1.0 Introduction and Objectives

Analytical methodology has been developed to determine residues of flumioxazin ([2-[7-fluoro-3,4-dihydro-3-oxo-4-(2-propynyl)-2H-1,4-benzoxazin-6-yl]-4,5,6,7-tetrahydro-1H-isoindole-1,3(2H0-dione)]) and its APF and 482-HA metabolites in pond water and pond sediment. The residue analytical method includes four sample preparation/extraction procedures; two each for water and sediment. Residues of flumioxazin in water were determined by addition of pH 5 buffer. Residues of APF and 482-HA in water were determined after adjusting the sample pH to 8. Residues of flumioxazin and APF in sediment were determined by sequential extraction with with acetone/water/formic acid and acetonitrile (ACN)/water/formic acid. Residues of 482-HA in sediment were determined by sequential extraction with acetone/water/ammonium hydroxide and ACN/water/formic acid. Prepared water samples and sediment extracts were analyzed by high performance liquid chromatography with tandem mass spectrometric detection (HPLC/MS/MS). The method limit of quantitation (LOQ) for each compound was 1 ppb for pond water and 10 ppb for pond sediment.

This study was conducted for Valent U.S.A. to provide independent laboratory validation data as required by OPPTS 860.1340. The study protocol and protocol amendment appear in Appendix A.

2.0 Materials and Methods

2.1 Test/Reference Substances

The reference substances (analytical reference standards) used in this study appear in Table 1. A retainer sample of each reference substance is being stored frozen in EPL-BAS archives.

2.1.1 Reference Substance Stock Solutions

All stock solutions were stored in a freezer (ca. -10°C) when not needed in the laboratory.

Individual stock solutions of flumioxazin and APF were prepared at target concentrations of 1000 µg/mL and 482-HA was prepared at a target concentration of 100 µg/mL. The reference substances flumioxazin and APF were weighed (0.01 g) into 10 mL class A volumetric flasks and brought to volume with ACN. The reference substance 482-HA was weighed (0.01g) into a 100 mL class A volumetric flask and brought to volume with acetone.

2.1.2 Reference Substance Fortification and Working Standard Solutions

Fortification Standard Solution, 30 µg/mL Flumioxazin and APF:
A 0.3 mL aliquot of each stock standard solution was transferred to its own 10 mL volumetric flask and diluted to volume with acetone. The solutions were stored frozen (ca. -10°C).

Fortification Standard Solution, 1 µg/mL Flumioxazin and APF:
A 0.1 mL aliquot of each stock standard solution was transferred to its own 100 mL volumetric flask and diluted to volume with acetone. The solutions were stored frozen (ca. -10°C).

Fortification Standard Solution, 30 µg/mL 482-HA:
A 3 mL aliquot of the stock standard solution was transferred to a 10 mL volumetric flask and diluted to volume with acetone. The solution was stored frozen (ca. -10°C).

Fortification Standard Solution, 1 µg/mL 482-HA:
A 1 mL aliquot of the stock standard solution was transferred to a 100 mL volumetric flask and diluted to volume with acetone. The solution was stored frozen (ca. -10°C).

Mixed Working Standard Solution, 1 µg/mL each Flumioxazin and APF:
A 0.1 mL aliquot of each stock standard solution was transferred to a100 mL volumetric flask and diluted to volume with methanol (MeOH). The solution was stored refrigerated (ca. 5°C).

Mixed Working Standard Solution, 100 ng/mL each Flumioxazin and APF:
A 1 mL aliquot of the 1 µg/mL mixed working solution was transferred to a10 mL volumetric flask and diluted to volume with MeOH. The solution was stored refrigerated (ca. 5°C).

Mixed Working Standard Solution, 1 µg/mL each APF and 482-HA:
A 0.1 mL aliquot of each stock standard solution was transferred to a 100 mL volumetric flask and diluted to volume with acetone. The solution was stored frozen (ca. -10°C).

Mixed Working Standard Solution, 100 ng/mL each APF and 482-HA:
A 1 mL aliquot of the mixed 1 µg/mL working solution was transferred to a10 mL volumetric flask and diluted to volume with acetone. The solution was stored frozen (ca. -10°C).

