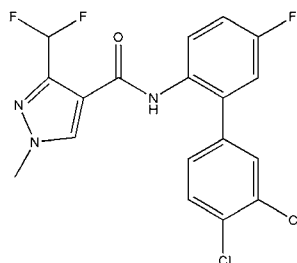# 2.0 REFERENCE SUBSTANCE

A summary of the compound information is listed below which includes structure, chemical name, purity and expiration date. The certificate of analysis is included in Appendix A.

2.1 REFERENCE STANDARD

- **Bixafen (F9650)**

Bixafen
(F9650)

Common Name:	Bixafen
Chemical Name:	N-(3',4'-dichloro-5-fluorobiphenyl-2-yl)-3-(difluorodimethyl)-1-methyl-1H-pyrazole-4-carboxamide
CAS No.:	581809-46-3
Molecular Formula:	$C_{18}H_{12}Cl_2F_3N_3O$
Molecular weight:	414.21 g/mol
Batch Number:	NLL7292-37
Purity:	98.9%
Expiration Date:	07/29/2015
Storage:	Frozen (~ -20 °C)

3.0   EXPERIMENTAL SECTION

3.1 TEST SYSTEM

The test system (New Jersey control soil UTC-SC-CTR-6) was homogenized with dry ice in a Hobart 8181D, and stored at ~ -20 ºC (in a freezer) until analysis. The dry ice was permitted to sublimate before the soil was weighed for analysis. The soil parameters are summarized in Table 2 below. The certificate of analysis is included in Appendix A.

Table 2: Characterization Data for Control Soil UTC-SC-CTR-6

Parameters	Result	
Percent Sand	33	
Percent Silt	44	
Percent Clay	23	
USDA Textural Class (Hydrometer method)	Loam	
Bulk Density (disturbed) gm/cc	1.09	
Cation exchange capacity (meq/100g)	7.9	
% Moisture at 1/3 Bar	26.4	
% Organic Matter –Walkley Black	1.4	
pH in 1:1 soil:water ratio	6.3	
Base Saturation Data		
Cation	**Percent**	**ppm**
Calcium	45.0	713
Magnesium	14.5	138
Sodium	0.8	14
Potassium	2.4	74
Hydrogen	37.4	30

3.2 MATERIALS

3.2.1   Chemicals
Acetic acid, Fisher HPLC Grade
Acetonitrile, Fisher Optima Grade
Water, Fisher HPLC Grade

3.2.2   Reagents
Soil extraction solvent: Acetonitrile-HPLC water (8:2, v:v)
Dilution Solvent: Acetonitrile-HPLC water (8:2, v:v)

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
Benchtop electronic balance with 0.01-g readability
Burrell Wrist-Action Shaker
Sorvall RC-5B Refrigerated Superspeed Centrifuge
Eppendorf micropipettes: 10-100 µL, 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

The following stock solution was prepared by weighing 12.6 mg of bixafen (purity 98.9%)
in a 25-mLvolumetric flask, and acetonitrile (ACN) was added to the mark:

• 498.5 µg/mL bixafen in ACN (purity corrected)

The following fortification and calibration solutions were prepared by serial dilution of the
stock solution with ACN-water (8:2, v:v) in volumetric flasks, as detailed in Table 3 below.

Table 3: Preparation Scheme for Standard Solutions

Standard Solution Used	Volume Taken (mL)	Final Volume (mL)	Nominal Concentration
498.5 µg/mL	5.02	50	50 µg/mL
50 µg/mL	10	50	10 µg/mL
498.5 µg/mL	0.2006	100	1000 ng/mL
1000 ng/mL	5	50	100 ng/mL
1000 ng/mL	2.5	50	50 ng/mL
1000 ng/mL	1	50	20 ng/mL
1000 ng/mL	1	100	10 ng/mL
50 ng/mL	5	50	5 ng/mL
20 ng/mL	5	50	2 ng/mL
20 ng/mL	2.5	50	1 ng/mL
10 ng/mL	2.5	50	0.5 ng/mL

All standard solutions were stored in a freezer (~-20 ℃) after preparation.

3.2.5 Soil Fortifications

For 0.005 ppm and 0.05 ppm spiking, control soil UTC-SC-CTR-6 (~20 g each) was fortified with the following solutions, as indicated in Table 4 below.

