II. MATERIALS

A. Equipment

The equipment that was used is listed below:

- Analytical balance: Mettler Toledo XS204
- Top Loading balance: Mettler Toledo MS3002S/03
- Volumetric flasks, glass: 25 and 500 mL
- Bottles, amber glass with Teflon lined cap: various sizes (including 250 mL)
- Volumetric glass pipettes: various sizes
- Polypropylene tubes: BD Falcon 15 mL
- Graduated cylinders: various volumes
- Micropipette, Drummond Wiretrol disposable: various volumes
- Disposable pasteur pipettes, glass
- Repeating pipette: Eppendorf Stream
- HPLC/GC vials and caps: 1.8 mL
- SPE cartridges: Bakerbond Octadecyl (C_{18}), 6 mL SPE column, 1000 mg (Cat# 7020-07)
- SPE Manifold: Burdick & Jackson (24 position)
- LC-MS/MS: AB Sciex API4000 LC-MS/MS with Shimadzu LC-20AD HPLC Pumps, Shimadzu SCL-10A VP controller, and SIL-20AC HT autosampler
- GC-MSD: Hewlett Packard HP5890 Series II GC with 5972 MSD and 7673 autosampler
- Rotary evaporators: Labconco rotary evaporators with a Welch W series model 8912A direct drive vacuum pump
- Platform shaker: Eberbach 2 speed shaker
- Centrifuge: Eppendorf Multipurpose Centrifuge 5810
- Sonicator: Branson 5510 Ultrasonic Cleaner
- Buchner funnels: 7 cm
- Graduated mixing cylinders: 250 mL
- Vacuum side-arm with adapter
- Filter paper, glass fiber: Whatman GF/A, 7 cm
- Separatory funnels: 250 mL
- Round-bottom flasks: 125 and 250 mL
- Glass filter funnels
- Glass wool

B. Reagents and Standards

The following chemicals were used:

Chemical	Distributer	Part No:
Acetone	VWR	MK243510
Acetonitrile	Fisher	A996-4
Ammonium Formate, 99%	VWR	AA14517-30
Ethyl Acetate	VWR	MKH07810
Formic Acid ≥ 99.5%	Fisher	A117-50
Hexane	VWR	MKH48710
Hydrochloric Acid	VWR	EMD-HX0603-3
Methanol	VWR	MK304110
Sodium Sulfate, Anhydrous	Fisher	MSX0760E3
Toluene	Fisher	T290-4
Water	Fisher	W5-4

Preparation of Reagent Solutions:

Acetonitrile/1% hydrochloric acid solution (8:2, v/v): Prepared by combining 1200 mL of acetonitrile with 300 mL of 1% hydrochloric acid solution and mixing well.

Acetone/1% hydrochloric acid solution (9:1, v/v): Prepared by combining 1350 mL of acetone with 150 mL of 1% hydrochloric acid solution and mixing well.

Acetone/water (1:1, v/v): Prepared by adding 100 mL of acetone to 100 mL of HPLC-grade water and mixing well.

1% Hydrochloric Acid Solution: Prepared by adding 13 mL of concentrated hydrochloric acid to approximately 200 mL of HPLC-grade water in a 500-mL volumetric flask. The solution was brought up to volume (500 mL) with HPLC-grade water and mixed well.

Methanol/water (3:7, v/v): Prepared by adding 300 mL of methanol to 700 mL of HPLC-grade water and mixing well.

Methanol/water (3:7, v/v; 0.005 M Ammonium Formate): Prepared by adding 0.16 g of ammonium formate into 500 mL methanol/water (3:7, v/v) and mixing well.

Methanol/water (1:9, v/v): Prepared by adding 50 mL of methanol to 450 mL of HPLC-grade water and mixing well.

Mobile Phase A:
Methanol Solution: Prepared by dissolving 0.16 g ammonium formate and 98 µL of formic acid (≥ 96%) in 500 mL of methanol and mixing well.

Mobile Phase B:
Aqueous Formate Buffer: Prepared by dissolving 0.16 g ammonium formate and 98 µL of formic acid (≥ 96%) in 500 mL of HPLC-grade water and mixing well.

Needle-Wash, acetonitrile/water (50:50, v/v): Prepared by combining 500 mL of acetonitrile with 500 mL of HPLC-grade water and mixing well.

1. Reference Substances

The flumioxazin, THPA 2-Na (THPA disodium salt), and HPA analytical reference standards were received in good condition on April 29, 2015, from Valent Technical Center, Dublin, CA. The certificate of analysis for each of the standards is in the archives at GPL. The following table contains detailed information for the analytical standard used in this study.

Analytical Standard	CAS #	Lot #	Purity (%)	Expiration Date
Flumioxazin	103361-09-7	AS 1663L	99.1	09/03/2015
THPA 2-Na	NA	AS 1987b	99.9	06/30/2016
HPA	NA	AS 2001a	95.1	06/23/2016

Upon receipt, the neat reference standards were stored in a freezer set to maintain ≤ -10 °C (frozen).

