ABSTRACT

Enviro-Test Laboratories performed an independent laboratory validation (ILV) of Syngenta Analytical Method No. 6179-04. This is a LC/MS/MS method for the analysis of Mesotrione and its degradates AMBA and MNBA in well water and surface water by direct injection. Water samples were fortified with three analytes and analyzed according to the Analytical Method No. 6179-04. Analysis sets consisted of one reagent blank, two controls, five control samples fortified at the limit of quantitation (LOQ) of 0.05 ppb, and five control samples fortified at 10 times the LOQ of (0.50) ppb.

The method was successfully validated for each of the analytes at their respective LOQ and 10 x LOQ concentration levels for both matrices. The mean recovery, relative standard deviation (RSD) and 95% confidence interval were reported for each sample set. In addition, the statistics for the overall (LOQ and 10 x LOQ) results for each analyte/matrix combination were reported.

For all analytes the average recoveries were between 70 and 120 percent of theoretical with relative standard deviations of less than 20 percent.

1.0 INTRODUCTION

This report describes the independent laboratory validation (ILV) of Analytical Method No. 6179-04 as performed by Enviro-Test Laboratories for the determination of the Mesotrione and its degradates AMBA and MNBA in well water and surface water by direct injection using High Performance Liquid Chromatography with Mass Spectrometric Detection (LC/MS/MS).

This study was conducted to satisfy guideline requirements described in the US EPA FIFRA Pesticide Assessment Guidelines for Subdivisions N, E, and K, and addenda for Data Reporting Guideline for Environmental Methods [2]. It also satisfies the requirements outlined in the harmonized guidelines from the OPPTS, "Public Draft" - Data Reporting for Environmental Chemistry Methods, OPPTS 850.7100 [3].

2.0 STUDY PERSONNEL

The following personnel from Enviro-Test Laboratories participated in the conduct of this study.

Gary Bruns, replaced by Norm McLean*	Study Director
Russell Gottschalk	Senior Scientist
Narinder Bains	Residue Analyst
Meselu Abetew	Residue Analyst
Susan Nelson	Project Manager
Danuta Raszek	Log-In and Sample Control

* as per amendment #1.

3.0 MATERIALS

3.1 Test and Reference Substances

Reference substances were shipped from Syngenta US to Enviro-Test Laboratories and were received on Feb. 6, 2001 and Aug. 27, 2004 (see the Standard disposition sheets in the Raw Data package). The following substances were used:

Compound	Lot Number	Purity (%)	Expiration Date
Mesotrione (ZA 1296)	ASW01788-01R	99.6	Jan. 2008
AMBA (NOA 422848)	DAH-XXIX-4	97.4	April 30, 2005
MNBA (NOA 437130)	DAH-XXIX-5	>99.9	Feb. 28, 2006

The reference substances were logged in and then kept stored in a freezer after arrival at ETL. Syngenta Crop Protection, Inc. maintains the characterization and stability data for the reference substances.

3.2 Control Waters

3.2.1 Control Well Water

The control well water was obtained from a well in Alberta on February 22, 2004, owned by Gary Bruns located in a rural area near Rimbey, Alberta. The water was characterized under GLP for pH, hardness, turbidity, calcium, magnesium, sodium conductivity, sodium absorption ratio (SAR), total suspended solids and total dissolved solids. A certified copy of the characterization report can be found in Appendix 2.

Ground Water Characterization	
pH	7.6
Sodium	263 ppm
Calcium	40 ppm
Magnesium	14 ppm
Hardness mg equivalent CaCO3/L	159 ppm
Conductivity	1.360 mmhos/cm
Sodium Absorption Ratio (SAR)	9.1
Total Dissolved Solids	470 ppm
Total Suspended Solids	<3 ppm
Turbidity	0.20 NTU
Alkalinity	572 mg CaCO3/L

1. Enviro-Test Laboratories Report – L162007-1, also identified as L1294-1 in report.

L1294-1 is also referred to as L1365-1.

3.2.2 Control Surface Water

The control surface water was obtained from Gull Lake in Alberta on February 2, 2005, located in a rural area near Rimbey, Alberta. The water was characterized under GLP for pH, hardness, turbidity, calcium, magnesium, sodium conductivity, sodium absorption ratio (SAR), total suspended solids and total dissolved solids. A certified copy of the characterization report can be found in Appendix 2.

