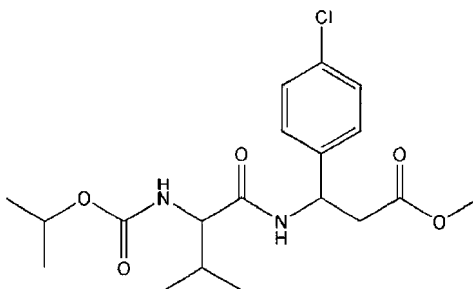




2.0 TEST SUBSTANCES

A summary of the information concerning the test substances is listed below which includes structure, chemical name, purity and expiration date. The certificates of analysis (CoA) are included in Appendix A.

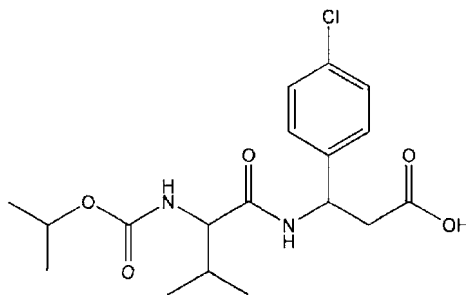
2.1 VALIFENALATE



Valifenalate

Common Name:	Valifenalate
Chemical Name:	Methyl N-(isopropoxycarbonyl)-L-valyl-(3 <i>R</i> , <i>S</i>)-2-(4-chlorophenyl)-β-alanine
CAS No.:	283159-90-0
Molecular Formula:	C ₁₉ H ₂₇ ClN ₂ O ₅
Lot No.:	G019/07 (ID in CoA: IR5885)
Purity:	99.52%
Expiration Date:	Nov 2017
Molecular Weight:	398.88 g/mole
Storage:	Ambient Temperature

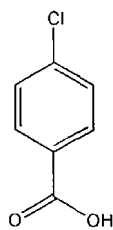
2.2 VALIFENALATE ACID



Valifenalate acid

Common Name:	Valifenalate acid
Chemical Name:	(<i>R,S</i>)- β -alanine, N-((1-methylethoxy)- <i>L</i> -valy-3-(4-chlorophenyl) acid)
CAS No.:	NA
Molecular Formula:	$C_{18}H_{25}ClN_2O_5$
Lot No.:	G029/08 (ID in CoA: IR5839)
Purity:	98.4%
Expiration Date:	Feb 21, 2017
Molecular Weight:	384.86 g/mole
Storage:	Ambient Temperature

2.3 *p*-CHLOROBENZOIC ACID (PCBA)



p-Chlorobenzoic acid (PCBA)

Common Name:	<i>p</i> -Chlorobenzoic acid (PCBA)
Chemical Name:	4-Chlorobenzoic acid
CAS No.:	74-11-3
Molecular Formula:	$C_7H_7ClO_2$
Lot No.:	LC07337V
Purity:	99.9%
Expiration Date:	Apr 2017
Molecular Weight:	156.57 g/mole
Storage:	Ambient Temperature



3.0 EXPERIMENTAL SECTION

3.1 TEST SYSTEM

The test system (Smokey Oaks Pond water, EFS#505, received from Penny Minor, North Bloomfield, Trumbull County, OH 44450, N41.495446, W80.747337,) was stored at 0-6 °C until analysis. The water parameters are summarized in Table 2 below. The water characterization report is included in Appendix A.

Table 2: Characterization Data of Smokey Oaks Pond Water (EFS#505)

Parameters	
pH	7.8
Calcium	28 ppm
Magnesium	6.6 ppm
Sodium	7.2 ppm
Hardness	98 mg equivalent CaCO ₃ /L
Conductivity	0.21 mmhos/cm
Sodium Adsorption Ratio (SAR)	0.32
Total Dissolved Solids	124 ppm
Turbidity	2.23 NTU

3.2 MATERIALS

3.2.1 Chemicals

Acetic acid, Fisher HPLC Grade
Formic acid, Fisher Optima LC-MS Grade
Hydrochloric acid 1 N solution, Fisher Scientific
Acetone, Fisher Optima Grade
Acetonitrile, Fisher Optima Grade
Methanol, Fisher Optima Grade
Water, Fisher HPLC Grade

3.2.2 Reagents

Dilution solvent: methanol -water (50:50) + 0.1% formic acid

Methanol (500 mL), HPLC water (500 mL), and formic acid (1 mL) were mixed.

