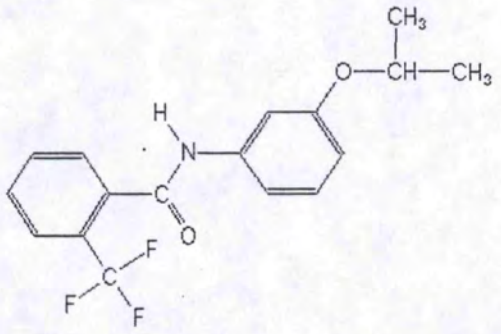
3.0 MATERIALS and METHODS

3.1 Test Item/Reference Substance

The analytical (reference) standard used in this study was:

Flutolanil:

Common Name: Flutolanil
Chemical Name:
CAS: *N*-[3-(1-methylethoxy)phenyl]-2-(trifluoromethyl) benzamide
IUPAC: α,α,α-trifluoro-3′-isopropoxy-*o*-toluanilide
Structural Formula:



Flutolanil

CAS No.:	66332-96-5
Molecular weight:	323.3 g/mole
Source:	Chem Service Inc.
Purity:	99.5%
Lot no.:	442-44A
Expiration date:	April 2013
Storage:	room temperature

Certificate of Analysis is provided in Appendix 6.

The test/reference substance (analytical standard) used in this study was purchased from ChemService, Inc. and stored as directed on the Material Data Safety Sheet. All solutions made from the reference substance (analytical standard) were stored according to the method.

3.2 Test System

One textural class of soil was evaluated in this study: sandy loam. This textural class of soil was chosen as it represents a typical type of soil analyzed using this method.

The soil was transferred from another Sponsor-related study (3). The soil, previously homogenized, was transferred to this study on November 12, 2010. No further processing was required. The soil remained in frozen storage (typically −20 ± 5 °C) pending analysis for suitability.

Characterization data for the soil is summarized below.

	Sandy Loam
Location	Porterville
State	CA
Sample ID	1651A
Depth:	0 - 12 inches
Characterization:	
% sand	64
% silt	30
% clay	6
USDA textural class	sandy loam
Bulk density (gm/cc)	1.15
Cation exchange capacity (meq/100g)	9.8
% Moisture at 1/3 bar	11.0
% organic matter	0.72
pH (1:1 soil:water)	7.9

A soil characterization report from Agvise Laboratories can be found in Appendix 5.

3.3 Equipment and Reagents/Supplies

The equipment and reagents/supplies used for the method validation were as outlined in the method. Identical or equivalent equipment and materials were used, as permitted by protocol. The equivalent equipment and reagents used were as follows:

3.3.1 Equipment

Balances:	Analytical: Mettler Toledo, Model AB104 (Mettler Instrument Corp., Hightstown, NJ) Top-loading: PE 1600 (Mettler Instrument Corp., Hightstown, NJ) Galaxy 4000 (OHAUS, Florham Park, NJ)
HPLC system:	Shimadzu LC-20AD high pressure liquid chromatograph system/DGU-20A5 vacuum solvent degasser, Shimadzu SIL-20AC autosampler, FIAtron CH-30 column heater, FIAtron TC-50 temperature controller, Shimadzu 6 port valve (diverter), Varian H560Z rough pump, Harvard single syringe pump, and Shimadzu CBM-20A communication bus module (system controller)
Mass spectrometer:	Applied BioSystems API 4000 (MS/MS) mass spectrometer (Applied Biosystems/MSD Sciex, Foster City, CA, USA) with Analyst Software for data collection and system control
Platform shaker:	Eberbach Model 6000 (Eberbach Corp., Ann Arbor, MI)
Rotary Evaporator:	Rotary evaporator equipped with a Dewar condenser (Labconco Corp., Kansas City, MS) Rotary evaporator equipped with Dewar condenser (Buchi, VWR Scientific, Bridgeport, NJ)
Auto pipettes:	Finnpipette digital pipettes, various sizes (VWR Scientific, Bridgeport, NJ)

3.3.2 Reagents

Reagents and standards used were of equivalent grade as that specified in the analytical method unless otherwise specified.

3.4 Standard Solution Preparation

The preparation of flutolanil standard solutions used for this study are described below. The solutions were stored as specified in the method when not in use.

3.4.1 Stock Standard Solution

Ten (10.0) mg (corrected for purity) of flutolanil analytical standard were accurately weighed and quantitatively transferred to a 10-mL volumetric flask. The contents were brought to volume with acetonitrile and mixed thoroughly. The concentration of the resulting solution was 1000 mg/L. The solution was stored under refrigerated conditions (typically 1 to 8 °C).

