution is F10291401.

The second level fortification standard solution (containing chlorothalonil at a concentration of 0.1 µg/mL) was prepared by adding 10 mL of the first level chlorothalonil fortification standard solution to a 100 mL volumetric flask and diluting to the mark with methanol. The ID of the resulting solution is F10291402.

3.4.3 Calibration Standard

Calibration Standards of chlorothalonil were prepared for LC-MS analysis as outlined below:

Stock Solution		Calibration Solution				
Standard Solution ID	Conc. (ng/mL)	Aliquot Taken (mL)	Final Volume (mL)	Solvent	Conc. (ng/mL)	Calibration Solution ID
F10291401	1004.2	2	100	20/80 (v/v) Methanol/ 0.1% Formic Acid	20.084	C10291401
F10291401	1004.2	1	100		10.042	C10291402
C10291401	20.084	25	100		5.021	C10291403
C10291401	20.084	10	100		2.008	C10291404
C10291402	10.042	10	100		1.004	C10291405
C10291402	10.042	5	100		0.502	C10291406
C10291402	10.042	2	100		0.2008	C10291407

3.5 Analytical Procedures and Modifications

Analytical Method GRM005.08A was independently validated as written. No modifications or deviations were made in this study.

3.5.1 Modifications

No modifications or deviations were made to the analytical method.

3.5.2 Fortifications

Untreated control soil samples were fortified using microliter amounts of the appropriate fortification standard to LOQ and 10×LOQ concentrations as per the method.

Fortifications for this method validation were performed as follows:

Matrix	Fortification Vol. (µL)	Fortification Conc. (µg/mL)	Sample Wt.(g)	Final Conc.	Replicates
Sandy Loam	1000	0.1	20	LOQ (5 µg/kg)	5
Sandy Loam	1000	1.0	20	10×LOQ (50 µg/kg)	5
Clay Loam	1000	0.1	20	LOQ (5 µg/kg)	5
Clay Loam	1000	1.0	20	10×LOQ (50 µg/kg)	5

3.5.3 Extraction Procedure

As indicated by the method, the extractions were performed as follows:

1. 20 g sub-samples of soil were weighed into 250 mL Nalgene plastic bottles. The recovery samples were then fortified with the fortification solution in methanol. The samples were allowed to stand for 5 minutes before the next step. Two untreated control and two control samples fortified with known amounts of analytes of interest were analyzed with each sample set, using the same procedure, to verify method performance.
2. To each of the samples, 100 mL of freshly prepared acidic acetone was added and the bottles were capped.
3. The sample bottles were placed horizontally on a mechanical shaker and shaken at a speed (300 cps) that visibly agitated the samples vigorously for 2 hours at room temperature.
4. Samples were then centrifuged at approximately 3500 rpm with refrigeration at 10°C for 10 minutes.
5. The supernatants were decanted carefully into brown plastic Nalgene bottles (125 mL).

3.5.4 SPE Clean-up Procedures

Agilent Bond Elut C18 SPE (500 mg, 6 mL) cartridges were used for sample clean up and concentration. The cartridges were kept from drying through the pre-conditioning, rinsing and final eluting procedures. The flow rates were controlled at < 20 drops per minute (approximately 1 mL/min). The SPE procedure is described below:

1. Bond Elut C18 SPE cartridges were pre-conditioned by passing 3 mL of methanol through the cartridges, followed by 3 mL of Milli-Q water, by gravity.
2. Aliquots (2 mL) were transferred from the extract into polypropylene centrifuge tubes (15 mL), then were diluted with 10 mL of ultrapure water.
3. The samples were loaded and passed through the SPE cartridges by gravity. The flow rate was controlled as described above.
4. The polypropylene centrifuge tubes were rinsed with 3 mL of 50/50 (v/v) methanol/Milli-Q water.
5. The samples were rinsed 3 times with 4 mL of 50/50 (v/v) methanol/Milli-Q water.
6. Chlorothalonil residues were eluted from the SPE using 5 mL of acetonitrile and were collected in a clean polypropylene tube (15 mL).
7. 1 mL of 0.1% formic acid was added to each of the tubes containing the sample eluent. The samples were then evaporated to just less than 1 mL under a stream of N₂ in a water bath set at approximately 40°C.
8. The samples were reconstituted to 2 mL with 40/60 (v/v) methanol/0.1% formic acid in Milli-Q water to yield final fractions.
9. The sample final fractions were sonicated for 5 minutes and aliquots (1 mL) were transferred into HPLC injection vials for LC-MS/MS analysis.

