2.0 INTRODUCTION

The purpose of this study was to conduct an independent laboratory validation on the flutolanil analytical method described in Nihon Nohyaku Co., Ltd. Final Report No. LSRC-A11-010A, Study Protocol No. GE-04, 11-0001, entitled "Validation of Analytical Method for Flutolanil in Water" as written.

The independent validation followed the U.S. EPA guidelines found in OPPTS 850.7100 (Data Reporting for Environmental Chemistry Methods) and PR Notice 2011-3 (1,2). In addition, 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 flutolanil in water.

Method Summary:

To a 50-mL aliquot of water sample, 5 mL of acetonitrile were added (to prevent sorption of flutolanil to glassware and keep wet the adsorbent during solid phase extraction). The sample was then loaded onto a Bond Elut® C18 solid phase extraction (SPE) cartridge that was previously washed with 6 mL of acetonitrile and equilibrated with 6 mL of 10% aqueous acetonitrile (v/v). After loading the water sample, the SPE cartridge was washed with 6 mL of 10% aqueous acetonitrile (v/v). Flutolanil was eluted from the SPE cartridge with 9 mL of acetonitrile. The eluate was evaporated to dryness under a nitrogen gas stream and the dried residue was reconstituted in 5 mL of 50% acetonitrile in water (v/v). The solution was 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 0.1 µg/L.

The method was used as written with the clarifications described in Section 3.7 of this report. Communication with the Method Developer/Study Representative consisted of discussions regarding use of equivalent equipment and reagents/solvents, clarification of some technical aspects of the method, application of a purity correction in preparation of reference material stock standard solution, clarification/modification of the calculations employed, the acceptability of the recoveries achieved in the first method validation trial, and the need to issue an amendment to update PR Notice 86-5 to 2011-3. These communications are thoroughly documented in Appendix 6.

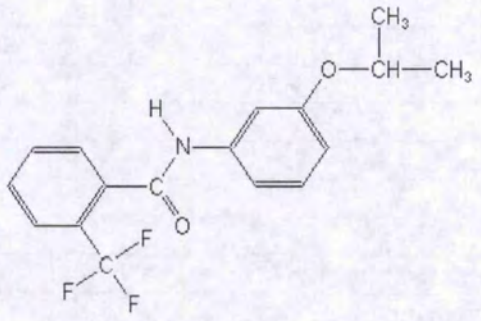
3.0 MATERIALS and METHODS

3.1 Test Item/Reference Substance

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

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:	ChemService Inc.
Purity:	99.5%
Lot no.:	442-44A
Expiration date:	April 2013
Storage:	room temperature

Certificate of Analysis is provided in Appendix 5.

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 water type was evaluated in this study: surface water (river). This water type was chosen as it represents a typical type of water analyzed using this method.

The surface (river) water sample was collected by Morse Labs personnel in a one-gallon plastic container on January 16, 2012 from the American River near Howe Avenue in Sacramento, California. Upon receipt of the sample at the laboratory (within 2 hours of collection) it was immediately placed in refrigerated storage (typically 1-8°C), where it remained pending use in analyses. An aliquot of the bulk sample was removed for subsequent characterization analysis.

Characterization data for the water sample is summarized below.

	River Water
Location	Sacramento
State	California
Sample ID	66925A
Characterization:	
pH	7.2
Calcium (ppm)	5.4
Magnesium (ppm)	1.7
Sodium (ppm)	2.1
Hardness (mg equiv. $CaCO_3$/L)	21
Conductivity (mmhos/cm)	0.05
Sodium Absorption Ratio (SAR)	0.20
Total Dissolved Solids (ppm)	58
Turbidity (NTU)	1.13

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)
HPLC system:	Waters Acquity UPLC System including Sample Organizer, Varian H560Z rough pump, Harvard Apparatus II plus syringe pump
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 (version 1.5)
N-Evap:	Laboratory sample evaporator, Model 113 attached to a nitrogen source (Organomation Associates, South Berlin, MA)
Auto pipettes:	EDP electronic pipets with 100-µL (0.10-mL) to 10.0-mL liquid ends and suitable pipet tips (Rainin Instrument Co., Inc., Ridgefield, 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 HPLC acetonitrile and mixed thoroughly. The concentration of the resulting solution was 1000 mg/L. The solution was stored under freezer conditions (typically -8 to -22 °C).

