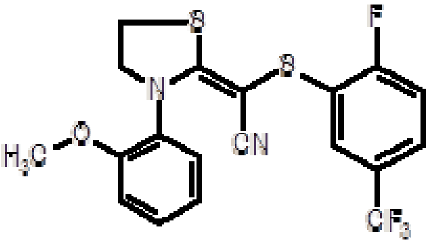
III. REFERENCE MATERIALS

A. Reference Substances

A reference substance of OK-5203 (common name flutianil) was received from LS Global Corporation on August 31, 2007. Upon receipt, the material was assigned Wildlife International substance number 8225 and stored refrigerated. An original Certificate of Analysis (COA) was received (Appendix I, May 15, 2011 analysis date, 98.7% purity), as well as an updated COA reporting a more recent analysis of the material extending the expiration date to November 26, 2016 (November 27, 2012 analysis date, 99.54% purity). Information received with the material 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: $C_{19}H_{14}F_4N_2OS_2$
Molecular Weight: 426.5

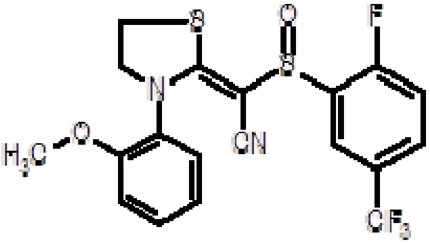
Structural Formula:



CAS Number: 958647-10-4
Lot Number: 05DF2
Purity: 98.7% (analyzed on May 15, 2011)
Expiration Date: May 14, 2013
Storage Conditions: Refrigerated in darkness

Reference standards of the following metabolites and/or degradates were received from Landis International, Inc. on behalf of OAT Agrio Co., Ltd. on July 19, 2012. Upon receipt, OC 53276, OC 56574, and OC 56635 were assigned Wildlife International test substance numbers 10627, 10628, and 10629, respectively, and placed under freezer conditions in darkness. Some chemical names, formulas, and structures were obtained from Sponsor contributed reports.[1] Purities, lot numbers and storage recommendations were obtained from the container labels and COAs. Expiration dates were obtained from COAs (Appendix I).

<u>OC 53276</u>
IUPAC Name: (Z)-2-[2-fluoro-5-(trifluoromethyl)phenylsulfinyl]-2-[3-(2-methoxyphenyl)thiazolidinylidene]acetonitrile
Molecular Formula: $C_{19}H_{14}F_4N_2O_2S_2$
Molecular Weight: 442.45
Structural Formula:



Lot Number: YaE0711005
Purity: 98.9% (analyzed on July 2, 2012)
Expiration Date: July 2, 2014

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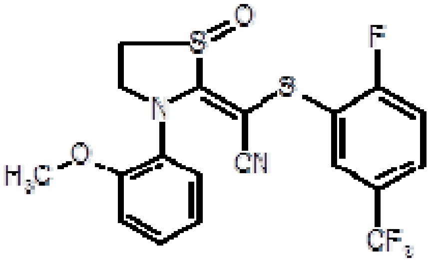
[1] <u>OK-5203 and its metabolites: Validation of an Analytical Method for the Determination of Residues in Soil</u>, Covance Laboratories Limited, Covance Report Number 2554/015-D2149, December 2009.

Storage Conditions: Freezer conditions in darkness

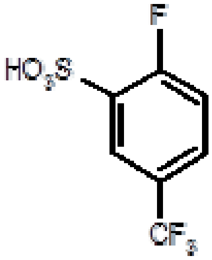
<u>OC 56574</u>
IUPAC Name: (Z)-2-[2-fluoro-5-(trifluoromethyl)phenylthio]-2-[3-(2-methoxypheny)-1-oxo-2-thiazolidinylidene]acetonitrile
Molecular Formula: $C_{19}H_{14}F_4N_2O_2S_2$

Molecular Weight: 442.45
Structural Formula:



Lot Number: TT0902015
Purity: 98.9% (analyzed on July 2, 2012)
Expiration Date: July 2, 2014
Storage Conditions: Freezer conditions in darkness

<u>OC 56635</u>
IUPAC Name: (2-fluoro-5-trifluoromethyl)benzenesulfonic acid
Molecular Formula: $C_7H_4F_4O_3S$
Molecular Weight: 244.16
Structural Formula:



Lot Number: 81010
Purity: 99.9% (analyzed on July 2, 2012)
Expiration Date: July 2, 2014
Storage Conditions: Freezer conditions in darkness

These reference substances were used for preparation of standard solutions for instrument calibration, and as fortification solutions for samples for method validation, for freezer storage stability, and for concurrent quality control samples (procedural recoveries). When not in use, the reference substance solutions were stored under refrigerated conditions at approximately 4 °C in darkness.

