EXPERIMENTAL

Sample Origin, Numbering, Preparation and Storage

Untreated control samples were obtained from EAG Laboratories' site. All samples were tracked in the EAG Laboratory Information Management System (LIMS) database. Unique sample numbers were assigned to the samples to track them during receipt, storage, and analysis. Complete source documentation was included in the study file.

No sample preparation was required for the water samples prior to analysis. Samples were stored refrigerated at approximately 2-8 °C after the time of sampling and during the course of the method validation study, except when they were removed for taking aliquots for sample analysis.

During the course of the study, the samples were stored in a temperature-monitored refrigerator at approximately 2-8 °C, except when removed for analysis.

Calculation of Standard Calibration Curve

Calculation of a standard curve is executed by injection of a series of calibration standards as described in Appendix I and acquisition of peak areas for oxyfluorfen and its internal standard.

Oxyfluorfen	*m/z* Q1/Q3 362.1/315.9 (quantitative)
Oxyfluorfen	*m/z* Q1/Q3 362.1/236.9 (confirmatory)
Oxyfluorfen d_5 (M+5) Stable Isotope	*m/z* Q1/Q3 367.1/236.9 (internal standard)

The linearity of detector response was evaluated using solvent standard solutions. In order to generate a standard curve, plot the analyte concentration on the abscissa (x-axis) and the respective quantitation ratio on the ordinate (y-axis) in Analyst. Using regression analysis, determine the equation for the curve with respect to the abscissa. Refer to Figure 2-Figure 3 for example calibration plots and to Figure 4 for example calculations. Individual calibration results can be found in Figure 7, Figure 12, and Figure 17.

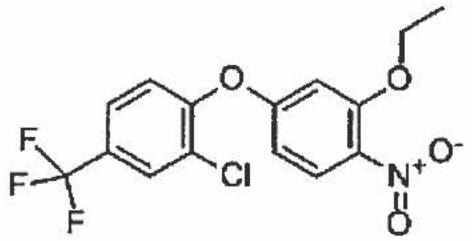
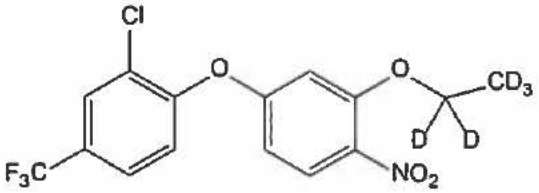
Full-Scan and Product-Ion Mass Spectra

A full scan and two product-ion mass spectra of oxyfluorfen and oxyfluorfen d_5 are illustrated in Figure 5 and Figure 6.

Statistical Treatment of Data

Statistical treatment of data included but was not limited to the calculation of regression equations, correlation coefficients (r) for describing the linearity of calibration curves, and means, standard deviations, and relative standard deviations of the results for the fortified recovery samples. In addition, the LOD and LOQ values were statistically generated.

Table 1. Identities and Structures of Oxyfluorfen and its Internal Standard

Common Name	Structural Formula and Chemical Name
Oxyfluorfen Molecular Formula: $C_{15}H_{11}ClF_3NO_4$ CAS Number: 42874-03-3	 2-Chloro-1-(3-ethoxy-4-nitrophenoxy)-4-(trifluoromethyl)benzene
Oxyfluorfen d_5 [M+5] Stable Isotope Molecular Formula: $C_{15}H_6D_5ClF_3NO_4$ CAS Number: NA	 2-Chloro-1-(3-ethoxy-d_5-4-nitrophenoxy)-4-(trifluoromethyl)benzene

APPENDIX I ANALYTICAL METHOD

Determination of Residues of Oxyfluorfen in Water Using LC-MS/MS

1.0. Scope

This method is applicable for the quantitative determination of residues of oxyfluorfen in water. The method is applicable for the concentration range of 0.0150-5.00 ng/mL with a limit of quantitation of 0.1 µg/L. Example recoveries can be found in Table 1. Example mass spectra can be found in Figure 1.

2.0. Safety Precautions

Each analyst must be acquainted with the potential hazards of the equipment, reagents, products, solvents, and procedures used in this method before commencing laboratory work. Sources of information include operation manuals, safety data sheets, literature, and other related data. Safety information should be obtained from the supplier. Disposal of waste materials, reagents, reactants, and solvents must be in compliance with applicable governmental requirements.

Acetonitrile and methanol are flammable and should be used in well-ventilated areas away from ignition sources. Formic acid is corrosive and can cause severe burns. It is imperative that proper eye and personal protection equipment be worn when handling these reagents.

