1.0 INTRODUCTION

This report describes the independent laboratory validation (ILV) of Analytical Method No. 108919 as performed by ALS Laboratory Group, Environmental Division for the determination of the MKH 6562 and its metabolites NODT, sulfonic acid, and sulfonamide in ground water using High Performance Liquid Chromatography with Triple Quadrapole Mass Spectrometric Detection (LC-MS/MS).

2.0 STUDY PERSONNEL

The following personnel from ALS Laboratory Group, Environmental Division participated in the conduct of this study.

Susan Nelson	Study Director
Narinder Bains	Residue Analyst
Jillian Devine	Log-In and Sample Control

3.0 MATERIALS

3.1 Test and Reference Substances

Reference substances were shipped from Arysta Life Science North America Corporation (formerly Arvesta Corporation) to ALS Laboratory Group, Environmental Division and were received on October 4, 2005. The following substances were used:

Compound	Lot Number	Purity (%)	Expiration Date
Flucarbazone (MKH 5730) MKH 6562 acid	0909200501 (K-1536)	95.5	Aug. 19/2010
Flucarbazone-methyl-d3	0621200502	99.8	Jan. 20/2015
NODT	K-751	86.7*	June 02/2008
NODT-d3	96B0330129 (K-704)	99.2	Mar. 25/2008
MKH 6562 Sulfonic acid (sodium salt)	0621200503	95.5	Aug. 11/2015
MKH 6562 Sulfonic acid -d3 (ammonium salt)	0909200504 (K 1545)	100	Aug. 12/2015
MKH 6562 Sulfonamide	95R-31-86C (K-826)	99.7*	June 02/2008
MKH 6562 Sulfonamide-d3	0909200503 (K-1537)	99.8	Aug. 23/2015

* These standards were received by ALS Laboratory Group, Environmental Division and had expired prior to the start of the study. Therefore they were re-certified by ALS Laboratory Group, Environmental Division before analysis was conducted.

The reference substances were logged in and then kept stored in a freezer after arrival at ALS Laboratory Group, Environmental Division. Arysta Life Science North America Corporation maintains the characterization and stability data for the reference substances.

On December 2, 2005, stock standards were prepared from the neat reference substances. On December 8, 2005 and January 3, 2006, fortification and instrument calibration standards were prepared from stock standards. All standards were prepared as per the method. The stock standards, calibration, and fortification standards were kept stored in a refrigerator when not in use. Fortification standards and internal standards prepared December 8, 2005 were used for sample extraction. Calibration standards prepared January 3, 2006 were not used, since some analytes were pending re-certification (see page 10).

On June 3, 2006, stock standards were prepared from the neat reference substances. On June 3, 2006 and June 6, 2006, instrument fortification and calibration standards were prepared from stock standards. All standards were prepared as per the method with the exception of the native sulfonic acid, which was made up in ACN only. The stock standards, calibration, and fortification standards were kept stored in a refrigerator when not in use. Calibration standards were prepared from neat, re-certified standards.

3.2 Control Ground Water

Control ground water obtained from Gary Bruns' water well, located near Rimby, Alberta, Canada was used to validate the method. The control ground water sample was characterized by ALS Laboratory Group, Environmental Division (formerly Enviro-Test Labs). The Lab I.D. number was L162007. The date received was April 4, 2005. The composite control ground water was characterized for selected inorganic parameters. A certified copy of this ground water characterization report can be found in Appendix 2.

3.3 Equipment and Reagents

The equipment and reagents used for the method validation were as outlined in Method 108919 (Section 3.0 Materials and Apparatus, see Appendix 4). Identical or equivalent apparatus and materials were used.

4.0 METHOD AND METHOD MODIFICATIONS

4.1 Modifications

No modifications were made to the extraction or cleanup sections of the method. They were performed exactly 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 3000 MS/MS system was used in place of the Thermo Separation's Consta Metric 3200 MS, 3500 MS. The instrument specifications are listed in Table 1 and Table 2.