B. Reagents and Solvents

Solvents used for the preparation of stock solutions, both primary and secondary, and instrument calibration standard solutions were prepared using HPLC-grade solvents, specifically Burdick & Jackson®, B & J Brand®, High Purity Solvent. Formic acid used for the 0.1% formic acid aqueous dilution solvent was obtained from Aldrich. The same formic acid was used for preparation of the acetonitrile:water:formic acid (10:90:0.1, v:v:v) dilution solvent.

C. Preparation of Stock Solutions

Standard solutions containing flutianil, OC 53276, OC 56574, and OC 56635 were prepared for fortification of control matrices, for determination of method recoveries and as calibration standards for the LC/MS/MS instrument. All standard solutions were stored refrigerated when not in use.

Primary stock solutions were quantitatively prepared using volumetric flasks with the aid of weigh paper and sonication. An individual 100-mL primary stock solution of flutianil and 50.0-mL primary stock solutions of OC 53276, OC 56574, and OC 56635 were prepared in methanol at concentrations of 1.00 mg/mL. The percent purity of each reference standard was taken into account such that the final concentrations were 1.00-mg/mL active ingredient (a.i.). Therefore, nomenclature of a.i./unit volume or mass (e.g., µg a.i./mL, mg a.i./Kg, etc.) is equivalent to the specific analyte per unit volume or mass (e.g., µg/mL, mg/Kg, etc.).

A combined secondary stock of flutianil, OC 53276, OC 56574, and OC 56635 was prepared in methanol from the individual primary stocks at a concentration of each component of 100 µg/mL. The combined stock was prepared by mixing equal 10.0-mL volumes of each primary stock in a 100-mL volumetric flask and bringing to volume with methanol. Additional 100-mL combined stocks at concentrations for each component of 10.0, 1.0, and 0.1 µg/mL in methanol were prepared by serial dilution. The secondary stock solutions were used for fortification of control soil samples to prepare procedural recoveries. Additionally, they were used to prepare calibration solutions for the LC/MS/MS instrument. All stock and calibration standard solutions were stored refrigerated at approximately 4 °C when not in use.

D. Preparation of Instrument Calibration Standard Solutions

Standard solutions containing known nominal concentrations of flutianil, OC 53276, OC 56574, and OC 56635 were prepared as calibration standards for the LC/MS/MS instrument. Solutions in methanol ranging in concentration of flutianil, OC 53276, OC 56574, and OC 56635 from 0.100 to 5.00 ng/mL of each compound were prepared from the 0.100- and 1.00-µg/mL combined stocks. Calibration solutions containing equal concentrations of flutianil and analytes were prepared at concentrations of 0.10, 0.25, 0.50, 1.00, 2.50, and 5.00 ng/mL. All standard solutions were stored refrigerated at approximately 4 °C when not in use.

IV. ANALYTICAL METHOD FOR SOIL ANALYSES

A. Analytical Method for the Determination of Flutianil, OC 53276, OC 56574, and OC 56635 in Soil

The analytical method applied to analysis of soil for flutianil, OC 53276, OC 56574, and OC 56635 is described below and presented schematically in Figure 1. The method was validated prior to use for recovery of flutianil, OC 53276, OC 56574, and OC 56635.

For each sample, 10.0 g of soil was weighed at the time of preparation into a 50-mL plastic centrifuge tube. Procedural recoveries were prepared at this step by fortification of 10.0 g of control soil (wet weight) using an appropriate combined stock solution of flutianil, OC 53276, OC 56574, and OC 56635. When applied to the analysis of field samples of unknown concentration, procedural recoveries were prepared at concentrations of 0.0100 mg/kg for each analyte and at a higher, typically 10x-LOQ concentration, with the intention of bracketing the magnitude of residue of flutianil and metabolites in field samples analyzed concurrently. In addition, a control sample (matrix blank) was prepared in the same manner consisting of 10.0 g of unfortified control soil.

