2.0 INTRODUCTION

The purpose of this study was to conduct an independent laboratory validation on the flutriafol analytical method for groundwater found in Appendix 1 of SGS Institut Fresenius GmbH Report for Study No. IF-04/00159540 entitled "Validation of Analytical Methods to Determine Flutriafol in Soil Water, Groundwater and Soil and Investigations on the Stability and the Adsorption Situation under Sampling Conditions," as written.

This study was designed to satisfy guideline requirements described in OPPTS 850.7100 (1). This study was conducted in compliance with EPA FIFRA Good Laboratory Practice Standards, 40 CFR Part 160 (3).

The residue analytical method is applicable for the detection and quantitation of flutriafol in groundwater.

To summarize, 30.0 g of groundwater were weighed into a 40-mL glass sample vial. Five (5.0) mL of dichloromethane were added. The vial was capped and placed on a

platform shaker and shaken at ~240 cycles per minute for approximately 1 minute. The vial was then centrifuged at 2500 rpm (at 10 °C) for 2 minutes. The lower organic phase was removed by means of a Pasteur pipette and transferred into another 40-mL glass sample vial. The solvent partition was repeated twice with fresh 5.0 mL aliquots of dichloromethane. The organic phases were combined and evaporated to dryness on an N-Evap evaporator at 40 °C. The remainder was reconstituted in 1.0 mL of acetonitrile/water/formic acid (50:50:0.2, v/v/v) and ultrasonically agitated for 1 minute for complete dissolution of the analyte. The solution was submitted to HPLC (high performance liquid chromatography) analysis. Determination and quantitation of flutriafol were performed using HPLC employing tandem mass spectrometric (MS/MS) detection. The limit of quantitation (LOQ) was 0.05 µg/L. The full text of the method can be found in Appendix 2.

The method was used as written. Communications with the Study Representative and/or Sponsor Technical Consultant consisted of discussions regarding use of equivalent equipment and reagents/solvents, permission to use fortification standards prepared at 0.1 µg/mL and 0.01 µg/mL, failure of the first validation trial and permission to start the second method validation trial, continuation of the second validation trial rather than starting a third validation trial (due to technician errors), and acceptability of the recoveries achieved in the second portion of the second method validation trial. These communications are thoroughly documented in Appendix 6.

3.0 MATERIALS and METHODS

3.1 Test Item/Reference Substance

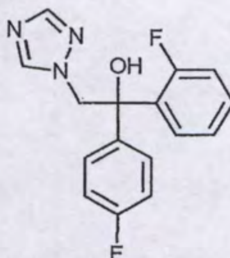
The analytical (reference) standard used in this study was:

Flutriafol:

Common Name: Flutriafol
Chemical Names: 1H-1,2,4-triazole-1-ethanol, alpha-(2-fluorophenyl)-α-(4-fluorophenyl)- [CAS]

(*RS*)-2,4′-difluoro-α-(1*H*-1,2,4-triazol-1-ylmethyl) benzhydryl alcohol [IUPAC]

Structural Formula:



CAS No.:	76674-21-0
Molecular weight:	301.29 g/mol
Source:	Cheminova A/S
Purity:	99.0%
Lot no.:	ASJ-10005-01
Receipt date:	November 30, 2009
Expiration date:	November 5, 2011
Reassay date:	November 4, 2011
Reassay purity:	99.0%
Expiration date:	November 4, 2013
Storage:	Freezer (approximately -8 to -22 °C)

Certificate of Analysis is provided in Appendix 5.

The test/reference substance (analytical standard) used in this study was provided by the Sponsor 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

Groundwater, collected from a well, was evaluated in this study. This type of water was chosen as it represents one of the water types the method was designed for.

The water was transferred from another Sponsor-related study (4) to this study on September 12, 2011. The water had been stored refrigerated prior to transfer and remained refrigerated (1 - 8°C) following transfer, pending analysis for suitability.

Characterization data for the water is summarized below:

	Ground Water
Location	Sacramento (Well)
State	CA
Sample ID	66799A
pH	7.7
Calcium (ppm)	48
Magnesium (ppm)	33
Sodium (ppm)	20
Hardness (mg equiv. $CaCO_3$/L)	259
Conductivity (mmhos/cm)	0.55
Sodium Absorption Ratio (SAR)	0.53
Total Dissolved Solids (ppm)	330
Turbidity (NTU)	0.93

A water characterization report from Agvise Laboratories can be found in Appendix 4.

