SUMMARY

An analytical method for the quantitation of Bixafen in a soil test system was independently validated. The method is described in "Ferguson, Ling-Jen, 2015: Method Validation of Bixafen in Soils, FMC Report No. PC-0848".

The test substance, Bixafen was analyzed using external standardization by Liquid Chromatography with Tandem Mass Spectrometry Detection (LC-MS/MS). Soil samples were fortified with 0.005 µg/g or 0.005 ppm (LOQ) and 0.05 µg/g or 0.05 ppm (10X LOQ). The limit of detection was defined as approximately 20% of LOQ using the current methodology.

The experiment for soil matrix was conducted with one reagent blank, two untreated controls, and five control samples spiked for each fortification level: one at the LOQ level and another at 10X LOQ level, and analyzed the soil samples by LC-MS/MS.

Two LC gradients (8 and 13 minutes) as described and explained in the original method validation report were used for analyte separation. Bixafen content was quantitated using both gradients against 1/x weighted linear curves of the reference substance Bixafen for both the quantitation and confirmation ions, with concentrations ranging from 0.25 ng/mL to 50 ng/mL. The calibration for Bixafen for both analysis methods yielded acceptable linearity (correlation coefficients r > 0.9999) over the range examined. The quantitation of Bixafen was based on the peak area response and concentrations of the calibration standards. The amount of Bixafen was determined with the quantitation MS/MS ion transition from m/z 414 to m/z 394 and the confirmation MS/MS ion transition from m/z 414 to m/z 266. Method recovery from fortified samples was determined by calculating the found concentration of Bixafen and dividing the concentration by the relevant fortification level.

The LOD in soil is estimated to be 0.001 ppm for Bixafen using either MS/MS transition.

No interferences or residues were detected in control soil with the MS/MS transitions selected for quantitation and confirmation.

The recovery results from 8 and 13 minute gradients are summarized in the tables below. All calculated mean recovery values for quantitation and confirmation ion transitions are within the acceptable range between 70% and 120%. The additional 5 min wash step was added to enhance signal response by further reduction of matrix effects, which was clearly demonstrated from the recovery results.

INTRODUCTION

The purpose of this study was to conduct an independent laboratory validation (ILV) for the determination of Bixafen in soil. The analysis of the test substance was performed by Liquid Chromatography with Tandem Mass Spectrometry Detection (LC-MS/MS) based on the method described in " Ferguson, Ling-Jen, 2015: Method Validation of Bixafen in Soils, FMC Report No. PC-0848" [1].

This study was designed to satisfy US EPA Guideline requirements described in OCSPP 850.6100. The study was initiated on April 29, 2015. The experimental work was conducted from May 4, 2015 through May 22, 2015 at PTRL West, 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.

MATERIAL AND METHODS

Test and Reference Substance

Common Name:	**Bixafen (F9650)**
Chemical Name: (IUPAC):	N-(3',4'-dichloro-5-fluorobiphenyl-2-yl)-3-(difluorodimethyl)-1-methyl-1H-pyrazole-4-carboxamide
CAS Registry No.:	581809-46-3

Chemical Structure:
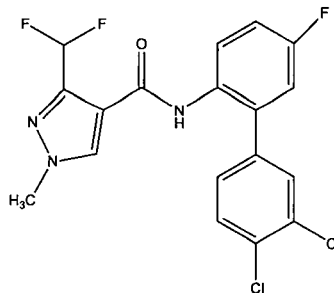
Molecular Formula:	$C_{18}H_{12}Cl_2F_3N_3O$
Molecular Mass:	414.21 g/mole
Supplier:	FMC
Batch No.:	PL14-0108
Purity:	99.7%
Date of Expiry:	April 30, 2019
Storage Conditions:	Ambient Temperature

Certificate of Analysis for the test/reference substance is provided in Appendix B.

Other Chemicals

HPLC grade water and acetonitrile were obtained from Burdick & Jackson; acetic acid was obtained from Fisher Scientific.