Surface Water Characterization	
pH	9.1
Sodium	246 ppm
Calcium	11.8 ppm
Magnesium	77.6 ppm
Hardness mg equivalent CaCO3/L	349 ppm
Conductivity	1540 uS/cm
Sodium Absorption Ratio (SAR)	5.9
Total Dissolved Solids	1020 ppm
Total Suspended Solids	<3 ppm
Turbidity	0.75 NTU
Alkalinity	824 mg CaCO3/L

2. Enviro-Test Laboratories Report – L243276-4, also identified as L1406-1.

3.3 Equipment and Reagents

The equipment and reagents used for the method validation were as outlined in 6179-04 (Section 2.0 Materials and Methods). Identical or equivalent apparatus and materials were used.

4.0 METHOD AND METHOD MODIFICATIONS

4.1 Modifications

Trial 1 - Well Water

No modifications were made to the fortification and sample preparation sections of the method during Trial 1 for well water. The method was performed as written. As a result of the difference in LC/MS/MS systems the following specific modifications to the method are noted:

1. A PE Sciex API 4000 MS/MS system was used in place of the Micromass Quatro Ultima. The instrument specifications are listed in Table 1 and Table 2.
2. Increase of injection volume to 75 µL.

Trial 2 - Well Water

The following modifications were made to the method for Trial 2 in Well Water:

1. Mixed analyte fortification solutions were prepared in methanol at 10.0, 0.100, 0.0500 and 0.00500 ppm not in 5% ACN/0.1% acetic acid. The new mixed standards in methanol at 50.0 and 5.00 ppb were used for fortification. The solubility of MNBA in 5% ACN/0.1% acetic acid was determined to be poor.
2. Increase of injection volume to 100 µL.

Trial 1 - Surface Water

The following modifications were made to the method for Trial 1 in Surface Water:

1. Mixed analyte fortification solutions were prepared in methanol at 10.0, 0.100, 0.0500 and 0.00500 ppm not in 5% ACN/0.1% acetic acid. The new mixed standards in methanol at 50.0 and 5.00 ppb were used for fortification. The solubility of MNBA in 5% ACN/0.1% acetic acid was determined to be poor.
2. Increase of injection volume to 100 µL.

4.2 Standard Solution Preparation

Standard solutions were prepared as indicated in the method. The exact scheme of preparation is listed below:

Stock Solutions

A separate stock solution for each analyte was prepared by weighing the analytical reference standard (10 mg weighed accurately) into 100 mL volumetric flasks and diluting with methanol to achieve an initial concentration of about 100 µg/mL. The exact concentration is determined based on the exact weight and purity in each case.

Trial 1 – Well Water

Mixed Analyte Solution

Aliquots of approximately 1 mL (used exact volume needed to give a final concentration of 10.0 ppm) of the 100 µg/mL individual stock solutions of Mesotrione, MNBA, and AMBA were combined and diluted with 5% ACN/0.1% acetic acid in water to 10.0 mL to prepare a 10.0 ppm mixed solution. The mixed solution was used to prepare the fortification solutions and the calibration standards solutions.

Mixed Analyte Fortification Solutions

A mixed analyte fortification solution of 100 ppb was prepared by taking a 0.100 mL aliquot of the 10.0 ppm mixed analyte solution (described in the preceding section) and diluting to 10.0 mL with 5% ACN/0.1% acetic acid in water.

A mixed analyte fortification solution of 50.0 ppb was prepared by taking a 0.050 mL aliquot of the 10.0 ppm mixed analyte solution (described in the preceding section) and diluting to 10.0 mL with 5% ACN/0.1% acetic acid in water.

A mixed analyte fortification solution of 5.00 ppb was prepared by taking a 1.00 mL aliquot of the 50.0 ppb mixed analyte solution and diluting to 10.0 mL with 5% ACN/0.1% acetic acid in water.