Working Standard Solution, 1 µg/mL 482-HA:
A 1 mL aliquot of the stock standard solution was transferred to a 100 mL volumetric flask and diluted to volume with acetone. The solution was stored frozen (ca. -10°C).

Working Standard Solution, 100 ng/mL 482-HA:
A 1 mL aliquot of the 1 µg/mL working solution was transferred to a10 mL volumetric flask and diluted to volume with acetone. The solution was stored frozen (ca. -10°C).

2.1.3 Reference Substance Calibration Solutions

The working standards for flumioxazin, APF and 482-HA were used to make the calibration standards. Mixed calibration standards containing flumioxazin and APF were diluted to final volume with MeOH in class A volumetric flasks and stored refrigerated (ca. 5°C) when not in use. Mixed calibration standards containing APF and 482-HA were diluted to a final volume with acetone in a class A volumetric flask and stored frozen (ca. -10°C) when not in use.

Volume Used (mL)	Standard Used	Final Volume (mL)	Target Concentration (ng/mL)
4	1 µg/mL	100	40
2	1 µg/mL	100	20
1	1 µg/mL	100	10
0.5	1 µg/mL	100	5
0.25	1 µg/mL	100	2.5
0.5 or 1	100 ng/mL 5 ng/mL	100 10	0.5

2.2 Test Systems

The test systems were control pond water and control pond sediment obtained from Eurofins Scientific, Inc. Several containers of water and sediment were received from the field. Containers of each matrix type were composited to yield a bulk control sample of each matrix type. The bulk sediment sample was ground and homogenized with dry ice. The bulk samples were stored frozen (ca. -20°C) until needed for laboratory analysis. The sample identification code used to uniquely identify laboratory sub-samples appears in Appendix B.

2.3 Study Design and Procedures

Preliminary Activities: Prior to fortification of the control samples, a set of samples consisting of a reagent blank and two unfortified controls were analyzed for each sample preparation/extraction procedure outline in method GPL-MTH-064.

Method Validation Trials: The residue method was validated with acceptable and reproducible method recoveries. A method validation set consisted of one reagent

blank, two control samples, and 10 fortified control samples. Fortification was accomplished by adding known amounts of flumioxazin, APF, and 482-HA standards in solvent directly to the control sample by glass syringe. After fortification, the samples were analyzed as part of the assay set with the reagent blank and unfortified control samples to determine the method recoveries.

2.4 Fortification Procedure

The adequacy of the analytical method was determined by the recoveries resulting from the analysis of fortified control samples. Control samples were fortified by adding known amounts of non-radiolabeled solutions of flumioxazin, APF, and 482-HA to water and sediment sub-samples using a glass syringe. Fortifications were evaluated at the LOQ and 10x LOQ levels. The fortified samples were carried through the same analytical procedures as the control samples and reagent blank. The amount of analyte found in the sample extract versus the known amount of standard added to the matrix sub-sample was used to calculate method recovery.

2.5 Equipment and Supplies

The following equipment and supplies were used in the independent laboratory analysis:

Balance, analytical, capable of weighing to the nearest 0.1 mg.
Balance, top loading, capable of weighing to the nearest 1 mg.
Disposal Pasteur pipettes, glass
Centrifuge, International Equipment Company
Centrifuge, Beckman Coulter
Centrifuge bottles, HDPE, 250 mL
Adjustable micropipetters, 100 – 1000 µL
Flasks, side-arm, 250 mL
Funnels, Buchner, 7 cm
Filters, 7 cm, Whatman GF/A
Class A volumetric pipets and flasks, various sizes
Test Tubes, glass, 15 mL
Graduated cylinders, various sizes
Shaker, variable speed, platform, New Brunswick Scientific
Syringes, glass, various sizes for sample fortification
HPLC autosampler vials, glass, screw cap
Vacuum pump

2.6 Reagents and Prepared Solutions

The following reagents were used in the independent laboratory analysis:

Acetic Acid, Glacial, Fisher
Acetone, Burdick & Jackson
Acetonitrile (ACN), Burdick & Jackson
Ammonium Acetate, Sigma-Aldrich
Ammonium Hydroxide, BDH
Formic Acid, Fisher
Methanol (MeOH), Burdick & Jackson
Water, Deionized (DI), as delivered by a Barnstead NANOpure water system

HPLC Mobile Phase 0.2% Formic Acid in DI Water:
To a 1 liter graduated cylinder, add 500 mL of DI water. Add 2.0 mL of formic acid. Adjust volume to 1000 mL using DI water. Mix thoroughly.

