1.0 PURPOSE

The purpose of this study was to validate methodology associated with the analysis of flutriafol in soil using methodology as described in Zeneca method RAM 057/04 with modifications by Morse Laboratories. The study followed the extraction procedure described in the method and used gas chromatography with mass spectrometry (GC/MS) detection.

2.0 SUMMARY

Zeneca method RAM 057/04 was evaluated for the analysis of flutriafol in soil. This evaluation was designed to meet requirements for independent laboratory validations (ILV) as described in U.S. EPA Ecological Effects Test Guidelines, OPPTS 850.7100 (1).

A total of fourteen matrix samples were analyzed as part of a method trial, consisting of two unfortified control samples and twelve control samples fortified with flutriafol. The fortification levels included seven samples fortified with flutriafol at the target limit of quantitation (LOQ) of 0.01 mg/kg and five samples fortified at 0.5 mg/kg.

The method was successfully validated during the third method trial.

3.0 EXPERIMENTAL DETAILS

3.1 Test Substance

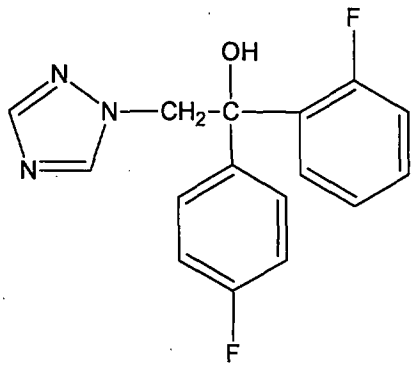
The test substance for this study was flutriafol.

3.2 Analytical Reference Substance

The test substance mentioned in the previous section also served as the analytical reference substance. Information regarding the test and reference substance is summarized below.

Flutriafol:

Chemical Name:	1H-1,2,4-Triazole-1-ethanol, α-(2-fluorophenyl)-α-(4-fluorophenyl)
CAS Number:	76674-21-0
Empirical Formula:	$C_{16}H_{13}F_2N_3O$
Molecular Weight:	301.29 g/mole
Lot No.:	ASJ-10005-01
GLP Purity:	99.0%
Expiration Date:	02 November 2009
Storage Conditions:	Freezer
Chemical Structure:	



A 0.994 mg/mL stock solution was prepared on 20 June 2008, by transferring 50.2 mg (uncorrected for purity) of flutriafol to a 50 mL volumetric flask and bringing to volume with acetone. Calibration standards were prepared from dilutions of the stock solution in acetone. A 1.01 mg/mL stock solution was prepared on 25 June 2008, by transferring 10.2 mg (uncorrected for purity) of flutriafol to a 10 mL volumetric flask and bringing to volume with acetone. Fortification solutions were prepared from dilutions of the stock solution in acetone.

All standard solutions were prepared using class A volumetric glassware and were stored in a refrigerator when not in use.

3.3 Method Summary

Control soil samples were weighed out at 20 ± 0.1 g per sample. The samples were fortified using the flutriafol spiking solutions in acetone at concentrations of 0.01 and 0.5 mg/kg. A volumetric pipette was used to add 100 mL of 70:30 acetonitrile:water (adjusted to pH 9 with ammonia solution) to each sample. The samples were refluxed for ~1 hour and allowed to cool. Aliquots of each sample were filtered under vacuum through 2 Whatman No. 5 filter papers, topped with ~10 g Celite® 545 filtering aid, in a funnel attached to a 500-mL side-arm flask. The filters were washed with 50 mL of 70:30 acetonitrile:water solution (pH 9). The samples were adjusted to 150 mL with the 70:30 acetonitrile:water solution (pH 9). An aliquot of each filtered sample was transferred to a 125-mL separatory funnel and 20 mL of pH 9 water plus 20 mL of methylene chloride were added for partitioning. The lower organic layer was filtered through anhydrous sodium sulfate and washed with further methylene chloride collected in a 125-mL flat-bottom flask. Partitioning was repeated twice for a total of three partitions. The sample was evaporated to dryness and the residual material redissolved in 2 mL of acetone. The samples were then sonicated and the acetone was transferred to a 15-mL graduated centrifuge tube. The flask was rinsed with 2 mL acetone and the two acetone fractions were combined. The acetone was evaporated with

nitrogen and 0.7 mL diethyl ether was added. The concentrated extract was passed through a conditioned SPE cartridge and the eluate discarded. Five, then fifteen milliliters of acetone:hexane (8:92, v/v) were passed through the SPE cartridge and the eluate discarded. Ten milliliters of ethyl acetate were eluted and collected in a test tube. The eluate was concentrated to ~0.2 mL using a nitrogen evaporator at ≤40°C and the remaining solvent was evaporated to dryness using manual nitrogen blowdown. Extracts were brought to final volume with acetone and analyzed by GC/MS.