3.4.2 Intermediate/Fortification Standard Solutions

The following concentrations of intermediate/fortification standard solutions were prepared. All solutions were stored under refrigerated conditions (typically 1 to 8 °C).

100 mg/L: 2.5 mL of the stock 1000 mg/L standard solution were transferred to a 25-mL volumetric flask and brought to volume with acetonitrile. The contents were mixed well.

10 mg/L: 2.5 mL of the stock 100 mg/L standard solution were transferred to a 25-mL volumetric flask and brought to volume with acetonitrile. The contents were mixed well.

1.0 mg/L: 2.5 mL of the stock 10 mg/L standard solution were transferred to a 25-mL volumetric flask and brought to volume with acetonitrile. The contents were mixed well.

100 µg/L: 50 µL of a 100 mg/L standard solution were transferred to a 50-mL volumetric flask and brought to volume with acetonitrile. The contents were mixed well.

20 µg/L: 5.0 mL of a 100 µg/L standard solution were transferred to a 25-mL volumetric flask and brought to volume with acetonitrile. The contents were mixed well.

10 µg/L: 2.5 mL of a 100 µg/L standard solution were transferred to a 25-mL volumetric flask and brought to volume with acetonitrile. The contents were mixed well.

2.0 µg/L: 500 µL of a 100 µg/L standard solution were transferred to a 25-mL volumetric flask and brought to volume with acetonitrile. The contents were mixed well.

1.0 µg/L: 250 µL of a 100 µg/L standard solution were transferred to a 25-mL volumetric flask and brought to volume with acetonitrile. The contents were mixed well.

0.40 µg/L: 100 µL of a 100 µg/L standard solution were transferred to a 25-mL volumetric flask and brought to volume with acetonitrile. The contents were mixed well.

3.4.3 Linearity Standard Solutions

The following concentrations of matrix-matched linearity (calibration) standard solutions were prepared. All solutions were stored under refrigerated conditions (typically 1 to 8 °C). Control matrix (Control 5) used in the preparation of the standard solutions was prepared during analysis of Set 3.

Calibration standards:

50 µg/L: 1.0 mL of a 100 µg/L standard solution + 1.0 mL of purified control matrix extract (0.2 grams sample/mL) were transferred to an HPLC vial and mixed well.

10 µg/L: 1.0 mL of a 20 µg/L standard solution + 1.0 mL of purified control matrix extract (0.2 grams sample/mL) were transferred to an HPLC vial and mixed well.

5.0 µg/L: 1.0 mL of a 10 µg/L standard solution + 1.0 mL of purified control matrix extract (0.2 grams sample/mL) were transferred to an HPLC vial and mixed well.

1.0 µg/L: 1.0 mL of a 2.0 µg/L standard solution + 1.0 mL of purified control matrix extract (0.2 grams sample/mL) were transferred to an HPLC vial and mixed well.

0.50 µg/L: 1.0 mL of a 1.0 µg/L standard solution + 1.0 mL of purified control matrix extract (0.2 grams sample/mL) were transferred to an HPLC vial and mixed well.

0.20 µg/L: 1.0 mL of a 0.40 µg/L standard solution + 1.0 mL of purified control matrix extract (0.2 grams sample/mL) were transferred to an HPLC vial and mixed well.

3.5 Analytical Methods

3.5.1 Soil Method

The flutolanil analytical method described in Nihon Nohyaku Co., Ltd. Final Report No. LSRC-A07-161A (Amended), Study Protocol No. GE-04, 07-0127, entitled "Validation of Analytical Method for Flutolanil in Soil," was used for the analyses in this study. See Appendix 2 for the complete text of the method. The following is a summary of that method:

To summarize, flutolanil was extracted from the soil matrix (20 grams, dry weight) with 50 mL of acetonitrile in a 250-mL HDPE centrifuge bottle by vigorous shaking for 15 minutes. The mixture was centrifuged at 2500 rpm for 5 minutes and the supernatant decanted into a 100-mL mixing cylinder. The solids remaining were re-extracted by shaking with 20 mL of acetonitrile. The mixture was vacuum-filtered through a Whatman GF/F (70 mm) glass fiber filter and the filtrate was combined with the supernatant from the first extraction in the mixing cylinder. The mixture was brought to a final volume of 100 mL with acetonitrile. Three (3) grams of NaCl and 12.5 mL of phosphate buffer solution (0.1M, pH 7) were added to 12.5 mL of diluted sample extract in a 60-mL separatory funnel, followed by vigorous shaking for 1 minute. The upper layer was removed and transferred to a 50-mL evaporation flask and evaporated to ~0.2 mL using rotary evaporation at ~35 °C, and on to dryness using manual nitrogen blow-down. The residue was redissolved in 3 mL of acetonitrile:water (4:1, v/v). The concentrate was loaded onto a carbon graphite/aminopropyl silica gel SPE (solid phase extraction) cartridge (previously conditioned with 5 mL of acetonitrile followed by 5 mL acetonitrile:water (4:1, v/v). The cartridge was washed with 2 mL of acetonitrile:water (4:1, v/v) followed by 2 mL of acetonitrile, then eluted with 5 mL of acetonitrile:acetic acid (95:5, v/v). The eluate was brought to a final volume of 25 mL with HPLC water. A portion of the diluted purified extract was transferred to an HPLC vial and submitted to HPLC analysis. Determination and quantitation of flutolanil were performed using HPLC (high performance liquid chromatography) employing tandem mass spectrometric (MS/MS) detection. The limit of quantitation (LOQ) was 0.01 ppm.