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:

Sartorius, Model B3100S (Sartorius Instruments, McGaw Park, IL)

Centrifuge:

Sorvall, Super *T*21 Refrigerated Tabletop Superspeed Centrifuge (Kendro Laboratory Products, Asheville, NC)

HPLC system:	Applied Biosystems/Sciex API 4000 LC/MS/MS System with ACQUITY UPLC System including Sample Organizer with Applied Biosystems/MDS Sciex Analyst Software for data collection and system control (version 1.5)
Pasteur Pipets:	Glass, 5 ¾ inches, disposable, (Fisher Scientific, Fair Lawn, NJ)
Pipets, Adjustable:	Finnipette Digital Pipettors:
	40-200 µL, Cat.#53575-052 (VWR Scientific, Arlington Heights, IL)
	100-1000 µL, Cat.#14-386-74 (Fisher Scientific, Fair Lawn, NJ)
	Finnipette pipet tips:
	1-200 µL, Cat.#02-681-140 (Fisher Scientific, Fair Lawn, NJ)
	101-1000 µL, Cat.#02-681-421 (Fisher Scientific, Fair Lawn, NJ)
Platform shaker:	Eberbach Model 6000 (Eberbach Corp., Ann Arbor, MI)
Nitrogen Evaporator:	N-Evap, Model 115 N-Evap Laboratory Sample Evaporator (Organomation Assoc. Inc., Berlin, MA)
Sample Vial:	Glass, 40 mL, 95 mm × 27 mm, Screw Cap Septum Vials, Cat.# TS-13075 (Thermo Scientific, Auburn, AL)
Sample Vial Cap:	Fluoropolymer Resin Liner, Thermoset screw caps, cap size 24-400, Cat.# 16161-213 (VWR Scientific, Arlington Heights, IL)
Ultrasonic Bath:	Branson Ultrasonic Bath, model 2210 (VWR Scientific, Arlington Heights, IL)
Volumetric Flask:	Glass, 50 mL, 25 mL, 10 mL (Fisher Scientific, Fair Lawn, NJ)

Volumetric Pipets:	Glass, 5.0 mL, 1.0 mL, 0.5 mL (Fisher Scientific, Fair Lawn, NJ)

3.3.2 Reagents

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

Acetonitrile:	Fisher, 99.9%, part# A998SK-4
Acetone:	Fisher, 99.9%, part# A929-4
Dichloromethane:	Fisher, 99.8%, part# D151-4
Formic Acid:	EMD, 98%, part# FX0440-5
Ultra Pure Water:	Fisher, 100%, part# W5-4

3.4 Standard Solution Preparation

The preparation of flutriafol 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

Twenty-five (25.0) mg (corrected for purity) of flutriafol analytical standard were accurately weighed and quantitatively transferred to a 25-mL volumetric flask. The contents were brought to volume with acetone and mixed thoroughly. The concentration of the resulting solution was 1000 µg/mL. 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 µg/mL: 5.0 mL of the stock 1000 µg/mL standard solution were transferred to a 50-mL volumetric flask and brought to volume with acetone. The contents were mixed well.

10 µg/mL: 0.5 mL of the stock 1000 µg/mL standard solution were transferred to a 50-mL volumetric flask and brought to volume with acetone. The contents were mixed well.

5.0 mL of the intermediate 100 µg/mL standard solution were transferred to a 50-mL volumetric flask and brought to volume with acetone. The contents were mixed well.

1.0 µg/mL: 5.0 mL of the intermediate 10 µg/mL standard solution were transferred to a 50-mL volumetric flask and brought to volume with acetone. The contents were mixed well.

2.5 mL of the intermediate 10 µg/mL standard solution were transferred to a 25-mL volumetric flask and brought to volume with acetone. The contents were mixed well.

0.1 µg/mL: 100 µL of the intermediate 10 µg/mL standard solution were transferred to a 10-mL volumetric flask and brought to volume with acetone. The contents were mixed well.

5.0 mL of the intermediate 1.0 µg/mL standard solution were transferred to a 50-mL volumetric flask and brought to volume with acetone. The contents were mixed well.

0.01 µg/mL: 100 µL of the intermediate 1.0 µg/mL standard solution were transferred to a 10-mL volumetric flask and brought to volume with acetone. The contents were mixed well.