Dilution solvent: methanol -water (10:90) + 0.1% formic acid

Methanol (100 mL), HPLC water (900 mL), and formic acid (1 mL) were mixed.

HPLC solvent A: 0.1% formic acid in water

HPLC water (1 L) and formic acid (1 mL) were mixed.

HPLC solvent B: 0.1% formic acid in acetonitrile

Acetonitrile (1 L) and formic acid (1 mL) were mixed.

HPLC solvent A: 0.1% acetic acid in water

HPLC water (1 L) and acetic acid (1 mL) were mixed.

HPLC solvent B: 0.1% acetic acid in acetonitrile

Acetonitrile (1 L) and acetic acid (1 mL) were mixed.



3.2.3 Equipment

The following contains a partial list of the equipment used in this study. Any equipment used in this study and not contained in the following list can be found in the appropriate sections of this report.

Analytical electronic balance with 0.1-mg readability

Eppendorf micropipettes: 20-200 μL , and 100-1000 μL

Glassware: Assorted beakers, bottles, graduated cylinders, pipettes, etc., which are routinely used for residue analysis.

3.2.4 Preparation of Standard Solutions and Calibration Solutions

The following standard solutions and calibration solutions were prepared by mixing of the following stock solutions, followed by serial dilution with methanol-water (50:50, v:v) + 0.1% formic acid or methanol-water (10:90, v:v) + 0.1% formic acid in "Class A" volumetric flasks, as detailed in Table 3 below. All solutions were stored in a freezer (~ -20 $^{\circ}\text{C}$) when not in use.

- 1005 $\mu\text{g}/\text{mL}$ Valifenalate in acetone stock solution (purity corrected)
- 1004 $\mu\text{g}/\text{mL}$ Valifenalate acid in acetone stock solution (purity corrected)
- 1009 $\mu\text{g}/\text{mL}$ p-Chlorobenzoic acid (PCBA) in acetone stock solution (purity corrected)

Table 3: Preparation Scheme for Standard Solutions and Calibration Solutions

Standard solutions in methanol -water (50:50) + 0.1% formic acid			
Solution used	Volume taken (mL)	Final volume (mL)	Nominal concentration ($\mu\text{g}/\text{mL}$)
1005 $\mu\text{g}/\text{mL}$ Valifenalate	0.249	50	5
1004 $\mu\text{g}/\text{mL}$ Valifenalate Acid	0.249		
1009 $\mu\text{g}/\text{mL}$ PCBA	0.248		
5 $\mu\text{g}/\text{mL}$	5	25	1
5 $\mu\text{g}/\text{mL}$	5	50	0.5
Calibration solutions in methanol -water (10:90) + 0.1% formic acid			
Solution used	Volume taken (mL)	Final volume (mL)	Nominal concentration (ng/mL)
1000 ng/mL	5	50	100
1000 ng/mL	2.5	50	50
1000 ng/mL	1	50	20
100 ng/mL	5	50	10
50 ng/mL	5	50	5
20 ng/mL	5	50	2
10 ng/mL	5	50	1
5 ng/mL	5	50	0.5
2 ng/mL	5	50	0.2
1 ng/mL	5	50	0.1



3.2.5 Water Fortifications

Using a graduated cylinder, twelve aliquots of Smokey Oaks Pond water samples (100 mL each) were transferred to 250-mL Erlenmeyer flasks. Ten water samples were fortified with valifenalate, valifenalate acid, and PCBA mixed solutions as described in Table 4 below. One “reagent blank” and two water samples serving as controls were not spiked.

Table 4: Preparation Scheme for Water Fortifications (0.1 ppb and 1 ppb)

