

### **Abstract/Summary**

This report summarizes a successful ILV of the analytical method for the analysis of valifenalate and its metabolites valifenalate acid and p-chlorobenzoic acid in soil.

### **Objective**

The objective was to independently validate an analytical method for the determination of valifenalate, valifenalate acid and p-chlorobenzoic acid in soil to achieve a limit of quantitation (LOQ) of 0.005 mg/kg for valifenalate and valifenalate acid, and a LOQ of 0.01 mg/kg for p-chlorobenzoic acid.

### **Method Principles**

Valifenalate, valifenalate acid and p-chlorobenzoic acid are extracted with mixtures of extraction solvents, acetone and 0.5 N HCl. The material is subjected to repeated extractions by shaking on a horizontal shaker table and, following centrifugation, a portion of the combined extract is reduced in volume by evaporation under an atmosphere of nitrogen and reconstituted in a mixed dilution solvent of CH<sub>3</sub>OH:H<sub>2</sub>O:HCOOH (10:90:0.1). An aliquot of the reconstituted extract is measured by LC-MS/MS, using MRM transitions for quantitation and confirmation. The limit of quantitation (LOQ) of the method is 0.005 mg/kg for valifenalate and valifenalate acid, and 0.01 mg/kg for p-chlorobenzoic acid.

## 1. Introduction

This project represents an Independent Laboratory Validation (ILV) of Precision Study Management, Protocol Amendment PSM-14-02-05, Amendment Number 2 for the Determination of Valifenalate and its metabolites valifenalate acid and p-chlorobenzoic acid. This method represents a highly selective LC-MS/MS method, as it employs both a quantitation and confirmation MRM transition for each analyte. The ILV was conducted on soil matrix. This ILV targeted an LOQ of 0.005 mg/kg and a corresponding LOD of 0.0015 mg/kg for both analytes, valifenalate and its metabolite valifenalate acid in soil and a targeted LOQ of 0.010 mg/kg and a corresponding LOD of 0.003 mg/kg for valifenalate's other metabolite p-chlorobenzoic acid in soil.

## 2. Experimental

### 2.1 Test System

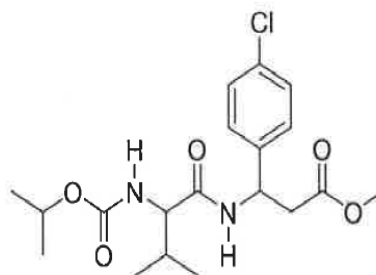
The validation study was carried out using soil purchased from Agvise Laboratories. The soil was homogenized in a Robot Coupe Blixer 3 prior to use.

### 2.2 Analytical Test and Reference Item

Standards of valifenalate, valifenalate acid and p-chlorobenzoic acid were provided by FMC Agricultural Solutions ([Appendix 1](#)).

#### Valifenalate (IR5885):

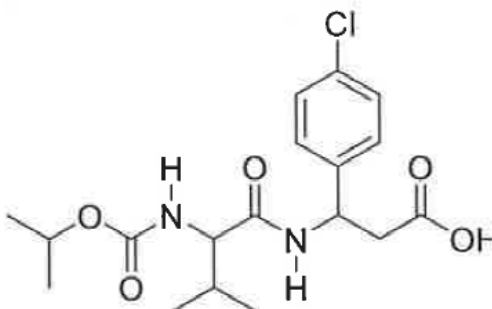
Structure:



Empirical formula:	C <sub>19</sub> H <sub>27</sub> ClN <sub>2</sub> O <sub>5</sub>
Molecular weight:	398.88 g/mol
CAS No.:	283159-90-0
Batch No.:	20071/77
Expiry Date:	November 2017
Purity:	99.27%

**Valifenalate acid (IR5839):**

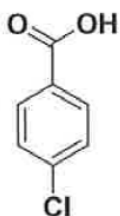
Structure



Empirical formula:	C <sub>18</sub> H <sub>25</sub> ClN <sub>2</sub> O <sub>5</sub>
Molecular weight:	384.86 g/mol
CAS No.:	NA
Batch No.:	G029/08
Expiry Date:	21 February 2017
Purity:	98.4%

**p-Chlorobenzoic acid:**

Structure:



Empirical Formula:	ClC <sub>6</sub> H <sub>4</sub> CO <sub>2</sub> H
CAS Number:	74-11-3
Molecular Weight:	156.57 g/mol
Batch No:	LC07337V
Expiry Date:	May 2017
Purity:	99.2%

## **2.3 Analytical Method**

The Precision Study Management, Protocol Amendment PSM-14-02-05, Amendment Number 2 Analytical Method was used to conduct the ILV.