4.2 Sample Preparation, Fortification, and Extraction

The validation trial consisted of one analytical set. This set consisted of 12 samples: two matrix blanks, five matrix blanks fortified at the LOQ (0.05ppb) and five matrix blanks fortified at 10X LOQ.

Twelve (50 mL) portions of ground water were used as samples. Samples designated as spikes were fortified with either 250 µL of a 10.0 µg/L a mixed standard fortification solution (for LOQ fortifications) or 250 µL of 100 µg/L (for 10X LOQ fortifications). Spikes were mixed and left standing 10 minutes. See detailed method below:

Extraction:

1. Measure twelve representative amounts of ground water (50 ± 0.1 mL) into separate 100 mL graduated cylinders. Five control samples fortified with known amounts of MKH 6562 and its three metabolites (NODT, sulfonic acid, and sulfonamide) in acetonitrile at 0.0500 ppb (LOQ), and five at 0.500 ppb (10X LOQ).

2. Add 50 µL of the 0.1 µg/mL mixed internal standard solution and shake to mix the solutions.

3. Add 1 mL of 1 N Hydrochloric acid and mix.

4. Condition a 2 g MegaBond C_{18} SPE cartridge by rinsing with 10 mL of methanol followed with 10-15 mL of HPLC grade water. Do not allow the cartridges to go to dryness.

5. Elute the water samples through the cartridge at a rate of 20-30 mL/min.

6. Add 10 mL of HPLC grade water onto the cartridge and elute. Allow clean air to pass through and dry the cartridge under vacuum for 1 – 2 minutes.

7. Elute the samples with 10 mL of methanol: 5% ammonium hydroxide (9:1, v:v) and collect in a 15 mL test tube.

8. Evaporate the samples to dryness at 40-45°C with a nitrogen or turbo evaporator.

9. Reconstitute the residue with 1.0 mL of 19:1 (v:v) water:100 mM ammonium acetate in methanol.

10. Filter the extract with 0.45 µm filter (Nylon Acrodisc, 13 mm and 0.45 µm Gelman) into a HPLC vial.

11. Store extract in a freezer until ready for LC-MS/MS.

4.3 LC-MS/MS Instrumentation

All samples were analyzed using an Applied Biosystems API-3000 Triple Quadrapole Mass Spectrometer with Turbo Ion Spray Interface. The following components completed the system:

HPLC: Two Perkin Elmer Series 200 Micropumps
Autoinjector: Perkin Elmer 200
Column Heater: Waters Temp. Control module Millipore
Data System Version: MacQuan 1.7.1

The HPLC operating parameters are shown in Table 1. The API 3000 LC-MS/MS operating parameters are shown in Table 2.

4.4 Data Acquisition and Reporting

Peak integration and quantitation were performed by using MacQuan, Version 1.7.1 (Apple). Quantitation of native analyte was based on five point calibration curve with a concentration range from 0.5 to 25 ppb. The peak area ratio of native to internal standard of each compound was plotted with its standard concentration. The slope and intercept from a weighted (1/X) linear regression curve was used for quantitation of MKH 6562, NODT, MKH 6562 sulfonamide and MKH 6562 sulfonic acid.

$$\text{Conc. (ppb)} = \frac{\dfrac{\text{Native Area}}{\text{Internal Standard Area}} - \text{Intercept}}{\text{Slope}} \times \text{Dilution Factor}$$

Recovery in Spiked Validation Samples

$$\%\,\text{Recovery} = \frac{\text{Conc.}_{\text{NAT}}}{\text{Spiked Level}} \times 100$$

8.0 TABLES

Table 1. HPLC System

Analytical Column:	Phenomenex-Synergi Max-RP column, 75 x 4.6 mm, 4µm, 80 °A, Part No. 00C-4337-EO
Mobile Phase Flow Rate:	400 µL/min
Mobile Phase A:	95:5 water/100 mM ammonium acetate in methanol
Mobile Phase B:	5 mM ammonium acetate in methanol
Run time:	16 minutes
Injection Volume:	20 µL

Mobile Phase Gradient Program:

