2 INTRODUCTION

The purpose of this study was to conduct an independent laboratory validation (ILV) for the determination of the cis- and trans isomers of d-Phenothrin in soil. The analysis of the reference/test substances was performed by gas chromatography coupled with positive-ion tandem mass spectrometry (GC-MS/MS) based on the method "Independent laboratory Validation (ILV) of an Analytical Method for the Determination of the cis- and trans Isomers of d-Phenothrin in Soil by GC-MS," PTRL Europe Study No. P3047G, November 28, 2013, provided by the sponsor.

This study was designed to satisfy US EPA Guideline requirements described in OCSPP 860.1340 and 850.6100. The study was initiated on July 28, 2017 at EAG Laboratories-Hercules, 625-B Alfred Nobel Drive, Hercules, CA 94547 under an approved protocol (Appendix A) according to the US EPA FIFRA Good Laboratory Practice Standards, 40 CFR §160. The experimental work was completed on October 16, 2017. This study was completed on July 3, 2019.

3 MATERIAL AND METHODS

3.1 Test/Reference Substances

d-Phenothrin (Sumithrin):

Chemical Name:	3-Phenoxybenzyl (1R)-*cis,trans*-chrysanthemate
CAS No.:	26002-80-2
Lot No.:	151108
Inventory No.:	2950W-002
Purity:	96.2%
Trans isomer ratio:	80.4%
1R isomer ratio:	96.1%

cis-d-Phenothrin:

Chemical Name:	3-Phenoxybenzyl (1RS)-*cis, trans*-chrysanthemate
CAS No.:	51186-88-0
Lot No.:	C170227
Inventory No.:	2950W-001
Purity:	99.8%

trans-d-Phenothrin:

Chemical Name:	3-Phenoxybenzyl (1R)- *trans*-chrysanthemate
CAS No.:	26046-85-5
Lot No.:	161102
Inventory No.:	2950W-003
Purity:	97.4%

d-Phenothrin, cis-d-Phenothrin and trans-d-Phenothrin were provided by Sumitomo Chemical Company, Ltd. on July 17, 2017. Upon receipt at EAG Laboratories-Hercules, the test/reference substances were assigned the inventory No. 2950W-001 through 2950W-003. The test/reference substances were stored refrigerated when not in use.

The certificates of analysis are provided in Appendix B.

3.2 Reagents

Water, HPLC grade (Fisher Chemical or equivalent manufacturer)
Methanol (MeOH), HPLC grade (Fisher Chemical or equivalent manufacturer)
Dichloromethane (DCM), HPLC grade (Fisher Chemical or equivalent manufacturer)
Hexane HPLC grade (Fisher Chemical or equivalent manufacturer)
Ethyl acetate, HPLC grade (Fisher Chemical or equivalent manufacturer)
Toluene, HPLC grade (Fisher Chemical or equivalent manufacturer)
Sodium chloride (NaCl), (EMD or equivalent manufacturer)
Sodium sulfate, (Fisher Chemical or equivalent manufacturer)
Florisil (Fisher Scientific or equivalent manufacturer)

3.3 Equipment/Materials List

Laboratory Balances
Orbital Shaker
Polypropylene bottles (250 mL capacity)
Glass fiber piper (GF/A)
Buchner funnels
Separatory funnels (500 mL capacity)
Funnels with glass wool
Flat bottom flasks (250 mL and 125 mL capacity)
Rotary evaporators with water bath
Glass columns (17 mm)
Sonicator
Graduated glass centrifuge tubes (15 mL capacity)
Nitrogen evaporator (N_2 Evap)
Volumetric flasks
Vortex
Variable volume pipetors with plastic disposable tips
Glass precision syringes
Volumetric pipettes
Oven
Total recovery vials and autosampler vials
Amber bottles and vials with Teflon® lined caps

Agilent 7000 Series Triple Quadrupole Mass Spectrometer (GC-QQQ) with Agilent 7890A Series gas chromatograph and ATLAS Combi-PAL auto sampler
Mass Hunter Data System Software

3.4 Test System

3.4.1 Source of the Test System

A sandy loam soil was collected in Othello, WA and received on April 22, 2016. Upon arrival at EAG Laboratories-Hercules, the test system was assigned the inventory No. 2705W-069 and stored refrigerated (typically between 4 °C and 10°C) in the dark when not in use.