LC/MS/MS Calibration Solutions

Instrumental calibration standards were prepared by dilution of the mixed standards according to the following scheme:

Starting Solution Concentration (ng/mL)	Volume Used (mL)	Final Volume (mL)	Final Concentration (ng/mL)
100	0.100	10.0	1.00
5.00	1.00	10.0	0.500
5.00	0.500	10.0	0.250
1.00	1.00	10.0	0.100
0.500	1.00	10.0	0.0500
0.500	0.500	10.0	0.0250

Dilutions were to 10.0 mL with 5% ACN/0.1% acetic acid in water. When stored the fortification solutions and calibration standards were placed in a cooler at 4°C.

Trial 2 – Well Water

Mixed Analyte Solution in Methanol

Aliquots of approximately 1 mL (used exact volume needed to give a final concentration of 10.0 ppm) of the 100 µg/mL individual stock solutions of Mesotrione, MNBA, and AMBA were combined and diluted with methanol to 10.0 mL to prepare a 10.0 ppm mixed analyte solution. The mixed standard solution was used to prepare the fortification solutions and the calibration standards solutions.

Mixed Analyte Fortification Solutions in Methanol

The solutions were prepared in the same way as Trial 1 except the solutions were diluted to volume with methanol.

Trial 1 – Surface Water

Mixed Analyte Solution

Aliquots of approximately 1 mL (used exact volume needed to give a final concentration of 10.0 ppm) of the 100 µg/mL individual stock solutions of Mesotrione, MNBA, and AMBA

were combined and diluted with 5% ACN/0.1% acetic acid in water to 10.0 mL to prepare a 10.0 ppm mixed solution. The mixed solution was used to prepare the fortification solutions and the calibration standards solutions.

Mixed Analyte Fortification Solutions

The solutions were prepared in the same way as Trial 1for Well Water except the solutions were diluted to volume with methanol.

LC/MS/MS Calibration Solutions

Instrumental calibration standards were prepared by dilution of the mixed standards according to the following scheme:

Starting Solution Concentration (ng/mL)	Volume Used (mL)	Final Volume (mL)	Final Concentration (ng/mL)
100	0.100	10.0	1.00
5.00	1.00	10.0	0.500
5.00	0.500	10.0	0.250
1.00	1.00	10.0	0.100
0.500	1.00	10.0	0.0500
0.500	0.500	10.0	0.0250

Dilutions were to 10.0 mL with 5% ACN/0.1% acetic acid in water. When stored the fortification solutions and calibration standards were placed in a cooler at 4°C.

LC/MS/MS Calibration Standards Solutions in 5 % ACN/0.1% Acetic Acid

Instrumental calibration standards were prepared by dilution of the mixed standards according to the following scheme:

Starting Solution Concentration (ng/mL)	Volume Used (mL)	Final Volume (mL)	Final Concentration (ng/mL)
100	0.100	10.0	1.00
5.00	1.00	10.0	0.500
5.00	0.500	10.0	0.250
1.00	1.00	10.0	0.100
0.500	1.00	10.0	0.0500
0.500	0.500	10.0	0.0250

Dilutions were to 10.0 mL with 5% ACN/0.1% acetic acid in water. When stored the fortification solutions and calibration standards were placed in a cooler at 4°C.

4.3 Sample Preparation, Fortification, and Extraction

A validation trial consisted of an analytical set. A set was comprised of 13 samples: one reagent blank, two matrix blanks, five matrix blanks fortified at the LOQ (0.050 ppb) and five matrix blanks fortified at 10 x LOQ (0.50 ppb).

Twelve 9.9-mL portions of the control water were used as samples. Samples designated as "spikes" were fortified with either 100 µL of a 5.00-ppb standard fortification solution (for LOQ fortifications) or 100 µL of 50.0 ppb (for 10 x LOQ fortifications). Spikes were mixed and let stand 10 minutes. See detailed method below:

Extraction:

1. Measure 10.0 mL sample of water into 15 mL screw top glass test tubes.
2. Add 1 drop of glacial acetic acid. Cap with teflon lined cap and mix.
3. Remove 100 uL of sample. Discard.
4. Fortify spike sample at this point.
5. Re-cap and mix well.
6. Transfer an aliquot of sample into autosampler vial.
7. Analyze by LC/MS/MS.