Table 4: Preparation Scheme for Soil Fortifications (0.005 ppm and 0.05 ppm)

Sample ID#	Soil Sample weight (g)	Spiking solution	Spiking amount
Control 1	20.11	ACN-water (8:2, v:v)	1000 µL
Control 2	20.19	ACN-water (8:2, v:v)	1000 µL
Control 3	20.03	ACN-water (8:2, v:v)	1000 µL
0.005 ppm-1	20.11	100 ng/mL bixafen*	1000 µL
0.005 ppm-2	20.11	100 ng/mL bixafen*	1000 µL
0.005 ppm-3	20.00	100 ng/mL bixafen*	1000 µL
0.05 ppm-1	20.17	1000 ng/mL bixafen*	1000 µL
0.05 ppm-2	20.15	1000 ng/mL bixafen*	1000 µL
0.05 ppm-3	20.10	1000 ng/mL bixafen*	1000 µL

*in ACN-water (8:2, v:v)

For 0.1 ppm spiking, control soil UTC-SC-CTR-6 (~20 g each) was fortified with the following solutions, as indicated in Table 5 below.

Table 5: Preparation Scheme for Soil Fortifications (0.1 ppm)

Sample ID#	Soil Sample weight (g)	Spiking solution	Spiking amount
Control	20.02	ACN-water (8:2, v:v)	1000 µL
Control	20.00	ACN-water (8:2, v:v)	1000 µL
0.1 ppm-1	20.01	10 µg/mL bixafen*	200 µL
0.1 ppm-2	20.06	10 µg/mL bixafen*	200 µL
0.1 ppm-3	20.02	10 µg/mL bixafen*	200 µL
0.1 ppm-4	20.09	10 µg/mL bixafen*	200 µL
0.1 ppm-5	20.07	10 µg/mL bixafen*	200 µL
0.1 ppm-6	20.08	10 µg/mL bixafen*	200 µL

*in ACN-water (8:2, v:v); ACN = acetonitrile

For 0.68 ppm and 3.9 ppm spiking, control soil UTC-SC-CTR-6 (~5 g each) was fortified with the following solutions, as indicated in Table 6 below.

Table 6: Preparation Scheme for Soil Fortifications (0.68 ppm and 3.9 ppm)

Sample ID#	Soil Sample weight (g)	Spiking solution	Spiking amount
Control 1	5.0159	ACN-water (8:2, v:v)	390 µL
Control 2	5.0140	ACN-water (8:2, v:v)	390 µL
0.68 ppm-1	5.0013	10 µg/mL bixafen*	340 µL
0.68 ppm-2	5.0001	10 µg/mL bixafen*	340 µL
0.68 ppm-3	5.0101	10 µg/mL bixafen*	340 µL
3.9 ppm-1	5.0078	50 µg/mL bixafen*	390 µL
3.9 ppm-2	5.0008	50 µg/mL bixafen*	390 µL
3.9 ppm-3	5.0060	50 µg/mL bixafen*	390 µL

*in ACN-water (8:2, v:v); ACN = acetonitrile

3.2.6 Soil Extraction (0.005 ppm, 0.05 ppm, and 0.1 ppm)

1. Approximately 20 g of the soil was weighed into a 125-mL polyethylene bottle and fortified with bixafen at 0.005 ppm, 0.05 ppm, and 0.1 ppm levels.
2. ACN- HPLC water (8:2, v:v, 40 mL) was added to the sample.
3. The sample was shaken using a Burrell Wrist-Action Shaker for 30 minutes.
4. The sample was sonicated for 10 minutes.
5. The sample was centrifuged for 5 minutes at ~3000 rpm.
6. The supernatant was transferred to a new 125-mL polyethylene bottle.
7. The solid residue was re-extracted with 40 mL of acetonitrile-HPLC water (8:2, v:v), as for step 2 to 5, combining the supernatants in the 125-mL polyethylene bottle.
8. An aliquot of ~3 mL of the extract was filtered through a 0.45 µm PTFE syringe filter and the filtrate was analyzed by LC-MS/MS to quantify bixafen.

3.2.7 Soil Extraction (0.68 ppm and 3.9 ppm)

1. Approximately 5 g of the soil was weighed into a 50-mL polypropylene centrifuge tube and fortified with bixafen at 0.68 ppm and 3.9 ppm levels.
2. ACN- HPLC water (8:2, v:v, 40 mL) was added to the sample.
3. The sample was shaken using a Burrell Wrist-Action Shaker for 30 minutes.
4. The sample was sonicated for 10 minutes.
5. The sample was centrifuged for 5 minutes at ~3000 rpm.
6. The supernatant was transferred to a new 125-mL polyethylene bottle.
7. The solid residue was re-extracted with 40 mL of acetonitrile-HPLC water (8:2, v:v), as for step 2 to 5, combining the supernatants in the 125-mL polyethylene bottle.
8. An aliquot of ~3 mL of the extract was filtered through a 0.45 µm PTFE syringe filter
9. All samples were diluted 20 times with ACN- HPLC water (8:2, v:v)
10. Diluted samples were analyzed by LC-MS/MS to quantify bixafen.