Sample #	Smokey Oaks Pond Water	Fortification Solution and Volume
Reagent Blank*	None	None
Water Control-1	100 mL	None
Water Control-2	100 mL	None
Water 0.1 ppb-1	100 mL	valifenalate, valifenalate acid, PCBA 0.1/0.1/0.1 µg/mL** 100 µL
Water 0.1 ppb-2	100 mL	valifenalate, valifenalate acid, PCBA 0.1/0.1/0.1 µg/mL 100 µL
Water 0.1 ppb-3	100 mL	valifenalate, valifenalate acid, PCBA 0.1/0.1/0.1 µg/mL 100 µL
Water 0.1 ppb-4	100 mL	valifenalate, valifenalate acid, PCBA 0.1/0.1/0.1 µg/mL 100 µL
Water 0.1 ppb-5	100 mL	valifenalate, valifenalate acid, PCBA 0.1/0.1/0.1 µg/mL 100 µL
Water 1 ppb-1	100 mL	valifenalate, valifenalate acid, PCBA 0.5/0.5/0.5 µg/mL*** 200 µL
Water 1 ppb-2	100 mL	valifenalate, valifenalate acid, PCBA 0.5/0.5/0.5 µg/mL 200 µL
Water 1 ppb-3	100 mL	valifenalate, valifenalate acid, PCBA 0.5/0.5/0.5 µg/mL 200 µL
Water 1 ppb-4	100 mL	valifenalate, valifenalate acid, PCBA 0.5/0.5/0.5 µg/mL 200 µL
Water 1 ppb-5	100 mL	valifenalate, valifenalate acid, PCBA 0.5/0.5/0.5 µg/mL 200 µL

*Reagent Blank constitutes the final SPE eluate and water dilution following the SPE clean-up procedure (excluding step 2)

**in methanol-water (10:90) + 0.1% formic acid

***in methanol-water (50:50) + 0.1% formic acid

3.2.6 SPE Clean-up

1. Waters OASIS SPE cartridges (HLB, 6cc, 500 mg) were placed on SPE vacuum manifolds (Supelco Visiprep) and conditioned with one 5 mL aliquot of methanol followed by one 5 mL aliquot of water.
2. Each sample was added with 5 mL of 1 N HCl and applied to a SPE cartridge with a reservoir adapter.
3. Each flask was rinsed once with a mixture of water (20 mL) and 1 N HCl (1 mL), and the rinse was applied to the SPE cartridge. All eluates from steps 1 to 3 were discarded.
4. Polypropylene centrifuge tubes (15 mL) were placed under the SPE cartridges.
5. Methanol (5 mL) was added to each cartridge to elute the analytes.
6. The centrifuge tube was filled to the 10-mL mark with HPLC water and the sample was mixed.
7. The final samples were transferred to auto sampler vials for analysis by LC-MS/MS.



3.3 LC-MS/MS ANALYSIS

3.3.1 Principle of Measurement

Separation of the analyte from water matrix was achieved by high performance liquid chromatography (HPLC). Quantitative LC-MS/MS analysis of valifenalate, valifenalate acid, and PCBA in the samples utilized highly specific and sensitive MRM (Multiple Reaction Monitoring) methods. Valifenalate precursor ion (m/z 399) was monitored in Q1 and the fragment ions were monitored in Q3 (primary MRM transition, m/z 155, and confirmatory MRM transition, m/z 116). Valifenalate acid precursor ion (m/z 385) was monitored in Q1 and the fragment ions were monitored in Q3 (primary MRM transition, m/z 116, and confirmatory MRM transition, m/z 144). PCBA precursor ion (m/z 155) was monitored in Q1 and the fragment ions were monitored in Q3 (primary MRM transition, m/z 111, and confirmatory MRM transition, m/z 35). The analytes were identified by the same retention times as in the calibration standards, and were quantitated by using the peak areas against the calibration curve.

3.3.2 Chromatography and Detection

The following are the LC-MS/MS parameters used. The MS parameters were obtained from the Eurofins Method No. RA034 (Reference 1).

Valifenalate and Valifenalate Acid

HPLC: Two Shimadzu LC20-AD pumps and a Shimadzu SIL-HTA

Controller/Autosampler

Column: Analytical Advantage ARMOR C18, 5 μ m, 100 x 2.1 mm, P/N ADV7009

Column Temperature: 40 $^{\circ}$ C

Injection Volume: 10 μ L

Solvent System:

Solvent A = 0.1% formic acid in water

Solvent B = 0.1% formic acid in acetonitrile

Solvent program:

Time (minutes)	Flow Rate (mL/min)	%A	%B
0.0	0.8	80	20
1.5	0.8	80	20
1.7	0.8	5	95
3.0	0.8	5	95
3.1	0.8	80	20
5.0	0.8	80	20

The LC flow was diverted to the MS between 1.0 and 3.9 min and to waste between 0.0 and 1.0 min and between 3.9 and 5.0 min.