3.4.2 Characterization of the Test System

The soil used in the study was characterized by Agvise Laboratories, Inc. (604 Highway 15 West, Northwood, North Dakota). The characterization report is presented in Appendix C.

3.5 Test Method

The analytical method for the analysis of d-Phenothrin isomers *cis*-d-Phenothrin and *trans*-d-Phenothrin in soil was independently validated at EAG Laboratories-Hercules by GC-MS and GC-MS/MS. Analysis of d-Phenothrin isomers was based on the analytical method described in "Independent laboratory Validation (ILV) of an Analytical Method for the Determination of the cis- and trans Isomers of d-Phenothrin in Soil by GC-MS", PTRL Europe, Method No. P 3047 G, November 28, 2013. There was no modification from the described analytical method.

The method for the analysis of the cis- and trans isomers of d-Phenothrin in soil samples consisted of extraction with methanol followed by liquid/liquid partition (adding 10% aqueous NaCl) with dichloromethane (DCM) and glass column chromatography using activated Florisil. Final extracts were analyzed by Gas Chromatography with Mass Spectrometry Detection (GC-MS) using Selected Ion Monitoring (SIM) and Gas Chromatography coupled with tandem mass spectrometry (GC-MS/MS) using Multiple Reaction Monitoring (MRM). The percent recovery of the compound was determined

using external standardization where separate linear curves of calibration standards for each isomer were analyzed along with the samples.

3.5.1 Preparation of Stock Solutions

Separate stock solutions of d-Phenothrin isomers (*cis*-d-Phenothrin and *trans*-d-Phenothrin) were prepared by weighing aliquots (approximately 25 mg) of the reference substances into 25 mL volumetric flasks and diluting to the mark with toluene. Additional solvent was added as necessary to achieve a nominal concentration of 1.0 mg/mL after adjusting for the purity of the reference substances as follows:

Compound	Weight (mg)	Final volume (mL)	Purity (%)	Theoretical conc. (µg/mL) [1]
cis d-Phenothrin	25.13	25.08	99.8	1,000
trans d-Phenothrin	25.92	25.24	97.4	1,000

[1]Theoretical conc. (µg/mL) = [weight (mg) x 1,000 µg/mg ÷ final volume (mL)] x [purity (%)]

The stock solutions were sonicated to ensure complete dissolution, transferred into amber bottles and stored refrigerated (typically between 4°C and 10°C) when not in use.

3.5.2 Preparation of Fortification Solutions

A stock solution containing d-Phenothrin was prepared by weighing an aliquot (26.47 mg) of the test substance into a 25 mL volumetric flask and diluting to the mark with toluene. Additional solvent (0.39 mL) was added to achieve a concentration of 1.003 mg/mL after adjusting for the purity of the test substance (96.2%).

Fortification solutions containing d-Phenothrin were prepared by measuring an appropriate volume of the source solution and transferring into separate 10 mL volumetric flasks. Solutions were diluted to the mark with toluene and transferred into amber bottles. Fortification solutions were stored refrigerated (typically between 4°C and 10°C) when not in use.

Aliquot (mL)	Solution used (µg/mL)	Volume (mL)	Theoretical concentration (µg/mL) [1]
1.0	1,003 stock	10	100
2.0	100 µg/mL	10	20
0.2	100 µg/mL	10	2

[1]Theoretical conc. (µg/mL) = {[theoretical conc. solution used x aliquot (mL)] ÷ volume (mL)}

3.5.3 Preparation of Intermediate Solutions

Intermediate solutions were prepared for each isomer by volumetrically combining proper aliquots of each stock (1.0 mg/mL) into separate 10 mL volumetric flasks. Final solutions were diluted to the mark with toluene, mixed and transferred to amber bottles.