2. Preparation of Standard Solutions

The flumioxazin, THPA 2-Na, and HPA reference substances were used in the preparation of the fortification and calibration solutions. Preparation and dilution data forms pertaining to the stock and working solutions are located in the raw data.

a. Stock Solution

On May 11, 2015, 25.7 mg of flumioxazin reference standard was weighed directly into a 25-mL volumetric flask and diluted to the mark with acetone. After correcting for purity, the stock solution contained 1.02 mg/mL flumioxazin (Solution A). For THPA, 32.4 mg of THPA disodium salt reference standard was weighed directly into a 25-mL volumetric flask and diluted to the mark with acetone/water (50:50, v/v). After correcting for purity and content (free base), the stock solution contained 1.03 mg/mL THPA (free acid) (Solution B). For HPA, 26.4 mg of reference standard was weighed directly into a 25-mL volumetric flask and diluted to the

mark with acetone. After correcting for purity, the stock solution contained 1.00 mg/mL HPA (Solution C).

b. Fortification Solutions

Flumioxazin

A 4-mL aliquot of Solution A was diluted to 102 mL with acetone, resulting in a solution that contained 40.0 µg/mL flumioxazin (Solution D). Further, a 5 mL aliquot of Solution D was diluted to 100 mL with acetone, resulting in a solution that contained 2.00 µg/mL flumioxazin (Solution E). Aliquots of Solution E were used to fortify soil at the LOQ level. Aliquots of Solution D were used to fortify soil at the 10x LOQ level. Solution D and E were given an expiration of three months. The fortification solutions were stored in a refrigerator set to maintain 4 ± 5 °C (refrigerated) when not in use.

THPA/HPA

A 3.9-mL aliquot of Solution B and a 4-mL aliquot of Solution C were diluted to 100 mL with methanol, resulting in a solution that contained 40.2/40.0 µg/mL THPA/HPA (Solution F). Further, a 5-mL aliquot of Solution F was diluted to 100 mL with methanol, resulting in a solution that contained 2.01/2.00 µg/mL THPA/HPA (Solution G). Aliquots of Solution G were used to fortify soil at the LOQ level. Aliquots of Solution F were used to fortify soil at the 10x LOQ level. Solution F and G were given an expiration of three months. The fortification solutions were stored refrigerated when not in use.

c. Intermediate and Calibration Solutions

Flumioxazin

A 2-mL aliquot of Solution A was diluted to 102 mL with toluene, resulting in a solution containing 20.0 µg/mL flumioxazin (Solution H). Solution H was diluted with toluene to produce calibration standards as follows:

Aliquot Volume (mL)	Final Volume (mL)	Final Concentration (µg/mL) Flumioxazin	Final Solution ID
10	100	2.00	I
5	100	1.00	J
5	200	0.500	K
0.5	100	0.100	L

The intermediate solution and calibration standards were given an expiration of three months and were stored refrigerated when not in use.

THPA/HPA

Intermediate solutions of THPA and HPA were prepared by diluting Solution F with methanol as follows:

Aliquot Volume (mL)	Final Volume (mL)	Final Concentration (µg/mL) THPA/HPA	Final Solution ID
5	100	2.01/2.00	M
5	200	1.01/1.00	N
1	80	0.503/0.500	O
0.4	200	0.0804/0.0800	P

Solutions M, N, O, and P were assigned an expiration of 3 months and were diluted 10-fold with methanol/water (3:7, v/v; 0.005 M ammonium formate) to prepare the calibration standards. The calibration standards were assigned an expiration of 2 weeks. The intermediate solutions and calibration solutions were stored refrigerated when not in use.

C. Safety and Health

Material Safety Data Sheets (MSDS) and/or Safety Data Sheets (SDS) were available. Proper personal protective equipment was worn during the execution of this method. Staff avoided breathing chemical vapor and avoided chemical contact with eyes and skin. Caution should be used when handling concentrated hydrochloric acid. There were no other procedural steps that required special precautions to avoid safety or health hazards.

III. METHODS

A. Principle of Analytical Method

The analysis of soil for flumioxazin, THPA, and HPA was performed according to the reference method titled "Determination of Flumioxazin, THPA, and HPA in Soil" (Method Number RM-30S-1-1). The limit of quantitation (LOQ) for flumioxazin, THPA, and HPA was 0.02 ppm (μg/g). The method defined limit of detection LOD was 0.01 ppm for all analytes.

The LOQ (0.02 ppm) samples were validated as part of set 597ILV01 on May 14, 2015. The 10x LOQ (0.2 ppm) samples were validated as part of set 597ILV02 on May 21, 2015. The complete method validation trial was divided into two analytical sets for efficient sample handling and as a result of a fortification error (the original 10x LOQ samples in 597ILV01 were inadvertently fortified at 100x LOQ rendering them unusable for the purposes of this study). All samples were successfully validated in one trial.

