

## 1.0 ABSTRACT

The purpose of the study was to demonstrate the draft analytical method entitled, “Determination of Flutianil and its Metabolites in Soil Using LC-MS/MS” could be performed successfully at an outside facility with no prior experience with the method. (Reference 1)

**Principle of the method.** Residues of Flutianil and its metabolites, OC-53276, OC-56574 and OC-56635 were extracted from soil samples with two extractions of acetonitrile; one shaking by hand and a second using a mechanical shaker. The combined sample extract was adjusted to 80 mL with acetonitrile and a 5-mL aliquot was filtered using a 0.2- $\mu$ m PURADISK 25 TF syringe filter into a glass scintillation vial. A 2.5-mL aliquot of the filtered portion was concentrated with a gentle stream of nitrogen and reconstituted in acetonitrile. The final sample volume was adjusted with 0.1% formic acid in water and transferred to an HPLC vial for analysis by LC-MS/MS.

**Limit of Quantification (LOQ) and Limit of Detection (LOD).** The limit of quantitation (LOQ) for Flutianil and its three metabolites, OC-53276, OC-56574 and OC-56635 as stated in the method was set at 0.01 ppm in soil. The limit of detection (LOD) for Flutianil and its three metabolites, OC-53276, OC-56574 and OC-56635 as stated in the method was set at 0.002 ppm.

**Selectivity.** The method was highly selective for analysis of the test items (mass transitions from the positively charged molecule ion to two typical fragment ions in MS/MS mode for Flutianil, OC-53276 and OC-56574 and negatively charged molecule ion to two typical fragment ions in MS/MS mode for OC-56635 as listed below). The two MRM transitions used to Flutianil and its metabolites were defined in the draft method provided. The retention time of the test item in matrix matched the retention times in fortified samples. No peak interferences occurred at the retention times of the test item.

	<u>Quantitation</u>	<u>Confirmatory</u>
Flutianil	427 $\rightarrow$ 192	427 $\rightarrow$ 132
OC-53276	443 $\rightarrow$ 192	443 $\rightarrow$ 132
OC-56574	443 $\rightarrow$ 136	443 $\rightarrow$ 181
OC-56635	243 $\rightarrow$ 179	243 $\rightarrow$ 80

**Linearity.** For analysis of Flutianil, OC 53726, OC-56574 and OC-56635 by LC-MS/MS, the detector response was linear ( $r > 0.99$ ) within the range from 0.05 ng/mL to 10.0 ng/mL for both transitions of each analyte.

**Controls.** No detectable residues were found in the control soil samples used for the study (i.e. residues of Flutianil, OC 53726, OC-56574 and OC-56635 were below the LOD).

## 2.0 INTRODUCTION

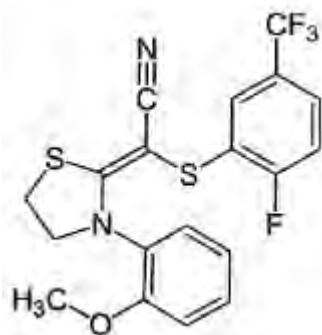
The purpose of the study was to demonstrate that the draft analytical method, “Determination of Flutianil and its Metabolites in Soil Using LC-MS/MS” could be performed successfully at an outside facility with no prior experience with the method.

## 3.0 REFERENCE SUBSTANCE AND SAMPLING HISTORY

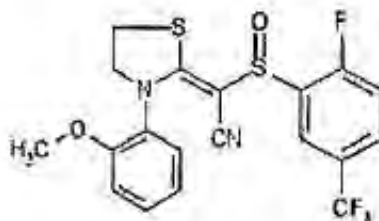
### 3.1 Reference Materials

Reference substances Flutianil, OC-53276, OC-56574 and OC-56635 were combined in solutions for fortifications and LC-MS/MS calibration. Concentrated (stock), fortification, and calibration standards were prepared according to the analytical draft method. The certificates of analysis for the reference substance is presented in Appendix A. Standard solutions prepared for this study were stored at in refrigerator E-109 which maintained an average temperature of 3 °C. A brief description of the reference standards used in this study is presented below.