Aliquot (mL)	Solution used (µg/mL)	Volume (mL)	Theoretical concentration *cis* d- Phenothrin (µg/mL) [1]	ID
0.1	1,000 stock cis	10	10	IS-A

Aliquot (mL)	Solution used (µg/mL)	Volume (mL)	Theoretical concentration *trans* d-Phenothrin (µg/mL) [1]	ID
1.0	1,000 stock trans	10	100	IS-B
0.1	1,000 stock trans	10	10	IS-C

The intermediate solutions were stored refrigerated (usually between 4°C and 10°C) when not in use..

3.5.4 Preparation of Calibration Solutions

Eight calibration solutions containing cis d-Phenothrin and seven calibration solutions containing trans d-Phenothrin were prepared by measuring appropriate volumes of the of the source solution and transferring into separate 10 mL volumetric flasks. Solutions were diluted to the mark with toluene. The concentration of cis d-Phenothrin ranged from 4 ng/mL to 1,000 ng/mL; the concentration of d-trans phenothrin ranged from 40 ng/mL to 2,500 ng/mL as shown below:

Aliquot (mL)	Solution used	Final volume (mL)	Theoretical concentration *cis* d- Phenothrin (ng/mL) [1]
1.000	IS-A	10	1,000
0.250	IS-A	10	250
0.150	IS-A	10	150
0.100	IS-A	10	100
0.500	1,000 ng/mL	10	50
0.250	1,000 ng/mL	10	25
0.100	1,000 ng/mL	10	10
0.400	100 ng/mL	10	4

Aliquot (mL)	Solution used	Final volume (mL)	Theoretical concentration *trans* d-Phenothrin (ng/mL) [1]
0.250	IS-B	10	2,500
0.150	IS-B	10	1,500
0.100	IS-B	10	1,000
0.500	IS-C	10	500
0.250	IS-C	10	250
0.100	IS-C	10	100
0.400	1,000 ng/mL	10	40

[1]Theoretical conc. (ng/mL) = {[theoretical conc. solution used (ng/mL) x aliquot (mL)] ÷ final volume (mL)}

The calibration solutions were stored refrigerated ((usually between 4 °C and 10 °C) when not in use.

3.6 Preparation of Spiked Solutions for Matrix Effects Assessment Solutions

3.6.1 Preparation of Matrix-Based Standard Solutions

A control soil extract was spiked at LOQ (0.01 mg/kg) by combining 0.18 mL aliquot of a control soil extract with 0.02 mL of d-Phenothrin fortification solution (2 µg/mL, section 3.5.2) to yield a nominal concentration of 200 ng/mL.

A control soil extract was spiked at 10XLOQ (0.1 mg/kg) by combining 0.49 mL aliquot of a control soil extract with 0.01 mL of d-Phenothrin fortification solution (100 µg/mL, section 3.5.2) to yield a nominal concentration of 2,000 ng/mL.

3.6.2 Preparation of Solvent-Based Standard Solutions

A reagent blank was spiked at LOQ (0.01 mg/kg) following the procedure described in section 3.6.1 except that 0.18 mL of the reagent blank was used instead of the control soil extract.

A reagent blank was spiked at 10XLOQ (0.1 mg/kg) following the procedure described in section 3.6.1 except that 0.49 mL of the reagent blank was used instead of the control soil extract.

3.7 Fortification Procedure

Fortification of untreated soil samples was conducted at two fortification levels as shown below:

Test system (Matrix)	Fortification Level (mg/kg)	Fortification volume (mL)	Solution used
Soil (20 g)	0.01	0.1	2 µg/mL Low fortification solution
	0.1	0.1	20 µg/mL High fortification solution

Fortification was conducted to determine the percent recovery within the Independent Laboratory Validation. This procedure was performed in quintuplicate during Independent Laboratory Validation at each fortification level.

3.8 Extraction Procedure for cis- and trans Isomers of d-Phenothrin in Soil

3.8.1 Extraction

1. Weigh soil (20 g) into a polypropylene bottle (250 mL).
2. Fortify the samples as needed.
3. Add 40 mL MeOH to soil.
4. Extract in an orbital shaker for 10 minutes.
5. Filter extract through glass fiber paper into a Buchner funnel, under vacuum.
6. Transfer extract into a 500 mL separatory funnel.
7. Repeat steps 3 thru 5.
8. Combine filtered extracts into the 500 mL separatory funnel.
9. Rinse the polypropylene tube and Buchner funnel with 30 mL MeOH.