The first set consisted of one reagent blank sample, two control samples, five LOQ laboratory fortification samples and five 100x LOQ laboratory fortification samples. The five 100x LOQ samples were dropped from the final analysis sets because they were unusable for the purposes of this study. The second set consisted of one reagent blank sample, two control samples, and five LOQ laboratory fortified samples. Prior to extraction, a unique laboratory code designation was assigned by GPL to each sample. The laboratory code consisted of the last three digits of the GPL study number, the sample set designation and a sample number (e.g., 597ILV01-1).

Sub-samples (20.0 ± 0.1 g) of control soil sample were fortified. Soil samples were extracted twice; once with an acetone/1% hydrochloric acid (9:1, v/v) mixture and once with an acetonitrile/1% hydrochloric acid (8:2, v/v) mixture. The combined extract was split.

Half of the combined extract was taken through the steps for determination of flumioxazin content. The organic solvent was evaporated from the extract using a rotary evaporator. The remaining aqueous extract was taken through a liquid-liquid extraction with hexane. The hexane was drained through sodium sulfate and evaporated to dryness using a rotary evaporator. The residues were re-suspended in toluene, and were vialed for flumioxazin analysis by gas chromatography with mass spectrometric detection (GC-MSD).

A fourth of the combined extract was taken through the steps to determine the THPA and HPA content. The organic solvent was evaporated from the extract using a rotary evaporator. The remaining aqueous extract was centrifuged and the supernatant was cleaned through the use of a C18 SPE cartridge. The resulting eluant was vialed for THPA and HPA analysis by liquid chromatography with

tandem mass spectrometric detection (LC-MS/MS).

B. Analytical Procedure

1. Control Matrix

The soil control matrix sample (V-38586-BS-C) was received from the Sponsor on April 27, 2015 in good condition. The sample was sourced from a previous Valent study. The sample was kept frozen when not in use. The GLP characterization data from a sample taken from an adjacent plot (V-38586-TRT 0-30CM) was provided by the Sponsor and is presented in Appendix B.

2. Preparation of Samples

Sub-samples (20.0 ± 0.1 g) of the control soil matrix were measured into 250-mL amber glass bottles.

3. Fortifications

Independent laboratory validation samples were fortified at the LOQ (0.02 ppm) or 10x the LOQ (0.2 ppm). Fortifications were performed using Wiretrol disposable micropipettes to directly fortify the 20-g samples as follows:

Fortification Level	Amount and Concentration of Spiking Solution Used
LOQ (0.02 ppm)	200 µL of a 2.00 µg/mL flumioxazin solution 200 µL of a 2.01/2.00 µg/mL THPA/HPA solution
10x LOQ (0.2 ppm)	100 µL of a 40.0 µg/mL flumioxazin solution 100 µL of a 40.2/40.0 µg/mL THPA/HPA solution

4. Extraction

After fortification, 80 mL of acetone/1% hydrochloric acid solution (9:1, v/v) was added to each sample. Each sample jar was capped and shaken on a plaform shaker for 30 minutes. A filter apparatus was assembled using a 250-mL graduated mixing cylinder fitted with an adapter connected to a filter side arm with a Buchner funnel on top. A Whatman GF/A filter was placed into the Buchner funnel and pre-wetted with acetone. After shaking, vacuum was applied to the filter apparatus; the sample supernatant was decanted into the funnel and filtered into the 250-mL graduated mixing cylinder. The filter was then transferred back into the jar.

An 80-mL portion of acetonitrile/1% hydrochloric acid solution (8:2, v/v) was then added to each sample. Each sample jar was capped and was

shaken on a platform shaker for 30 minutes. After shaking, the extract was filtered through a new Whatman GF/A filter, combining the extracts. The filtrate was brought up to 160 mL with acetone, stoppered, and mixed well. The extract was stored frozen overnight before the start of the fraction B procedure (LOQ samples) or fraction A procedure (10x LOQ samples).

Flumioxazin Analysis (Fraction A)

An 80-mL aliquot of the extract filtrate was measured into a 250-mL round-bottom flask. The 250-mL round bottom flask was attached to a rotary evaporator system with the water bath temperature set to ≤ 40 °C. The solvent was evaporated to obtain an aqueous residue (approximately 15 mL).

The aqueous mixture was poured into a 250-mL separatory funnel and the flask was rinsed with 50 mL of HPLC-grade water. *Note: For the purposes of this ILV study, all DI water was replaced with HPLC-grade water.* The rinse was added to the separatory funnel. The round-bottom flask was rinsed further with 75 mL of hexane, and the hexane rinse was also added to the separatory funnel. The separatory funnel was stoppered, inverted and vented, and then shaken vigorously for 1 minute with occasional venting. The phases were allowed to separate and then most of the aqueous layer was drained back into the 250-mL round bottom flask (approximately 5 mL of the aqueous layer was left behind). The separatory funnel was swirled and briefly shaken (with venting) to clarify the hexane layer. The solids (if any were present) were allowed to settle and the remaining aqueous and any interface layer were drained into the same round-bottom flask.

