INTRODUCTION

Verification samples were fortified and analyzed to evaluate the performance of a method developed by Wildlife International, Ltd. for the analysis of Flutriafol in freshwater and saltwater. A mixing trial was also conducted to determine the functional solubility of Flutriafol in saltwater. This study was conducted by Wildlife International, Ltd. and identified as Project Number 232C-105. The analyses of the samples were performed at Wildlife International, Ltd. using High Performance Liquid Chromatography (HPLC) with ultraviolet detection (UV). Verification samples were prepared and analyzed on May 17 and 18, 2006. Solubility samples were prepared and analyzed between June 6 and June 8, 2006. Raw data generated by Wildlife International, Ltd. and a copy of the final report are filed under Project Number 232C-105 in archives located on the Wildlife International, Ltd. site.

PURPOSE

The purpose of this study was to verify the performance of methodology for the analysis of Flutriafol in freshwater and saltwater and to determine the functional water solubility of Flutriafol to be used by Wildlife International, Ltd. to perform environmental effects studies.

EXPERIMENTAL DESIGN

Freshwater and saltwater were fortified at four different concentrations and analyzed based on methodology developed by Wildlife International, Ltd. Three reagent and three matrix blank samples were analyzed with each fortification set to evaluate potential analytical method interferences. Calibration curves were generated from analyses of standard solutions of Flutriafol analyzed with the series of method verification samples.

MATERIALS AND METHODS

This study was conducted according to the protocol "Analytical Method Verification for the Determination of Flutriafol in Freshwater and Saltwater" (Appendix 1).

Test Substance

The test substance, Flutriafol Technical (Code Number CHA 131), was received from Cheminova A/S on February 6, 2006. It was assigned Wildlife International, Ltd. identification number 7497 upon receipt. The test substance was described as a white powder and was identified as: Flutriafol Technical Dry; Batch Number: UPL Bx 1; CAS Number: 76674-21-0. The test substance had an expiration date of

October 12, 2006 and a reported purity of 95.1% w/w. The test substance was stored under ambient conditions. A Certificate of Analysis for the test substance is presented in Appendix 2.

Analytical Standard

The analytical standard was received from Cheminova A/S on February 6, 2006. It was assigned Wildlife International, Ltd. identification number 7496 upon receipt. The analytical standard was described as a white powder and was identified as: Flutriafol Analytical Standard; Batch Number: ASJ-10005-01; CAS Number: 76674-21-0. The analytical standard had an expiration date of November 2, 2009 and a reported purity of 99.0% w/w. The analytical standard was stored under frozen conditions. A Certificate of Analysis for the analytical standard is presented in Appendix 2.

Reagents and Solvents

All solvents used in this study were HPLC grade or equivalent.

Test Systems

Freshwater

The freshwater used to prepare the freshwater method verification samples was obtained from a well approximately 40 meters deep located on the Wildlife International, Ltd. site. The well water was characterized as moderately-hard water. The means and ranges of specific conductance, hardness, alkalinity and pH measurements of the well water during the four-week period immediately preceding the test are presented in Appendix 3.

The well water was passed through a sand filter to remove particles greater than approximately 25 µm, and pumped into a 37,800-L storage tank and aerated with spray nozzles. Prior to use, the water again was filtered to 0.2 µm in order to remove microorganisms and fine particles. The results of periodic analyses performed to measure the concentrations of selected contaminants in well water used by Wildlife International, Ltd. are presented in Appendix 4.

Saltwater

The saltwater used to prepare the saltwater method verification samples was natural seawater collected at Indian River Inlet, Delaware, and diluted to a salinity of approximately 20‰ with well water. Mean salinity and pH measurements taken during the four-week period immediately preceding the test are presented in Appendix 5.

The freshly-collected seawater was passed through a sand filter to remove particles greater than approximately 25 µm, and pumped into a 37,800-L storage tank and aerated with spray nozzles. Prior to delivery to the end user system, the water again was filtered (0.45 µm) to remove microorganisms and particles. The results of periodic analyses performed to measure the concentrations of selected contaminants in saltwater used by Wildlife International, Ltd. are presented in Appendix 6.

Analytical Method

The methods used for the analyses of Flutriafol in freshwater and saltwater were developed by Wildlife International, Ltd. Freshwater and saltwater verification samples were fortified, diluted with either freshwater or saltwater and analyzed by High Performance Liquid Chromatography with UV detection.