10. Add MeOH from step 9 to the separatory funnel (step 8)
11. Partition the extract with 80 mL of 10% NaCl (aqueous) with 40 mL DCM
12. Pass DCM layer thru a bed of sodium sulfate supported by a glass wool in a funnel into a 250 mL flat bottom flask
13. Partition extract with additional 40 mL DCM and repeat step 12.
14. Concentrate DCM extract using a rotary evaporator to dryness at 30°C.
15. Reconstitute residues with 3 mL of mixed solvent (hexane: ethyl acetate (20:1)) and sonicate.

3.8.2 Florisil Clean-up

16. Load 3 mL extract from step 15 onto a glass column (17 mm diameter) packed with 15 g of activated Florisil.[1]
17. Use three additional 3 mL washes of mixed solvent (hexane: ethyl acetate (20:1)) to the 250 mL flat bottom flask.
18. Load washes onto the column sonicating the flask in between each of the 3 mL washes.
19. Allow extract and washes to percolate through the column and discard eluate.
20. Add 45 mL hexane: ethyl acetate (20:1) to the column and discard the first 5 mL.
21. Collect remaining 40 mL of eluate in a 125 mL flat bottom flask.
22. Concentrate eluate to 1 – 2 mL using a rotary evaporator at 30°C and sonicate flask.
23. Transfer concentrated extract into a 15 mL graduated glass centrifuge tube.
24. Rinse flat bottom flask with 2 x 5 mL hexane sonicating in between and transfer rinses into the 15 mL centrifuge tube.
25. Concentrate extract under nitrogen at 30°C to dryness.
26. Reconstitute residues with 1.0 mL toluene and sonicate.
27. Transfer final extract into a total recovery vial.
28. Aliquot final extract in an autosampler vial and analyze by GC-MS and GC-MS/MS.

Notes: Glass columns were packed with activated Florisil as follows:

- Create a slurry with 15 g Florisil (activated overnight 130°C) in a mixed solvent (20:1 hexane: ethyl acetate)
- Transfer small portions of the slurry at a time onto the column and tap glass in order to pack the column

[1] Activate Florisil in an oven at 130°C overnight prior to use.

- Drain the mixed solvent after all the slurry is transferred
- Add approximately 1 g of sodium sulfate on top of the Florisil column

3.9 GC-MS and GC-MS/MS Analytical Methods

3.9.1 GC conditions

Column: DB-5MS, 30 m x 0.25 mm, 0.25 µm film thickness

Injection mode: splitless

Liner: Single Goose Neck

Injection volume: 2 µL

Needle rinse: Toluene

Injector temperature: 225°C

Oven temperature program:

Initial conditions: 95°C hold for 0.75 minutes
Ramp 1: 15°C/minute to 250°C hold for 0 min
Ramp 2: 10°C/minute to 275°C hold for 7 min

Run time: 20.6 minutes

Column flow rate (He): 1.5 mL/min (constant flow)

Approximate retention times:

- d-Phenothrin: 14.0 minutes

3.9.2 MS conditions (SIM Mode)

Electron Impact mode (EI)

MSD transfer line: 275°C

MS source: 275°C

Solvent delay: 6 min

Scan type: Selected Ion Monitoring (SIM)

Time segments

Time segment	Time (min)	Scan Type	Gain
1	3	MS1 SIM	100

Time Events

Time segment 1

Compound name	Mass (m/z)	MS1 resolution	Dwell (ms)
d-Phenothrin	184	unit	150
d-Phenothrin	183	unit	150
d-Phenothrin	123	unit	150

3.9.3 MS/MS conditions (MRM Mode)

Electron Impact mode (EI)

MSD transfer line: 275°C

MS source: 275°C

Solvent delay: 6 min

Scan type: Multiple Reaction Monitoring (MRM)

Time segments

Time segment	Time (min)	Scan Type	gain
1	6	MRM	100

Time Events

Time segment 1

Compound name	Precursor ion (m/z)	MS1 resolution	Product ion (m/z)	MS2 resolution	Dwell (ms)	CE (V)
d-Phenothrin	183	unit	168	unit	20	10
d-Phenothrin	183	unit	165	unit	20	10
d-Phenothrin	183	unit	153	unit	20	10