Equipment List

Laboratory Balances
Beakers
Pipetmen with plastic disposable tips
Vortex mixer
Centrifuge
Ultrasonic bath
Plastic Bottles
Wrist Action Shaker

AB Sciex API 5500 Series Tandem Mass Spectrometer with Agilent 1200 HPLC system (LC-MS/MS)

Test System

Source of Test System

The same soil test system that was used in the validation study was provided by the Sponsor. The bulk soil was received frozen and stored under frozen conditions (Inventory No. 2763W-001).

Test Method

The analytical method for the analysis of Bixafen validated at PTRL West by Liquid Chromatography with Tandem Mass Spectrometry Detection (LC-MS/MS) was described in the method validation report [1]

The soil samples were spiked with known concentrations of Bixafen. An aliquot of the final sample solution was injected onto the high performance liquid chromatography and subjected to reversed phase chromatography coupled with tandem mass spectrometry (MS/MS) with electro spray ionization. The percent method recovery was determined

using external standardization where linear curve for calibration standards was generated along with the samples.

Preparation of Stock Solutions

Duplicate stock solutions were prepared by weighing an aliquot of the reference substance into a 25 mL volumetric flask. The stock solutions were dissolved and diluted with acetonitrile to yield a nominal concentration of 0.5 mg/mL. The concentration of stock solution was corrected for the purity of the reference substance as shown in the following table. The stock solution was transferred into an amber bottle and stored in the freezer (typically < -10°C) when not in use.

Ptrl West No.	Stock ID	Standard Name	Weight (mg)	Final volume (mL)	Purity (%)	Theoretical Conc. (µg/mL)
2763W-002	Stock A	Bixafen	12.72	25.364	99.7	500
*2763W-002	Stock B	Bixafen	12.82	25.563	99.7	500

*Stock B prepared for information only to confirm accuracy of weighing

Preparation of Bixafen Fortification Solutions

A 1000 ng/mL fortification solution was prepared by measuring 0.2 mL of stock solution "A" using a glass-tight syringe into a 100 mL volumetric flask. Final solution was diluted to the mark with acetonitrile:water solution (8:2, v:v).

A 100 ng/mL fortification solution was prepared by measuring 5.0 mL of the 1000 ng/mL fortification solution using a glass-tight syringe into a 50 mL volumetric flask. Final solution was diluted to the mark with acetonitrile:water solution (8:2, v:v).

The stock and fortification solutions were vortexed to mix, transferred into amber bottles and stored in the freezer (typically < -10°C) when not in use.

Preparation of Bixafen Standard Solutions

Eight calibration standard solutions were prepared by mixing appropriate volumes of stock/calibrant solutions via gas-tight syringes with appropriate volumes of acetonitrile:water solution (8:2, v:v) into the volumetric flask/HPLC vials described below. Final calibrants were transferred into 60 or 125 mL glass amber bottles and stored in the freezer (typically < -10°C) when not in use, except for the 0.25 ng/mL calibrant, which was prepared directly into an HPLC vial prior to analysis and disposed of after injection. The standard solutions ranged from 0.25 ng/mL to 50 ng/mL were prepared as shown below:

Theoretical Conc. (ng/mL)	Solution Used (ng/mL)	Volume of Solution (mL) (mL)	Final Volume (mL)
50	1000	2.5	50
20	1000	1	50
10	1000	1	100
5.0	50	5	50
2.0	20	5	50
1.0	20	2.5	50
0.5	10	2.5	50
0.25	50	0.005	1

Fortification Procedure

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

Fortification Level (ppm or µg/g)	Fortification Solution
0.005	1 mL of 100 ng /mL in 20 g of soil
0.05	1 mL of 1000 ng /mL in 20 g of soil

Fortification was conducted to determine the percent recovery, and accuracy within the method validation. This procedure was performed in quintuplicate during method validation at each fortification level.