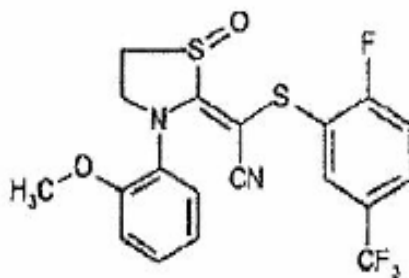
<b>Name:</b>	OK-5203
<b>Common Name:</b>	Flutianil
<b>Batch No.:</b>	05DF2
<b>CAS Number:</b>	958647-10-4
<b>IUPAC Name:</b>	(Z)-2-[2-fluoro-5-(trifluoromethyl)phenylthio]-2-[3-(2-methoxyphenyl)-1,3-thiazolidin-2-ylidene]acetonitrile
<b>Molecular Formula:</b>	C <sub>19</sub> H <sub>14</sub> F <sub>4</sub> N <sub>2</sub> OS <sub>2</sub>
<b>Molecular Weight:</b>	426.5 g/mol
<b>Purity:</b>	99.54%
<b>Expiration Date:</b>	November 26, 2016
<b>Storage:</b>	Refrigerated in darkness
<b>Chemical Structure:</b>	



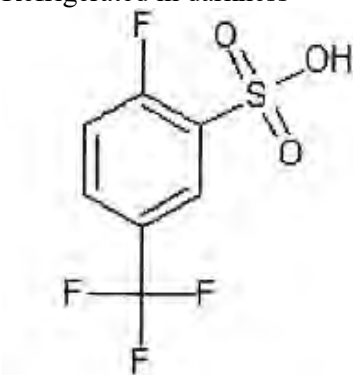
**Common Name:** OC-53276  
**Batch No.:** TT1005019  
**IUPAC Name:** (Z)-2-[2-fluoro-5-(trifluoromethyl)phenylsulfinyl]-2-[3-(2-methoxyphenyl)thiazolidinylidene]acetonitrile  
**Molecular Formula:** C<sub>19</sub>H<sub>14</sub>F<sub>4</sub>N<sub>2</sub>O<sub>2</sub>S<sub>2</sub>  
**Molecular Weight:** 442.45 g/mol  
**Purity:** 96.54%  
**Expiration Date:** July 06, 2018  
**Storage:** Refrigerated in darkness  
**Chemical Structure:**



**Common Name:** OC-56574  
**Batch No.:** TT0908011  
**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 g/mol  
**Purity:** 98.72%  
**Expiration Date:** July 06, 2018  
**Storage:** Refrigerated in darkness  
**Chemical Structure:**



<b>Common Name:</b>	OC-56635
<b>Batch No.:</b>	81010
<b>IUPAC Name:</b>	(2-fluoro-5-trifluoromethyl)benzenesulfonic acid
<b>Molecular Formula:</b>	C <sub>7</sub> H <sub>4</sub> F <sub>4</sub> O <sub>3</sub> S
<b>Molecular Weight:</b>	244.16 g/mol
<b>Purity:</b>	97.3%
<b>Expiration Date:</b>	04/22/18
<b>Storage:</b>	Refrigerated in darkness
<b>Chemical Structure:</b>	



### 3.2 Test System

An untreated composite soil sample was shipped via FedEx to ADPEN Laboratories, Inc., Jacksonville, FL on January 26, 2015 and received on January 27, 2015. The sample received at ADPEN, arrived frozen with dry ice, intact and in good condition. Upon receipt, the sample was stored in freezer E-16 which operated at an average temperature of -18 °C during the course of this study.

The Laboratory Information Management System (LIMS) provided a unique laboratory analysis code (i.e. 150721001-001) for the control sample, which is cross-referenced on detailed analytical data reports to the sample identification number.

## 4.0 ANALYTICAL METHOD

Analytical Draft Method, "Determination of Flutianil and its Metabolites in Soil Using LC-MS/MS" was used for the analysis of the composite soil sample.