3.10 GC-MS and GC-MS/MS Analyses

Each of the SIM and MRM sequences consisted of *cis* and *trans*-d-Phenothrin calibrants (section 3.5.4) which were analyzed from the lowest concentration to the highest concentration interspersed among the sample set in single injection. Toluene was analyzed as the solvent blank during each sequence . Toluene and a mid-level calibrant were injected multiple times at the beginning of the sequence to stabilize response of the instrument. A mid-level calibrant of each isomer was reanalyzed as quality control standard to ensure good chromatography and consistent instrument performance at the end of each sequence. All samples were analyzed in single injection.

The stability of the signal was monitored by comparing the response (analyte peak area) of a quality control standard injection with that of a comparable standard from the linear curve of the corresponding isomer within the sequence(s).

To assess if the responses of the calibration solutions had been affected by matrix either by signal suppression or enhancement, the matrix-based and solvent-based standard solutions (200 ng/mL and 2,000 ng/mL, section 3.6) were injected each in single injection.

3.11 Methods of Calculation

3.11.1 Quantitation

Cis and *trans* d-Phenothrin was quantitated by the external standard method using at least a six-point linear curve regression for each isomer. Separation of these compounds was achieved by GC-MS in SIM mode and by GC-MS/MS in MRM mode. The isomers were identified by the coincidence of their retention times with their respective reference standards and MS characteristics. The quantitation of *cis* and *trans* d-Phenothrin was conducted by peak area of each fragment ion (SIM)/transition ion (MRM) of each isomer to the theoretical concentration of the calibration standard solutions.

The content of *cis* and *trans* d-Phenothrin in the MRM sequence was quantitated against weighted (1/x) linear curves ($y = mx + b$) of the corresponding isomer calibration solutions where:

y = peak area
x = theoretical concentration (ng/mL) *cis* or *trans* d-Phenothrin

m = slope
b = intercept

The content of *cis* and *trans* d-Phenothrin in the SIM sequence was quantitated against weighted (1/x) quadratic curves ($y = ax^2 + bx + c$) of the corresponding isomer calibration solutions where:

y = peak area
x = theoretical concentration (ng/mL) *cis* or *trans* d-Phenothrin
a = quadratic coefficient
b= linear coefficient
c = constant

Weighting of the calibration curve was applied so as to provide better curve fit at the lower concentration levels. The calculation of the linear and quadratic curve regressions and found concentration (ng/mL) present in the samples and calibrants was conducted using Mass Hunter software.

The percent recovery of *cis* or *trans* d-Phenothrin from fortified samples was determined by dividing the found amount (ng/g) of each isomer (corrected for mean control contribution, if necessary) by the relevant theoretical fortified amount (ng/g) of the corresponding isomer. The theoretical fortified amount (ng/g) of each isomer was calculated by multiplying the fortification level of d-Phenothrin by the respective cis (19.6%) or trans (80.4%) ratio[2]. The percent recovery of the sum of *cis* and *trans* d-Phenothrin was also determined by summing the found amount of both isomers and dividing by the sum of the total theoretical fortified amount.

[2] 80.4%; ratio value from COA; see Appendix B; therefore, cis ratio = 100% – 80.4% = 19.6%

3.11.2 Residue in soil

% Recovery (%) = [(ng/g recovered isomer – ng/g isomer mean control) ÷ ng/g isomer fortified] x 100

Where:
ng/g recovered = [isomer calculated concentration (ng/mL) x dilution factor x final volume (mL)] ÷ sample mass (g)

ng/g fortified = fortification level (µg/g) x 1,000 ng/µg

Cis d-Phenothrin fortified (ng/g) = Fortification level (ng/g) x [trans ratio (%) ÷ 100]
Cis d-Phenothrin fortified (ng/g) = 10.0 ng/g x (19.6 ÷ 100) = 1.96

Trans d-Phenothrin fortified (ng/g) = Fortification level (ng/g) x [trans ratio (%) ÷ 100]
Trans d-Phenothrin fortified (ng/g) = 10.0 ng/g x (80.4 ÷ 100) = 8.04

Sum cis + trans recovered (ng/g) = cis recovered (ng/g) + trans recovered (ng/g)

Calculated concentration (ng/mL) was determined by Mass Hunter software

Note: Control residues were not subtracted if below LOD.