To each field sample, control sample and procedural recovery, 25 mL of acetonitrile was added and the sample and solvent mixed well by hand shaking. This was followed by further agitation for approximately one minute on a BRANSONIC ultrasonic disruption sample processor at an approximately 45% amplitude setting. Samples were centrifuged at approximately 3500 rpm (revolutions per minute) for approximately five minutes. The supernatant was decanted into a 100-mL glass graduated cylinder. The soil pellet was extracted a second time by addition of 25 mL of acetonitrile:water (75:25, v:v) followed by extraction with the ultrasonic processor for an additional approximately one minute followed by centrifuging for five minutes at approximately 3500 rpm. The second supernatant was removed and combined with the first in the 100-mL graduated cylinder. An additional 25 mL of acetonitrile:water (75:25, v:v) was added to the soil pellet and the tube was capped and securely placed on a gyratory shaker table. The soil/solvent mixture was shaken at a setting of approximately 250 for approximately fifteen minutes. The mixture was centrifuged at approximately 3500 rpm for approximately five minutes. The supernatant was removed and added to the previous extracts in the 100-mL graduated cylinder.

The final volume of extract was adjusted to 80.0 mL with acetonitrile, transferred to a 150-mL beaker and swirled to mix. A 5.0-mL aliquot was filtered using a plastic, disposable BD syringe tipped with 0.2-µm PURADISK 25 TF filter into a 20-mL glass scintillation vial. A second portion, approximately 20 mL, of each unfiltered extract was transferred to an appropriate storage vessel. The remainder of the extract was discarded. An aliquot of 2.50 mL of the filtered aliquot was transferred to a 15-mL plastic centrifuge tube and evaporated to the aqueous remainder, approximately 0.5-mL aliquot of acetonitrile, using a nitrogen evaporator with a bath temperature of 40 to 50 ºC. A 1.00-mL aliquot of acetonitrile was added volumetrically to each aqueous remainder and the solution mixed well. The final solution was diluted to 10.0 mL with a solution of 0.1% formic acid in water to achieve a final extract of acetonitrile:water:formic acid (10:90:0.1, v:v:v). The solution was mixed well and an aliquot transferred to an autosampler vial and submitted for LC/MS/MS analysis. If required, samples were subsequently diluted with acetonitrile:water:formic acid (10:90:0.1, v:v:v).

Each analytical sequence consisted of injection of the calibration standard solution series, followed by sample injections interspersed with standard solution injections and concluding with the same complete series of calibration standard solutions. A calibration standard solution was injected following no more than five sample extract injections.

B. Quantitation of Flutianil, OC 53276, OC 56574, and OC 56635 by LC/MS/MS

An aliquot of each sample extract was transferred to an autosampler vial for subsequent separation of analytes and quantitation by LC/MS/MS. The liquid chromatograph was connected to the mass spectrometer through a Valco valve that diverted only the eluate from 5.5 to 11.0 minutes post-injection to the LC/MS/MS. Flutianil, OC 53276, and OC 56574 were quantified in the positive-ion multiple reaction monitoring (MRM) mode. OC 56635 was quantified in the negative ion MRM mode. Quantitation was performed monitoring the transitions noted below. For each analyte, a confirmatory transition was also monitored.

	Quantitation Transition	Confirmatory Transition
Flutianil	427 → 192	427 → 132
OC 53276	443 → 192	443 → 132
OC 56574	443 → 136	443 → 181
OC 56635	243 → 179	243 → 80

Instrument operating conditions for the LC/MS/MS are presented in Table 1.

V. EXPERIMENTAL DESIGN – METHOD VALIDATION

Control soil from the 0-3 inch horizon of the Iowa field site was selected as the control soil for method validation. This sample was a pre-qualification soil collected two days prior to the first application (Sample Number LA12-2217). A surface soil was selected under the assumption that it would provide a soil with greater organic matter than a lower soil horizon thereby providing a worst case for residue extractability and maximum co-extractives.