Retention times: valifenalate ~3.3 min, valifenalate acid ~3.2 min;



Mass Spectrometer: SCIEX API 4000

Scan Type:	MRM
Polarity:	Positive
Ion Source:	Turbo Spray
Resolution Q1	Unit
Resolution Q3	Unit
Ion Source Gas 1 (GS1):	50 psi
Ion Source Gas 2 (GS2):	70 psi
Curtain Gas (CUR):	20 psi
Collision Gas (CAD):	6 psi
IonSpray Voltage (IS):	5500 V
Temperature (TEM):	400 °C
Entrance Potential (EP):	10 V
Collision Gas Exit Potential (CXP):	12 V

MRM Transition

MRM Transition Method	Analyte ID	Q1 Mass (amu)	Q3 Mass (amu)	Declustering Potential (DP)	Collision Energy (CE)	Dwell Time (msec)
Primary	Valifenalate	399	155	66 V	47 V	100
Confirmatory	Valifenalate C	399	116	66 V	33 V	100
Primary	Valifenalate acid	385	116	51 V	31 V	100
Confirmatory	Valifenalate acid C	385	144	51 V	21 V	100

Calibration Standards for Analysis of Valifenalate and Valifenalate Acid

For analysis of valifenalate and valifenalate acid, a series of calibration standards containing a mixture of valifenalate, valifenalate acid, and PCBA at 0.2, 0.5, 1, 2, 5, 10, and 20 ng/mL were prepared to quantify the residues in the water samples.

PCBA

HPLC: Two Shimadzu LC20-AD pumps and a Shimadzu SIL-HTA Controller/Autosampler

Column: Analytical Advantage ARMOR C18, 5 µm, 100 x 2.1 mm, P/N ADV7009

Column Temperature: 40 °C

Injection Volume: 20 µL

Solvent System:

Solvent A = 0.1% acetic acid in water

Solvent B = 0.1% acetic acid in acetonitrile

Solvent Program:



Time (minutes)	Flow Rate (mL/min)	%A	%B
0.0	0.8	90	10
2.5	0.8	10	90
3.0	0.8	10	90
3.1	0.8	90	10
5.0	0.8	90	10

The LC flow was diverted to the MS between 1.0 and 4.0 min and to waste between 0.0 and 1.0 min and between 4.0 and 5.0 min.

Retention time: PCBA ~2.6 min

Mass Spectrometer: SCIEX API 4000

Scan Type:	MRM
Polarity:	Negative
Ion Source:	Turbo Spray
Resolution Q1	Unit
Resolution Q3	Unit
Ion Source Gas 1 (GS1):	60 psi
Ion Source Gas 2 (GS2):	50 psi
Curtain Gas (CUR):	19 psi
Collision Gas (CAD):	8 psi
IonSpray Voltage (IS):	-4500 V
Temperature (TEM):	550° C
Declustering Potential (DP):	-30 V
Entrance Potential (EP):	-10 V

MRM Transition

MRM Transition Method	Analyte ID	Q1 Mass (amu)	Q3 Mass (amu)	Collision Energy (CE)	Collision Gas Exit Potential (CXP)	Dwell Time (msec)
Primary	PCBA	155	111	-16 V	-7 V	500
Confirmatory	PCBA-C	155	35	-52 V	-3 V	500

Calibration Standards for Analysis of PCBA

For analysis of PCBA, a series of calibration standards containing a mixture of valifenalate, valifenalate acid, and PCBA at 0.2, 0.5, 1, 2, 5, 10, and 20 ng/mL were prepared to quantify the residues in the water samples.

3.4 METHODS OF CALCULATION

3.4.1 Recoveries

The recoveries of analytes (valifenalate, valifenalate acid, and PCBA) from fortified samples were calculated based on the linear calibration curves generated with the analytical set:



Linear regression formula from calibration curve $y = mx + b$

$$x \text{ (analyte in ng/mL)} = \frac{y-b}{m}$$

Where y = Sample peak area

b = Calibration curve intercept

m = Calibration curve slope

$$\text{Sample concentration (ng/mL)} = \frac{\text{Sample peak area} - \text{intercept}}{\text{Slope}}$$

$$\text{ppb analyte} = \frac{\text{Sample conc. (ng/mL)} \times \text{Extract vol. (mL)} \times \text{Dilution factor}}{\text{Sample weight (grams)}}$$

where ng/g is equivalent to $\mu\text{g/kg}$ and ppb.

$$\text{Percent recovery} = \frac{\text{Conc. of analyte in fortified sample (ppb)} - \text{Conc. of control (ppb)}}{\text{analyte fortification level (ppb)}} \times 100\%$$