3.5.2 Moisture Analysis

Moisture determination was conducted on the control sample used to prepare all validation fortifications. The method used was Morse Labs' SOP# Pest-17a, Revision #7, entitled, "Determination of Moisture in Various Matrices". See Appendix 3 for SOP.

3.6 Fortification Procedures

Aliquots of untreated control soil sample were fortified with microliter amounts of flutolanil analytical standard solution. During fortification, the standard solution was evenly distributed over the exposed sample as much as possible.

Untreated control samples were fortified according to the following scheme:

Matrix	Sample Type	Fortifying Compound	Fortification Level (ppm)	# of Samples
---	Reagent Blank	None	0.0	1
Soil	Control	None	0.0	2
Soil	Fortified control	Flutolanil	0.01 (LOQ)	5
Soil	Fortified control	Flutolanil	0.10 (10 × LOQ)	5

3.7 Modifications, Interpretations, and Critical Steps

The analytical method was run exactly as written using equivalent equipment and materials where permitted and with the following clarifications:

1) Section 3.3.2, Step 2: Extraction.

Flutolanil was extracted from the soil matrix (20 grams, dry weight) with 50 mL of acetonitrile in a 250-mL HDPE centrifuge bottle by vigorous shaking for 15 minutes. The mixture was centrifuged at 2500 rpm for 5 minutes and the supernatant decanted into a 100-mL mixing cylinder. The solids remaining were re-extracted by shaking with 20 mL of acetonitrile. The mixture was vacuum-filtered through a Whatman GF/F (70 mm) glass fiber filter and the filtrate was combined with the supernatant from the first extraction in the mixing cylinder. The mixture was brought to a final volume of 100 mL with acetonitrile.

2) Section 3.3.2, Step 3. Purification.

Three (3) grams of NaCl and 12.5 mL of phosphate buffer solution (0.1M, pH 7) were added to 12.5 mL of diluted sample extract in a 60-mL separatory funnel, followed by vigorous shaking for 1 minute. The upper layer was removed and transferred to a 50-mL evaporation flask and evaporated to ~0.2 mL using rotary evaporation at ~35 °C, and on to dryness using manual nitrogen blow-down. The residue was redissolved in 3 mL of acetonitrile:water (4:1, v/v). The concentrate was loaded onto a carbon graphite/aminopropyl silica gel SPE (solid phase extraction) cartridge (previously conditioned with 5 mL of acetonitrile followed by 5 mL acetonitrile:water (4:1, v/v). The cartridge was washed with 2 mL of acetonitrile:water (4:1, v/v) followed by 2 mL of acetonitrile, then eluted with 5 mL of acetonitrile:acetic acid (95:5, v/v). The eluate was brought to a final volume of 25 mL with HPLC water. A portion of the diluted purified extract was transferred to an HPLC vial and submitted to HPLC analysis.

3.8 Instrumentation

All samples were analyzed by HPLC employing tandem mass spectrometric (MS/MS) detection (LC-MS/MS). Typical conditions were as follows:

- **Operating conditions**

Instrument:	Applied Biosystems/Sciex API 4000 LC/MS/MS System with Shimadzu LC-20AD Liquid Chromatographs and Shimadzu SIL-20AC Autosampler, Shimadzu DGU-20A5 degasser, and Shimadzu CBM-20A Communications Bus Module (System Controller) with Applied Biosystems/MDS Sciex Analyst Software for data collection and system control (version 1.5)
HPLC column:	50-mm × 2.0-mm i.d. Cadenza CD-C18, 3 µm particle size
Mobile phase:	Fisher water, Fisher methanol, and EM Science formic acid (all solvents HPLC grade)
	Component A: 0.1% formic acid in water
	Component B: 0.1% formic acid in methanol

Gradient:

Time (min)	% A	% B
0.0	50	50
0.5	30	70
5.5-7.5	0	100
8.0-11.0	50	50

Divert Valve: Programmed to divert LC flow from column to waste (bypassing detector) from 0.0 to 3.0 minutes and again from 6.0 to 11.0 minutes. LC flow is directed to detector during the 3.0 to 6.0 minute window. Diversion time settings can be adjusted as necessary depending on the retention times of the analytes.

