

## INTRODUCTION

Method validation samples were prepared and analyzed to assess the performance of a residue analytical method developed by Wildlife International for the analysis of the Flutianil and metabolites (OC56635, OC56574, OC53276, and OC53279) in surface and ground water. This study was conducted under Wildlife International, Project Number 181C-116. The study was performed based on U.S. Environmental Protection Agency Residue Chemistry Test Guideline, OPPTS 860.1340, entitled “*Residue Analytical Method (1)*”, and on the procedures outlined in the European Commission Working Document SANCO/3029/99 rev.4, 11/07/00, entitled “*Residues: Guidance for Generating and Reporting Methods of Analysis in Support of Pre-registration Data Requirements for Annex II (Part A, Section 4) and Annex III (Part A, Section 5) of Directive 91/414 (2)*” . The analyses of the samples were performed utilizing a dilute and direct injection technique, with analysis by High Performance Liquid Chromatography (HPLC) coupled with Tandem Mass Selective Detection (MS/MS). Surface and ground water validation samples were prepared and analyzed between July 13 and 14, 2015. The raw data generated by Wildlife International and a copy of the final report are filed under Project Number 181C-116 in archives located on the Wildlife International site.

## OBJECTIVE

This study was conducted to fulfill EPA requirements set forth in guidelines OPPTS 860.1340 and SANCO/3029/99 rev. 4 dated 11/07/00.

## MATERIALS AND METHODS

This study was conducted according to the protocol “Validation of a Method for the Determination of Flutianil and Metabolites (OC56635, OC56574, OC53276, and OC53279) in Surface and Ground Water” (Appendix 1).

### Reference Substances

The Flutianil reference substance was received from LS Global Corp. on August 31, 2007 and was assigned Wildlife International identification number 8225 upon receipt. The reference substance, described as a solid, was identified on the label as: OK-5203 Technical Grade; Lot/Batch Number: 05DF2. A Certificate of Analysis for the reference substance is presented in Appendix 2. Information received with the material and subsequent to its receipt is summarized below:

OK-5203

Common Name: Flutianil

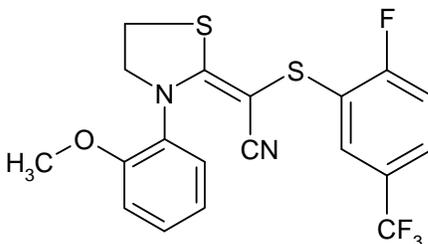
Name or Code Number: OK-5203

IUPAC Name: (Z)-2-[2-fluoro-5-(trifluoromethyl)phenylthio]-2-[3-(2-methoxyphenyl)-1,3-thiazolidin-2-ylidene]acetonitrile

Molecular Formula: C<sub>19</sub>H<sub>14</sub>F<sub>4</sub>N<sub>2</sub>OS<sub>2</sub>

Molecular Weight: 426.5

Structural Formula:



CAS Number: 958647-10-4

Lot Number: 05DF2

Purity: 99.54%

Expiration Date: November 26, 2016

Storage Conditions: Refrigerated conditions in darkness

The metabolite OC56635 reference substance was received from Landis International, Inc. on July 07, 2015 and was assigned Wildlife International identification number 12467 upon receipt. The reference substance, described as a solid, was identified on the label as: OC56635; Lot/Batch Number: 81010. A Certificate of Analysis for this reference substance is presented in Appendix 3. Information received with the material and subsequent to its receipt is summarized below:

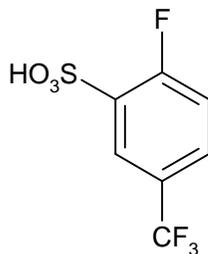
OC56635

IUPAC Name: 2-fluoro-5-(trifluoromethyl)benzenesulfonic acid (dihydrate)

Molecular Formula: C<sub>7</sub>H<sub>4</sub>F<sub>4</sub>O<sub>3</sub>S (2 H<sub>2</sub>O)

Molecular Weight: 244.2

Structural Formula:



(\*2H<sub>2</sub>O)

Batch Number: 81010

Purity: 99.9%

Expiration Date: February 25, 2017

Storage Conditions: Ambient conditions (cool, dry area as per C.O.A.)