For validation, seven replicates of soil were fortified at 10.0 µg/Kg (0.0100 mg/Kg) and three replicates at 100 µg/Kg (0.100 mg/Kg) with flutianil, OC 53276, OC 56574, and OC 56635. Seven fortified soil replicates, consisting of 10.0 g each (wet weight), were prepared by fortification of control soil with 0.100 mL of a stock solution of 1.00-µg/mL concentration of flutianil and each metabolite (combined stock solution). Analogously, three 10.0-g fortified soil samples were prepared by fortification of control soil with 0.100 mL of a 10.0-µg/mL combined stock solution. Samples were prepared on August 30, 2012 and injected immediately following application of the analytical method on an API 3000 Sciex instrument. Samples were stored refrigerated before they were again injected on an API 5000 Sciex instrument on September 13, 2012, which was adopted as the platform to analyze all samples.

D. Quantitation of Flutianil, OC 53276, OC 56574, and OC 56635 Residue Concentrations in Soil

The analytical method used for determination of residue concentrations of flutianil, OC 53276, OC 56574, and OC 56635 was the same as the validated method presented schematically in Figure 1. LC/MS/MS operating conditions for the final quantitation of residues are presented in Table 1. As noted, when applied to analyses of field samples, procedural recoveries were prepared at concentrations of 0.0100 mg/L for each analyte and at a higher concentration, typically 10x the LOQ, with the intention of bracketing the magnitude of residue of flutianil and metabolites in field samples analyzed concurrently. In addition, a control sample (matrix blank) was prepared in the same manner consisting of 10.0 g of unfortified control soil. Procedural recovery samples containing flutianil and metabolites were prepared with each analytical set of field samples. Analytical sequences consisted of injection of the six calibration standard solutions, followed by an injection of dilution solvent, a matrix blank, procedural recovery samples, field samples, periodic calibration standard solutions and conclusion with injection of the same six calibration standard solutions. Periodic calibration standard solutions were injected at a minimum following injection of five samples.

VIII. CALCULATIONS

A. Standard Curves by Linear Regression

For flutianil, OC 53276, OC 56574, and OC 56635, regression analysis was applied to the chromatographic peak area responses for the calibration standard solutions versus the respective nominal concentrations of the analytes. Standard curves were generated by plotting the regression functions consisting of the analyte concentration (µg/mL) on the abscissa and the respective peak area responses on the ordinate.

Representative standard curves for flutianil, OC 53276, OC 56574, and OC 56635 are presented in Figures 2, 3, 4, and 5, respectively. Representative chromatograms for low- (0.100 mg/mL) and high-level calibration standard solutions (5.00 mg/mL) are presented in Figures 6, 7, 8, and 9, respectively. A linear, 1/x weighted, regression analysis was used for quantitation.

Regression equations, derived from linear regression of peak areas and nominal concentrations of calibration standard solutions, were expressed as follows:

$$\text{Peak Area} = (\text{Slope})(\text{Concentration}) + (\text{y-Intercept})$$

Concentrations of flutianil, OC 53276, OC 56574, and OC 56635 in final extract solutions of samples were calculated using a rearrangement of the above equation:

$$\text{Concentration} = \frac{\text{Peak Area} - (\text{y-Intercept})}{\text{Slope}}$$

Calculations of concentrations for injected calibration standards (ng/mL) and soil (mg/Kg) were performed using Analyst Version 1.5.1 Applied Biosystems/MDS Sciex software. Entry of dilution factors, conversion factors for soil (relating mass extracted and equivalent final volumes) and sample identifiers were entered into the software.

Excel 2000, in full-precision mode, was used for calculation of means, standard deviations, etc., using un-rounded values generated by Analyst Version 1.5.1. Values calculated using rounded numbers as presented in the text and tables might differ slightly. The following calculation section is illustrative of the procedure used by Analyst Version 1.5.1.