### 4.1 Preparation of Solutions

All solutions used for the conduct of the ILV were prepared as described in the analytical method. Below are the solutions and solvent mixtures used for this study and their composition:

Solutions	Composition and preparation method
Extraction solvent	<b>Acetonitrile: water (75: 25, v/v)</b> mix 250 mL of water and 750 mL of ACN in a 1 L volumetric flask
Final volume for LC-MS/MS analysis	<b>Acetonitrile: water (10: 90, v/v) + 0.1% formic acid</b> mix 900 mL of water and 100 mL of ACN in a 1-Lglass storage bottle and add 1 mL of formic acid.
HPLC mobile phase A	<b>0.2% formic acid in HPLC water</b> Add 500 mL of water and 2 mL of concentrated formic acid into a 1L volumetric flask, bring up to volume, mix well and sonicate.
HPLC mobile phase B	<b>0.2% formic acid in acetonitrile</b> Add 500 mL of ACN and 2 mL of concentrated formic acid into a 1L volumetric flask, bring up to volume, mix well and sonicate.

## 4.2 Preparation of Standard Solutions

All stock, fortification and calibration standard solutions were stored at refrigerated temperatures in amber bottles. An example of the standard preparation solutions is presented in Table 10.

### 4.2.1 Stock Standard Solutions

The individual stock standard solutions were prepared as described in the method in a 10-mL volumetric flask. Each of the standards was dissolved, using sonication and/or vortexing and then diluted to the mark with methanol and having a concentration of approximately 1.0 mg/mL.

### 4.2.2 Fortification Standard Solutions

Four mixed-analyte fortification solutions were prepared at 10 and 1.0 µg/mL as described in the method. The two volumetric flasks containing the fortification standards were diluted volumetrically with methanol as described in the method and complete homogeneous solutions were accomplished by sonication and/or vortexing.

### 4.2.3 Calibration Standard Solutions

Calibration standard solutions for LC-MS/MS analysis were prepared using intermediate solutions prepared separately from the fortification standards as described in the method. Dilutions were prepared directly in HPLC vials for LC-MS/MS injection using acetonitrile: water (10:90, v/v) + 0.1% formic acid. A description of the standard preparation for calibration is shown below.

Parent Standard (ng/mL)	Aliquot Volume of Parent Standard (mL)	Final volume (mL)	Concentration (ng/mL)
100	0.100	1.0	10.0
100	0.050		5.0
100	0.025		2.5
10	0.100		1.0
10	0.050		0.5
10	0.025		0.25
1	0.100		0.10
1	0.050		0.05

### 4.3 Extraction and Analysis

**Extraction:** A 10 g of soil sample was weighed into a 50 mL disposable centrifuge tube. Fortifications were performed to all necessary samples where recovery samples were fortified at LOQ and 10× LOQ, as described on the lab bench sheet. 25 mL of HPLC grade acetonitrile was added to the samples and then they were shaken by hand to suspend the soil in solution. The samples were sonicated for one minute, centrifuged at 3500 rpm for 5 minutes and the acetonitrile extract was decanted into a 100-mL graduated cylinder. The sample extraction was repeated using acetonitrile: water (75:25, v/v), adding the sample extract to the same graduated cylinder. An additional 25-mL of acetonitrile: water (75:25, v/v) was added to the soil, the sample was placed on a mechanical shaker for 15 minutes at approximately 250 rpm for adequate mixing. The sample was centrifuged, decanted and the extract solvent combined in the same graduated cylinder. The volume of the sample was adjusted to 80 mL with acetonitrile. To ensure a homogeneous sample extract, the sample was transferred to a 150-mL glass beaker and gently swirled. A 5-mL portion of the extract was filtered using a 0.2 µm PURASDISK 25 TF syringe filter into a glass scintillation vial. A 2.5-mL aliquot of the filtered portion was transferred to a graduated disposable centrifuge tube. The sample was concentrated with a gentle stream of nitrogen to about 0.5 mL, with only the aqueous solvent remaining. Upon removal of the sample from the nitrogen evaporator, 1.0 mL of acetonitrile was added to the sample. The final sample volume was adjusted to 10 mL using 0.1% formic acid in water.