HPLC Mobile Phase 0.2% Formic Acid in ACN:
To a 1 liter graduated cylinder, add 500 mL of ACN. Add 2.0 mL of formic acid. Adjust volume to 1000 mL using ACN. Mix thoroughly.

50:50 Acetonitrile:DI Water:
Combine equal volumes of ACN and DI water. Mix thoroughly.

100 mM Ammonium Acetate pH 5 Buffer:
To a 100 mL graduated cylinder, add approximately 0.4625 g of ammonium acetate and approximately 0.2290 mL of glacial acetic acid. Adjust volume to 100 mL using DI water. Mix thoroughly.

2.5% Formic Acid in DI Water:
To a 1 liter graduated cylinder, add 500 mL DI water. Add 25 mL of formic acid. Adjust volume to 1000 mL using DI water. Mix thoroughly.

2.5% Ammonium Hydroxide in DI Water:
To a 1 liter graduated cylinder, add 500 mL DI water. Add 25 mL of ammonium hydroxide. Adjust volume to 1000 mL using DI water. Mix thoroughly.

90:10 Acetone:2.5% Formic Acid in DI Water::
For 100 mL, combine 90 mL of acetone with 10 mL 2.5% formic acid in DI water. Mix thoroughly.

80:20 Acetonitrile:2.5% Formic Acid in DI Water::
For 100 mL, combine 80 mL of ACN with 20 mL 2.5% formic acid in DI water. Mix thoroughly.

90:10 Acetone:2.5% Ammonium Hydroxide in DI Water::
For 100 mL, combine 90 mL of acetone with 10 mL 2.5% ammonium hydroxide in DI water. Mix thoroughly.

80:20 Acetonitrile:2.5% Ammonium Hydroxide in DI Water::
For 100 mL, combine 80 mL of ACN with 20 mL 2.5% ammonium hydroxide in DI water. Mix thoroughly.

2.7 Sample Preparation/Extraction

Pond Water

Flumioxazin: Ten mL aliquots of pond water were transferred to 15 mL glass test tubes. To each tube, 1 mL of pH 5 buffer was added. Samples designated as laboratory fortifications were spiked at this point in the procedure. An aliquot of each sample solution was transferred to an autosampler vial and analyzed by HPLC/MS/MS.

APF and 482-HA: Prior to APF and 482-HA sample preparation, an aliquot of the bulk control sample was adjusted to pH 8.0 by drop-wise addition of 2.5% ammonium hydroxide solution. The pH of the bulk control water sample prior to pH adjustment was 7.5. The pH adjustment was not specified in method GPL-MTH-064. However, the Sponsor directed the ILV test facility to make the pH adjustment as a precaution to assure that the water was basic. Ten mL aliquots of basic pond water were transferred to 15 mL glass test tubes. Samples designated as laboratory fortifications were spiked at this point in the procedure. An aliquot of each sample solution was transferred to an autosampler vial and analyzed by HPLC/MS/MS.

Pond Sediment

Flumioxazin and APF: Pond sediment samples were weighed (20 g) into 250 mL centrifuge bottles. Samples designated as laboratory fortifications were spiked at this point in the procedure. To each sample, 80 mL of 90:10 Acetone:2.5% formic acid in DI water was added. The samples were shaken on a platform shaker for 15 minutes then centrifuged at 3,000 rpm for four minutes. The sample extracts were vacuum filtered through Whatman GF/A filter papers. The extraction, centrifugation and filtration steps were repeated using 80 mL of 80:20 ACN:2.5% formic acid in DI water with the second extract being combined with the first in a 250 mL graduated cylinder. The cylinders were brought to a final volume of 200 mL with DI water. Each sample

extract was then diluted 1:1 by mixing 0.5 mL of extract with 0.5 mL of DI water in an HPLC autosampler vial. Samples were then submitted for HPLC/MS/MS analysis.

482-HA: With the exception of different extraction solvents, the extraction procedure for 482-HA was identical to that described above for flumioxazin and APF. Eighty mL each of 90:10 acetone:2.5% ammonium hydroxide in DI water and 80:20 ACN:2.5% ammonium hydroxide in DI water were used sequentially for 482-HA extraction.