A funnel was prepared with approximately 30 g of sodium sulfate suspended on glass wool. The sodium sulfate was freshly washed with 25 mL of hexane and the funnel was placed on top of a clean 250-mL round-bottom flask. The hexane layer was drained through the sodium sulfate. The sodium sulfate was rinsed with 25 mL of hexane combining the rinse with the extract.

The 250-mL round-bottom flask was attached to a rotary evaporator system with the water bath temperature set to ≤ 40 °C. The solvent was evaporated to a target of between 30 and 40 mL. *Note: For the first extraction set containing the LOQ samples, the 250-mL round-bottom flasks were allowed to return to room temperature in the middle of this procedure, stoppered, and were stored refrigerated overnight. In the morning, the samples were allowed to return to room temperature and the flasks were re-attached to the rotary evaporator systems and the solvent was allowed to evaporate to a target of between 30 and 40 mL.* The

solvent was then transferred into a 125-mL round-bottom flask. The 250-mL round-bottom flask was rinsed with 10 to 15 mL of ethyl acetate, and the rinse was added to the 125-mL round bottom flask.

The 125-mL round-bottom flask was attached to a rotary evaporator system and rotary evaporation of the sample extract was continued just to dryness. The residues were re-suspended in 1 mL of toluene. The flask was stoppered and sonicated for approximately 30 seconds to dissolve the residues. The sample was vialed and submitted for analysis by GC-MSD.

THPA and HPA Analysis (Fraction B)

An approximately 40-mL aliquot (aliquot volumes were recorded when different than 40 mL) of the extract filtrate was measured into a 125-mL round bottom flask. The 125-mL round bottom flask was attached to a rotary evaporator system with the water bath temperature set to < 30 °C. The solvent was evaporated to obtain an aqueous residue (< 5 mL).

The round-bottom flask was briefly swirled and then sonicated for approximately 15 seconds to dislodge residues on the flask. The aqueous residue was transferred into 15-mL BD Falcon disposable plastic centrifuge tube using a Pasteur pipette. The round-bottom flask was rinsed twice with 2-mL portions of HPLC-grade water, and each rinse was transferred into the centrifuge tube. The volume in the centrifuge tube was adjusted to 10 mL. The centrifuge tube was centrifuged in a centrifuge set to 4000 rpm and 10 minutes.

SPE (C_{18}) cartridges were conditioned with 5 mL of methanol followed by equilibration using four 5-mL aliquots of HPLC-grade water. No vacuum was used.

The supernatant was withdrawn from the centrifuge tube with a Pasteur pipette and transferred into the SPE cartridge. Care was taken to not disturb the pellet at the bottom of the centrifuge tube. The sample was allowed to drain through using gravity (i.e., no vacuum was used).

Once the sample was loaded onto the cartridge, the cartridge was rinsed sequentially with a 2-mL aliquot of HPLC-grade water, and then with a 1.5-mL aliquot of methanol/water (10:90, v/v). Each rinse was added to the cartridge after the previous rinse had completely passed through the cartridge. The accumulated eluant was discarded.

A 15-mL BD Falcon disposable plastic centrifuge was placed under the cartridge to collect the eluent. The cartridge was eluted with 6 mL of methanol/water (3:7, v/v; 0.005 M ammonium formate). The cartridge was allowed to elute by gravity. After the eluent stopped flowing (by

gravity), vacuum was applied to pull any remaining liquid through the cartridge. The sample was brought up to a final volume of 7 mL with methanol/water (3:7, v/v; 0.005 M ammonium formate). When withdrawing an aliquot for analysis, the sample was mixed using a Pasteur pipette and vialed for analysis by LC-MS/MS.

C. Instrumentation

Flumioxazin Analysis

Instrument: Hewlett Packard 5890 Series II with Hewlett Packard 7673 Autosampler and Hewlett Packard 5972 MSD

GC Column: Supelco SPB-1 length 30 m, diameter 0.32 mm, film 0.25 μm
Catalog #: 2-4044
Serial #: 775704B

Guard Column: None

Data System: HP Chemstation Chromatography Data System

GC Analysis Conditions	
He Flow Rate	EPP at 1 mL/min
Septum Purge	1 mL/min
Split/Splitless Injection	Split 20:1
Injection Volume	1 μL
Injector Temperature	280 °C
MS Transfer Line Temperature	280 °C
Injector Liner	Direct Injection Single Gooseneck, Restek #20799

Temperature Program	
Initial Oven Temp	200 °C
Initial Time	1 min
Rate	15 °C/min
Temp	320 °C
Final Time	5.0 min
Total Run Time	14.0 min