3.6 Instrumentation

LC-MS/MS Instrument Description:

LC System	Waters Acquity UPLC Sample Manager and Column Manager
MS Detector	Applied Biosystems Sciex Triple Quad 6500 mass spectrometer with Analyst™ software version 1.6.2

Flow Rate	0.6 mL/min
Column	Zorbax SB-Aq, 4.6 x 50 mm, 1.8 mm
Column Oven Temp.	Ambient
Injection Vol.	20 µL
Run Time	15 minutes
Mobile Phase A	0.1 mM ammonium acetate in Milli-Q water
Mobile Phase B	Methanol

Isocratic/Gradient Flow:

Time (minutes)	A%	B%	Flow Rate (mL/min)
0	80	20	0.6
0.5	80	20	0.6
8	5	95	0.6
12	5	95	0.6
12.1	80	20	0.6
15	80	20	0.6

Mass Spectrometer Conditions:

Interface	Turbo Ion Spray
Polarity	Negative
Curtain gas (CUR)	Nitrogen set at 30
Temperature (TEM)	500°C
Ionspray voltage	-4500
Collision gas setting (CAD)	Medium
Gas 1 (GS1)	Air set at 55
Gas 2 (GS2)	Air set at 55
Interface heater (ihe)	On
Scan type	MRM

MRM Conditions	Chlorothalonil Primary Transition	Chlorothalonil 1 st Confirmatory Transition	Chlorothalonil 2 nd Confirmatory Transition
Q1 <i>m/z</i>	244.900	244.900	244.900
Q3 <i>m/z</i>	181.900	209.900	174.900
Dwell time	150	150	150
Resolution Q1	Unit	Unit	Unit
Resolution Q3	Unit	Unit	Unit
Declustering potential (DP)	-50	-52	-51
Entrance potential (EP)	-10	-10	-10
Collision energy (CE)	-39	-37	-38
Collision cell exit potential (CXP)	-9	-9	-11

3.7 Data Acquisition

Peak integration and peak area count quantitation were performed by AB Sciex Analyst 1.6.2. A best-fit, linear regression equation was derived and used in conjunction with the analyte response in each sample to calculate the concentration of analyte. The correlation coefficient (R) for the calibration curve for each analytical set was greater than 0.99. Recovery results were computed for each sample.

A statistical treatment of the data includes the calculation of averages, standard deviations, relative standard deviations. Mean percent recoveries, standard deviations, and relative standard deviations were calculated using a current Microsoft Office Excel package.

TABLE 1 Characterization Data of Soil Types used for Method Validation

Soil Type	pH (0.01 M CaCl ₂)	Sand Content (% w/w)	Silt Content (% w/w)	Clay Content (% w/w)	Organic Carbon (%)
Clay Loam ^[1]	7.6	21	42	37	N/A
Sandy Loam ^[2]	7.3	55	28	17	N/A

[1] Soil from Underwood farm 0-6: Syngenta Report TK0002309

[2] Soil from Madera CA 1-15-13 0-6: Syngenta Report TK0002309

Note: The GLP characterization of these soil types was performed by AGVISE Laboratories, 604 Highway 15, P.O. Box 510, Northwood, ND 58267.

TABLE 2 Soil Fortification Levels

Soil Type	Fortification Level	Number of Recovery Samples
Clay Loam	Control	2
	LOQ (5 µg/kg)	5
	10×LOQ (50 µg/kg)	5

Soil Type	Fortification Level	Number of Recovery Samples
Sandy Loam	Control	2
	LOQ (5 µg/kg)	5
	10×LOQ (50 µg/kg)	5