**Final Sample Preparation:** A portion of the final sample was transferred to an HPLC vial for analysis by LC-MS/MS.

A method flow diagram of the method used is presented in Table 1.

### 4.4 LC-MS/MS Instrumentation and Conditions

The LC-MS/MS instrumentation and conditions including the both the quantitation and confirmatory transitions (m/z) monitored during this validation study are presented in Table 11.

## 5.0 LIMITS OF QUANTITATION AND DETECTION

For Flutianil and its three metabolites OC-53276, OC-56574 and OC-56635, the limit of quantitation (LOQ) was set to 0.01 ppm. For Flutianil and its three metabolites OC-53276, OC-56574 OC-56635, the limit of detection (LOD) was set to 0.002 ppm (20% of LOQ).

## 6.0 CALIBRATION, CALCULATIONS, AND STATISTICS

Quantitation of residues in all samples was achieved using an external calibration curve calculated by linear regression of instrument responses for the reference substance at multiple concentrations.

A calibration standard curve was prepared using all four analytes in a mixed standard solution by injecting standard solutions prepared as described in Section 3.2.3 of this report; calibration standard concentrations ranged from 0.05 ng/mL to 10 ng/mL. The calibration standards were interspersed with sample injections. Analyst® (version 1.6.2) software was used to create the standard curve based on linear regression using 1/x weighting as well as peak integration and quantitation as obtained from the instrument used. The regression functions were used to calculate the best-fit line by plotting the analyte found (ng) on the x-axis versus the detector's peak response (peak area) on the y-axis. The calibration curve equation was used to determine the amount of analyte found (ng) during sample analysis. Typical

quantitation and confirmatory calibration curves for Flutianil, OC-53276, OC-56574 and OC-56635 are presented in Figures 1 and 2, Figures 9 and 10, Figures 17 and 18, and Figures 25 and 26, respectively. Representative chromatograms of calibration standards for both mass transitions (m/z) are presented in Figures 3 and 4, Figures 11 and 12, Figures 19 and 20, and Figures 27 and 28. Recovery results and additional sample concentrations were calculated for each set of samples within the LIMS and reported in Microsoft® Excel spreadsheet as detailed analytical data reports, which are presented in Appendix B.

### 6.1 Residue Calculations for Flutianil

The following equations are for residue and recovery calculations for soybean samples:

a) Calibration curve:  $y = mx + b$       Solving for x:  $x = \frac{y-b}{m}$

Where,      m =      slope  
              b =      y-intercept  
              x =      Amount found (ng)  
              y =      Peak area

b) Amount of sample injected (mg) =  $\frac{(\text{injection size (mL)} \times \text{sample wt. (g)})}{\text{final sample volume (mL)}} \times \frac{1000\text{mg}}{\text{g}}$

c) ppm found =  $\frac{\text{Amount found (ng)}}{\text{Amount of sample injected (mg)}}$

d) Recovery (%) =  $\frac{\text{ppm found}}{\text{ppm added}} \times 100$

As an example, to calculate recovery results for Flutianil (quantitation transition, 427 → 192 *m/z*) using sample 15072804-Recovery1-1 from work order WO-15072804, where the peak area counts were 698730, are shown below:

a) Calibration curve:  $y = (4.86e+007)x - 215$

Solving for x:  $x = \frac{698730 + 215}{4.86e+007} = 0.0144 \text{ ng}$

b) Amount of sample injected (mg) =  $\frac{0.06 \text{ mL} \times 10 \text{ g}}{320 \text{ mL}} \times \frac{1000 \text{ mg}}{1 \text{ g}} = 1.87500 \text{ mg}$