Transitions and Corresponding Mass Spectrometry Parameters				
Ionization Source/Polarity		Electron Impact, Positive		
Resolution		Low		
Analyte	**Detection Ion, *m/z***	**Ion Mode**	**Dwell Time (msec)**	**Retention Time (min.)**
	Target			
Flumioxazin	354	+	100	~8.5

THPA and HPA Analysis

Instrument: AB Sciex API4000 LC/MS/MS with Shimadzu LC-20AD HPLC Pumps, Shimadzu SCL-10A VP Controller, Shimadzu SIL-20AC HT Autosampler

HPLC Column: Phenomenex Luna C18 (2)
50 x 3.0 mm, 3 µm, 100 Å
Part # 00B-4521-Y0
Serial # 761825-4

Guard Column: Phenomenex Security Guard Cartridge C18
4 x 2.0 mm
Part #: AJ0-4286

Data System: Analyst Chromatography Data System version 1.5.2, AB Sciex

Mobile Phases:

A) Methanol Solution, Formate Buffer
B) Aqueous Formate Buffer

Flow Rate: 0.3 mL/minute
Run Time: 15.0 minutes
Injection Volume: 10 µL
Gradient Program:

Time (minutes)	%A	%B
0.0	30	70
1.0	30	70
7.0	80	20
9.0	80	20
10.0	30	70
15.0	30	70

Column Heater: 50 °C

Approximate Retention Times:

THPA: 3.0 minutes

HPA: 4.3 minutes

Mass Spectrometer Parameters (operated in LC-MS/MS mode):

AB Sciex API 4000 Acquisition Parameters (TurboIonSource, ESI interface, MRM mode, negative mode, Unit/Unit Resolution)					
Analyte	**Q1 (m/z)**	**Q3 (m/z)**	**Dwell (msec)**	**CE**	**CXP**
THPA	168.9	124.8	150	-17	-4
HPA	170.9	126.9	150	-19	-5

Parameter	Setting
DP:	-40
CUR:	25
GS1:	40
GS2:	40
IS:	-1500
TEM:	550
CAD:	6
EP:	-12

D. Potential Interferences

1. Matrix Interference

No matrix interferences were observed for flumioxazin.

Although the detection technique is highly selective for this method, chromatographic interference was observed intermittently for both THPA and HPA. The interference was not visible in the chromatograms; however, enhancement of the analyte signal was confirmed.

The interference was confirmed with an enhancement experiment where both a control extract (that had been taken through the entire metabolite method) and an aliquot of methanol/water (3:7, v/v; 0.005 M ammonium formate) were fortified with the same amount of THPA and HPA. Both solutions were vialed and were analyzed in succession. There was significant enhancement to the THPA analyte signal (peak to peak

comparison recovery was approximately 200%) and to the HPA analyte signal (approximately 120%).

Two approaches were considered to resolve the chromatographic interferences. On the API 4000, the signal to noise ratio was large at the LOD, so there was significant room for dilution of both the calibration standards and the sample extracts. However, in the interest of using the calibration range that was used for the development of the method, it was determined that if the column heater temperature setting was increased from 35 °C to 50 °C, the unseen additional resolving power alleviated the observed matrix enhancement.

2. Reagent and Solvent Interference

High purity solvents and reagents were used for this assay. No interferences were observed.

3. Labware Interference

This method uses disposable labware and washable glassware. No interferences from the labware or glassware use were observed.

E. Confirmatory Techniques

The independent laboratory validation sets were run by LC-MS/MS and GC-MSD. These methods of analysis are highly selective; no additional confirmatory technique was used.

F. Time Required for Analysis

For the 10x LOQ samples: one eight-hour day was required to extract eight samples and prepare fraction B for the analysis of THPA and HPA by LC-MS/MS. An additional 6 hours were needed the following day to prepare fraction A for the analysis of flumioxazin by GC-MSD. The LC-MS/MS and GC-MSD analysis sets were run overnight with approximately 1 hour of data processing the following day for each analytical run.
Note: The LOQ samples were validated as part of a 13 sample set. It is suggested that sample sets be limited to 8 samples until the analyst is familiar with the method and gains efficiency.

It takes approximately 3 calendar days to prepare, analyze, and tabulate an eight sample analytical set. However, once an analyst is familiar with the method, it is likely the time requirements would be diminished.

G. Modification or Potential Problems

There were several minor modifications to the method.

For all three analytes, any time the method specified the use of DI water, HPLC-grade water was used in its place. GPL ordinarily uses HPLC-grade water as its water source for the extraction and analysis of samples. Additionally, due equipment availability, the filtering apparatus that was used was different than the apparatus described in the method, but had the same functionality.

For flumioxazin, the following changes were made due to the equipment availability at GPL: the HP system that was used was a 5890 Series II GC rather than a 6890. An equivalent Supelco SPB-1 column was substituted in place of the suggested DB-1 (J & W Scientific, Inc.) or Rtx-200 (Restek) columns. A different liner was used and the injection volume was 1 µL rather than 0.5 µL.