An example calculation for the recovery of valifenalate (0.1 ppb fortification) from water (sample ID#: **Water 0.1 ppb-1**, Figure C-8) is shown below:

The calibration curve equation was $y = 12100x + 320$ ($r = 0.9998$):

$$\text{ng/mL valifenalate} = \frac{14450 - 320}{12100} = 1.168 \text{ ng/mL}^*$$

The ppb valifenalate in water was calculated as follows:

$$\text{ppb valifenalate} = \frac{1.168 \text{ ng/mL} \times 5 \text{ mL} \times 2}{100 \text{ grams}} = 0.1168 \text{ ppb}^*$$

*slight difference from the reported data due to rounded numbers were used for calculation.

$$\text{Percent recovery} = \frac{0.1168 \text{ ppb} - 0 \text{ ppb}}{0.1 \text{ ppb}} \times 100\% = 117\%$$



APPENDIX B

LC-MS/MS method for analysis of valifenalate, valifenalate acid, and PCBA residues in soil (Eurofins Method No. RA034)



LC-MS Method

Valifenalate and valifenalate acid analysis

HPLC

Column: Analytical Advantage ARMOR C18, 5 µm, 100 x 2.1 mm, P/N ADV7009

Column Temperature: 40 °C

Injection Volume: 50 µL

Solvent System:

Solvent A = 0.1% formic acid in water

Solvent B = 0.1% formic acid in methanol

Solvent program:

Time (minutes)	Flow Rate (mL/min)	%A	%B
0.0	0.8	95	5
1.0	0.8	95	5
1.2	0.8	5	95
3.0	0.8	5	95
3.1	0.8	95	5
3.5	0.8	95	5

Mass Spectrometer: SCIEX API 4000

Scan Type:	MRM
Polarity:	Positive
Ion Source:	Turbo Spray
Resolution Q1	Unit
Resolution Q3	Unit
Ion Source Gas 1 (GS1):	50 psi
Ion Source Gas 2 (GS2):	70 psi
Curtain Gas (CUR):	20 psi
Collision Gas (CAD):	6 psi
IonSpray Voltage (IS):	5500 V
Temperature (TEM):	400 °C
Entrance Potential (EP):	10 V
Collision Gas Exit Potential (CXP):	12 V

MRM Transition

Analyte ID	Q1 Mass (amu)	Q3 Mass (amu)	Decustering Potential (DP)	Collision Energy (CE)	Dwell Time (msec)
Valifenalate	399	155	66 V	47 V	100
	399	144	66 V	21 V	100
	399	116	66 V	33 V	100
Valifenalate acid	385	116	51 V	31 V	100
	385	186	51 V	17 V	100
	385	144	51 V	21 V	100



PCBA analysis

HPLC

Column: Analytical Advantage ARMOR C18, 5 µm, 100 x 2.1 mm, P/N ADV7009

Column Temperature: 40 °C

Injection Volume: 50 µL

Solvent System:

Solvent A = 5 mM ammonium acetate in water

Solvent B = 5 mM ammonium acetate in methanol

Solvent Program:

Time (minutes)	Flow Rate (mL/min)	%A	%B
0.0	0.8	90	10
0.5	0.8	90	10
1.0	0.8	10	90
2.0	0.8	10	90
2.1	0.8	90	10
3.5	0.8	90	10

Mass Spectrometer: SCIEX API 4000

Scan Type:	MRM
Polarity:	Negative
Ion Source:	Turbo Spray
Resolution Q1	Unit
Resolution Q3	Unit
Ion Source Gas 1 (GS1):	60 psi
Ion Source Gas 2 (GS2):	50 psi
Curtain Gas (CUR):	19 psi
Collision Gas (CAD):	8 psi
IonSpray Voltage (IS):	-4500 V
Temperature (TEM):	550° C
Dclustering Potential (DP)	-30 V
Entrance Potential (EP):	-10 V

MRM Transition

Analyte	Q1 Mass (amu)	Q3 Mass (amu)	Collision Energy (CE)	Collision Gas Exit Potential (CXP)	Dwell Time (msec)
PCBA	155	111	-16 V	-7 V	150
	155	35	-52 V	-3 V	150

Calibration Standards

For each analysis, a series of calibration standards containing a mixture of valifenalate, valifenalate acid, and PCBA at 0.3, 0.5, 1, 2, 5, 10, and 25 ng/mL were prepared to quantify the observed residues in the soil samples.