The metabolite OC56574 reference substance was received from Landis International, Inc. on July 07, 2015 and was assigned Wildlife International identification number 12465 upon receipt. The reference substance, described as a solid, was identified on the label as: OC56574; Batch Number: TT0902015. A Certificate of Analysis for this reference substance is presented in Appendix 4. Information received with the material and subsequent to its receipt is summarized below:

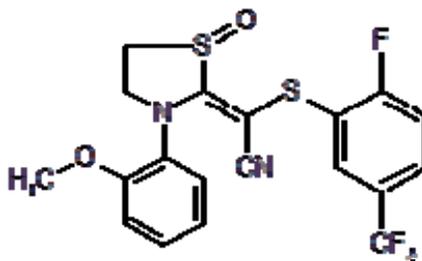
#### OC56574

IUPAC Name: (Z)-2-(2-fluoro-5-(trifluoromethyl)phenylthio)-2-[3-(2-methoxyphenyl)-1-oxo-2-thiazolidinylidene]acetonitrile

Molecular Formula: C<sub>19</sub>H<sub>14</sub>F<sub>4</sub>N<sub>2</sub>O<sub>2</sub>S<sub>2</sub>

Molecular Weight: 442.45

Structural Formula:



Batch Number: TT0902015

Purity: 98.93%

Expiration Date: February 25, 2017

Storage Conditions: Ambient conditions (cool, dry area as per C.O.A.)

The metabolite OC53276 reference substance was received from Landis International, Inc. on July 07, 2015 and was assigned Wildlife International identification number 12464 upon receipt. The reference substance, described as a solid, was identified on the label as: OC53276; Batch Number: YaE0711005. A Certificate of Analysis for this reference substance is presented in Appendix 5. Information received with the material and subsequent to its receipt is summarized below:

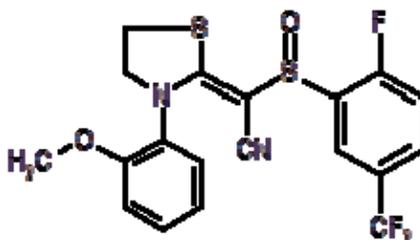
#### OC53276

IUPAC Name: (Z)-2-[2-fluoro-5-(trifluoromethyl)phenylsulfinyl]-2-[3-(2-methoxyphenyl)thiazolidinylidene]acetonitrile

Molecular Formula: C<sub>19</sub>H<sub>14</sub>F<sub>4</sub>N<sub>2</sub>O<sub>2</sub>S<sub>2</sub>

Molecular Weight: 442.45

Structural Formula:



Batch Number: YaE0711005

Purity: 98.9%

Expiration Date: February 25, 2017

Storage Conditions: Ambient conditions (cool, dry area as per C.O.A.)

The metabolite OC53279 reference substance was received from Landis International, Inc. on July 07, 2015 and was assigned Wildlife International identification number 12466 upon receipt. The reference substance, described as a solid, was identified on the label as: OC53279; Batch Number: HN0510003. A Certificate of Analysis for this reference substance is presented in Appendix 6. Information received with the material and subsequent to its receipt is summarized below:

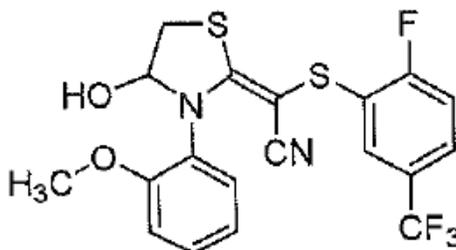
OC53279

IUPAC Name: (Z)-2-[2-fluoro-5-(trifluoromethyl)phenylthio]-2-[4-hydroxy-3-(2-methoxyphenyl)thiazolidinylidene]acetonitrile

Molecular Formula: C<sub>19</sub>H<sub>14</sub>F<sub>4</sub>N<sub>2</sub>O<sub>2</sub>S<sub>2</sub>

Molecular Weight: 442.45

Structural Formula:



Batch Number: HN0510003

Purity: 99.6%

Expiration Date: February 25, 2017

Storage Conditions: Ambient conditions (cool, dry area as per C.O.A.)

### **Laboratory Equipment/Glassware**

- Applied Biosystems Triple Quad 5500 LC/MS/MS System
- Agilent Technologies 1260 Infinity HPLC System
- Analytical Balance
- Eppendorf 2500 Reference Pipettor and Associated Tips
- Assorted Class A Volumetric Flasks and Pipettes
- Disposable 15-mL Graduated Plastic Centrifuge Tubes
- Hamilton Gas-tight Syringes, Assorted Sizes
- Disposable Glass Pasteur Pipettes
- 2-mL Auto-sampler Vials

Alternative equipment may be substituted as long as it is considered equivalent in function and generates successful method outcome.

