III. MATERIALS AND METHODS

A. Test and Reference Substances

The analytical reference standards were received in good condition on September 22, 2005, from:

Valent Technical Center
6560 Trinity Court
Dublin. California 94568-2628

The following standards along with Certificates of Analyses and MSDS' were received and stored frozen for the duration of the study:

Analytical Standards	Description	Lot #	Purity (%)	Expiration Date
Fluopicolide CAS# 239110-15-7	Beige crystalline solid	AS 2174a	98.9	08/16/06
BAM CAS# 2008-58-4	Beige crystalline solid	0315200501	96.2	09/15/07
PCA CAS# 80914-68-9	White crystalline solid	0926200304	97.2	04/14/08

Upon receipt, the neat fluopicolide, BAM and PCA standards were stored in a freezer maintained at <0°C. As per the analytical method, solutions of fluopicolide, BAM and PCA were prepared in acetonitrile to serve as the stock solutions. Subsequent dilutions of the stock solutions were prepared in acetonitrile for use as the spiking solution and in water:acetonitrile:formic acid (95:5:0.1) for use as HPLC reference standards. The spiking solution contained all three compounds. Preparation and dilution data forms pertaining to the stock and working solutions are located in the raw data. All stock and working solutions were stored refrigerated (~4°C ± 5°C) when not in use.

B. Reagents and Equipment

See Appendix A – Study Protocol and Analytical Method for a list of Reagents and Equipment used for this study.

C. Untreated Control (UTC) Material

Golden Pacific Laboratories, LLC provided the water samples suitable for use as untreated control material. Water samples were taken as needed from the tap.

D. Processing Procedures

No homogenization of the untreated water sample was required.

E. Analysis Method

1. Method Summary: Water

All samples for the independent lab validation were analyzed according to Bayer Analytical Method AR 307-03 entitled "Analytical method AR 307-03 for the determination of AE C638206 and its metabolites AE C653711 and AE 657188 in water" dated November 13, 2003 (Appendix A). The procedure is summarized as follows:

Twenty-mL portions of tap water were transferred to 60-mL glass tubes. Aliquots of 25 µL of formic acid were added to the water samples. Control samples were fortified with fluopicolide, BAM and PCA at appropriate levels (i.e. 0.1 µg/L or 1.0 µg/L).

Twenty-mL portions of ethyl acetate were added to the water samples. Samples were placed on a platform shaker at room temperature for 5 minutes at ~200 rpm. Samples were removed from the platform shaker and the phases were allowed to separate. After phase separation, 10-mL aliquots of the ethyl acetate were transferred to 15-mL culture tubes. Samples were evaporated to dryness using a gentle stream of nitrogen.

Samples were re-constituted in 2-mL of water:acetonitrile:formic acid (95:5:0.1) and sonicated for two to five minutes. The samples were transferred to chromatography vials and submitted for analysis by HPLC with MS/MS detection.

2. Instrument Parameters for Fluopicolide and BAM

Instrument:	Sciex API 3000 HPLC/MS/MS with two Shimadzu LC-10AD HPLC Pumps, Shimadzu SCL-10A Controller and Perkin Elmer PE-200 Autosampler

HPLC Column:	Phenomenex Aqua 3µ C18 125A (150 mm x 2.0 mm), Catalog #00F-4311-B0
Data System:	Analyst Chromatography Data System version 1.3
Ionization and MS Mode:	Turbo Ion Spray in Positive Ion Mode MS/MS with multiple reaction monitoring (MRM)
Mobile Phase Flow Rate:	300 µL/minute
Mobile Phase Program:	at 0.0 minutes 50% acetonitrile:50% 0.1% formic acid in water at 3.0 minutes 60% acetonitrile:40% 0.1% formic acid in water at 5.0 minutes 60% acetonitrile:40% 0.1% formic acid in water at 5.2 minutes 50% acetonitrile:50% 0.1% formic acid in water

Mass Spectrometer Parameters:

Transition Ions:

Fluopicolide	383.0/173.0
BAM	191.1/173.0

Nebulizer Gas (NEB):	8
Curtain Gas (CUR):	8
Collision Gas (CAD):	9
Ionspray Voltage (IS):	5500
Temperature (TEM):	475
Entrance Potential (EP):	10
Declustering Potential (DP):	51
Focusing Potential (FP):	275
Collision Energy (CE):	31
Collision Cell Exit Potential:	10

Run Time: 9.0 min

Retention Times:

Fluopicolide	~6.20 min
BAM	~2.18 min

Injection Volume: 50 µL

3. Instrument Parameters for PCA

Instrument:	Sciex API 3000 HPLC/MS/MS with two Shimadzu LC-10AD HPLC Pumps, Shimadzu SCL-10A Controller and Perkin Elmer PE-200 Autosampler
HPLC Column:	Thermo Electron BetaBasic 18 3µ (100 mm x 2.1 mm), Catalog #71503-102130
Data System:	Analyst Chromatography Data System version 1.3
Ionization and MS Mode:	Turbo Ion Spray in Negative Ion Mode MS/MS with multiple reaction monitoring (MRM)
Mobile Phase Flow Rate:	200 µL/minute
Mobile Phase Program:	Isocratic 10% acetonitrile:90% water

Mass Spectrometer Parameters:

Transition Ions:

PCA	224.0/180.0

Nebulizer Gas (NEB):	8
Curtain Gas (CUR):	8
Collision Gas (CAD):	9
Ionspray Voltage (IS):	-4500
Temperature (TEM):	475
Entrance Potential (EP):	-10
Declustering Potential (DP):	-31
Focusing Potential (FP):	-170
Collision Energy (CE):	-14
Collision Cell Exit Potential:	-8

Run Time: 10.0 min

Retention Time:

PCA	~5.28 min

Injection Volume: 100 µL

F. Quantitation Procedures

Analyst Chromatography Data System version 1.3, a product of PE Sciex, was used to acquire, integrate and calculate the concentrations of fluopicolide, BAM and PCA in water using Analyst's linear regression function with weighting. For the regression calculations, concentration was designated as the independent variable and plotted on the x-axis. Peak response was designated as the dependent variable and plotted on the y-axis. From this regression curve, a slope, y-intercept and correlation coefficient of the standard curve run with each analytical set were calculated. The correlation coefficients were all greater than 0.990. Fluopicolide, BAM and PCA calibration standards were injected every one to five sample injections, as well as at the beginning and ending of the injection sequence. Six different standard concentrations ranging from 0.189 to 24.7 ng/mL were injected with each analysis set. The analyte concentrations in the sample extracts were extrapolated from the curve equation. The concentration, as µg/L, of residue found in the samples were then calculated by Microsoft Excel using the following equation:

$$\mu g/L = \frac{(ng/mL \text{ from curve}) \times (\text{Final Volume in mL}) \times (1000 \text{ mL}) \times (1 \mu g)}{(\text{Aliquot Volume in mL}) \times (1 \text{L}) \times (1000 \text{ ng})}$$

Recovery of fluopicolide, BAM and PCA from fortified samples was calculated as follows:

$$\%Recovery = \frac{(\text{Measured Concentration, } \mu g/L)}{(\text{Theoretical Concentration, } \mu g/L)} \times 100$$

An example calculation for a low level fluopicolide laboratory fortification in set 203ILV02, sample 203ILV02-6, Low Spike C at 0.0988 µg/L, is as follows:

standard curve equation: y = 1.3e+005 (x) – 6.55e+003, (1/x) weighting
where x = fluopicolide concentration in ng/mL and
y = peak response = 57482.1
fluopicolide concentration from the curve = 0.491 ng/mL

$$\mu g/L = \frac{(0.491 \text{ ng/mL fluopicolide}) \times (2 \text{ mL}) \times (1000 \text{ mL}) \times (1 \mu g)}{(10 \text{ mL}) \times (1 \text{L}) \times (1000 \text{ ng})} = 0.0982 \mu g/L$$

$$\%\text{ recovery} = \frac{0.0982 \mu g/L}{0.09880 \mu g/L} \times 100 = 99.4\%$$

Fortification samples were not corrected for residues detected in control samples. All unfortified control samples were reported as <LOQ.