2.8 Instrumental Analysis

HPLC/MS/MS was used to quantify residues of flumioxazin, APF and 482-HA. Representative calibration curves and chromatograms appear in Figures 1-3 and 4-30, respectively. The following instrument parameters were used for the successful method validation trials.

HPLC System:	Waters 2695 Separations Module
MS Detector:	Micromass Quattro micro
HPLC Column:	Luna C18, 50 mm x 3 mm x 3 µm, Phenomenex
Column Temperature:	40°C
Injection Volume:	Pond Water: 30 µL (flumioxazin), 10 µL (APF and 482-HA)
	Pond Sediment: 10 µL for all calibration standards, 20 µL for all samples (flumioxazin, APF and 482-HA)
Mobile Phase:	A: 0.2% Formic Acid B: 0.2% Formic Acid in ACN
Flow Rate:	0.5 mL/minute

Pond Water Gradient Program (flumioxazin, APF and 482-HA)

Time (min)	%A	%B
0.00	90.0	10.0
5.00	5.0	95.0
6.00	5.0	95.0
6.01	90.0	10.0
10.00	90.0	10.0

Pond Sediment Gradient Program (flumioxazin and APF)

Time (min)	%A	%B
0.00	90.0	10.0
5.00	5.0	95.0
6.00	5.0	95.0
6.01	90.0	10.0
10.00	90.0	10.0

Pond Sediment Gradient Program (482-HA)

Time (min)	%A	%B
0.00	80.0	20.0
5.00	10.0	90.0
5.01	80.0	20.0
8.00	80.0	20.0

MS Interface: Electrospray, Positive Ion
Data System: Masslynx version 4.0 or 4.1
Scan Type: Multiple Reaction Monitoring (MRM)

MRM Program:

Compound	Parent (m/z)	Daughter (m/z)	Cone Voltage	Collision Energy
Flumioxazin	355	327	35	20
APF	221	163	21	18
482-HA	373	221	15	20

Cone voltage and collision energy values appearing in the table are only provided for guidance. Actual instrument parameters varied based on optimum voltages established during tuning. The actual tune files used were documented with each HPLC/MS/MS analysis sequence.

The HPLC/MS/MS system was calibrated by analysis of standard solutions ranging in concentration from approximately 0.5-40 ng/mL. A linear regression calibration curve was constructed using peak area (y-axis) and the inverse (1/x) of the standard solution concentration (x-axis). Sample solution concentrations were calculated using the straight line equation from the calibration curve. Peak areas, calibration curves and sample solution concentrations were determined using the HPLC/MS/MS data system (Masslynx 4.0 or 4.1).

2.9 Interferences

Inference peaks were not observed when the highly selective triple quadrupole mass spectrometer was used as the detection system.

2.10 Confirmatory Technique

No additional confirmation for flumioxazin and its APF and 482-HA metabolites was necessary since the triple quadrupole mass spectrometer provides unique selectivity by detecting the parent ion and the product ion which are specific for each compound. The selected molecular ions (m/z, mass/charge) used for the HPLC/MS/MS were 355>327 for flumioxazin, 221>163 for APF, and 373>221 for 482-HA.

2.11 Time Required for Analysis

For a set of 12 pond water samples and one reagent blank, the analytical procedure for flumioxazin can be completed from the time of sample aliquoting up to HPLC injection within two hours. A set of samples for APF and 482-HA can also be completed up to HPLC injection within two hours. For a set of 12 pond sediment samples and one reagent blank, the analytical procedure for flumioxazin and APF can be completed from the time of sample weighing up to HPLC injection within six hours. A set of samples for 482-HA can also be completed up to HPLC injection within six hours.

2.12 Modifications or Potential Problems

No major modifications were made to method GPL-MTH-064. Minor changes to HPLC parameters were used for the analysis of flumioxazin and APF. Larger injection volumes than those specified in method GPL-MTH-064 were used. A weaker mobile phase was used at Time 0 in the gradient program. The starting gradient composition was 90% A (0.2 % formic acid) and 10% B (0.2% formic acid in ACN). The alternate parameters were necessary to achieve adequate sensitivity to detect the lowest calibration standard (0.5 ng/mL). This modification was communicated to the Study Monitor. A communications log between Study Director and Study Monitor appears in Appendix D.