5.0 mL of the intermediate 0.1 µg/mL standard solution were transferred to a 50-mL volumetric flask and brought to volume with acetone. The contents were mixed well.

3.4.3 Linearity Standard Solutions

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

Calibration standards:

10 ng/mL: 250 µL of a 1.0 µg/mL intermediate standard solution were transferred to a 25-mL volumetric flask and brought to volume with HPLC acetonitrile/HPLC water/formic acid (50:50:0.2, v/v/v). The contents were mixed well.

5.0 ng/mL: 125 µL of a 1.0 µg/mL intermediate standard solution were transferred to a 25-mL volumetric flask and brought to volume with HPLC acetonitrile/HPLC water/formic acid (50:50:0.2, v/v/v). The contents were mixed well.

5.0 mL of a 10 ng/mL calibration standard solution were transferred to a 10-mL volumetric flask and brought to volume with HPLC acetonitrile/HPLC water/formic acid (50:50:0.2, v/v/v). The contents were mixed well.

2.0 ng/mL: 50 µL of a 1.0 µg/mL intermediate standard solution were transferred to a 25-mL volumetric flask and brought to volume with HPLC acetonitrile/HPLC water/formic acid (50:50:0.2, v/v/v). The contents were mixed well.

2.0 mL of a 10 ng/mL calibration standard solution were transferred to a 10-mL volumetric flask and brought to volume with HPLC acetonitrile/HPLC water/formic acid (50:50:0.2, v/v/v). The contents were mixed well.

0.4 ng/mL: 1000 µL of a 10 ng/mL calibration standard solution were transferred to a 25-mL volumetric flask and brought to volume with HPLC acetonitrile/HPLC water/formic acid (50:50:0.2, v/v/v). The contents were mixed well.

0.2 ng/mL: 500 µL of a 10 ng/mL calibration standard solution were transferred to a 25-mL volumetric flask and brought to volume with HPLC acetonitrile/HPLC water/formic acid (50:50:0.2, v/v/v). The contents were mixed well.

3.5 Analytical Methods

The flutriafol analytical method for groundwater described in Appendix 1 of SGS Institut Fresenius GmbH Report for Study No. IF-04/00159540 entitled "Validation of Analytical Methods to Determine Flutriafol in Soil Water, Groundwater and Soil and Investigations on the Stability and the Adsorption Situation under Sampling

Conditions," 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, 30.0 g of groundwater were weighed into a 40-mL glass sample vial. Five (5.0) mL of dichloromethane were added. The vial was capped and placed on a platform shaker and shaken at ~240 cycles per minute for approximately 1 minute. The vial was then centrifuged at 2500 rpm (at 10 °C) for 2 minutes. The lower organic phase was removed by means of a Pasteur pipette and transferred into another 40-mL glass sample vial. The solvent partition was repeated twice with fresh 5.0 mL aliquots of dichloromethane. The organic phases were combined and evaporated to dryness on an N-Evap evaporator at 40 °C. The remainder was reconstituted in 1.0 mL of acetonitrile/water/formic acid (50:50:0.2, v/v/v) and ultrasonically agitated for 1 minute for complete dissolution of the analyte. The solution was submitted to HPLC (high performance liquid chromatography) analysis. Determination and quantitation of flutriafol were performed using HPLC employing tandem mass spectrometric (MS/MS) detection. The limit of quantitation (LOQ) was 0.05 µg/L. The full text of the method can be found in Appendix 2.

3.6 Fortification Procedures

Aliquots of untreated control groundwater were fortified with microliter amounts of flutriafol analytical standard solution. Following fortification, the sample was hand-shaken for about 30 seconds so that the analyte was homogeneously distributed in the water sample.

Untreated control samples were fortified according to the following scheme:

Matrix	Sample Type	Fortifying Compound	Fortification Level (µg/L)	# of Samples
---	Reagent Blank	None	0.0	1
Groundwater	Control	None	0.0	2
Groundwater	Fortified control	Flutriafol	0.05 (LOQ)	5
Groundwater	Fortified control	Flutriafol	0.50 (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.