4.4 LC/MS/MS Instrumentation

All samples were analyzed using an Applied Biosystems Sciex API-4000 Triple Quadrupole Mass Spectrometer with APCI Interface. The following components pleted the system:

HPLC:	Two Perkin Elmer Series 200 Micropumps
Autoinjector:	CTC HTS PAL
Column Heater:	Waters Temp. Control module
Data System:	Dell Precision 360, Intel(R) Pentium Computer, 4 CPU 3.00 GHz running Microsoft Windows 2000 Version 5 and Analyst Version 1.4

The HPLC operating parameters are shown in Table 1. The API 4000 MS/MS operating parameters are shown in Table 2.

4.5 Data Acquisition and Reporting

Peak integration and quantitation were performed by using Analyst, Version 1.4 (Applied Biosystems). Analytes were quantitated by external calibration. The MS detector response (peak area) was plotted versus the standard concentration and used to generate calibration curves for each analyte. Best-fit linear regression equations for the curves were derived and these equations were used to calculate the concentration of analytes in the samples. Mesotrione and AMBA calculations were based on a linear regression. MNBA calculations were based on a linear through zero regression for well water and

linear regression for surface water. LOQ fortifications were unacceptable for well water with a linear regression as a result of a negative intercept contribution. Recovery results were computed for each sample. The equations used for quantitation are presented in Table 10.

Statistical treatment of the data includes calculation of averages, standard deviations, relative standard deviations and confidence limits. The calculations were performed using Excel 97 SR-2. Results were rounded off for reporting purposes but not during calculations.

During the establishment phase the API 4000 was optimized for the analytes. Standards were injected to determine the analyte retention times, their instrument detection limits and their linearity of response. It was noted on the API 4000 system that the primary ion transition listed for AMBA (214->170) showed significant interference and that it was very difficult to reduce the background signal present in the ion trace. The alternate transition 214->155 was much cleaner and gave superior signal to noise. Both transitions were monitored, but the 214->155 trace was chosen for quantitation.

After the method was determined to be under control and the instrument performance assessed the first method trial was initiated.

It was subsequently determined that MNBA is poorly soluble in a 5% ACN/0.1% acetic acid solution. The fortification stock at 50.0 ppb was analyzed and the concentration of MNBA was similar to the concentration in the 5.00 ppb stock. After consultation with the Study Monitor it was concluded that the solubility of MNBA in 5%ACN/0.1% acetic acid was probably poor and that dilutions of the fortification stocks should be made in methanol rather than in a 5% ACN/0.1% acetic acid solution.

5.5 Critical Steps

There are two critical steps noted in the method:

1. The optimization of the ESI interface may require some time and patience. Position of the spray tip and the interface temperature were of particular importance. The conditions detailed in Table 2 are suitable starting points but they may require changes to achieve the required sensitivity.

2. It was determined that the solubility of MNBA in 5% ACN/0.1% acetic acid was variable at a level in excess of 5.00 ppb. Fortification standards were prepared in methanol as opposed to the 5% ACN/0.1% acetic acid listed in the method. Solutions suitable for instrument calibration at levels lower than 5.00 ppb can be prepared in 5% ACN/0.1% acetic acid.

5.6 Time Required for Analysis

For each matrix, a total of 2 person-hours are required to fortify and complete one set of 13 samples. The HPLC instrumental data analysis requires approximately 15 minutes per run. A sample set can be analyzed in about 6 hours.

5.7 Communication with Study Sponsor

The Independent Laboratory Validation of Analytical Method 6179-04 required communication with the Study Monitor to clarify the optimization of the API 4000 instrument and to clarify issues regarding the solubility of MNBA in the fortification solutions. See Table 9.

8.0 TABLES

Table 1. HPLC System

PE LC-200 Micro Pumps
Minimum Pressure (psi): 0.0
Maximum Pressure (psi): 3500.0
Shutdown Time (min): 30.0
Guard Column: Phenomenex Polymerx RP-1
Analytical Column: Phenomenex PLRP-S, 4.6 x 50 mm, 5 µm
Mobile Phase: A = 0.1% acetic acid in HPLC water
B = Acetonitrile (ACN)

Step Table:

Step	Total Time (min)	Flow Rate (µl/min)	Gradient Profile	A (%)	B (%)
0	1.0	500.00	0.0	98.0	2.0
1	0.5	500.00	0.0	98.0	2.0
2	5.0	500.00	1.0	5.0	95.0
3	8.0	500.00	0.0	5.0	95.0
4	12.0	500.00	0.0	98.0	2.0

Gradient Profile: 0.0 = isocratic
1.0 = linear
Injection Volume: 100 uL

Analyte Retention times:

Analyte	Min.
Mesotrione	5.20
MNBA	3.38
AMBA	4.12

Table 2. LC/MS/MS Operating Parameters

The samples were analyzed using negative ion detection. The MRM (multiple reaction monitoring) scan mode was used for the signal acquisition.