Example:
Sample set: MRM
Sample: F1A (m/z 183 → 153)
Fortification level (ng/g) = 0.01 µg/g x 1000 ng/µg = 10.0 ng/g
Cis d-Phenothrin fortified (ng/g) = 10.0 ng/g x 19.6% = 1.96
Trans d-Phenothrin fortified (ng/g) = 10.0 ng/g x 80.4% = 8.04
Sample mass (g) = 20
Final volume (mL) = 1.0
Dilution factor = 1

Cis d-Phenothrin calculated concentration (ng/mL) = 42.6384
Cis d-Phenothrin ng/g recovered = (42.6384 ng/mL x 1.0 mL x 1) ÷ 20 g = 2.13
Cis d-Phenothrin % Recovery = [(2.13 ng/g - 0.0 ng) ÷ 1.96 ng/g] x 100 = 109%

Trans d-Phenothrin calculated concentration (ng/mL) = 185.2682
Trans d-Phenothrin ng/g recovered = (185.2682 ng/mL x 1.0 mL x 1) ÷ 20 g = 9.26
Trans d-Phenothrin % Recovery = [(9.26 ng/g - 0.0 ng) ÷ 8.04 ng/g] x 100 = 115%

Sum cis + trans recovered (ng/g) = 2.13 + 9.26 = 11.4
Sum cis + trans % Recovery = (11.4 ng/g ÷ 10.0 ng/g) x 100 =114%

Note: values rounded for presentation and may differ slightly from reported values.

3.11.3 LOQ theoretical/expected concentration (ng/mL) in soil samples

LOQ (ng/mL) = (ng fortified ÷ final volume mL) ÷ dilution factor

Where:
ng fortified = fortification level (ng/g) x sample mass (g)
Fortification level (ng/g) = 0.01 µg/g x 1000 ng/µg = 10.0 ng/g
Sample mass (g) = 20
Final volume (mL) = 1.0
Dilution factor = 1

d-Phenothrin fortified (ng) = 10.0 ng/g x 20 g = 200
LOQ (ng/mL) = [(200 ng ÷ 1 mL)] ÷ 1 = 200 ng/mL

Trans d-Phenothrin LOQ (ng/mL) = 200 ng/mL x [trans ratio (%) ÷ 100]
Trans d-Phenothrin LOQ (ng/mL) = 200 ng/mL x (80.4 ÷ 100) = 160.8

cis d-Phenothrin LOQ (ng/mL) = 200 ng/mL x [cis ratio (%) ÷ 100]
cis d-Phenothrin LOQ (ng/mL) = 200 ng/mL x (19.6 ÷ 100) = 39.2

3.11.4 LOD theoretical/expected concentration (ng/mL) in soil samples

Theoretical LOD (ng/mL) = (200 ng x 20%) ÷ 100% = 40

Trans d-Phenothrin LOD (ng/mL) = 40 ng/mL x [trans ratio (%) ÷ 100]
Trans d-Phenothrin LOD (ng/mL) = 400 ng/mL x (80.4 ÷ 100) = 32.2

cis d-Phenothrin LOD (ng/mL) = 40 ng/mL x [cis ratio (%) ÷ 100]
cis d-Phenothrin LOD (ng/mL) = 40 ng/mL x (19.6 ÷ 100) = 7.8

3.12 Calibration Range

The calibration curves were generated by Mass Hunter software for *cis* and *trans* d-Phenothrin in each validation. The calibration ranges for each isomer in soil by GC-MS in SIM mode and by GC-MS/MS in MRM mode were as follows:

Analysis	Mode	Isomer	concentration range (ng/mL)	
			low	high
GC-MS	SIM	cis	4	1,000
		trans	40	2,500
GC-MS/MS	MRM	cis	10*	1,000
		trans	40	2,500