For the THPA and HPA (Fraction B) work up, samples were pipetted into 15-mL centrifuge tubes for centrifugation rather than the 7-mL tubes described in the method. Two 2-mL aliquots of HPLC-grade water were used the rinse the round-bottom flask and subsequently each sample was brought up to 10 mL with HPLC-grade water rather than 7 mL. This was advantageous because any remaining solvent left over from the rotary evaporation step would be weakened with the addition of extra water, thus ensuring that the extra solvent (if any) would not affect the retention of THPA and HPA on the SPE cartridge.

At the end of the SPE clean-up for THPA and HPA, the cartridges were allowed to elute by gravity flow until no further flow was seen. Subsequently, vacuum was applied to pull off the liquid that was remaining in the cartridge and the samples were all brought up to a final volume of 7 mL. Although the method did not state to use vacuum at the end of the SPE, it likely ensured that no analyte remained on the cartridge.

The only potential problem that was observed during this study was enhancement of the analyte signal for THPA and HPA. This was resolved by changing the column heater temperature from 35 °C to 50 °C.

Due to equipment availability at GPL, a Sciex API 4000 was used rather than a Sciex API 2000. The difference in instrumentation may have resulted in a larger effect from the chromatographic matrix interference than was previously observed on the Sciex API 2000 in the method development trial. Additionally, the injection volume was changed from 15 µL to 10 µL to avoid detector overload at the high end of the calibration curve because THPA and HPA had greater sensitivity on the Sciex API 4000.

H. Methods of Calculation

Analyst Chromatography Data System version 1.5.2, a product of AB Sciex, was used to acquire and integrate the chromatographic peaks for THPA and HPA. Hewlett Packard ChemStation Software G1701BA version B.01.00 was used to acquire and integrate the chromatographic peaks for flumioxazin. A linear regression with weighting was generated in Microsoft Excel® from the peak area of the calibration standards. The regression was not forced through the origin. For the regression calculations, concentration was designated as the independent variable and plotted on the x-axis. Peak area response was designated as the dependent variable and plotted on the y-axis. To generate a weighted regression, the flumioxazin GC-MSD data was weighted as follows:

Standard Concentration (µg/mL)	Number of Entries in Data Set
2.0	1
1.0	2
0.5	4
0.1	20

To generate a weighted regression, the THPA and HPA LC-MS/MS data was weighted as follows:

Standard Concentration (ng/mL)	Number of Entries in Data Set
200	1
100	2
50	4
8	25

From this regression, a slope, intercept, a correlation coefficient and other parameters of the standard curve were calculated. The slope and intercept of the weighted regression were used to determine the amount of residues in each sample. Calibration standards were injected every four sample injections as well as at the beginning and end of the injection sequence. Four different standard concentrations were injected within the analytical set. A continuing calibration (reference standard) was injected at the beginning, middle, and end of the injection sequence.

The concentration as ppm of flumioxazin residue found in samples was calculated with Microsoft® Excel using the following equation:

$$ppm = \frac{(\mu g/mL\ from\ curve) \times (Aliquot\ Factor) \times (Final\ Volume\ (mL))}{Sample\ Amount\ (g)}$$

Where the aliquot factor is:

$$Aliquot\ Factor = Extraction\ Volume\ (mL) \div Aliquot\ Volume\ (mL)$$

The concentration as ppm of THPA and HPA residue found in samples was calculated with Microsoft® Excel using the following equation:

$$ppm = \frac{(ng/mL\ from\ curve)\ x\ (Aliquot\ Factor)\ x\ (Final\ Volume\ (mL))\ x\ 1\ \mu g}{(Sample\ Amount\ (g))\ x\ 1000\ ng}$$

Where the aliquot factor is:

Aliquot Factor = Extraction Volume (mL) ÷ Aliquot Volume (mL)

Recovery of each of the analytes from fortified samples was calculated as follows:

$$\%\ Recovery = \frac{Measured\ Concentration\ (ppm)}{Theoretical\ Concentration\ (ppm)}\ X\ 100$$

An example calculation for soil, for a flumioxazin laboratory fortification sample in set 597ILV01, sample 597ILV01-6 LOQ sample fortified at 0.0200 ppm, is as follows:

standard curve equation: y = 5034.8 (x) + (-121.36)

where x = flumioxazin concentration in µg/mL and

y = peak response = 839

Flumioxazin concentration from the curve =0.191 µg/mL

Aliquot Factor = 160 mL ÷ 80 mL = 2

$$ppm = \frac{(0.191 \mu g/mL)\ x\ (2)\ x\ (1\ mL)}{(20.04\ g)} = 0.0191\ ppm$$

$$\%\ Recovery = \frac{0.0191\ ppm}{0.0200\ ppm}\ X\ 100 = 95.5\%$$

No detectable residues were measured in any control samples. Laboratory fortification samples were not corrected for control responses (no responses were observed). Rounding differences result in minor variations in values between the results obtained using the standard curve equation and peak area response above in the calculations versus those values in the report tables and raw data.