Interface:	ESI
Polarity:	Negative
Nebuliser Gas (GS1):	60
Turbo Gas (GS2):	60
Curtain Gas (CUR):	10 (arbitrary units)
Temperature (TEM):	700
IS:	-4000
Collision gas (CAD):	Nitrogen 6 (arbitrary units)
Scan type:	MRM

Table 2 cont'd

Period 1:

Scans in Period:	357
Relative Start Time:	2 minutes
Experiments in Period:	1

Period 1 Experiment 1:

Scan Type:	MRM (MRM)
Polarity:	Negative
Scan Mode:	N/A
Ion Source:	Turbo Spray
Resolution Q1:	Unit
Resolution Q3:	Unit
Intensity Thres.:	0 cps
Settling Time:	0 msec
MR Pause:	10 msec
MCA:	No
Step Size:	0.00 amu

Q1 Mass (amu)	Q3 Mass (amu)	Dwell(msec)	Parameters	
337.90	291.00	200.00	DP	-55.00
			EP	-10.00
			CE	-14.00
			CXP	-7.00
243.70	199.80	200.00	DP	-30.00
			EP	-6.00
			CE	-10.00
			CXP	-11.00
213.80	170.10	200.00	DP	-20.00
			EP	-6.00
			CE	-20.00
			CXP	-9.00
213.80	155.10	200.00	DP	-25.00
			EP	-6.00
			CE	-28.00
			CXP	-1.00

Table 13. Calculations

All results were reported on a weight/volume basis.

- Calculation of Analyte Concentration

$$\text{Results (ng/mL)} = \left[\frac{(\text{P.A.} - \text{y intercept})}{\text{Slope}}\right] \times \frac{\text{F.V.}}{\text{Volume}}$$

Where:
P.A. = peak area of the analyte of interest
y-intercept = y-intercept of a linear plot of concentration vs. peak area
Slope = slope of a linear plot of concentration (ng/mL) vs. peak area
F.V. = final volume of extract (mL)
Volume = Volume of sample (mL)

Calibration standards covering a minimum of six concentration levels for each of the analytes were included in an analysis set. The injected standard solutions were comprised of the combined analytes at the same concentration. The concentration of the standards bracketed the concentration expected for the analytes in the sample extracts injected. The correlation coefficient, r, determined from linear least squares curve fit of the standard calibration curve was at least 0.9950 for each analyte.

- Calculation of Percent Recovery (Fortification Only)

$$\%\,\text{Recovery} = \frac{\text{ppb Found in Sample Extract}}{\text{Fortification Level (ppb)}} \times 100$$

- Example Calculation for a LOQ fortification

Compound: Mesotrione
Lab Sample #: L1365+T1 10 mL (GB Well Water Sep/03)
Analysis Date: Nov. 18/04
Chrom. I.D.: RG112404A\run09.wiff

$$\text{Results (ng/mL)} = \left[\frac{(5234 - 317)}{1.03E5}\right] \times \frac{10\,\text{mL}}{10.0\,\text{mL}} = 0.0479\,\text{ng/mL}$$

$$\%\,\text{Recovery} = \frac{0.0479\,\text{ppb}}{0.050\,\text{ppb}} \times 100 = 95.8$$

- Statistical Methods Used to Evaluate Data

The statistical methods used were limited to calculations of the average, standard deviation, and percent relative standard deviation.

The standard deviation was determined using the following formula:

$$\sigma\ (n-1) = \sqrt{\frac{\sum_{i=1}^{n} (x_i - \bar{x})^2}{n-1}}$$

Where the sum of the squares of the individual deviations from the mean (x_i - x) is divided by n-1.

Percent relation standard deviation was determined by dividing the standard deviation by the mean and multiplying by 100.