### **2.3.1 Apparatus**

#### **2.3.1.1 Laboratory Equipment**

- Balances:
  - Ohaus Explorer EOD120, SN D2771118362156
  - Mettler AT201, SN L92660
- Centrifuges:
  - Sorvall Legend XF
  - Eppendorf 5810
- Turbovap – Zymark TurboVap II
- Pipettors: Rainin Pipet-plus, various sizes
- Fisherbrand 50 mL polypropylene centrifuge tube
- Fisher glass vials, 25 mL screw-cap and 2 mL crimp top
- Fisher 15 mL PP centrifuge tubes
- Gas tight syringes – Hamilton, various sizes

All reusable glassware was cleaned in a laboratory dishwasher, solvent rinsed, and air-dried before use. Consumable glassware (injection vials, glass pipettes) was baked at 400°C for at least 30 minutes before use.

#### **2.3.1.2 LC-MS/MS System**

- Shimadzu LC2080 UHPLC system, including a vacuum solvent degasser, binary UHPLC pump, column oven, autosampler
- Applied Biosystems MDS Sciex API 6500 linear ion trap MS/MS system with TurboIonspray (ESI) source
- Thermo Betasil C18 100 x 2.1 mm, 5 µm Catalog # 70105102130

#### **2.3.2 Solvents, Chemicals and Consumables**

- Methanol, HPLC grade, Fisher, lot #152153
- Formic Acid, Acros Organics, lot #B0527746
- Acetone, Fisher, lot # 150933
- Hydrochloric acid 0.5N, Fisher lot # 153033

### 2.3.3 Preparation of Standard Solutions

#### 2.3.3.1 Stock and Working Solutions

Valifenalate, valifenalate acid and p-chlorobenzoic acid were received as neat compounds from FMC Agricultural Solutions. The neat materials were stored under ambient conditions when not in use. The stock solutions were prepared in methanol and stored in the freezer at -20 °C.

Stock solutions:

Compound	Battelle ID	Neat material ID	Mass (mg)	Final Volume (mL)	Solution Concentration (µg/mL)**
Valifenalate	IM13	150624-04 (Val*)	10.78	10	1070
Valifenalate Acid	IN93	150624-05 (ValA*)	10.74	10	1057
p-Chlorobenzoic Acid	IO10	150624-03 (p-CBA*)	10.19	10	1011
Valifenalate	IN38	150624-04 (Val)	10.11	10	1004
Valifenalate Acid	IN98	150624-05 (ValA)	10.45	10	1028
p-Chlorobenzoic Acid	IO11	150624-03 (p-CBA)	9.05	10	898

\* “Val” refers to valifenalate, “ValA” refers to valifenalate acid, and p-CBA refers to p-chlorobenzoic acid.

\*\* Concentration corrected for purity of neat material

Working solutions: The working solutions were prepared with methanol:water (50:50 v:v) with 0.1 % formic acid and stored in the freezer at -20 °C when not in use:

Working solution: valifenalate and valifenalate acid					
Battelle ID	Use solution	Stock conc. (µg/mL)	Stock volume (µL)	Total volume (mL)	Conc. (µg/mL)
IN94	IM13	1070	467	10	50.0 (val)
	IN93	1057	473		50.0 (valA)

Working solution: p-CBA					
Battelle ID	Use solution	Stock conc. (µg/mL)	Stock volume (µL)	Total volume (mL)	Conc. (µg/mL)
IO12	IO10	1011	990	10	100