I. Statistical Procedures

Laboratory statistical procedures included calculation of arithmetic mean, the corresponding standard deviation (where n ≥ 3), coefficient of variation, and 95% confidence interval for analyte recovery data. Linear regression analysis using weighting as defined in the method (see section H above) was applied to generate GC-MSD and LC-MS/MS calibration curves for the determination of slope,

y-intercept and correlation coefficient values.

STUDY PROTOCOL

Independent Laboratory Validation of Valent U.S.A. Corporation's Residue Analytical Method for the Determination of Flumioxazin, THPA, and HPA in Soil
(Method Number: RM-30S-1-1)

Guideline Requirements

USA EPA Ecological Effects Test Guidelines, OCSPP 850.6100

Study Sponsor

Valent U.S.A. Corporation
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Dublin, CA 94568

Sponsor Representative

Charles A. Green, Ph.D.
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GPL Study Number

150597

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I. STUDY OBJECTIVE

The objective of this study is to independently validate U.S.A. Valent Corporation's Residue Analytical Method (Method Number: RM-30S-1-1) for the determination of Flumioxazin, THPA, and HPA in soil as an enforcement method in accordance to Environmental Protection Agency (EPA) Ecological Effects Test Guidelines, OCSPP 850.6100. The study will be conducted in accordance with EPA's Good Laboratory Practice (GLP) Standards, 40 CFR Part 160.

II. JUSTIFICATION OF TEST SYSTEM

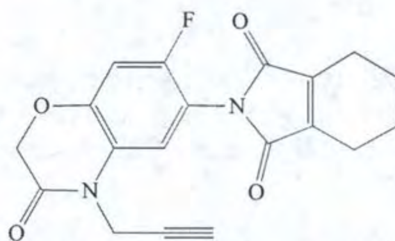
The test system consists of a representative control (untreated) sample of Washington soil. The representative control sample will be analyzed to determine matrix background of unfortified controls and procedural recoveries of fortified samples.

Analysis of this test system is required by independent laboratory analyst(s) according to EPA's Ecological Effects Test Guidelines, OCSPP 850.6100 to demonstrate method ruggedness prior to the Sponsor's submission of the method to the EPA.

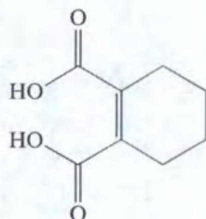
III. MATERIALS AND METHODS

A. Test/Reference Substances

The following test/reference substances will be used for this study and will be the same.

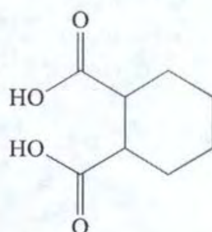


Name: Flumioxazin
Molecular Weight: 354.1
CAS Number: 103361-09-7
Purity: 99.1%
Lot Number: AS1663L



Name:	THPA
Molecular Weight:	170.1
CAS Number:	NA
Purity:	99.9%
Lot Number:	AS1987b

Note: THPA Disodium Salt (MW = 214.0) will be used to prepare the THPA reference solutions. A molecular weight factor will be used to calculate the equivalent concentration of THPA from the THPA Disodium Salt concentration.



Name:	HPA
Molecular Weight:	172.1
CAS Number:	NA
Purity:	95.1%
Lot Number:	AS2001a

The test/reference substances will be supplied by the Sponsor and will be accompanied by a Certificate of Analysis (COA), which includes the purity and expiration date, and a Safety Data Sheet or Material Safety Data Sheet.

Characterization (such as identity, purity, synthesis documentation) and stability data of the test/reference substances will be the responsibility of the Sponsor. Results and supporting raw data of the characterization and stability data will be maintained by the Sponsor. Retention samples of the reference materials will also be retained by the Sponsor. All reference substances will be stored as specified by the Sponsor or certificate of analysis when not in use.

B. Sample Identification and Storage Conditions

The control (untreated) soil will be obtained according to Golden Pacific Laboratories, LLC (GPL) Standard Operating Procedures (SOPs).

New control samples undergo log-in according to GPL's SOPs. Control samples will be labeled (or re-labeled as necessary) with the following minimum information:

Study Number
Sample Number/Code
Sample Description
Initials of Responsible Analyst

Each sample will be identified with a unique sample number/code. This number/code will be used to track the use of the sample throughout the validation process. The sample will be kept isolated from reference substances during storage. The sample will be stored in a freezer set to maintain ≤ -10 °C (frozen) when not in use. Storage temperatures will be monitored and recorded per GPL's SOPs.