It is not explicitly stated in method GPL-MTH-064 that the pH of the water samples must be in the range of 7.5-9 for the determination of APF and 482-HA. The method only refers to "basic pond water". It is recommended that all water samples be adjusted to pH 8 as a precaution to assure that the sample is basic.

3.0 Calculations

The following formulas were used for calculation of analyte residues and recoveries: A calibration equation was obtained using linear regression analysis of the peak area values (y-axis) and the inverse (1/x) of the concentrations (x-axis) of standard solutions injected over the course of an analysis sequence. The amount found (ng/mL) of each sample solution analyzed was calculated from the linear regression equation:

$y = mx + b$

Where y is the peak area, m is the slope of the linear regression calibration curve, x is the amount found (ng/mL) and b is the y-intercept of the linear regression curve.

Amount Found (ng/mL) = Peak Area – y-intercept / slope

Example:	Set V005, Flumioxazin
Lab Sample ID:	159G410S003
Sample Peak Area:	460.406
y-Intercept:	65.6427
Slope:	449.163

Amount Found = 460.406 – 65.6427 / 449.163 = 0.879 ng/mL

Masslynx software (Waters, Version 4.0 or 4.1) was used to calculate linear regression calibration curves and amount found (ng/mL). All other calculations were performed using Microsoft Office Excel 2003.

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ppb Found =

$$\frac{\textit{Amount Found (ng/mL)} * \textit{Final Volume (mL)}}{\textit{Sample Amount (mL) (grams for sediment)}}$$

Example:	Set V005, Flumioxazin, Pond Water
Lab Sample ID:	159G410S003
Amount Found:	0.879 ng/mL
Final Volume:	11 mL (10 mL sample plus 1 mL pH 5 buffer)
Sample Amount:	10 mL

ppb Found = 0.879 * 11 / 10 = 0.967 ppb

Fortification Level (ppb) =

$$\frac{\textit{Volume Spiking Soln. (mL)} * \textit{Spiking Soln. Concn. (ng/mL)}}{\textit{Sample Amount (mL) (grams for sediment)}}$$

Example:	Set V005, Flumioxazin, Pond Water
Lab Sample ID:	159G410S003
Volume Spiking Soln:	0.01 mL
Spiking Soln Concn.:	1000 ng/mL
Sample Amount:	10 mL

Fortification Level = 0.01 * 1000 / 10 = 1.000 ppb

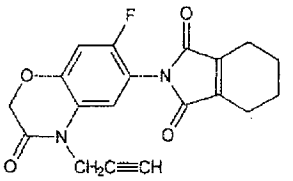
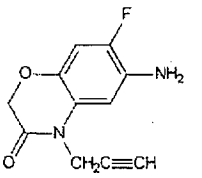
Recovery (%) =

*ppb Found / Fortification Level (ppb) * 100*

Example:	Set V005, Flumioxazin, Pond Water
Lab Sample ID:	159G410S003
ppb Found:	0.967
Fortification Level:	1.000 ppb

Recovery = 0.967 / 1.000 * 100 = 96.700% (Due to rounding, the recovery value for this sample appearing in the Excel data table (96.690%) differs slightly.)

Table 1. Reference Substances

Common Name.....: Chemical Name (CAS).....: CAS Number.....: Lot Number.....: Purity (%) .....: Assay Date.....: Expiration Date.....: Storage Conditions..: Structure:	Flumioxazin [2-[7-fluoro-3,4-dihydro-3-oxo-4-(2-propynyl)-2H-1,4-benzoxazin-6-yl]-4,5,6,7-tetrahydro-1H-isoindole-1,3(2H0-dione)] 103361-09-7 AS1663i 99.2 April 11, 2006 and April 9, 2008 April 9, 2010 Frozen under nitrogen 
Common Name.....: Lot Number.....: Purity (%) .....: Assay Date.....: Expiration Date.....: Storage Conditions..: Structure:	APF AS1981b 99.7 June 13, 2007 June 13, 2009 Frozen under nitrogen 
Common Name.....: Lot Number.....: Purity (%) .....: Assay Date.....: Expiration Date.....: Storage Conditions..: Structure	482-HA AS1997b 98.4 April 26, 2007 April 26, 2009 Frozen under nitrogen