B. Problems Encountered

There were no problems with the extraction procedure as written in the method. A few problems occurred on the HPLC/MS/MS during the analysis of the samples for PCA. The high standard for PCA had to be dropped due to poor peak shape. It was later determined that the high standard did not chromatograph very well due to the amount of acetonitrile present in the standard (~25%). When the standard was prepared for the first trial, it had ~2.5% of acetonitrile and a nice peak shape was obtained. This was later confirmed when the standard was prepared again using ~2.5% of acetonitrile, a nice peak was obtained and it was linear with the other calibration standards.

Another problem occurred with the linearity of PCA. The lowest standard response was high when back calculated with the standard curve (RPD = 13.8%). Since this low standard was heavely weighted, the responses for low spike samples and the second lowest standard (approximately equivalent to the low spike samples) were low. If this standard were to fit better on the curve, then the low spike samples would have a higher recovery (~10% higher).

C. Critical Steps

1) A critical step in this method is during the fortification procedure. When following the method as written when preparing the fortification solution, a very small amount (2 µL) of this solution has to be used to prepare the low laboratory fortification samples. For the second trial, a more dilute solution was prepared for use as the fortification solution so a larger volume (20 µL) could be used for fortifying the low fortification samples.

2) A second critical step as indicated by the method is that the calibration solutions need to be prepared weekly.

D. Time Requirement

Five hours are required for one person to prepare an analysis set from the time samples are prepared to HPLC analysis. Automated HPLC analysis can be performed overnight. An additional 1.5 hours may be spent on data calculation and tabulation the following day.

E. Description of Contact

The initial contact with the Sponsor Monitor occurred upon completion of the first validation trial. Some samples had recoveries greater than 120%, and responses, though less than the LOQ, were found in the Reagent Blank and one Control sample, which indicated laboratory contamination. After demonstrating that the reagent blanks were clean (triplicate samples), the Sponsor Representative approved of a second attempt at the validation. The fortifying standards and calibration standards were prepared again from the stock solution. The Sponsor Monitor also approved the preparation of a more dilute fortifying solution so a larger volume could be used to fortify the water samples.

Upon completion of the second validation trial, one compound, PCA, had recoveries less than 70% in three low spike samples. The calibration standard at which the samples were supposed to be at had a RPD value of 14.1%. By performing a point-to-point calculation with the calibration standard and the low spike samples, the recoveries of the low spike samples all increased by ~10%, resulting in all of the samples having acceptable recoveries. The Sponsor Monitor was contacted and after reviewing the data, it was decided to run the samples again to see if a better curve for this compound could be obtained. A second analysis of the water samples for PCA yielded similar results. The results were sent to the Sponsor Monitor for review. On October 24, 2005, the Sponsor Monitor decided that the first analysis of PCA would be accepted.

VI. CONCLUSIONS

In conclusion, Bayer Crop Science Analytical Method entitled "Analytical method AR 307-03 for the determination of AE C638206 and its metabolites AE C653711 and AE 657188 in water" was successfully validated within the guidelines of EPA Ecological Effects Test Guidelines, OPPTS 850.7100, Data Reporting for Environmental Chemistry Methods (EPA Draft Guideline for the Independent Laboratory Validation) in two attempts for fluopicolide, BAM and PCA. The Limit of Quantitation stated in the method (0.1 µg/L each analyte) was validated by spiking samples at this level and achieving an average of greater than 70% recovery

for all three analytes. The low recoveries of PCA for some of the low spike samples was accepted since the average for the recoveries was >70% with a Standard Deviation of 5.61%.

VII. CHANGES TO THE PROTOCOL

A protocol deviation occurred when the high standard was dropped from the calibration curve during the analysis of PCA on the second attempt. All samples were quantitated using a curve generated from the remaining standards.

VIII. DATA STORAGE AND RECORDS RETENTION

All original raw data, or certified copies thereof, and summaries of data specific to this study will be transferred to the Sponsor upon issuance of the final report for archiving. Original standard preparation, compound receipt and usage, equipment maintenance logs, personnel training records, GPL SOP's, and quality assurance records will be archived at GPL. A copy of the final report and raw data will also be archived at GPL.