3.4.2 Intermediate/Fortification Standard Solutions

The following concentrations of intermediate/fortification standard solutions were prepared. All solutions were stored under freezer conditions (typically -8 to -22 °C).

10 mg/L: 250 µL of the stock 1000-mg/L standard solution were transferred to a 25-mL volumetric flask and brought to volume with HPLC acetonitrile. The contents were mixed well.

500 µg/L: 1.25 mL of the stock 10-mg/L standard solution were transferred to a 25-mL volumetric flask and brought to volume with HPLC acetonitrile:HPLC water (50:50, v/v). The contents were mixed well.

100 µg/L: 5.00 mL of a 500-µg/L standard solution were transferred to a 25-mL volumetric flask and brought to volume with HPLC acetonitrile:HPLC water (50:50, v/v). The contents were mixed well.

50 µg/L: 12.5 mL of a 100-µg/L standard solution were transferred to a 25-mL volumetric flask and brought to volume with HPLC acetonitrile:HPLC water (50:50, v/v). The contents were mixed well.

10 µg/L: 5.00 mL of a 50-µg/L standard solution were transferred to a 25-mL volumetric flask and brought to volume with HPLC acetonitrile:HPLC water (50:50, v/v). The contents were mixed well.

5.0 µg/L: 12.5 mL of a 10-µg/L standard solution were transferred to a 25-mL volumetric flask and brought to volume with HPLC acetonitrile:HPLC water (50:50, v/v). The contents were mixed well.

2.0 µg/L: 10.0 mL of a 5.0-µg/L standard solution were transferred to a 25-mL volumetric flask and brought to volume with HPLC acetonitrile:HPLC water (50:50, v/v). The contents were mixed well.

3.4.3 Linearity Standard Solutions

The following concentrations of calibration standard solutions were prepared. All solutions were stored under freezer conditions (typically -8 to -22 °C).

<u>Calibration standards:</u>

50 µg/L: 2.50 mL of a 500-µg/L standard solution were transferred to a 25-mL volumetric flask and brought to volume with HPLC acetonitrile:HPLC water (50:50, v/v). The contents were mixed well.

10 µg/L: 2.50 mL of a 100-µg/L standard solution were transferred to a 25-mL volumetric flask and brought to volume with HPLC acetonitrile:HPLC water (50:50, v/v). The contents were mixed well.

5.0 µg/L: 2.50 mL of a 50-µg/L standard solution were transferred to a 25-mL volumetric flask and brought to volume with HPLC acetonitrile:HPLC water (50:50, v/v). The contents were mixed well.

1.0 µg/L: 2.50 mL of a 10-µg/L standard solution were transferred to a 25-mL volumetric flask and brought to volume with HPLC acetonitrile:HPLC water (50:50, v/v). The contents were mixed well.

0.50 µg/L: 2.50 mL of a 5.0-µg/L standard solution were transferred to a 25-mL volumetric flask and brought to volume with HPLC acetonitrile:HPLC water (50:50, v/v). The contents were mixed well.

0.20 µg/L: 2.50 mL of a 2.0-µg/L standard solution were transferred to a 25-mL volumetric flask and brought to volume with HPLC acetonitrile:HPLC water (50:50, v/v). The contents were mixed well.

3.5 Analytical Method

The flutolanil analytical method described in Nihon Nohyaku Co., Ltd. Final Report No. LSRC-A11-010A, Study Protocol No. GE-04, 11-0001, entitled "Validation of Analytical Method for Flutolanil in Water." 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 a 50-mL aliquot of water sample, 5 mL of acetonitrile were added (to prevent sorption of flutolanil to glassware and keep wet the adsorbent during solid phase extraction). The sample was then loaded onto a Bond Elut® C18 solid phase extraction (SPE) cartridge that was previously washed with 6 mL of acetonitrile and equilibrated with 6 mL of 10% aqueous acetonitrile (v/v). After loading the water sample, the SPE cartridge was washed with 6 mL of 10% aqueous acetonitrile (v/v). Flutolanil was eluted from the SPE cartridge with 9 mL of acetonitrile. The eluate was evaporated to dryness under a nitrogen gas stream and the dried residue was reconstituted in 5 mL of 50% acetonitrile in water (v/v). The solution was 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 0.1 µg/L.