Extraction Method for Bixafen in Soil

1. Transfer 20 g of soil into a 125 mL polyethylene bottle.
2. Fortify the sample as necessary.
3. Add 40 mL of 8:2 (v:v) ACN:H_2O (extraction solvent) to each sample
4. Place samples on wrist action shaker for 30 minutes
5. Sonicate samples for 10 minutes.
6. Centrifuge samples for 5 minutes at ~3000 rpm.
7. Repeat steps 3-7, combining supernatant into the same bottle.
8. Filter sample through 0.45 µm PTFE syringe filter prior to analysis.
9. Aliquot in autosampler vials for analysis by LC-MS/MS.

A schematic diagram of the soil extraction method is presented in Figure 1.

Liquid Chromatography with Tandem Mass Spectrometry Analytical Method (LC-MS/MS)

LC conditions (8 minute gradient)

Agilent 1200 HPLC system (LC-MS/MS)
Column: Thermo Betasil C18, 2.1 mm × 100 mm, 5 μm
Injection volume: 5 μL
Flow rate: 0.5 mL/min
Run time: 13 minutes

Mobile Phase:
- A: 0.01% Acetic acid in 90:10 (v:v) HPLC H_2O:ACN
- B: 0.01% Acetic acid in HPLC grade ACN

Gradient Program:

Time (minutes)	%A	%B	Flow rate (mL/min)
0	80	20	0.5
5.5	25	75	0.5
5.6	80	20	0.5
8	80	20	0.5

LC conditions (13 minute gradient)

Agilent 1200 HPLC system (LC-MS/MS)
Column: Thermo Betasil C18, 2.1 mm × 100 mm, 5 μm
Injection volume: 5 μL
Flow rate: 0.5 mL/min
Run time: 13 minutes
Mobile Phase:
- A: 0.01% Acetic acid in 90:10 (v:v) HPLC H_2O:ACN
- B: 0.01% Acetic acid in HPLC grade ACN

Gradient Program:

Time (minutes)	%A	%B	Flow rate (mL/min)
0	80	20	0.5
5.5	25	75	0.5
5.6	80	20	0.5

10.0	5	95	0.5
10.1	80	20	0.5
13.0	80	20	0.5

MS conditions

An Applied Biosystems API 5500 tandem mass spectrometer was used with electrospray ionization (ESI) in positive polarity mode to acquire data by Multiple Reaction Monitoring (MRM).

API 5500:

Q1 Mass (amu)	Q3 Mass (amu)	Dwell (msec)	DP	CE	CXP
Bixafen					
414.0	394.0	200	76	21	40
414.0	266.0	200	76	21	40

DP = Declustering Potential
CE = Collision Energy
CXP = Collision Cell Exit Potential

Source Dependent Settings:

Temperature (TEM):	400°C
Nebulizer Gas (GS1):	60.0
IonSpray Gas (GS2):	60.0
Curtain Gas (CUR):	15.0
Collision Activated Dissociation Gas (CAD):	6.0
Ionization Spray (IS)	5500
Entrance Potential (EP)	10.0

Post-column effluent was diverted into the mass spectrometer between 4.6 and 6.1 minutes.

LC-MS/MS Analysis

Samples were analyzed in a set consisting of a solvent blank, reagent blank, two control extracts, five 0.005 ppm fortified controls, and five 0.05 ppm fortified controls interspersed between the calibrants. In addition, to ensure accuracy was maintained over

the course of the analytical set, quality control (QC) calibrants were added to the sequence. The final QC calibrant's response was within 10% when compared to its initial response. Calibrants and samples were analyzed in a single sequence of injections.

Methods of Calculation

Quantitation

Separation of Bixafen was achieved by LC-MS/MS. The compound was identified by the coincidence of the retention time with the respective reference standard and MS characteristics. The quantitation of Bixafen was conducted using peak area relative to the theoretical concentrations of the calibrants. The content of Bixafen in sample was quantitated against 1/x weighted linear curve of Bixafen calibrants where:

$$\text{ng/mL analyte} = \frac{y - b}{m}$$

y = peak area
x = ng/mL compound injected
m = slope
b = intercept

Weighting of the calibration curve was applied to provide better curve fit at the lower concentration levels of bixafen.

The calculation of weighted curve equations (linear regression) and concentrations (ng/mL) present in samples and calibrants was conducted using Analyst® software.