Concentrations of Flutriafol in the samples were determined using an Agilent Series 1100 High Performance Liquid Chromatograph (HPLC) equipped with an Agilent Series 1100 Variable Wavelength Detector. Chromatographic separations were achieved using a YMC Pack ODS-AM column (150 mm x 4.6 mm, 3 µm particle size). The instrument parameters are summarized in Table 1 and a method flowchart is provided in Figure 1.

Stocks/Standards Preparation

A stock solution of Flutriafol test substance was prepared by weighing 0.1052 grams (weight corrected for purity) of the test substance on an analytical balance. The test substance was transferred to a 100-mL class A volumetric flask, and brought to volume using methanol. The primary stock solution (1.00 mg a.i./mL) was serially diluted in methanol to prepare 0.100 and 0.0100 mg a.i./mL stock solutions. The 1.00, 0.100 and *0.0100 mg a.i./mL stock solutions were used to prepare the method verification samples.*

For the verification trial, a stock solution of Flutriafol analytical standard was prepared by weighing 0.1010 grams (weight corrected for purity) of the analytical standard on an analytical balance. The analytical standard was transferred to a 100-mL class A volumetric flask, and brought to volume using methanol. The primary stock solution (1.00 mg a.i./mL) was diluted with methanol to prepare 0.100 and 0.0100 mg a.i./mL stock solutions. Calibration standards were prepared in freshwater and saltwater using the 0.0100 mg a.i./mL stock solution. The following shows the dilution scheme for a set of calibration standards for the verification trial:

Stock Concentration (mg a.i./mL)	Aliquot (µL)	Final Volume (mL)	Standard Concentration (mg a.i./L)
0.0100	20.0	10.0	0.0200
0.0100	50.0	10.0	0.0500
0.0100	100	10.0	0.100
0.0100	150	10.0	0.150
0.0100	200	10.0	0.200

For the solubility trial, a stock solution of Flutriafol analytical standard was prepared by weighing 0.1010 grams (weight corrected for purity) of the analytical standard on an analytical balance. The analytical standard was transferred to a 100-mL class A volumetric flask, and brought to volume using methanol. The primary stock solution (1.00 mg a.i./mL) was used to prepare the calibration standard in saltwater. The following shows the dilution scheme for a set of calibration standards for the solubility trial:

Stock Concentration (mg a.i./mL)	Aliquot (µL)	Final Volume (mL)	Standard Concentration (mg a.i./L)
1.00	40.0	10.0	4.00
1.00	100	10.0	10.0
1.00	200	10.0	20.0
1.00	300	10.0	30.0
1.00	400	10.0	40.0

Calibration Curves

For the verification trial, calibration standards of Flutriafol for both freshwater and saltwater, ranging in concentration from 0.0200 to 0.200 mg a.i./L were analyzed with each respective sample set. Linear regression equations were generated using the peak area responses versus the respective concentrations of the

calibration standards. The calibration curves are presented in Figures 2 and 8. The concentration of Flutriafol in the samples was determined by substituting the peak area responses of the samples into the linear regression equation. Representative chromatograms of low and high-level calibration standards for freshwater and saltwater are presented in Figures 3, 4, 9 and 10, respectively.

For the solubility trial, calibration standards of Flutriafol in saltwater, ranging in concentration from 4.00 to 40.0 mg a.i./L were analyzed with each respective sample set. Linear regression equations were generated using the peak area responses versus the respective concentrations of the calibration standards. A representative calibration curve is presented in Figure 14. The concentration of Flutriafol in the samples was determined by substituting the peak area responses of the samples into the linear regression equation. Representative chromatograms of low and high-level calibration standards for freshwater and saltwater are presented in Figures 15 and 16, respectively.

Limit of Quantitation (LOQ)

The limit of quantitation (LOQ) for the freshwater and saltwater method verification was calculated to be 0.0200 mg a.i./L based upon the product of the concentration of the lowest calibration standard (0.0200 mg a.i./L) and the dilution factor of the matrix blank samples (1.00).

Reagent and Matrix Blank Samples

Concurrent with each series of method verification samples, three reagent and three matrix blank samples were analyzed to determine possible interferences. No interferences were observed at or above the LOQ during the sample analyses for each matrix (Tables 2 and 3, respectively). Representative chromatograms of reagent blanks in freshwater and saltwater are presented in Figures 5 and 11, respectively. Representative chromatograms of matrix blanks for freshwater and saltwater are presented in Figures 6 and 12, respectively.