Flow Rate: 0.2 mL/min.

Interface: TIS (Turbo Ion Spray)

Ionization Mode: Positive (+)

Acquisition Mode: MRM

Source Temperature: 450 °C

Curtain Gas: Nitrogen @ setting of "30"

Collision Gas: Nitrogen @ setting of "6"

Injection Volume: 2 µL

Column Temperature: 40 °C

Resolution: Q1-Unit, Q3-Unit (Note: Unit is equivalent to medium)

Transitions Monitored:	Ion, *m/z* Q1	Q3	Time, *ms*	CE, *v*	
Flutolanil:	324.2	262.0	100	27	(quantitation)
	324.2	282.1	100	19	(confirmation)
	324.2	242.0	100	35	(confirmation)

Retention Time: Flutolanil: ~4.3 minutes

3.9 Calculations

Due to an incomplete description in the method regarding the calculation of residues, the calculations to be performed and equations to be used were provided by the laboratory. This approach was approved by the Sponsor by e-mail (January 9, 2011).

Calculations for instrumental analysis were conducted using a validated software application to create a standard curve based on linear regression. The regression functions were used to calculate a best-fit line (from a set of standard concentrations in µg/L versus peak response) and to determine concentrations of the analyte found during sample analysis from the calculated best-fit line. Per the method, the curve was forced through the origin (zero).

The equation used for the least squares fit with a 0-intercept is:

$$y = mx$$

where:

y = peak response
m = slope
x = µg/L found for peak of interest

Note: A standard curve was generated by plotting the standard concentration (in µg/L) on the x-axis and the respective peak response on the y-axis.

Standard (calibration) curves generated for each analytical set were used for the quantitation of flutolanil in the samples. For this study, the correlation coefficient (r) for each calibration curve was equal to or greater than 0.990 (r^2 equal to or greater than 0.98).

The calculations for ppm found and percent recovery (for fortified samples) were:

$$ppm = \mu g/L\ found \times \frac{HPLC\ final\ vol.(mL)}{sample\ wt.(g)} \times \frac{ext.solv.(mL)}{aliq.(mL)} \times \frac{1\ L}{1000\ mL} \times HPLC\ dil.\ factor$$

1. The amount of analyte (in ppm) found in the sample was calculated according to the following equation:

   where:

   µg/L found = µg/L of analyte found in sample injected from standard curve

HPLC final vol. (mL)	=	volume of final extract submitted to HPLC (typically 25.0 mL)
sample wt. (g)	=	dry weight, in grams, of sample extracted (typically 20.0 g)
ext. solv. (mL)	=	final volume of extraction solvent (typically 100 mL)
aliq. (mL)	=	volume of sample extract processed through the method (typically 12.5 mL)
1 L/1000 mL	=	conversion factor
HPLC dil. factor	=	dilution of sample extract required to produce an analyte response bracketed by standards

2. The percent recovery for fortified control samples is calculated as follows:

$$\%\ Recovery = \frac{ppm\ found\ in\ fortified\ control}{ppm\ added} \times 100$$

Example Calculations

1. ML ticket #87278, Flutolanil, Soil, Set #3, 1651A,
   Control 6 (Figure 8):

$$0\ peak\ response\ (area) \rightarrow\ 0.00\ \mu g/L$$

$$ppm = 0.00\ \mu g/L\ found \times \frac{25.0\ mL}{20.0\ g} \times \frac{100\ mL}{12.5\ mL} \times \frac{1\ L}{1000\ mL} \times 1$$

$$ppm = 0.000$$

$$Reported\ ppm = ND$$

2. ML ticket #87278, Flutolanil, Soil, Set #3, 1651A, **Fortified Control** 14 @ 0.01 ppm (Figure 9):

$$27100 \text{ peak response (area)} \rightarrow 0.792 \text{ µg/L}$$

$$ppm = 0.792 \text{ µg/L found} \times \frac{25.0 \text{ mL}}{20.0 \text{ g}} \times \frac{100 \text{ mL}}{12.5 \text{ mL}} \times \frac{1 \text{ L}}{1000 \text{ mL}} \times 1$$

$$ppm = 0.00792$$

$$\text{Reported } ppm = 0.00792$$

$$\% \text{ Rec.} = \frac{0.00792 \text{ ppm}}{0.01 \text{ ppm}} \times 100$$

$$= 79\%$$