3.3 LC-MS/MS ANALYSIS

3.3.1   Principle of Measurement

Separation of the analyte from soil matrix was achieved by high performance liquid chromatography (HPLC). Quantitative LC-MS/MS analysis of bixafen in the samples utilized a highly specific and sensitive MRM (Multiple Reaction Monitoring) method. Bixafen precursor ion (*m/z* 414) was monitored in Q1 and the fragment ions were monitored in Q3 (primary MRM transition, *m/z* 394, and confirmatory MRM transition, *m/z* 266). The analyte was identified by the coincidence of its retention time with the calibration standards, and quantitated by integration of the peak area relative to the calibration curve.

3.3.2   Chromatography and Detection

The following are the LC-MS/MS parameters used.

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

  Column: Synergi 4 µm Hydro-RP 50 mm x 2 mm
  Column Temperature: Ambient
  Injection Volume: 5 µL

Mobile phase:

  Solvent A = 0.1 mL/L acetic acid in HPLC water-acetonitrile (90:10, v:v)
  Solvent B = 0.1 mL/L acetic acid in acetonitrile
  Wash solvent = acetonitrile- HPLC water (50:50, v:v)

Mobile phase gradient for 0.005 ppm, 0.05 ppm, 0.68 ppm, and 3.9 ppm analysis:

Time (minutes)	Flow Rate (mL/min)	%A	%B
0.0	0.5	80	20
5.5	0.5	25	75
5.6	0.5	80	20
8.0	0.5	80	20

The LC flow was diverted to the MS between 1.5 and 6.5 min, and to waste between 0.0 and 1.5 min and between 6.5 and 8.0 min. The retention time of bixafen was ~ 4.6 min

Mobile phase gradient for 0.1 ppm analysis:

Time (minutes)	Flow Rate (mL/min)	%A	%B
0.0	0.5	80	20
5.5	0.5	25	75
5.6	0.5	5	95
10.0	0.5	5	95
10.1	0.5	80	20
13.0	0.5	80	20

The LC flow was diverted to the MS between 1.5 and 5.5 min, and to waste between 0.0 and 1.5 min and between 5.5 and 13.0 min. The retention time of bixafen was ~ 4.6 min.

Solvent program for daily LC-MS wash:

Time (minutes)	Flow Rate (mL/min)	%A	%B
0.0	0.5	80	20
5.5	0.5	5	95
40.0	0.5	5	95
41.0	0.5	80	20
60.0	0.5	80	20

The LC flow was diverted to the MS between 1.5 and 40.0 min, and to waste between 0.0 and 1.5 min and between 40.0 and 60.0 min.

MS Parameters

Scan Type:	MRM
Polarity:	Positive
Ion Source:	Turbo Spray
Resolution Q1	Unit
Resolution Q3	Unit
Ion Source Gas 1 (GS1):	60 psi
Ion Source Gas 2 (GS2):	60 psi
Curtain Gas (CUR):	15 psi
Collision Gas (CAD):	6 psi
IonSpray Voltage (IS):	5500 V
Temperature (TEM):	400 ℃
Declustering Potential (DP):	76 V
Entrance Potential (EP):	10 V
Collision Gas Exit Potential (CXP):	40 V

MRM Transition	Analyte ID	Q1 Mass (amu)	Q3 Mass (amu)	Dwell Time (msec)	Collision Energy (CE)
Primary	Bixafen	414	394	200	21 V
Confirmatory	BixafenC	414	266	200	33 V

3.4 METHODS OF CALCULATION

3.4.1 Recoveries

The recoveries of bixafen from fortified samples were calculated based on the linearity curve generated with each set:

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

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

Where y = Sample peak area

b = Calibration intercept

m = Calibration slope

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

$$\text{ppm bixafen} = \frac{\text{Sample conc. (ng/mL) x Extract vol. (mL) x Dilution factor x 0.001 } \mu\text{g/ng}}{\text{Sample weight (grams)}}$$

where µg/g is equivalent to mg/kg and ppm.

$$\text{Percent recovery} = \frac{\text{Conc. of bixafen fortified sample (ppm) - Conc. of control (ppm)}}{\text{bixafen fortification level (ppm)}} \text{ x 100}$$

An example calculation for the recovery of bixafen (0.1 ppm fortification) from soil (sample ID#: **NJ_UTC-SC-1-CTR-6 0.1 ppm R-1_11/12/13,** Figure C - 11) is shown below:

The calibration curve equation was $y = 4010x + 131$ (r = 0.9997)

$$\text{ng/mL bixafen} = \frac{101400 - 131}{4010} = 25.25 \text{ ng/mL}$$

Calculation of the ppm bixafen in soil was calculated as shown:

$$\text{ppm bixafen} = \frac{25.25 \text{ ng/mL x 80 mL x 1 x 0.001}}{20.09 \text{ grams}} = 0.1005 \text{ ppm}$$

$$\text{Percent recovery} = \frac{0.1005 \text{ ppm - 0 ppm}}{0.1 \text{ ppm}} \text{ x 100} = 101\%$$