### **Reagents and Solvents**

All solvents used in this study were HPLC grade or equivalent.

### **Test System - Surface and Ground Water**

The surface water used to prepare the method validation samples was collected locally from Tuckahoe Lake in Tuckahoe State Park near Ridgely, Maryland on September 19, 2014 and stored under refrigerated conditions. The water was categorized as “surface” type water. Upon receipt, the surface water was logged in as an external sample at the testing facility and was characterized by measuring specific conductance, hardness, alkalinity, and pH. The specific conductance, hardness, alkalinity and pH of the surface water are provided in Appendix 7.

The ground (well) water used to prepare the method validation samples was obtained from a well approximately 40 meters deep located on the Wildlife International site. The ground (well) water is characterized as moderately-hard water. The specific conductance, hardness, alkalinity and pH of the well water during the four-week period immediately preceding the test are provided in Appendix 8.

The ground (well) water was passed through a sand filter to remove particles greater than approximately 25 µm, and pumped into a 37,800-L storage tank and aerated with spray nozzles. Prior to use, the water was filtered to 0.45 µm in order to remove fine particles. The results of periodic analyses performed to measure the concentrations of selected organic and inorganic constituents in the ground (well) water used by Wildlife International are summarized in Appendix 9.

### Reference Substance Primary Stock Preparation

A primary stock solution of Flutianil was prepared by weighing 0.1005 grams (weight corrected for purity) of the reference substance on an analytical balance. The reference substance was transferred to a 100-mL class A volumetric flask, and brought to volume using methanol to achieve a 1.00 mg/mL stock solution. This primary stock solution (1000 µg/mL) was serially diluted in the same solvent solution to prepare 100 µg/mL working stock solution as shown below:

Stock Concentration (µg/mL)	Aliquot (mL)	Final Volume (mL)	Stock Concentration (µg/mL)
1000	5.00	50.0	100

A primary stock solution of OC56635 was prepared by weighing 0.01001 grams (weight corrected for purity) of the reference substance on an analytical balance. The reference substance was transferred to a 100-mL class A volumetric flask, and brought to volume using methanol to achieve a 100 µg/mL stock solution.

A primary stock solution of OC56574 was prepared by weighing 0.01011 grams (weight corrected for purity) of the reference substance on an analytical balance. The reference substance was transferred to a 100-mL class A volumetric flask, and brought to volume using methanol to achieve a 100 µg/mL stock solution.

A primary stock solution of OC53276 was prepared by weighing 0.01011 grams (weight corrected for purity) of the reference substance on an analytical balance. The reference substance was transferred to a 100-mL class A volumetric flask, and brought to volume using methanol to achieve a 100 µg/mL stock solution.

A primary stock solution of OC53279 was prepared by weighing 0.01004 grams (weight corrected for purity) of the reference substance on an analytical balance. The reference substance was transferred to a 100-mL class A volumetric flask, and brought to volume using methanol to achieve a 100 µg/mL stock solution.

The 100 µg/mL stock solutions were used to prepare combined reference substance fortification/calibration stock solutions for this study as described in the next section below.

### Combined Reference Substance Fortification/Calibration Stock Preparation

Aliquots of the individual 100 µg/mL stock solutions of Flutianil and metabolites (OC56635, OC56574, OC53276, and OC53279) were combined by diluting in the methanol solvent to prepare 10.0, 1.00 and 0.100 µg/mL combined working fortification/calibration stock solutions. The following shows the dilution scheme for the combined working stocks containing Flutianil and its metabolites:

Stock Concentration (µg/mL)	Aliquot (mL)	Final Volume (mL)	Combined Stock Concentration (µg/mL)
100 (Flutianil)	5.00	50.0	10.0
100 (OC56635)	5.00	--	--
100 (OC56574)	5.00	--	--
100 (OC53276)	5.00	--	--
100 (OC53279)	5.00	--	--
10.0 (combined)	5.00	50.0	1.00
1.00 (combined)	5.00	50.0	0.100

The 1.00 and 10.0 µg/mL combined stock solutions were used to prepare the method validation samples for this study.