3.6 Fortification Procedures

Aliquots of untreated control river water were fortified with microliter amounts of flutolanil analytical standard solution. Following fortification, the sample was hand-swirled 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
Water (river)	Control	None	0.0	2
Water (river)	Fortified control	Flutolanil	0.1 (LOQ)	5
Water (river)	Fortified control	Flutolanil	1.0 (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 clarification/modifications:

1) Section 3.5: Calculation.

1. An equation was added to calculate the actual residue found in the sample. Following was the equation used:

$$\mu g/L\ sample = \mu g/L\ analyte\ found \times \frac{HPLC\ final\ vol.\ (mL)}{sample\ vol.\ (mL)} \times HPLC\ dil.\ Factor$$

where:

µg/L sample = residue found in sample (in µg/L)

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

HPLC final vol. (mL) = volume of final extract submitted to HPLC (5.0 mL)

sample vol. (mL) = volume of sample of sample taken through procedure (50.0 mL)

HPLC dil. factor = dilution of sample extract required to produce an analyte response bracketed by standards

2. Recovery (%) was calculated using the following equation:

$$\%\ Recovery = \frac{\mu g/L\ found\ in\ fortified\ control}{\mu g/L\ added} \times 100$$

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: 50-mm × 2.0-mm i.d. Imtakt 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
7.6-12.0	50	50

Divert Valve: Not used.

Flow Rate: 0.2 mL/min.

Interface: TIS (Turbo Ion Spray)

Ionization Mode: Positive (+)

Acquisition Mode:	MRM
Source Temperature:	700 °C
Curtain Gas:	Nitrogen @ setting of "20"
Collision Gas:	Nitrogen @ setting of "7"
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:	324.1	262.1	100	27	(quantitation)
	324.1	282.1	100	19	(confirmation)
	324.1	242.0	100	35	(confirmation)

Retention Time: Flutolanil: ~2.3 minutes

3.9 Calculations

The calculations described in the method were modified slightly by the laboratory. The modifications were approved by the Sponsor by e-mail (March 5, 2012).

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 ng/mL versus peak response) and to determine concentrations of the analyte found during sample analysis from the calculated best fit line. Weighting (1/x) was used.

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

where:

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

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:

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

$$\mu g/L\ sample = \mu g/L\ analyte\ found \times \frac{HPLC\ final\ vol.(mL)}{sample\ vol.(mL)} \times HPLC\ dil.\ Factor$$

where:

µg/L sample	=	residue found in sample (in µg/L)
µg/L analyte found	=	µg/L of analyte found in sample injected from standard curve
HPLC final vol. (mL)	=	volume of final extract submitted to HPLC (5.0 mL)
sample vol. (mL)	=	volume of sample of sample taken through procedure (50.0 mL)
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:

$$\%\ Recovery = \frac{\mu g/L\ found\ in\ fortified\ control}{\mu g/L\ added} \times 100$$

Example Calculations

1. ML ticket #88226, Flutolanil, Water, Set #3, 66925A, **Control 3** (Figure 8):

$$0 \text{ peak response (area)} \rightarrow 0.00 \text{ µg/L}$$

$$\text{µg/L sample} = 0.00 \text{ µg/L found} \times \frac{5.0 \text{ mL}}{50.0 \text{ mL}} \times 1$$

$$\text{µg/L sample} = 0.000$$

$$\text{Reported µg/L sample} = ND$$

2. ML ticket #88226, Flutolanil, Water, Set #3, 66925A, **Fortified Control** 2 @ 0.1 µg/L (Figure 9):

$$5830 \text{ peak response (area)} \rightarrow 0.864 \text{ µg/L}$$

$$\text{µg/L sample} = 0.864 \text{ µg/L found} \times \frac{5.0 \text{ mL}}{50.0 \text{ mL}} \times 1$$

$$\text{µg/L sample} = 0.0864$$

$$\text{Reported µg/L sample} = 0.0864$$

$$\% \text{ Rec.} = \frac{0.0864 \text{ µg/L}}{0.1 \text{ µg/L}} \times 100$$

$$= 86\%$$