3.0. Stability of Working Solutions

A stock solution of oxyfluorfen prepared in acetonitrile was tested after 45 days of storage at approximately 2-8 °C, and was found to be stable. Fortification standard solutions of oxyfluorfen in acetonitrile were tested after 32 days of storage (19 days for 0.100 µg/mL and 0.0100 µg/mL) at approximately 2-8 °C, and were found to be stable. Calibration standard solutions of oxyfluorfen prepared in acetonitrile/water (50/50) were tested after 19 days of storage (9 days for 0.0150 ng/mL) at approximately 2-8 °C, and were found to be stable.

4.0. Stability of Sample Extracts

Final sample extracts containing oxyfluorfen from ground, surface, and drinking water in acetonitrile/water (50/50) were tested after 9-13 days of storage at approximately 2-8 °C, and were determined to be stable.

Matrix	Demonstrated Stability (days)
Ground Water	9 and 12
Surface Water	11 and 13
Drinking Water	10 and 12

5.0. Matrix Effects

To correct for matrix effects, a stable isotope internal standard was used in the preparation of samples.

6.0. Laboratory Equipment

Balance, analytical, Model XP205DR, Mettler-Toledo, Inc.

Vortex Mixer, Model M16715, Thermos Scientific

Sonicator, Branson 8800 Bransonic series, Model CPX8800H

Falcon™ 15-mL polypropylene conical centrifuge tubes, part number 14-959-70C, Fisher Scientific (used for standard stability determination only)

Pipet, positive-displacement, 3-25 µL capacity, Model M25, Gilson Inc.

Pipet, positive-displacement, 10-100 µL capacity, Model M100, Gilson Inc.

Pipet, positive-displacement, 50-250 µL capacity, Model M250, Gilson Inc.

Pipet, positive-displacement, 100-1000 µL capacity, Model M1000, Gilson Inc.

Repeater Xstream, positive-displacement, Eppendorf

7.0. Chromatographic System

Column, analytical, HSS T3, 2.1 mm x 50 mm, 1.8 µm particle size, Waters Acquity

Mass spectrometer, Model QTRAP 6500, AB SCIEX

Mass spectrometer data system, Analyst v.1.6.2, AB SCIEX

Liquid chromatography, System Controller CBM-20A, Degasser DGU-20A3R and DGU-20A5R, Pump LC30AD, Column Heater CTO-20AC and CTO-20A, Autosampler

SIL-30ACMP, Shimadzu

Column Heater CTO-20AC and CTO-20A, Autosampler SIL-30ACMP, Shimadzu

8.0. Glassware and Materials

Vial, 4-dram, amber glass with Teflon-lined screw caps, catalog number 03-339-23D, Fisher Scientific

Vial, 8-dram, amber glass with Teflon-lined screw caps, catalog number 03-339-23E, Fisher Scientific

Combitips 1 mL, Eppendorf

Combitips 5 mL, Eppendorf

Combitips 10 mL, Eppendorf

Combitips 50 mL, Eppendorf

Graduated Cylinder, 500-, 1000- and 2000-mL

Vial, autosampler, 2 mL, 11 mm amber glass crimp/snap top, catalog number C4011-6W, Thermo Scientific™

Autosampler vial inserts, 9 mm, 500 µL, catalog number 03-375-3B, Fisher Scientific

Volumetric flask, 10-, 20-, and 25-mL

Glass Bottle, 2-L

Carboy, 2- and 20-L, autoclavable polypropylene with spigot, catalog number 02-963-2B, Fisher Scientific

9.0. Reagents

Acetonitrile, OPTIMA grade, catalog number A996, Fisher Scientific

Formic acid, OPTIMA LC/MS grade, ≥99.5% purity, catalog number A117, Fisher Scientific

Methanol, OPTIMA, A.C.S. grade, catalog number A454, Fisher Scientific

Methanol, ACS/Reagent, catalog number AH230, Honeywell Burdick & Jackson

Water, HPLC grade, catalog number W5, Fisher Scientific

10.0. Prepared Solutions

Solution volumes may be scaled to accommodate alternate preparations.

10.1. Acetonitrile/Water (50/50)

To a 2-L glass bottle, add 1000 mL of acetonitrile followed by 1000 mL of water. Cap and shake bottle well to mix.

10.2. Acetonitrile/Methanol/Water (1/1/1) - Wash #1, Strong Wash

To a 20-L carboy add one unopened 4-L bottle of acetonitrile followed by one unopened 4-L bottle of water and finally, one unopened 4-L bottle of methanol. Cap and shake carboy well to mix.