### Combined Reference Substance Calibration Standards Preparation

Calibration standards of Flutianil and its metabolites (OC56635, OC56574, OC53276, and OC53279), ranging in concentration from 0.000200 to 0.0100 µg/mL were prepared in a solution of acetonitrile: HPLC water: formic acid (20:80:0.10, v/v/v) using the 0.100 and 1.00 µg/mL stock solutions. The following shows the dilution scheme for a set of calibration standards:

Combined Stock Concentration (µg/mL)	Aliquot (mL)	Final Volume (mL)	Combined Calibration Standard Concentration (µg/mL)
0.100	0.200	100	0.000200
0.100	0.500	100	0.000500
0.100	1.00	100	0.00100
1.00	0.200	100	0.00200
1.00	0.500	100	0.00500
1.00	1.00	100	0.0100

### **Analytical Method**

Both surface and ground water matrices (100 mL) were fortified at two different concentrations (0.00100 and 0.0100 mg /L) and analyzed based on methodology developed by Wildlife International. Two matrix blanks for each water type were also prepared for analysis to evaluate potential analytical method interferences.

The method validation samples were processed using a dilute/direct injection approach. An aliquot (8.00 mL) of each aqueous sample was initially combined/diluted volumetrically in a 15-mL graduated tube with an aliquot of acetonitrile: 0.5% formic acid solution (2.00 mL) to achieve a final solvent composition of acetonitrile: HPLC grade water: formic acid (20:80:0.1, v/v/v). The final diluted samples were transferred to auto-sampler vials and submitted for analysis.

Concentrations of flutianil and its metabolites (OC56635, OC56574, OC53276, and OC53279) in water samples were determined using an Agilent Technologies 1200 Infinity Series High Performance Liquid Chromatograph (HPLC) coupled with an AB Sciex Triple Quad 5500 Tandem Mass Spectrometer (MS/MS) using a Turbo-V Ion source operated in both negative and positive ion, multiple reaction monitoring (MRM) mode. Chromatographic separations were achieved using a Phenomenex LUNA 5 C-18(2) column (150 mm x 2.0 mm, 5 µm particle size), preceded by a Phenomenex SECURITY C-18 guard column (4 mm x 3mm) utilizing a gradient elution profile. The High Performance Liquid Chromatography/ Mass Spectrometer (HPLC/MS/MS) operating parameters are summarized in Table 1. A detailed analytical method outline is provided in Figure 1.

Calibration curves were generated from analyses of combined calibration standard solutions of Flutianil and its metabolites (OC56635, OC56574, OC53276, and OC53279) analyzed concurrently with each series of method validation samples.

### **Method Limit of Quantitation (LOQ)**

The limit of quantitation (LOQ) for the surface and ground water method validation was set at 0.00100 mg/L, the lowest level fortified and analyzed during the validation sets. Matrix blank samples were further screened to confirm any potential interference to be < 30% of the fortified LOQ level. The theoretical LOQ was 0.000250 mg/L, calculated as the product of the lowest calibration standard (0.000200 µg/mL) and the dilution factor of the matrix blank samples (1.25).

**Table 1**

Typical High Performance Liquid Chromatography/ Mass Spectrometer (LC/MS/MS)  
 Operational Parameters for the Analysis of Flutianil and its Metabolites  
 (OC56635, OC56574, OC53276, and OC53279)

Instrument:	Hewlett-Packard Series 1200 High Performance Liquid Chromatograph (HPLC) coupled with an AB SCIEX TRIPLE QUAD™ 5500 Tandem Mass Spectrometer (MS/MS) operated in both negative and positive ion, multiple reaction monitoring (MRM) modes.		
Ion Source:	Turbo V Ion Spray		
Analytical Column: Guard Column:	Phenomenex LUNA 5 C-18(2) - (150 x 2.0 mm, 5- $\mu$ m particle size) Phenomenex SECURITY C-18 (4 x 3 mm)		
Column Oven Temperature:	40 °C		
Mobile Phase:	A - 0.2% formic acid in water B - 0.2% formic acid in acetonitrile		
Flow Rate:	500 $\mu$ L/minute		
Elution Profile:	<u>Time</u>	<u>A (%)</u>	<u>B (%)</u>
	0.00	80.0	20.0
	2.00	80.0	20.0
	9.00	5.0	95.0
	10.0	5.0	95.0
	10.5	80.0	20.0
	15.0	80.0	20.0
Injection Volume:	25.0 $\mu$ l		
Parameter Table: Period 1 - 0.0 to 8.0 minutes (Negative-Ion Mode)	CUR: 25.00	IS: -4500.00	
	GS1: 35.00	DP: -100.00	
	GS2: 45.00	EP: 10.00	
	CAD: 6.00	TEM: 500.00	
	<u>Quantitation Ion Transition</u>		
	OC56635 CE: -34.0, CXP: -11		
	<u>Confirmation Ion Transition</u>		
	OC56635 CE: -60.0, CXP: -11		