Abbreviations and Symbols

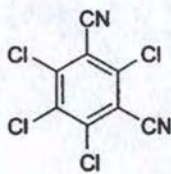
Abbreviation	Definition
°C	degrees Celsius or Centigrade
amu	atomic mass unit
CAS	Chemical Abstract Services
cm	centimetre
EPA	Environmental Protection Agency (U.S.)
EC	European Commission
EU	European Union
g	gram
GRM	Global Residue Method
HPLC	high performance liquid chromatography
i.d.	internal diameter
IUPAC	International Union of Pure and Applied Chemistry
kg (or Kg)	kilogram
L	litre
LC-MS/MS	tandem liquid chromatography/mass spectrometry/mass spectrometry
LOD	limit of detection
LOQ	limit of quantification
m	metre
MeOH	methanol
µg	microgram
µL	microlitre
µm	micrometre
mg	milligram
mL	millilitre
mm	millimetre
mmol	millimole
min	minute
mol	mole
ms	millisecond
MS/MS	tandem mass spectrometry/mass spectrometry
mV	millivolt
MW	molecular weight
<i>m/z</i>	mass to charge ratio

Abbreviation	Definition
N/A (or n/a)	not applicable
ND (or nd)	not detectable (below limit of detection)
ng	nanogram
No.	number
OES	Occupational Exposure Standards
OECD	Organisation for Economic Co-operation and Development
OPPTS	Office of Prevention, Pesticides and Toxic Substances
ppb	parts per billion or micrograms per kilogram or micrograms per litre
ppm	parts per million or milligrams per kilogram or milligrams per litre
pg	picogram
R (or r)	correlation coefficient
R ² (or r ²)	coefficient of determination or square of correlation coefficient
RSD	relative standard deviation
Rt (or RT)	retention time
s (or sec)	second
SD (or STDEV)	standard deviation
SHC	Syngenta Hazard Category
SPE	Solid Phase Extraction
UPW	ultra pure water
V	volt
Vol (or vol)	volume

CHEMICAL STRUCTURES

FIGURE 1 Chlorothalonil

Compound Code Number : R44686
Alternative compound code number : SDS2787
CAS Number : 1897-45-6
IUPAC Name : 2,4,5,6-Tetrachloro-isophthalonitrile
Molecular Formula : $C_8Cl_4N_2$
Molecular Weight : 265.91
Molecular Mass : 263.88



APPENDIX 1 Apparatus**Recommended Suppliers [Need update]**

Equipment	Description	Supplier
General lab glassware	General lab glassware	www.thermoscientific.com
General lab plastic-ware	General lab plastic-ware	www.thermoscientific.com
Autosampler vials	Snap cap, 2 mL size	www.thermoscientific.com
LC-MS/MS system Includes HPLC and autosampler units	AB Sciex 5500 Qtrap	www.thermoscientific.com
HPLC column	Zorbax SB-Aq; 1.8 μm 4.6 mm i.d \times 50 mm	www.agilent.com
SPE Cartridges	Bond Elut C18 500 mg, 6-mL	www.agilent.com
PTFE Syringe Filter	13mm, 0.45 μm	www.thermoscientific.com

APPENDIX 2 Reagents

Recommended Suppliers

Reagent	Description	Supplier
Ultrapure water	Optima grade	www.thermoscientific.com
MeOH	HPLC grade	www.thermoscientific.com
Acetonitrile	HPLC grade	www.thermoscientific.com
Acetone	HPLC grade	www.thermoscientific.com
Formic Acid	A.C.S. grade	www.thermoscientific.com
Sulfuric Acid	A.C.S. grade	www.thermoscientific.com
Ammonium Acetate	A.C.S. grade	www.thermoscientific.com
Chlorothalonil (R44686)	GLP certified	Syngenta Crop Protection, LLC, P.O. Box 18300, Greensboro, NC 27419-8300.

Preparation of Reagents

- a) 0.1% formic acid in ultrapure water, prepared by mixing 1 mL of formic acid in 999 mL of ultrapure water;
- b) 40/60 (v/v) MeOH/0.1% formic acid in ultrapure water, prepared by mixing 200 mL of MeOH with 300 mL of 0.1% formic acid in ultrapure water;
- c) 50% H₂SO₄ in ultrapure water, prepared by mixing 200 mL of ultrapure water with 200 mL of concentrated H₂SO₄;
- d) Fresh extraction solvent, prepared by mixing 60 mL of 50% H₂SO₄ with 1540 mL of acetone before adding to the soil sample;
- e) 50/50 (v/v) MeOH/ultrapure water, prepared by mixing 500 mL of MeOH with 500 mL of ultrapure water;
- f) 20/80 (v/v) MeOH/0.1% formic acid in ultrapure water, prepared by mixing 200 mL of MeOH with 800 mL of 0.1% formic acid in ultrapure water;
- g) "0.05%" formic acid in ultrapure water, prepared by mixing 0.5 mL of formic acid with 1000 mL of ultrapure water;
- h) 1 M ammonium acetate aqueous solution, prepared by dissolving 7.7 grams of ammonium acetate into 100 mL of ultrapure water;
- i) 0.1 mM ammonium acetate aqueous solution, prepared by mixing 100 µL of 1 M ammonium acetate solution with 1000 mL optima grade water.