10.3. Acetonitrile/Methanol/Water (1/1/2) - Wash #2, Weak Wash

To a 20-L carboy add one unopened 4-L bottle of methanol followed by one unopened

4-L bottle of acetonitrile and finally, two unopened 4-L bottles of water. Cap and shake carboy well to mix.

10.4. Water with 0.01% formic acid – HPLC Mobile Phase A

Pipette 0.400 mL of formic acid into an unopened 4 L bottle of water. Cap and shake the bottle well to mix.

10.5. Methanol with 0.01% formic acid – HPLC Mobile Phase B

Pipette 0.400 mL of formic acid into an unopened 4 L bottle of methanol. Cap and shake the bottle well to mix.

Add 2000 mL of methanol to a 2-L carboy followed by 0.200 mL of formic acid. Cap and shake the bottle well to mix.

11.0. Preparation of Fortification Solutions

11.1. Weigh 10.00 mg (corrected for purity) of oxyfluorfen analytical standard and quantitatively transfer to a 10.0-mL volumetric flask with acetonitrile. Sonicate to dissolve any residual material (05-Jan-18 stock not sonicated) and then dilute to volume to obtain a 1000-µg/mL stock solution. Invert several times to mix thoroughly and transfer to a 4-dram amber vial.

11.2. Pipet 1.00 mL of the above stock solution into a 10-mL volumetric flask and adjust to volume with acetonitrile to obtain a solution containing 100 µg/mL of oxyfluorfen. Invert several times to mix thoroughly and transfer to a 4-dram amber vial.

11.3. Pipet 0.100 mL of the above stock solution into a 10-mL volumetric flask and adjust to volume with acetonitrile to obtain a solution containing 10.0 µg/mL of oxyfluorfen. Invert several times to mix thoroughly and transfer to a 4-dram amber vial.

11.4. Pipet 0.100 mL of the 100-µg/mL solution into a 10-mL volumetric flask and adjust to volume with acetonitrile to obtain a solution containing 1.00 µg/mL of oxyfluorfen. Invert several times to mix thoroughly and transfer to a 4-dram amber vial.

11.5. Pipet 1.00 mL of the 10.0-µg/mL solution into a 10-mL volumetric flask and adjust to volume with acetonitrile to obtain a solution containing 1.00 µg/mL of oxyfluorfen. Invert several times to mix thoroughly and transfer to a 4-dram amber vial

11.6. Pipet 1.00 mL of the 1.00-µg/mL solution into a 10-mL volumetric flask and adjust to volume with acetonitrile to obtain a solution containing 0.100 µg/mL of oxyfluorfen. Invert several times to mix thoroughly and transfer to a 4-dram amber vial.

11.7. Pipet 0.100 mL of the 1.00-µg/mL solution into a 10-mL volumetric flask and adjust to volume with acetonitrile to obtain a solution containing 0.0100 µg/mL of oxyfluorfen. Invert several times to mix thoroughly and transfer to a 4-dram amber vial.

12.0 Preparation of Oxyfluorfen Intermediate Calibration Standard Solutions

12.1. Pipet 1.00 mL of the 1.00-µg/mL solution into a 10-mL volumetric flask and adjust to volume with acetonitrile/water (50/50) to obtain a solution containing 100 ng/mL of oxyfluorfen. Invert several times to mix thoroughly and transfer to a 4-dram amber vial.

12.2. Pipet 0.500 mL of the 100-ng/mL solution into a 10-mL volumetric flask and adjust to volume with acetonitrile/water (50/50) to obtain a solution containing 5.00 ng/mL of oxyfluorfen. Invert several times to mix thoroughly and transfer to a 4-dram amber vial.

12.3. Pipet 0.050 mL of the 1.00-µg/mL solution into a 10-mL volumetric flask and adjust to volume with acetonitrile/water (50/50) to obtain a solution containing 5.00 ng/mL of oxyfluorfen. Invert several times to mix thoroughly and transfer to a 4-dram amber vial.

13.0. Preparation of Oxyfluorfen d_5 Internal Standard (IS) Solutions

13.1. Weigh 2.50 mg of oxyfluorfen d_5 analytical standard and quantitatively transfer to a 25-mL volumetric flask with acetonitrile. Dilute to volume to obtain a 100-µg/mL stock solution. Invert several times to mix thoroughly and transfer to an 8-dram amber vial.

13.2. Pipet 0.050 mL of the 100-µg/mL solution into a 10-mL volumetric flask and adjust to volume with acetonitrile to obtain a solution containing 0.500 µg/mL of the oxyfluorfen d_5 IS standard. Invert several times to mix thoroughly and transfer to a 4-dram amber vial.