### 2.3.3.2 Fortification Solutions

Fortification solutions of the analytes were prepared in methanol:water (50:50 v:v) with 0.1 % formic acid and stored in the freezer at -20 °C when not in use:

Fortification solutions: valifenalate and valifenalate acid					
Battelle ID	Use solution	Stock conc. (µg/mL)	Stock volume (µL)	Total volume (mL)	Conc. (µg/mL)
IN95	IN94	50.0	1000	10	5.00 (val)
		50.0			5.00 (valA)
IN96	IN95	5.00	1000	10	0.500 (val)
		5.00			0.500 (valA)

Fortification solutions: p-CBA					
Battelle ID	Use solution	Stock conc. (µg/mL)	Stock volume (µL)	Total volume (mL)	Conc. (µg/mL)
IO13	IO12	100	1000	10	10.0
IO14	IO13	10.0	1000	10	1.00

### 2.3.3.3 Solvent Calibration Solutions:

Intermediate solutions for calibrations were prepared in methanol:water (50:50 v:v) with 0.1 % formic acid and stored in the freezer at -20 °C when not in use:

Intermediate calibration solutions: valifenalate and valifenalate acid					
Battelle ID	Use solution	Stock conc. (µg/mL)	Stock volume (µL)	Total volume (mL)	Conc. (µg/mL)
IO01	IN38	1004	500	10	50.2 (val)
	IN98	1028	485		49.9 (valA)
IO02	IO01	50.2	500	10	2.51 (val)
		49.9			2.49 (valA)
IO03	IO02	2.51	500	10	0.125 (val)
		2.49			0.125 (valA)

Intermediate Calibration solutions:p-CBA					
Battelle ID	Use Solution	Stock conc. (µg/mL)	Stock volume (µL)	Total volume (mL)	Conc. (µg/mL)
IO65	IO11	898	110	10	9.88
IO66	IO65	9.88	100	10	0.0988

FMC Tracking Number: 2015RES-VAL1916

Solvent calibration solutions were prepared by diluting working calibration standards in methanol:water (20:80 v:v) with 0.1% formic acid. Solvent calibration solutions were stored in a freezer at -20 °C when not in use:

Calibration solutions: valifenalate and valifenalate acid					
Battelle ID	Use solution	Stock conc. (µg/mL)	Stock volume (µL)	Total volume (mL)	Conc. (ng/mL)
IO04	IO03	0.125	20	10	0.251 (val)
		0.125			0.249 (valA)
IO05	IO03	0.125	40	10	0.502 (val)
		0.125			0.499 (valA)
IO06	IO03	0.125	120	10	1.51 (val)
		0.125			1.50 (valA)
IO83	IO04	0.000	1000	10	0.251 (val)
		0.000			0.249 (valA)
IO84	IO05	0.001	1000	10	0.502 (val)
		0.000			0.499 (valA)
IO85	IO06	0.002	830	10	0.125 (val)
		0.001			0.124 (valA)

p-CBA calibration solutions, prepared in 20:80 Methanol/H2O w/0.1% Formic Acid					
Battelle ID	Use solution	Stock conc. (µg/mL)	Stock volume (µL)	Total volume (mL)	Conc. (ng/mL)
IO67	IO66	0.0988	7.5	10	0.0741
IO68	IO66	0.0988	25	10	0.247
IO69	IO66	0.0988	75	10	0.741
IO70	IO66	0.0988	150	10	1.48
IO71	IO66	0.0988	300	10	2.96
IO72	IO66	0.0988	500	10	4.94

Matrix matched calibration solutions of the analytes were prepared by diluting 1000 µL of untreated control (UTC) extract with a combination of 10:90 methanol:water with 0.1% formic acid and standard solution. Matrix-matched calibration solutions were stored refrigerated with samples at 0-4 °C when not in use:

Battelle ID	Stock solution ID	Volume taken of stock solution (µL)	Volume of diluent 10:90 methanol:water with 0.1% HCOOH (µL)	Final Volume (µL)	Solution Conc. (ng/mL)
CG959UTC-AG(7)	IO04	1000	7950	10000	0.0251 (val)
					0.0249 (valA)
	IO74	50			0.0741 (p-CBA)
CG959UTC-AG(9)	IO05	1000	7975	10000	0.0502 (val)
					0.0499 (valA)
	IO66	25			0.247 (p-CBA)
CG959UTC-AG(11)	IO06	830	8095	10000	0.125 (val)
					0.124 (valA)
	IO66	75			0.741 (p-CBA)
CG959UTC-AG(13)	IO03	20	8830	10000	0.251 (val)
					0.249 (valA)
	IO66	150			1.48 (p-CBA)
CG959UTC-AG(15)	IO03	40	8660	10000	0.502 (val)
					0.499 (valA)
	IO66	300			2.96 (p-CBA)
CG959UTC-AG(17)	IO03	120	8380	10000	1.51 (val)
					1.50 (valA)
	IO66	500			4.94 (p-CBA)
CH022UTC-AG(7)	IO06	167	8833	10000	0.0250 (valA)
CH022UTC-AG(9)	IO05	1000	8000	10000	0.0499 (valA)
CH022UTC-AG(11)	IO06	830	8170	10000	0.124 (valA)
CH022UTC-AG(13)	IO03	20	8980	10000	0.249 (valA)
CH022UTC-AG(15)	IO03	40	8960	10000	0.499 (valA)
CH022UTC-AG(17)	IO03	120	8880	10000	1.50 (valA)



### 2.3.4 Extraction

#### Extraction Method

1. Measure 10 g of homogenized sample into a 50 mL screw-capped polypropylene container
2. Fortify samples if necessary
3. Add 20 mL of extraction solvent, acetone: 0.5 N HCl (50:50, v/v)
4. Shake on a shaker table for 30 minutes at 3000 rpm
5. Centrifuge at 3000 rpm for 10 min
6. Transfer supernatant to a clean 50 mL centrifuge tube.
7. Vortex to break up pellet
8. Add 20 mL of extraction solvent, acetone: 0.5 N HCl (50:0, v/v) to the solid sample
9. Shake on a shaker table for 30 minutes at 3000 rpm
10. Centrifuge at 3000 rpm for 10 min
11. Combine supernatant from second extraction with that of first extraction
12. Transfer 10 mL aliquot of supernatant to a 15 mL centrifuge tube
13. Reduce the volume down to about 6.5 mL on turbovap with water bath at 40 °C
14. Add CH<sub>3</sub>OH:H<sub>2</sub>O:HCOOH (50:50:0.1, v/v/v) to bring the volume to 10 mL
15. Filter through a Teflon syringe filter
16. Transfer 1 mL to a 12 mL vial and diluted with 9 mL of 10:90 methanol: water (v/v) with HCOOH at 0.1%
17. Transfer an aliquot to an autosampler vial
18. Analyze by LC-MS/MS

## 2.4 LC-MS/MS Analysis

Calibration solutions, matrix-matched calibration solutions, blank extracts, control sample extracts and fortified sample extracts were analyzed by liquid chromatography with tandem mass spectrometry (LC-MS/MS). The following LC/MS/MS conditions were used for valifenalate and valifenalate acid analysis in positive ionization mode:

LC System	Shimadzu LC2080 UHPLC system, including a vacuum solvent degasser, binary UHPLC pump, column oven, autosampler			
LC Column	Thermo Betasil C18 100 x 2.1 mm, 5 µm Catalog # 70105102130			
Injection Vol.	20 µL			
HPLC Method	Mobile Phase A: 0.1 % formic acid in water			
HPLC Method Ret. Times	Mobile Phase B: 0.1 % formic acid in acetonitrile			
	Mobile Phase Composition			
	Time (min)			
	0.0	Flow rate (mL/min)	% A	% B
	1.5	0.8	80	20
	1.7	0.8	5	95
	3.0	0.8	5	95
	3.1	0.8	5	95
	5.0	0.8	80	20
	~ 2.2 – 2.4 minutes	0.8	80	20
MS/MS System	Applied Biosystems MDS Sciex API 6500 linear ion trap MS/MS system with TurboIonspray (ESI) source			
Ion Source Conditions ESI Positive Polarity	Source temperature:	550°C		
	Gas supply (GS 1):	70 (arbitrary units)		
	Gas supply (GS 21):	70 (arbitrary units)		
	Curtain gas (CUR):	45 (arbitrary units)		
	Collision gas (CAD):	medium (arbitrary units)		
	Entrance potential:	10 V		
	IonSpray voltage:	4000 V		
	Resolution:	Q1: Unit, Q3 Unit		