B. Determination of Flutianil, OC 53276, OC 56574, and OC 56635 Concentrations in Soil

Linear regression was applied to measured peak areas of analytes in solutions of known concentrations to derive equations relating peak areas and concentrations. The concentrations of flutianil, OC 53276, OC 56574, and OC 56635 in injected soil extracts were calculated using a rearrangement of the regression equation as follows:

$$\text{Extract Concentration in ng/mL} = \frac{\text{Peak Area} - (\text{y-Intercept})}{\text{Slope}}$$

Residue concentrations (mg/Kg) of flutianil, OC 53276, OC 56574, and OC 56635 were determined as the product of the solution concentration determined above, the equivalent final extract volume for the soil mass extracted and the initial extracted mass of the sample as follows:

$$\text{Sample Concentration in ng/g} = \text{Extract Concentration} \times \frac{(\text{Equivalent Final Volume})}{(\text{Initial Sample Weight})}$$

where the Initial Sample Weight was 10.0 g and the Equivalent Final Volume was 320 mL. The initial 10.0-g sample was extracted, rotary evaporated and brought to 80-mL total volume. A 2.50-mL aliquot of this extract was further processed ending with a final volume of 10.0 mL. Thus, the Equivalent Final Volume was calculated as follows:

$$\text{Equivalent Final Volume (mL)} = 80\text{mL} \times \frac{10.0\text{ mL}}{2.5\text{ mL}} = 320$$

and

$$\text{Sample Concentration in ng/g} = \text{Extract Concentration} \times \frac{320\text{ mL}}{10\text{ g}}$$

or

$$\text{Sample Concentration in ng/g} = \text{Extract Concentration} \times 32.0\,\frac{\text{mL}}{\text{g}}$$

It is noted that this sample concentration is a soil wet-weight residue concentration. To normalize soil residue values to dry weight, the wet-weight residue concentrations were corrected as follows:

$$\text{Dry-Weight Residue (ng/g)} = \frac{\text{Wet-Weight Residue Concentration}}{(1.00 - \text{Fractional Soil Moisture})}$$

C. Representative Calculation of a Soil Residue Concentration

A representative calculation is presented consisting of quantitation of flutianil in soil collected immediately following the first application to the Iowa field site (Sample Number LA12-2308). Concentrations of OC 53276, OC 56574, and OC 56635 residues in soil were quantitated analogously. Using the results from the linear regression analysis as follows:

Slope = 7.01928×10^5
y-Intercept = 16078.1
r = 0.999924 equivalent to r^2, the coefficient of determination, = 0.9998

$$\text{Concentration} = \frac{\text{Peak Area} - 16078.1}{7.01928 \times 10^5}$$

The concentration of flutianil in the extract solution was determined by substituting the resulting analyte peak area into the above equation. Using the peak area for Sample Number LA12-2308 (1336100 counts), the concentration in the final sample solution was calculated as:

$$\text{Concentration in ng/mL} = \frac{1.336100 \times 10^6 - 16078.1}{7.01928 \times 10^5}$$

$$\text{Concentration in ng/mL} = 1.881\text{ ng/mL}$$

The residue concentration of flutianil in soil was determined as the product of the extract solution concentration and the conversion factor from extract concentration to soil concentration:

Sample Concentration in ng/g = Extract Concentration x 32.0 mL/g

= 1.881 ng/mL x 32.0 ml/g

= 60.2 ng/g

Dry-Weight Residue = $\frac{60.2 \text{ ng/g}}{(1.00 - 0.117)}$, for 11.7% soil moisture

Dry-Weight Residue = 68.2 ng/g

Note: Calculations of concentrations for injected calibration standards were performed using Analyst Version 1.5.1 Applied Biosystems/MDS Sciex software algorithms following entry of the conversion factor and sample identifiers. Excel 2010, in full-precision mode, was used for calculation of means, standard deviations, etc. Values calculated using rounded numbers, either as presented above or in the tables, will differ slightly.

Table 1. Representative LC/MS/MS Operating Parameters for the Analysis of Flutianil, OC 53276, OC 56574, or OC 56635.

Instrument:	Hewlett-Packard Series 1200 High Performance Liquid Chromatograph (HPLC) coupled with an Applied Biosystems MDS SCIEX API 5000 Tandem Mass Spectrometer (MS/MS) with QJet Ion Guide Technology in reaction monitoring (MRM) mode.
Ion Source:	Turbo V Ion Spray
Analytical Column: Guard Column:	Phenomenex LUNA 5 C-18 (150 x 2.0 mm, 5-µm 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 µL/minute
Elution Profile:	Time A (%) B (%) 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:	50.0 µl
Parameter Table:	CUR: 25.00 IS: 5500.00 GS1: 35.00 DP: 100.00 GS2: 45.00 EP: 10.00 CAD: 4.00 TEM: 500.00 Quantitation Ion Transition Flutianil CE: 37.0; CXP: 12.0 (positive-ion mode) OC 53276 CE: 33.0; CXP: 12.0 (positive-ion mode) OC 56574 CE: 31.0; CXP: 14.0 (positive-ion mode) OC 56635 CE: -34.0 (negative-ion mode) Confirmation Ion Transition Flutianil CE: 65.0; CXP: 12.0 (positive-ion mode) OC 53276 CE: 75.0; CXP: 12.0 (positive-ion mode) OC 56574 CE: 31.0; CXP: 14.0 (positive-ion mode) OC 56635 CE: -60.0 (negative-ion mode)