The residue of the analyte in the sample is determined as follows:

$$\text{Residue (mg/kg)} = \frac{\text{ng/mL analyte} \times \text{Initial Extract Volume (mL)} \times \text{Dil. Factor}}{1,000 \text{ ng/µg} \times \text{sample weight (g)}}$$

where mg/kg = µg/g or ppm and

Initial Extract Volume = 80 mL (volume of ACN:H_2O added)

The Percent Recovery of a fortified sample is determined as follows:

$$\frac{\text{Residue } (mg/kg) - \text{Average Residue of Controls } (mg/kg)}{\text{Fortification Level } (mg/kg)} \times 100$$

An example calculation from analysis method 2 for the recovery of Bixafen (m/z 414.0/394.0 ion transition) in soil fortified at 0.005 mg/kg (sample designated F1-A) is given in following:

Linear regression equation: $y = 42255.543548x + 1169.2701227$ $(r = 0.999958435257)$

The calculated concentration in F1-A final extract:

$$\text{ng bixafen/mL} = \frac{54593.44430 - 1169.2701227}{42255.543548} = 1.26 \text{ ng/mL}$$

where 54593.44430 is the peak area of Bixafen (m/z 414.0/394.0) for F1-A

The Bixafen residue (μg/g) for F1-A =

$$\frac{1.26 \text{ ng/mL} \times 80 \text{ mL (Initial Extract Volume)} \times 1 \text{ (Dil. Factor)}}{1,000 \text{ ng/}\mu\text{g} \times 20 \text{ g (sample weight)}}$$

$= 0.00504$ mg/kg

The percent recovery of fortified sample F1-A (Bixafen):

{[0.00504 mg/kg – 0.000 mg/kg (avg. control residue)] ÷ 0.005 mg/kg (fort. level)} x 100%

$= 101\%$

Calibration Range

The calibration curve, ranging from 0.25 ng/mL to 50 ng/mL, was generated by Analyst® software for the soil method validation.

Limit of Quantitation

The limit of quantitation (LOQ) was set at 0.005 ppm for soil which was equivalent to 1.25 ng/mL Bixafen, when compared to the calibration standard curve as validated in this study.

Limit of Detection

The limit of detection (LOD) was defined as approximately 20% of LOQ which was equivalent to 0.25 ng/mL of Bixafen in the calibration standard curve. The LOD for soil was estimated to be 0.001 ppm for Bixafen.

Time Required for Completion of a Sample Set

A sample set consisted of a reagent blank (extraction solvent), two controls (untreated soil samples), and five fortified soil samples (at each level i.e. LOQ and 10X LOQ). Time required for one set from preparation of standard solutions, initiation of extraction, until the completion of instrumental analysis and data evaluation is as follows:

- Preparation of standard solutions takes approximately 6 hours

- Sample preparation takes approximately 4 hours

- LC-MS/MS analysis and data processing (two MS/MS transitions) take approximately 5 hours

TOTAL = approximately 15 hours for one analyst to complete a set to satisfy the validation requirements.

Statistical Methods

Means, standard deviation, relative standard deviation, and 1/x linear regression fit were the only statistical methods employed in this study.

Method Modification

Only one method modification occurred:

The original method indicated the LOD was set at 0.001 ppm (0.25 ng/mL equivalent); however, the standard curve didn't include an LOD standard. The standard curve range was modified to include an LOD calibrant (0.25 ng/mL).

Figure 1.   Schematic Diagram for the Extraction of Bixafen from Soil.

2763W Extraction Scheme

Weigh 20 g of Soil into 125 mL polyethylene bottle

Fortify as necessary

(1)Add 40 mL of 8:2 (v:v) ACN:H_2O

(2)Place samples on wrist action shaker for 30 minutes

(3)Sonicate Samples for 10 minutes

(4)Centrifuge at 3,000 rpm for 5 minutes

Repeat steps 1-4 and combine supernatants in same bottle

Filter supernatant through 0.45 um PTFE syringe filter