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 ACQUITY UPLC System including Sample Organizer with Applied Biosystems/MDS Sciex Analyst Software for data collection and system control (version 1.5)

HPLC column: 150-mm × 3.0-mm i.d. Thermo Hypurity C8, 5 µm particle size

Mobile phase: Fisher water, Fisher acetonitrile, and EM Science formic acid (all solvents HPLC grade)

Component A: 0.2% formic acid in water

Component B: 0.2% formic acid in acetonitrile

Gradient:

Time (min)	% A	% B
0.0	90	10
5.0-8.0	50	50
8.1-11.0	10	90
11.1-13.0	90	10

Divert Valve: Not used.

Flow Rate: 0.6 mL/min.

Interface: TIS (Turbo Ion Spray)

Ionization Mode: Positive (+)

Acquisition Mode: MRM

Source Temperature: 350 °C

Curtain Gas: Nitrogen @ setting of "30"

Collision Gas: Nitrogen @ setting of "6"

Injection Volume: 10 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*	
Flutriafol:	302.0	233.0	150	23	(quantitation)
	302.0	123.0	150	39	(confirmation)

Retention Time: Flutriafol: ~6.1 minutes

3.9 Calculations

Residue calculations, as described in the method, were employed in this study. The calculations 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 masses (pg) versus peak response (area)] and to determine concentrations of the analyte found during sample analysis from the calculated best-fit line.

The equation used for the least squares fit is: $y = mx + b$

where:

y	=	peak response (area)
m	=	slope
x	=	pg found for peak of interest
b	=	y-intercept

Note: A standard curve was generated by plotting the standard mass (in pg) on the x-axis and the respective peak response (area) on the y-axis.

Standard (calibration) curves generated for each analytical set were used for the quantitation of flutriafol 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:

$$\mu g/L = \frac{pg\ found \times HPLC\ final\ vol.(mL)}{sample\ wt. \times inj.\ vol.(\mu L)} \times HPLC\ dil.\ factor$$

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

   where:

µg/L	=	amount of analyte found in sample
pg found	=	mass amount of analyte found in sample injected, from standard curve
HPLC final vol. (mL)	=	volume of final extract submitted to HPLC analysis (1.0 mL)
sample wt.	=	weight of water sample taken through analysis (30.0 g). Assuming the density of water is d = 1.00, **30.0 g** water = **30.0 mL** water. 30.0 mL is the value and unit used in the calculation.
inj. vol. (µL)	=	amount of prepared sample extract injected in HPLC (10 µL)
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 #87885, Flutriafol, Groundwater, Set #4, 66799A, **Control** 6 (Figure 7):

$$0\ peak\ response\ (area) \rightarrow 0.00\ pg$$

$$\mu g/L = \frac{0.00\ pg\ found \times 1.0\ mL}{30.0\ mL \times 10\ \mu L} \times HPLC\ dil.\ factor$$

$$\mu g/L = 0$$

$$Reported = ND$$

2. ML ticket #87885, Flutriafol, Groundwater, Set #4, 66799A, **Fortified Control** 24 @ 0.05 µg/L (Figure 8):

$$7050\ peak\ response\ (area) \rightarrow 15.5\ pg$$

$$\mu g/L = \frac{15.5\ pg\ found \times 1.0\ mL}{30.0\ mL \times 10\ \mu L} \times HPLC\ dil.\ factor$$

$$\mu g/L = 0.051666667$$

$$Reported = 0.0517$$

$$\%\ Rec. = \frac{0.0517\ ppm}{0.05\ ppm} \times 100$$

$$= 103\%$$

4.2 LOQ and LOD

The method under evaluation has a stated Limit of Quantitation (LOQ) of 0.05 µg/L for groundwater. For this study, the Limit of Detection (LOD) of the method, based on a signal/noise ratio of 3, was 0.004 µg/L. See Appendix 6.

4.3 Communications

Communications with the Study Representative and/or Sponsor Technical Consultant consisted of discussions regarding use of equivalent equipment and reagents/solvents, permission to use fortification standards prepared at 0.1 µg/mL and 0.01 µg/mL, failure of the first validation trial and permission to start the second method validation trial, continuation of the second validation trial rather than starting a third validation trial (due to technician errors), and acceptability of the recoveries achieved in the second portion of the second method validation trial. These communications are thoroughly documented in Appendix 7.

4.4 Time Requirements

A single analyst completed a sample set consisting of 13 samples in approximately 5.5 hours with LC/MS/MS analysis performed in approximately 5 hours.