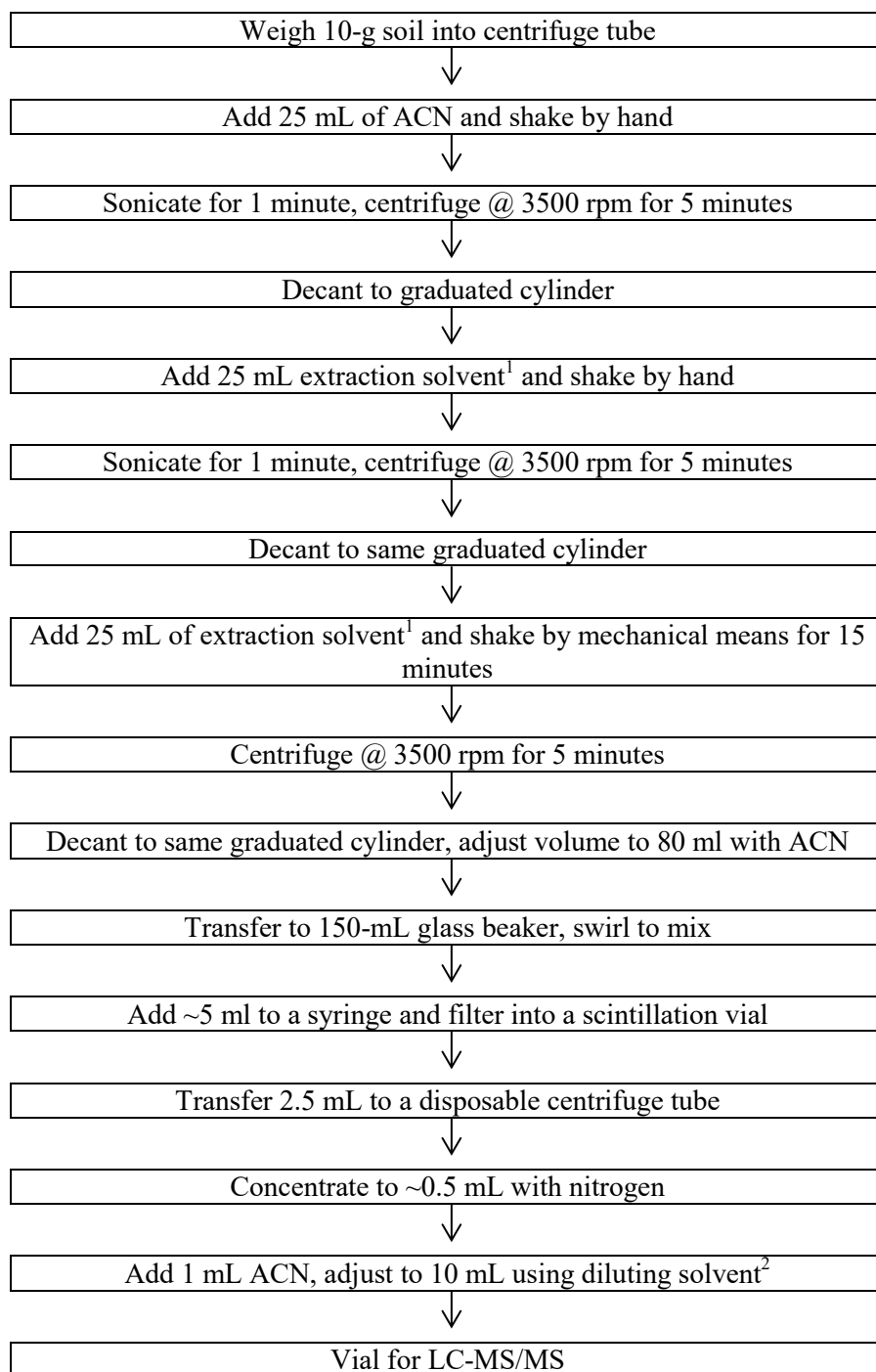
c) ppm found =  $\frac{0.0144 \text{ ng}}{1.87500 \text{ mg}} = 0.00768 \text{ ppm}$

d) Recovery (%) =  $\frac{0.00768 \text{ ppm}}{0.010 \text{ ppm}} \times 100 = 77\%$

## 6.2 Statistical Analysis

Statistical treatment of the data included calculation of means, standard deviations (SD), and percent relative standard deviations (RSD). These calculations were performed using Microsoft® Excel and LIMS software. Results were rounded only for reporting purposes. No calculations were made with rounded numbers.