14.0. Preparation of Calibration Standards

Prepare calibration standard solutions by diluting the fortification solutions with acetonitrile/water (50/50) to obtain calibration standards over the concentration range of 0.0150-5.00 ng/mL as follows:

Concentration of Standard Solution[a] (ng/mL)	Aliquot of Standard Solution (mL)	Final Soln. Volume[d] (mL)	Calibration Soln. Final Conc.[b] (ng/mL)	Equivalent Sample Conc.[c] (µg/mL)
100	1.00; 0.500	20.0; 10.0	5.00	0.0100
100	0.500; 0.250	20.0; 10.0	2.50	0.0050
100	0.200; 0.100	20.0; 10.0	1.00	0.0020
5.00	2.00; 1.00	20.0; 10.0	0.500	0.0010
5.00	0.200; 0.100	20.0; 10.0	0.0500	0.0001
5.00	0.0300	10.0; 10.0	0.0150	0.000015

[a] Intermediate calibration standard

[b] All standards were inverted several times to mix and transferred to an 8-dram amber vial, with the exception of the 0.0150-ng/mL standard, which was transferred to a 4-dram amber vial.

[c] The equivalent sample concentration is based on fortifying a 5.0-mL water sample and a final volume of 10.0 mL.

[d] Prior to bringing to volume, add 0.040 mL of 0.500-µg/mL internal standard (1.00 ng/mL) to all standards in a 20.0-mL volumetric; add 0.020 mL of a 0.500-µg/mL internal standard (1.00 ng/mL) to all standards in a 10.0-mL volumetric.

15.0. Analytical Procedure

15.1. For control, reagent blank, and treated samples only: pipet 5.00 mL of water sample into an 8-dram amber glass vial.

For fortified control samples only: pipet 4.95 mL of control water into an 8-dram amber glass vial.

15.2. For preparing fortified samples, add 0.050-mL aliquots of the appropriate fortification solutions to the applicable samples.

15.3. Add 0.020 mL of the 0.500-µg/mL internal standard to the sample vial; cap and vortex to mix.

15.4. Add 5.00 mL of acetonitrile to the sample vial; cap and vortex to mix. Allow sample to equilibrate to room temperature.

15.5. Vial a portion of the sample into a 2-mL glass autosampler vial and submit for analysis.

15.6. Vial a portion of each calibration standard into a 2-mL glass vial with a 500-µL glass insert and submit for analysis.

15.7. Analyze the calibration standards and samples by LC-MS/MS with positive-ion electrospray tandem mass spectrometry liquid chromatography. Determine the suitability of the chromatographic system using the following performance criteria:

15.7.1. Standard curve linearity: Determine that the correlation coefficient equals or exceeds 0.995 for the least squares equation which describes the detector response as a function of standard curve concentration.

15.7.2. Peak resolution: Visually determine that sufficient resolution has been achieved for the analyte and internal standard relative to background interferences.

15.7.3. Appearance of chromatograms: Visually determine the chromatograms with respect to peak response, baseline noise, and background interference. Visually determine that a minimum signal-to-noise ratio of 10:1 has been attained for each analyte in the 0.0150-ng/mL calibration standard.

15.8. If any sample concentration exceeds the range of the standard calibration curve, dilute with acetonitrile containing the internal standard to obtain a response within the range of the calibration curve.

16.0. Supplemental Notes

16.1. Equipment, glassware, materials, reagents, and chemicals considered to be equivalent to those specified may be substituted with the understanding that their performance must be confirmed by appropriate tests. Common laboratory glassware and supplies are assumed to be readily available. Unless specified otherwise, Class A volumetric glassware is used to prepare analytical standards, fortification solutions, and calibration standards.

16.2. The instrumental conditions may be modified to obtain optimal chromatographic separation and sensitivity.

16.3 Two occasions of sensitivity loss during analysis appeared to be attributable to the mobile phase; preparation of new mobile phase resulted in the return of sensitivity.

16.4. Based on availability of material, weighing of the analytical standard and internal standards can be modified and the subsequent solution preparation scheme adjusted.