APPENDIX 3 LC-MS/MS Tuning Procedure

Calibration of Instrument

The instrument must be mass calibrated on a regular basis using polytyrosine-1,3,6 solutions according to the manufacturer's instructions. Calibrate both mass resolving quadrupoles (Q1 and Q3).

Tuning Instrument for Analytes

For example, infuse a standard solution of chlorothalonil (0.1 to 1.0 µg/mL) in mobile phase (see section 4) directly into the mass spectrometer interface at a rate of approximately 5-20 µL/min in the presence of toluene (infused at a flow rate of 5 µL/min) as dopant under APPI conditions. Roughly adjust interface parameters (sprayer position and temperature, spray, heater/auxiliary gas flows, as well as voltages of spray, orifice, and focusing ring) for a sufficiently high parent ion signal at m/z 244.9 for chlorothalonil in negative ionization mode. The m/z of 244.9 represents a product ion of $[M-Cl+O]^-$ from chlorothalonil as reported in the literature.^[See references below]

Using the Xcalibur™ Software optimization routine, tune the instrument for chlorothalonil for MS/MS transitions and ensuring that the correct ion is selected. If desired, manual tuning of the ion optics and collision energy can be carried out to ensure maximum sensitivity.

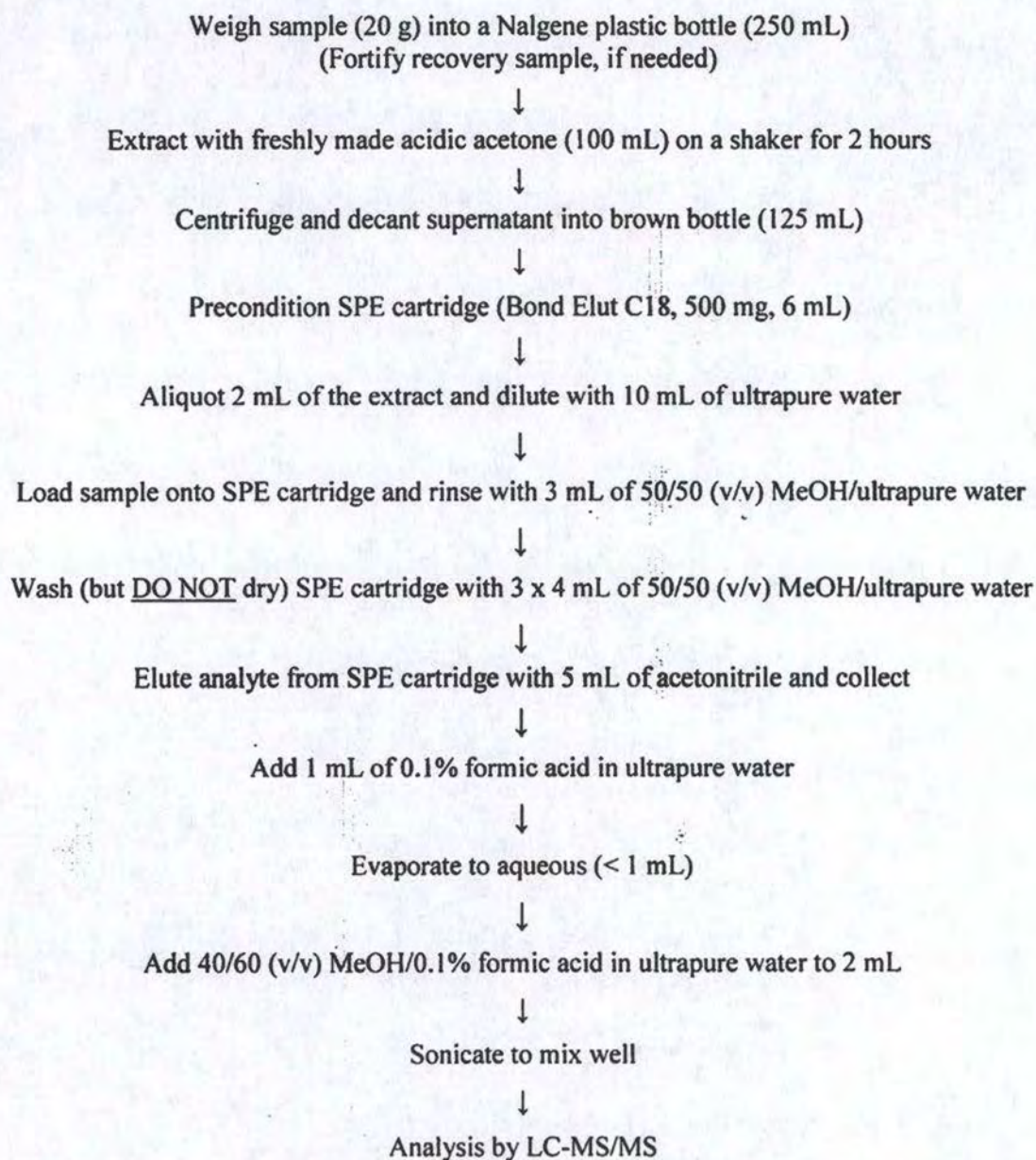
Finally, connect the LC-pump via the autosampler directly to the MS/MS instrument. Perform repetitive flow injection of standards using mobile phase at the flow rate to be used. Tune the interface parameters (sprayer position and temperature, spray and heater gas flows, spray, orifice, and focusing ring voltages and the collision gas pressure) for maximum sensitivity.