3.4 Sample Receipt and Storage

ABC Laboratories received approximately 0.1488 g of flutriafol from Cheminova A/S, Lemvig, Denmark on 07 March 2008. The test substance was stored at –20°C.

Soil obtained from a terrestrial field trial in Georgia (America Agricultural Services, Inc., Study No.: AA060709) was used as the untreated control.

3.5 Instrument Conditions

GC/MS Analysis:

Analysis of samples was accomplished using an Agilent 6890 GC/MS System. The instrument parameters were as follows:

Instrument:	Agilent gas chromatograph Model 6890 equipped with a mass selective detector (SIM mode), and ChemStation software.
Column:	10 m x 0.18-mm i.d. fused silica column crossbonded with 0.40 µm film thickness, Rtx-35
Inlet liner:	4 mm i.d. single gooseneck splitless liner lightly packed with glass wool
Injection volume:	2 µL
Carrier gas:	Helium at 0.8 mL/min (constant flow)
Temperatures:	Column: Initial: 80°C Rate: 30°C/min. Final: 300°C, hold 5.50 minutes Detector: 300°C Injector: 250°C
Ions monitored:	Flutriafol: 164 m/z (quantitation) 219 m/z (confirmation) 123 m/z (confirmation)
Dwell:	100 ms
Retention time:	Flutriafol: ~6.7 minutes

3.6 Assignment of Sample Identification

All samples were assigned a unique sample identification number beginning with the five-digit ABC study number (63578) followed by a number that was assigned consecutively as samples were prepared, beginning with the sample number -1. For example, the first sample was assigned the unique identification of 63578-1, the second 63578-2, and so on.

3.7 Calculations

Concentrations of the test substance, flutriafol, were determined using the external standard analysis function of Analyst software. The concentrations of flutriafol in the samples prepared for GC/MS analysis were determined directly from the standard curve of flutriafol as shown in the equation below:

$$Y = mX + b$$

where:

Y = peak area in mVs

m = the slope of the line from the calibration curve

X = concentration of injected sample in ng/mL

b = the Y-intercept of the calibration curve

Example:

For the GC/MS analysis of the definitive low fortification Sample Low Spike-A (63578-30), the equation for the standard curve was:

$$Y = 88{,}900X - 83.5$$

The peak area (Y) for the injection was 907 mVs. Substituting the peak area (Y) in the following equation and solving for X gave the concentration of flutriafol:

$$X = (907 + 83.5) / (88{,}900)$$

A concentration value (X) of 0.00926 µg/mL was calculated by the Analyst. The concentration value (X) in ng/mL was then multiplied by the analysis volume (20 mL) and divided by the sample mass (20 g) to result in a final concentration of 0.00926 µg/mL.

The percent recovery was calculated by dividing the residue found by the actual fortification level:

$$\%\text{Recovery} = \frac{0.00926\ \text{ppm}}{0.010\ \text{ppm}} = 93\ \%$$

The linear regression curve of the method trial is presented in Figure 1. Representative chromatograms for the quantitation standards are presented in Figure 2 through Figure 6. A representative chromatogram for the control is presented in Figure 7. Representative chromatograms for control soil fortified at 0.01 and 0.5 ppm are presented in Figure 8 and Figure 9. Raw data summary sheets from the method trial are provided in Appendix 1.

5.0 COMMUNICATIONS

The following is a listing of communications, regarding performance of the method, which took place between the confirmatory laboratory and the Sponsor Representative. Included are reasons for the contact, any changes that resulted, and time of this communication with respect to the progress of the confirmatory trial (i.e., before the first trial, during the first trial, etc.):

1) 25 January 2008: Paul Whatling, Study Monitor, Cheminova, Inc., sent an email to Del Koch, Principal Scientist-Chemical Services, ABC Laboratories, Inc. providing the ILV analytical method to be used in the study. (Before first trial)

2) 04 February 2008: An email message was sent from Del Koch to Paul Whatling requesting confirmation that the method was to be performed as it had been applied by Morse Laboratories to soil dissipation study samples, based upon Morse's modifications to RAM 057/04. In particular, the determinative step would be by GC/MSD rather than GC/NPD. (Before first trial)

3) 05 May 2008: An email message was sent from Paul Whatling to Tom Leak, Study Director and Senior Chemist-Chemical Services, ABC Laboratories, Inc. requesting the protocol be modified to accommodate an additional modification made to the method. (Before first trial)

4) 05 May 2008: Paul Whatling copied Tom Leak on an email to Robert Butz, Staff Scientist, Keller and Heckman requesting authorization to use soil from Georgia for the ILV work. (Before the first trial)