**Table 1**  
**(Continued)**

Parameter Table: Period 2 – 8.00 to 15 minutes (Positive-Ion Mode)	<p>CUR: 25.00      IS: 4500.00  GS1: 35.00      DP: 100.00  GS2: 45.00      EP: 10.00  CAD: 4.00        TEM: 500.00</p> <p><u>Quantitation Ion Transition</u>  Flutianil CE: 37, CXP: 12  OC53279 CE: 35, CXP: 16  OC56574 CE: 31, CXP: 14  OC53276 CE: 33, CXP: 12</p> <p><u>Confirmation Ion Transition</u>  Flutianil CE: 65, CXP: 12  OC53279 CE: 17, CXP: 28  OC56574 CE: 31, CXP: 14  OC53276 CE: 75, CXP: 14</p>
Monitored Transitions:	<p><u>Quantitation Ion Transition</u>  Flutianil: 427 → 192 amu (dwell time 150 msec)  OC 53279: 443 → 190 amu (dwell time 150 msec)  OC 56574: 443 → 136 amu (dwell time 150 msec)  OC 53276: 443 → 192 amu (dwell time 150 msec)  OC 56635: 243 → 179 amu (dwell time 500 msec)</p> <p><u>Confirmation Ion Transition</u>  Flutianil: 427 → 132 amu (dwell time 150 msec)  OC 53279: 443 → 425 amu (dwell time 150 msec)  OC 56574: 443 → 136 amu (dwell time 150 msec)  OC 53276: 443 → 132 amu (dwell time 150 msec)  OC 56635: 243 → 80 amu (dwell time 500 msec)</p>
Valco Diverter Valve	<p>Time - Position  0.00 - A (Waste)  5.00 - B (Detector)  11.0 - A (Waste)</p>
Approximate Retention Times:	<p>Flutianil – 10.0 minutes  OC 53279 – 9.2 minutes  OC 56574 – 8.8 minutes  OC 53276 – 8.7 minutes  OC 56635 – 6.0 minutes</p>

**METHOD OUTLINE FOR THE DIRECT INJECTION ANALYSIS OF  
FLUTIANIL AND METABOLITES (OC 56635, OC 53276, OC 56574, OC 53279  
IN AQUEOUS SAMPLES**

Prepare calibration standards in acetonitrile (ACN): HPLC Grade Water: formic acid (20:80:0.1, v/v/v) using volumetric flasks and gas-tight syringes from combined flutianil and metabolites stock solutions,

**STORE REFRIGERATED**



Prepare matrix fortification samples in appropriate aqueous matrix using flutianil and metabolites stock, volumetric flasks and gas-tight syringes. Mix well by inversion. Prepare matrix blank samples the same way, with the exception of fortification step.



Volumetrically combine/dilute 8.00 mL of all aqueous samples initially with 2.00 mL volumes of acetonitrile/0.5% formic acid in 15-mL plastic graduated centrifuge tubes or equivalent (i.e.; 8.00 mL H<sub>2</sub>O + 2 mL of ACN/0.5% formic acid = 10.0 mL final volume = 1.25X dilution factor; to achieve a final solvent composition of acetonitrile: HPLC grade water: formic acid (20:80:0.1, v/v/v).



Volumetrically dilute above samples further, if necessary, using a solution of acetonitrile: HPLC grade water; formic acid (20:80:0.1, v/v/v). Mix well by inversion.



Transfer aliquots of final sample dilutions and calibration standards to auto-sampler vials and submit for analysis by HPLC/MS/MS.

**Figure 1.** Analytical method outline for the analysis of Flutianil and its Metabolites (OC56635, OC56574, OC53276, and OC53279) in Surface and Ground Water.