In general, the two most sensitive MS/MS transitions are selected and used for quantitative and confirmative analysis. The tuned m/z values and corresponding MRM transitions for the analytes are listed in Section 4.3.

Final determination by LC-MS/MS with two transitions is considered to be highly specific; hence no further confirmatory conditions are included.

The instrument must be mass calibrated on a regular basis using polytyrosine-1,3,6 solutions according to the manufacturer's instructions. Calibrate both mass resolving quadrupoles (Q1 and Q3).

APPENDIX 4 Method Flow Chart



APPENDIX 3 Example Calculations

Typical Calculations for LC/MS/MS Quantitation

Sample No. 10-19-10 CASS ND SL 0-6", Sandy Loam Soil, LOQ-1
 Primary Transition (m/z 244.9 → 181.9), Filename: Batch2 10302014.rdb

$$\text{Concentration of Analyte (C}_A\text{)}(\text{ng/mL}) = \frac{\text{peak area} - \text{intercept}}{\text{slope}}$$

	Chlorothalonil
Peak Area	4808
Intercept	-296
Slope	6040
C _A (ng/mL)	0.845

$$\text{Matrix injected (mg)} = \left(\frac{\text{Sample Weight (g)}}{\text{Extract Vol. (mL)}} \right) \times \left(\frac{\text{Aliquot Vol. (mL)} \times \text{Inj. Vol. (}\mu\text{L)}}{\text{Final Volume (mL)}} \right)$$

$$\text{Extract Volume (mL)} = \text{extraction solvent volume (mL)} + \text{sample weight (g)} * \text{moisture (\%)}$$

	Chlorothalonil
Sample Weight (g)	20.73
Extract Volume (mL)	100
Aliquot Volume (mL)	2
Injection Volume (μL)	20
Final Volume (mL)	2
Matrix Injected (mg)	4.1460

$$\text{Residues (}\mu\text{g/kg)} = \frac{\text{Chlorothalonil Found on Column (pg)}}{\text{Sample Injected on Column (mg)}}$$

	Chlorothalonil
Chlorothalonil Injected on Column (pg)	0.0169
Sample Matrix Injected on Column (mg)	4.1460
Residues (ppb)	0.004

NOTE: Slight rounding differences may be noted when using a hand calculator. Full computer/calculator precision was used for any intermediate calculations. Only the final value was rounded.