Duration (min.)	%A	%B
0	90	10
1.0	90	10
8.0	10	90
1.0	10	90
1.0	90	10
4.0	90	10

Analyte Retention times:

Analyte	Min.
MKH 6562 (flucarbazone)	7.37
NODT	4.54
MKH 6562 sulfonic acid	6.54
MKH 6562 sulfonamide	8.17

Table 2. LC-MS/MS Operating Parameters

NODT and NODT-d3 using Positive Ion detection:

	NODT	NODT-d3
Q1 Mass	130	133
Q3 Mass	114.5	114.5

The samples were analyzed using positive ion detection for NODT and NODT-d3. The MRM (multiple reaction monitoring) scan mode was used for the signal acquisition.

Interface:	Turbo Ion-Spray
Polarity:	Positive
Nebuliser Gas (GS1):	10
Turbo Gas (GS2):	60
Curtain Gas (CUR):	12 (arbitrary units)
Temperature (TEM):	300
Ion-Spray voltage:	3000
Collision gas (CAD):	Nitrogen 6 (arbitrary units)
Scan type:	MRM
Dwell time (msec)	200
OR	46
RNG	300
Q0	-10
IQ1	-11
Electron multiplier setting (CEM)	2400

MKH 6562 acid, MKH 6562 acid-d3, MKH 6562, MKH 6562-d3, MKH 6562 amide, and MKH 6562-d3 using Negative Ion detection.

	MKH 6562 acid	MKH 6562 acid-d3
Q1 Mass	241	244
Q3 Mass	85	85

	MKH 6562	MKH 6562-D3
Q1 Mass	395	398
Q3 Mass	127.6	130.6

	MKH 6562 amide	MKH 6562 amide-d3
Q1 Mass	240	243
Q3 Mass	85	85

The samples were analyzed using negative ion detection for MKH 6562 acid, MKH 6562 acid-d3, MKH 6562, MKH 6562-d3, MKH 6562 amide, and MKH 6562-d3. The MRM (multiple reaction monitoring) scan mode was used for the signal acquisition.

Interface:	Turbo Ion-Spray
Polarity:	Positive
Nebuliser Gas (GS1):	10
Turbo Gas (GS2):	60
Curtain Gas (CUR):	12 (arbitrary units)
Temperature (TEM):	300
Ion-Spray voltage:	3000
Collision gas (CAD):	Nitrogen 6 (arbitrary units)
Scan type:	MRM
Dwell time (msec)	200
OR	-20
RNG	-140
Q0	10
IQ1	11
Electron multiplier setting (CEM):	2400

Table 4. Clarifications, Communication, and Recommendations to perform Analytical Method No. 108919

Clarifications, Communication and Recommendations:

The Independent Laboratory Validation of Analytical Method 108919 did not require communication regarding the method with the Sponsor and the Study Director at ALS Laboratory Group, Environmental Division. Communications with the sponsor dealt with standard re-certification requirements and personnel/location changes.

Table 5. Calculations

Peak areas and external calibrations were used for data analysis. The Mac Quan Version 1.7.1 quantitation software package was used to calculate a best fit, 1/x weighted line of the standards. Extract concentration found was determined from the analyte peak area versus the calibration.

a) Calculated Concentration in Samples:

$$\text{Calc.Conc.(ppb)} = \frac{(x - b)}{m} \times \text{D.F.}$$

Where:
x = Peak Area of the analyte
b = Intercept from weighted 1/x regression analysis (Peak Area)
m= Slope from weighted 1/x regression analysis (response per concentration)
D.F. = Dilution Factor

$$\text{D.F.} = \frac{\text{Final Volume (mL)}}{\text{Sample Volume (mL)}} = \frac{1.0\,\text{mL}}{50\,\text{mL}} = 0.0200$$

The Analyst data processing software generates both the slope and intercept.

The calculation of averages, standard deviations, relative standard deviations and 95% confidence limits were performed in Excel.

The report percent recoveries shown on Table 3 may not exactly match the corresponding recoveries on the Analyst Result tables shown in Appendix 3. This is because Analyst uses a large string of un-rounded numbers to calculate the percent recoveries.