The following LC/MS/MS conditions were used for p-chlorobenzoic acid analysis in negative ionization mode:

LC System	Shimadzu LC2080 UHPLC system, including a vacuum solvent degasser, binary UHPLC pump, column oven, autosampler			
LC Column	Thermo Betasil C18 100 x 2.1 mm, 5 µm Catalog # 70105102130			
Column Temp	40 °C			
Injection Vol.	50 µL			
HPLC Method	Mobile Phase A: 0.1 % formic acid in water			
	Mobile Phase B: 0.1 % formic acid in acetonitrile			
	Mobile Phase Composition			
	Time (min)	Flow rate (mL/min)	% A	% B
	0.0	0.8	90	10
	2.5	0.8	10	90
	3	0.8	10	90
	3.1	0.8	90	10
	5	0.8	90	10
Ret. Times	~ 2.2 – 2.4 minutes			
MS/MS System	Applied Biosystems MDS Sciex API 6500 linear ion trap MS/MS system with TurboIonspray (ESI) source			
Ion Source Conditions ESI Positive Polarity	Source temperature:	550°C		
	Gas supply (GS 1):	50 (arbitrary units)		
	Gas supply (GS 21):	50 (arbitrary units)		
	Curtain gas (CUR):	20 (arbitrary units)		
	Collision gas (CAD):	medium (arbitrary units)		
	Entrance potential:	-10 V		
	IonSpray voltage:	-4500 V		
	Resolution:	Q1: Unit, Q3: Unit		

**MRM Transitions for Valifenalate, Valifenalate acid and p-chlorobenzoic acid.**

MS/MS Conditions for Valifenalate	399 m/z > 155 m/z (used for quantitation)			
	Dwell time:	100 msec	DP:	80 V
	CE:	39 V	CXP:	11 V
	399 > 116 m/z (used for confirmation)			
	Dwell time:	100 msec	DP:	80 V
	CE:	25 V	CXP:	10 V
MS/MS Conditions for Valifenalate Acid	385 > 116 m/z (used for quantitation)			
	Dwell time:	100 msec	DP:	85 V
	CE:	27 V	CXP:	10 V
	385 > 144 m/z (used for confirmation)			
	Dwell time:	100 msec	DP:	85 V
	CE:	19 V	CXP:	9 V
MS/MS Conditions for Valifenalate	155 > 111 m/z (used for quantitation)			
	Dwell time:	500 msec	DP:	-27
	CE:	-16 V	CXP:	-10 V
	155 > 35 m/z (used for confirmation)			
	Dwell time:	500 msec	DP:	-19 V
	CE:	-45 V	CXP:	-16 V

**2.5 Calculations**

The following equation was used to calculate the individual residues R in mg/kg:

$$R = C_{End} \times \left(\frac{V_{Ex}}{W}\right) \times DF \times \frac{1}{1000}$$

Where:

*R*: Residue in mg/kg.

*C<sub>End</sub>*: Final concentration of analyte in extract in ng/mL.

*V<sub>Ex</sub>*: Extraction volume (40 mL).

*W*: Weight of sample (10 g)

*DF*: Dilution factor – final volume/aliquot volume

1/1000: mass conversion from ng/g to mg/kg

The values reported in the tables are calculated with full precision, but displayed with three significant figures. Therefore minor discrepancies may occur when recalculated with a pocket calculator.

Recoveries (*Rec.*) were calculated for the fortified specimens as follows:

$$Rec. = \frac{R}{R_{fort.}} \times 100$$

Where

*Rec.*: Recovery

*R<sub>fort.</sub>*: Residue fortified, in mg/kg.