Method Verification Samples

Freshwater and saltwater verification samples were fortified at 0.0400, 0.400, 4.00 and 40.0 mg a.i./L. Freshwater samples yielded mean recoveries of 98.9%, 98.6%, 99.6% and 102%, respectively (Table 2). A representative chromatogram of a freshwater matrix fortification sample is presented in Figure 7. Saltwater samples yielded mean recoveries of 101%, 99.4%, 101% and 101%, respectively (Table 3). A representative chromatogram of a saltwater matrix fortification is presented in Figure 13.

Solubility Samples

Three solubility samples were prepared at 15, 30 and 40 mg/L using stock solutions in Dimethylformamide (DMF). The concentration of DMF in the samples was 0.1 mL/L. Duplicate subsamples from each of these solutions were collected at test initiation and approximately hour 24 (Table 4). One subsample from each concentration was analyzed directly, and one was centrifuged at 14000 rpm for approximately five minutes prior to analysis. At 0 hours, mean recoveries ranged from 50.9% to 94.5% and were inversely related to nominal concentration. At nominal concentrations of 30 and 40 mg/L, the measured concentration was approximately 20 mg/L at 0 hour. After 24 hours of mixing, mean recoveries ranged from 93.6% to 94.3% of nominal (Table 4). Additionally, three samples were prepared at 15, 30 and 50 mg/L without a solvent. Duplicate subsamples from each of the solutions were collected at approximately 18 and 24 hours. One subsample from each concentration was analyzed directly and one was centrifuged prior to analysis (Table 5). Mean recoveries of samples collected at 18 hours ranged from 95.1% to 96.9% and after 24 hours of mixing, mean recoveries ranged from 97.6% to 98.7% (Table 5). A representative chromatogram of a test sample is presented in Figure 17.

Example Calculations

The analytical result and percent recovery for freshwater verification sample number 232C-105-VMAS-3, nominal concentration of 0.0400 mg a.i./L, were calculated using the following equations:

$$\text{Flutriafol (mg a.i./L) in sample} = \frac{\text{Peak area - (Y-intercept)}}{\text{Slope}} \text{ X Dilution factor}$$

Peak area = 4.58033
Y-intercept = 0.0088
Slope = 113.8393
Dilution Factor = 1.00

$$\text{Concentration of Flutriafol (mg a.i./L) in sample} = \frac{4.58033 - 0.0088}{113.8393} \text{ X } 1.00$$

Concentration of Flutriafol in sample (mg a.i./L) = 0.0402

$$\text{Percent of nominal concentration} = \frac{0.0402 \text{ (mg a.i./L)}}{0.0400 \text{ (mg a.i./L)}} \text{ X } 100$$

Percent of nominal concentration = 100%*

* Results were generated using Excel 2000 in the full precision mode. Manual calculations may differ slightly.

Table 1

Typical HPLC Operational Parameters

INSTRUMENT:	Agilent Model 1100 High Performance Liquid Chromatograph (HPLC) equipped with an Agilent Series 1100 Variable Wavelength Detector			
ANALYTICAL COLUMN:	YMC-Pack ODS-AM (150 x 4.6 mm, 3-µm particle size)			
STOP TIME:	15.00 minutes			
FLOW RATE:	1.000 mL/min			
SOLVENT A:	0.1% H_3PO_4			
SOLVENT B:	CH_3CN			
GRADIENT ELUTION PROFILE:	Time (min)	%A	%B	Flow (mL/min)
	0.01	90.0	10.0	1.000
	1.00	90.0	10.0	1.000
	10.00	5.0	95.0	1.000
	11.00	5.0	95.0	1.000
	11.10	90.0	10.0	1.000
	15.00	90.0	10.0	1.000
OVEN TEMPERATURE:	40°C			
INJECTION VOLUME:	100.0 µL			
APPROXIMATE FLUTRIAFOL RETENTION TIMES:	Freshwater – 8.6 minutes Saltwater – 8.9 minutes			
WAVELENGTH:	220 nm			

METHOD OUTLINE FOR THE ANALYSIS OF FLUTRIAFOL IN FRESHWATER AND SALTWATER

Prepare calibration standards in both freshwater and saltwater using gas tight syringes and volumetric flasks.

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Prepare verification samples in freshwater and saltwater using gas tight syringes, volumetric flasks, volumetric pipettes and culture tubes. Freshwater and saltwater serves as the matrix blanks. HPLC-grade bottled water serves as the reagent blanks.

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Dilute samples, as necessary, with freshwater and saltwater such that the final sample concentrations fall within the calibration standard range.

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Transfer samples and standards to autosampler vials for analysis by HPLC/UV.

Figure 1. Analytical method flowchart for the analysis of Flutriafol in freshwater and saltwater.