**Table 1 Method Flow Diagram**

<sup>1</sup> Extraction solvent = acetonitrile: water (75:20, v/v)

<sup>2</sup> Diluting solvent = 0.1% formic acid (aq.)

**Table 11 Instrument Conditions and Parameters**

<b>HPLC Conditions</b>			
Chromatographic System:	Agilent 1290 Liquid Chromatograph		
Column:	Luna 5 C18; 5.0 $\mu\text{m}$ , 2.0 x 150 mm		
Temperature:	40 °C		
Flow rate (mL/min)	0.500		
Gradient:	Time (min)	Mobile Phase A (%)	Mobile Phase B (%)
	0.0	80	20
	2.0	80	20
	9.0	5	95
	10.0	5	95
	10.5	80	20
	15.0	80	20
Mobile Phase A:	Water with 0.2% formic acid		
Mobile Phase B:	Acetonitrile with 0.2% formic acid		
Injection Volume:	60 $\mu\text{L}$		

<b>MS/MS Conditions</b>						
Detection System:	AB SCIEX Qtrap 5500					
Ionization:	TurboSpray Ionization					
Polarity:	Positive					
Curtain gas (CUR):	25.0					
Temperature (TEM):	500 °C					
Collision gas setting (CAD):	Medium					
GS1:	35.0					
GS2:	45.0					
Entrance potential (EP):	10.0					
Scan type:	MRM					
<b>MRM Conditions</b>	Transition* (m/z)	Dwell (msec)	DP	CE	CXP	Retention Time (min)
Flutianil	427 $\rightarrow$ 192	250	106.00	35.00	10.00	9.00
	427 $\rightarrow$ 132			67	6.00	
OC-53276	443 $\rightarrow$ 192	250	106.00	27.00	10.00	7.69
	443 $\rightarrow$ 132			63.00	6.00	
OC-56574	443 $\rightarrow$ 136	250	61.00	27.00	8.00	7.73
	443 $\rightarrow$ 181				10.00	

\*The quantification mass ion transition as specified in the analytical method.

**Table 11 Instrument Conditions and Parameters (continued)**

<b>HPLC Conditions</b>			
Chromatographic System:	Agilent 1290 UPLC		
Column:	Luna 5 C18; 5.0 $\mu$ m, 2.0 x 150 mm		
Temperature:	40 °C		
Flow rate (mL/min)	0.500		
Gradient:	Time (min)	Mobile Phase A (%)	Mobile Phase B (%)
	0.0	80	20
	2.0	80	80
	9.0	5	5
	10.0	5	5
	10.5	80	20
	15.0	80	20
Mobile Phase A:	Water with 0.2% formic acid		
Mobile Phase B:	Acetonitrile with 0.2% formic acid		
Injection Volume:	60 $\mu$ L		

<b>MS/MS Conditions</b>					
Detection System:	AB SCIEX 5500 QT				
Ionization:	TurboSpray Ionization				
Polarity:	Negative				
Curtain gas (CUR):	25.0				
Temperature (TEM):	500 °C				
Collision gas setting (CAD):	Medium				
GS1:	35.0				
GS2:	45.0				
Entrance potential (EP):	-10.0				
Scan type:	MRM				
<b>MRM Conditions</b>	Transition* (m/z)	Dwell (msec)	CE	CXP	Retention Time (min)
OC-56635	243 $\rightarrow$ 179	500	-34.00	-15.00	7.59
	243 $\rightarrow$ 80		-60.00	-7.00	

\*The quantitation mass ion transition specified in the analytical method.