The calculation is exemplified with the soil sample CG961LOQ-AG(5) fortified at 0.005 mg/kg (LOQ) for valifenalate. The final extract was examined by LC-MS/MS run to give a peak area of 13290 counts for the transition 399 m/z > 155 m/z. Using the respective calibration curve (see [Figure 4](#)) a final concentration of 0.0928 ng/mL was calculated (see [Table 1](#)).

Thus:

$$\begin{aligned} R &= C_{End} \times \left(\frac{V_{Ex}}{W}\right) \times Dilution\ Factor = 0.0928 \frac{\text{ng}}{\text{mL}} \times \left(\frac{40 \text{ mL}}{10.07 \text{ g}}\right) \times \frac{10 \text{ mL}}{1 \text{ mL}} \times \frac{1}{1000} \\ &= 0.00369 \frac{\text{mg}}{\text{kg}} \end{aligned}$$

And:

$$Rec. = \frac{R}{R_{fort.}} \times 100 = \frac{0.00369 \frac{\text{mg}}{\text{kg}}}{0.00496 \frac{\text{mg}}{\text{kg}}} \times 100 = 74\%$$

## 2.6 Deviations from the Method Validation

There were deviations from the method described in document Precision Study Management, Protocol Amendment PSM-14-02-05, Amendment Number 2 and included in the Study Plan.

1. The Study plan states that the laboratory will follow the extraction procedure as stated in Eurofins method RA034 v07, but the laboratory followed the extraction procedure as stated in Precision Study Management, Protocol Amendment Number 2 to be consistent with the method used to analyze field samples. The Eurofins method RA034 v07 MRM transitions were very unusual, used both positive and negative ionization for a given analyte, hence the laboratory alerted the Study Monitor and agreed upon to follow the Precision Study Management, Protocol Amendment Number 2 in its entirety. This deviation did not have any serious impact on the study.
2. Solvent based calibrations were prepared using 20:80:.1% (methanol:water with 0.1% HCOOH) and matrix matched calibrations were prepared using 10:90:.1% (methanol:water with 0.1% HCOOH) hence the solution makeup for the solvent based calibrations and matrix calibrations were slightly different. However, this deviation did not have any serious impact on the study.
3. During the sample extraction step, after centrifugation the procedure instructs to transfer the supernatant liquid to a clean 50 mL centrifuge tube followed by the addition of 20 mL of extraction solvent to the remaining solid residue in the extraction tube. The project team decided to vortex the extraction tube before the addition of 20 mL of extraction solvent so that the residue pellet can break up. Although this step was neither stated in the Protocol Amendment nor in the Study Plan, yet the laboratory included this step and it did not cause any serious impact on the study.

## 3.1 Specificity, Calibration, Matrix Effects and Sensitivity

The highly specific LC-MS/MS method utilizing two mass transitions was confirmed. The product ion spectra valifenalate, valifenalate acid and p-chlorobenzoic acid are shown in [Figures 1, 2, 3 and 3A](#), respectively. The project team was able to confirm that 399 m/z > 155 m/z and 399 > 116 m/z were the most appropriate transitions for quantitation and confirmation of valifenalate, respectively, using the instrument conditions described herein. Also, it was confirmed that 385 m/z > 116 m/z and 385 m/z > 144 m/z were the most appropriate transitions for quantitation and confirmation of valifenalate acid, respectively, both in the positive ionization mode. The laboratory confirmed that 155 m/z > 111 m/z and 155 m/z > 35 m/z were the most appropriate transitions for quantitation and confirmation of p-chlorobenzoic acid, respectively, both in the negative ionization mode.

The project team was able to confirm that for valifenalate and its metabolite valifenalate acid the LC-MS/MS method afforded detection of the analyte at concentrations of 0.025 ng/mL with a 20  $\mu$ L injection, providing sufficient sensitivity to quantify residues of the analyte in the final extracts. Also, the project team confirmed for the second metabolite, p-chlorobenzoic acid the LC/MS/MS method was capable of detecting the analyte at concentration of 0.075 ng/mL with a 50  $\mu$ L injection, providing adequate sensitivity to quantify residues of p-chlorobenzoic acid in the final extract. An instrument calibration for each transition was generated using six