- Continued -

Table 1 (continued). Representative LC/MS/MS Operating Parameters for the Analysis of Flutianil, OC 53276, OC 56574, or OC 56635.

Monitored Transitions:	<u>Quantitation Ion Transition</u> Flutianil: 427 → 192 amu (dwell time 250 msec) OC 53276: 443 → 192 amu (dwell time 250 msec) OC 56574: 443 → 136 amu (dwell time 250 msec) OC 56635: 243 → 179 amu (dwell time 500 msec) <u>Confirmation Ion Transition</u> Flutianil: 427 → 132 amu (dwell time 150 msec) OC 53276: 443 → 132 amu (dwell time 150 msec) OC 56574: 443 → 181 amu (dwell time 150 msec) OC 56635: 243 → 80 amu (dwell time 500 msec)
Approximate Retention Times: Retention times were abstracted from the method validation sequence.	Flutianil – 10.3 minutes OC 53276 – 9.1 minutes OC 56574 – 9.2 minutes OC 56635 – 7.2 minutes

Prepare samples and procedural recoveries by weighing 10.0 g of homogenized soil into 50-mL graduated centrifuge tubes or equivalent apparatus. Prepare procedural recoveries by fortification of control soil with an appropriate mixed reference standard solution. Add 25 mL of acetonitrile to the soil samples and mix well by hand shaking.

↓

Extract samples for approximately one minute using a BRANSONIC ultrasonic disruption sample processor at an approximate 45% amplitude setting. Centrifuge the samples at approximately 3500 rpm for approximately five minutes. Decant the supernatant into a 100-mL glass volumetric cylinder.

↓

Repeat the extraction process with 25 mL of acetonitrile:water (75:25, v:v) added to the residual soil pellet. Following the untrasonic disruption at approximately 45% amplitude setting, centrifuge the samples at approximately 3500 rpm for approximately five minutes. Decant and add the supernatant to the same 100-mL cylinder containing the original extract.

↓

Add 25 mL of acetonitrile:water (75:25, v:v) to each soil pellet. Cap and place securely on a gyratory shaker table. Shake the samples at a setting of approximately 250 for approximately 15 minutes. Centrifuge at apprximately3500 rpm for approximately 5 minutes and combine the extract supernatant in the 100-mL graduated cylinder from above.

↓

Adjust extract to final volume of 80.0 mL with acetonitrile. Transfer to a 150-mL beaker or equivalent and gently swirl to mix. Filter a 5.0-mL volume of each extract using a BD disposable 5.0-mL plastic syringe and a 0.2-µm PURADISK 25 T syringe filter into a 20-mL glass vial. Transfer an aliquot (~20 mL) of remaining unfiltered final intermediate extract to appropriate storage container. Discard the each remainder of each extract.

↓

Volumetrically transfer a 2.50-mL aliquot of each filtered extract to a 15-mL plastic centrifuge tube and evaporate to aqueous remainder (~ 0.5 mL) using a nitrogen evaporator set at 40 to 50 ˚C bath temperature.

↓

Add 1.00 mL of acetonitrile to each aqueous remainder. Mix well. Adjust to 10.0 mL final value using a solution of 0.1% formic acid in water to achieve a final extract of acetonitrile: water:formic acid (10:90:0.1, v:v:v). Mix well. Transfer an aliquot of the final extract to an autosampler vial and submit for LC/MS/MS analysis. Dilute samples further, if necessary, using acetonitrile:water:formic acid (10:90:0.1, v:v:v).

Figure 1. Method outline for the analysis of flutianil, OC 53276, OC 56574, and OC 56635 in soil.