C. Sample Characterization

GLP characterization of the control soil was conducted as part of a previous Valent study. The characterization data from the previous study will be included in the final report.

D. Analytical Procedure

The analysis for flumioxazin, THPA, and HPA residues in the WA soil sample will be performed according to Valent U.S.A. Corporation's residue analytical method entitled "DETERMINATION OF FLUMOXAZIN, THPA, AND HPA IN SOIL" method number RM-30S-1-1. The Limit of Quantitation (LOQ) for all three analytes will be 0.02 ppm (µg/g). Soil samples are extracted with both an acetone/1% Hydrochloric Acid (9:1, v/v) mixture and an acetonitrile/1% Hydrochloric Acid (8:2, v/v) mixture. The combined extract is split. Half of the extract is used for flumioxazin analysis by gas chromatography with mass spectrometric detection (GC-MSD) and a fourth of the extract is cleaned up on a C18 cartridge for THPA and HPA analysis by liquid chromatography with tandem mass spectrometric detection (LC-MS/MS). The soil analysis procedure will be validated in one analytical set. If after any validation attempt, the flumioxazin mean recovery is acceptable, but the degradedates' mean recoveries are not acceptable;

an additional validation attempt must be made for the degredates only. If after any validation attempt, the mean recoveries for both of the degredates are acceptable, but the mean recovery for the flumioxazin is unacceptable; an additional validation attempt must be made for flumioxazin only. Three validation attempts will be allowed to validate the soil method for each analyte.

Calibration standards will be prepared to evaluate detector response, linearity, and preparation technique. Fortification solutions will also be analyzed against calibration standards to check preparation technique. Background responses from reagents, solvents, glassware, and all specified procedures within the method will be checked alongside with the analysis of the first set of validation samples (i.e., a reagent blank sample will be analyzed) to insure that interferences will not hinder the quantitation of the LOQ fortification recoveries.

1. Method Validation

Each validation set will include one reagent blank, duplicate controls (unfortified), five controls spiked at the LOQ and five controls spiked with at 10x LOQ. Total number of samples per analytical batch will be 13.

Spiking levels are as follows:

Sample Description	Flumioxazin, THPA, HPA Fortification Level (ppm)
Reagent Blank	NA
Duplicate Untreated Controls	NA
Five LOQ Spikes	0.02
Five (10x LOQ) Spikes	0.2

Control (untreated) samples prepared as described in the analytical method are to be fortified with an appropriate amount of flumioxazin, THPA, and HPA. Recovery of all three analytes will be determined.

The method will be considered successfully validated if mean recoveries are in the range of 70-120%. Individual recoveries falling outside the 70-120% range will require approval from the

Sponsor Representative. Mean recoveries outside 70-120% will require review of the methodology with the sponsor representative and reanalysis. The first method trial will be conducted following Valent U.S.A. Corporation's analytical method as written. Discussions prior to and following the first attempt between the Sponsor Representative and the performing laboratory group will be documented. If the first trial is not successful, a second (and third trial, if necessary) will be conducted following collaboration with the Sponsor Representative (i.e., to determine potential problem areas). All discussions will be documented and a summary of these discussions will be presented in the final report to the Sponsor.

Control of procedural bias is achieved by analysis of both reagent blanks and unfortified (control) samples along with the fortified samples. This enables ongoing assessment of method/sample background in the region of analyte response and thus ensures that analyte recovery is determined without offset (bias) from background.

2. Quantitation

A standard curve will be used for quantitation of all samples. The standard curve will be derived from linearity standards injected within the analysis set. The standard curve will be generated from the GC-MSD or LC-MS/MS run using the resulting peak area versus 1/concentration (1/x weighted linear fit). Any standard chromatogram not used in the generation of the standard curve will have a documented reason for its omission.

The data must meet all acceptance criteria included in the reference method. The run sequences must be set up as required in the reference method. Where the reference method and GPL's SOPs conflict, the reference method will be followed.

Any data that does not meet the criteria of this section will not be considered acceptable unless approved by both the Study Director and Sponsor Representative. Written documentation of data acceptance must be in the raw data.

3. Reporting of Results

Results will be reported in units of µg/g or ppm, and percent recovery. The mean percent recovery, standard deviation, relative

standard deviation (RSD), and 95 or 99% Confidence Intervals for the true average recoveries will also be reported for flumioxazin, THPA, and HPA at each spiking level.

The reagent blank and control samples should be chromatographically clean at the retention time of the analytes of interest. Residues measured in matrix controls will not be used to correct fortified sample recoveries. RSDs of replicate measurements of recoveries should not exceed the target level of less than or equal to 20% for each spiking level.

E. Statistical Procedures

Statistical procedures include the calculation of mean percent recovery, the corresponding standard deviation (where $n \geq 3$), RSD, and 95 or 99% Confidence Intervals. In addition, a weighted linear regression analysis will be applied to the calibration curve to determine the slope, intercept values, and correlation coefficient.