16.5. Sample dilutions should be prepared using the acetonitrile/water (50/50) 0.0500-µg/mL IS solution.

16.6. The following is taken directly from Dow AgroSciences Analytical Method GRM 03.04 (Reference 1): "Due to its low water solubility, oxyfluorfen is readily adsorbed from water samples onto glass or plastic containers. Consequently, field water samples must be collected in the glass sample vial that will be used for sample extraction, and the entire sample should be extracted as described in Section 9.3. The exact sample volume or mass will need to be determined at sampling or by difference following analysis. Samples may be stored at ambient temperature, refrigerated, or frozen prior to analysis." Because the analysis conducted at EAG is for a Method Validation study, the potential for oxyfluorfen to stick to field sample containers will not exist. Method development recoveries appear to confirm that oxyfluorfen did not adhere to the glass extraction vial.

16.7. During method development, we assessed 0.45-µm and 0.2-µm nylon, Teflon and polypropylene syringe filters for analyte adherence due to the potential that field samples might contain particulate matter. It was found that oxyfluorfen adheres to all three types of syringe filters, although it is possible to remove the analyte from both Teflon and polypropylene syringe filters with acetonitrile. A 1-mL water sample can be filtered; however, it must be followed by a 2-mL acetonitrile rinse of the filter with collection of both fractions. Organic solvent is critical to removal of all oxyfluorfen from the Teflon and polypropylene filter membranes and did not remove oxyfluorfen from the polypropylene syringe filter. Filtration of the sample without an additional rinse of

acetonitrile results in significant analyte losses.

17.0. Instrumental Conditions

Typical HPLC Operating Conditions

Instrumentation:	Shimadzu System Controller CBM-20A, Degasser DGU-20A3R and DGU-20A5R, Pump LC30AD, Column Heater CTO-20AC and CTO-20A, Autosampler SIL-30ACMP
Column:	Waters Acquity HSS T3 2.1 x 50 mm, 1.8 µm
Guard Column:	Waters, In-line frit
Column Temperature:	40 °C
Autosampler Temperature:	10 °C
Injection Volume:	20 µL
Injection Wash:	Wash #1, Strong Wash: 1/1/1 ACN/MeOH:H_2O, 5 sec Wash #2, Weak Wash: 1/1/2 ACN/MeOH:H_2O, 5 sec
Run Time:	~8 minutes
Mobile Phase:	A – 0.01% Formic Acid (aq) B – 0.01% Formic Acid in Methanol

Gradient:

Time (min)	Flow Rate (mL/min)	Solvent A (percent)	Solvent B (percent)
0:50	0.50	40	60
5:00	0.50	5	95
7:00	0.50	5	95
7:01	0.50	40	60
8:00	0.50	stop	

Flow Diverter

Flow to Waste:	0.0 min → 2.6 min
Flow to Source:	2.6 min → 4.1 min
Flow to Waste:	4.1 min → end of run

Typical Mass Spectrometry Operating Conditions

Instrumentation:	AB SCIEX QTRAP 6500MS System AB SCIEX Analyst version 1.6.2 data system
Ionization Mode:	electrospray

Polarity:	positive
Scan Type:	MRM
Resolution:	Q1 – unit, Q3 – unit
Curtain Gas (CUR):	10 psi
Collision Gas (CAD):	High
IonSpray Voltage (IS):	5500 volts
Temperature (TEM):	600 °C
Ion Source Gas 1 (GS1):	40 psi
Ion Source Gas 2 (GS2):	70 psi
Entrance Potential (EP):	10 volts
Period Duration:	5.00 minutes

Analyte:	Precursor Ion, Q1 *m/z*	Product Ion, Q3 *m/z*	Declustering Potential V	Collision Energy V	Cell Exit Potential V	Retention Time (min)	
Oxyfluorfen 362.1/315.9	362.1	315.9	50	19	10	~3.5	(quant)
Oxyfluorfen 362.1/236.9	362.1	236.9	50	33	10	~3.5	(confirm)
Oxyfluorfen 362.1/298.0	362.1	298.0	50	25	10	~3.5	(confirm)
Oxyfluorfen 362.1/274.0	362.1	274.0	50	24	10	~3.5	(confirm)
Oxyfluorfen IS 367.1/320.9	367.1	320.9	50	19	10	~3.5	(quant)
Oxyfluorfen IS 367.1/236.9	367.1	236.9	50	33	10	~3.5	(confirm)
Oxyfluorfen IS 367.1/298.0	367.1	298.0	50	25	10	~3.5	(confirm)
Oxyfluorfen IS 367.1/274.0	367.1	274.0	50	24	10	~3.5	(confirm)

18.0 Reference

Edward L. Olberding, W. L. (2003). *Determination of Residues of Oxyfluorfen in Drinking Water, Groundwater and Surface Water by Gas Chromatography with Negative-Ion Chemical Ionization Mass Spectromety.* Indianapolis, IN: Dow AgroSciences LLC.