5) 05 May 2008: Robert Butz copied Tom Leak on an email reply to Paul Whatling indicating that the Georgia soil should be fine for the ILV work. (Before the first trial)

6) 30 May 2008: Kevin Lucash, Chemist-Chemical Services, ABC Laboratories, Inc. sent an email to Paul Whatling requesting clarification on a few items in the method, including the type of ammonia solution to use, whether activated or non-activated SPE cartridges are required, and what exactly is meant by "manual nitrogen blowdown". (Before the first trial)

7) 02 June 2008: Paul Whatling responded to Kevin Lucash in a forwarded email from Frances Brookey, Morse Laboratories, Inc. with answers to all questions. (Before the first trial)

8) 04 June 2008: Kevin Lucash sent an email to Paul Whatling requesting confirmation on the type of flask to be used. (Before the first trial)

9) 04 June 2008: Paul Whatling responded to Kevin Lucash in an email indicating that a 250-mL flat-bottom flask should be used. (Before the first trial)

10) 06 June 2008: Tom Leak emailed Paul Whatling asking if the Florisil SPE cartridges could be baked prior to use. (Before the first trial)

11) 09 June 2008: Paul Whatling forwarded an email to Tom Leak and Kevin Lucash with a response from Frances Brookey indicating that the florisil SPE cartridges should not be baked but recommended other items that could prove helpful to the analyst. (Before the first trial)

12) 09 July 2008: Paul Whatling sent an email to Tom Leak requesting an update on the ILV study. (Before the first trial)

13) 14 July 2008: Tom Leak replied to Paul Whatling's email attaching a summary of the first run of the ILV and indicating that the first trial was unsuccessful due to uniformly low recoveries for the 0.01 mg/kg level and the fact that two of the 0.5 mg/kg levels did not meet acceptance criteria. Tom laid out a plan for improving the second trial. (After the first trial)

14) 17 July 2008: Tom Leak sent Paul Whatling an email message summarizing the results of a previous phone conference regarding the method changes and details for the next set of samples. It was specified that samples should be run from spiking to instrument-ready in one day with no overnight storage. It was also noted that each set would contain at least one control, two low spikes, and 2 high spikes. Also stipulated was the fact that the sample should be reconstituted and applied to the florisil cartridges immediately after complete evaporation, and dry air evaporation should be replaced with nitrogen. (Before the second trial)

15) 05 August 2008: Bob Plastridge forwarded a summary of the results from the second trial of the ILV to Paul Whatling via email. Bob indicated that the results were mostly within the acceptable range

Bob requested a reply as to whether or not this data was acceptable for the ILV. (After the second trial)

21) 19 September 2008: Paul Whatling responded via email authorizing performance of a third trial for the ILV and asked that ABC proceed as soon as possible. He reiterated that per Tom's discussion with Cheminova's consultant, the GC column conditioning for the trial would involve the use of multiple injections of a spiked sample until a constant response was obtained from the GC before proceeding with the ILV samples. (Before the third trial)

22) 01 October 2008: Tom Leak attached results from the third ILV trial to an email sent to Paul Whatling and indicated that the set looked good. He stated his plan to draft a report by October 17 and hoped to have the final report by October 31. (After the third trial)

6.0 DISCUSSION AND CONCLUSION

Recoveries for the first and second trials proved to be unacceptable. Acceptable recoveries were obtained for fortified untreated control samples at three fortification levels on the third method trial using the method of extraction as modified under Sponsor supervision and these results were communicated to the Study Monitor.

7.0 COMMENTS ON THE METHOD

Method modifications were required to yield acceptable results. First, samples needed to be run from spiking to instrument-ready in one day with no overnight storage. This was to ensure that no storage-related issues, such as evaporation of solvents, affected the results. Secondly, each set had to contain at least one control, two low spikes, and 2 high spikes. This was done to ensure that no bias occurred relative to fortification level. Third, the samples had to be reconstituted and applied to the florisil cartridges immediately after complete evaporation, and dry air evaporation was replaced with nitrogen. Finally, the GC column conditioning for the trial involved the use of multiple injections of a spiked sample until a constant response was obtained from the GC before proceeding with the ILV samples. After these modifications, the third trial yielded acceptable results.

Approximately 9.25 person-hours were required to aliquot, fortify, and extract a set of 8 samples. Instrumental analysis of the validation samples was based on an injection sequence starting with three calibration standard injections, followed by four sample injections between calibration standard injections, and ending with four calibration standard injections. With a per sample run time of approximately 17 minutes, instrumental acquisition time is approximately 7 hours for a 25-injection run sequence. Therefore, a total of 16.25 hours was required to complete one set of samples, or two calendar days. Time associated with standard preparation, laboratory cleanup, data work up, and data checking is not included in this total.

8.0 PROTOCOL DEVIATIONS

None