2. EXPERIMENTAL DETAILS

Study initiation date: December 5, 2011
Experimental Completion Date: November 13, 2012
See Appendix 1 for complete Bayer Method GL-003-S11-01 and Appendix 2 for modification of same.

2.1 Test and Analytical Reference Substances

The test substances for this study were Glufosinate Ammonium (GA) and three of its metabolites; N-acetylglufosinate (NAG), Glufosinate Propanoic Acid (MPP) and Glufosinate Acetic Acid (MPA). They were obtained from Bayer CropScience. The test substances also served as the analytical reference substances. For complete nomenclature and information for the reference substances as well as the isotopic internal standards see Appendix 3. The test and reference substances were stored in a freezer until used to prepare fortification and calibration solutions.

2.2 Test System

The test system consists of soil and water and is representative of the matrices for which this method will be used. The soil was provided by Bayer CropScience. The river water samples were obtained locally by JRF America. Soil and water samples were logged-in, stored, and handled in accordance with applicable standard operating procedures. See Appendix 4 for details.

2.3 Reagents and Consumables

The reagents and consumables used in this validation are listed in the analytical method. Functional equivalents were used as needed.

2.4 Preparation of Standard Solutions

Analytical standard solutions of GA, NAG, MPP, and MPA as well as the isotopic internal standards GA-d3, NAG-d3, and MPP–d3 were prepared in Class A volumetric glassware using calibrated pipets. All standard solutions were stored in a refrigerator in amber glass bottles when not in use. Solutions were allowed to warm to room temperature prior to use. Corrections for standard purities were applied when expressing standard concentrations. All stock, fortification and calibration solutions were stored in a refrigerator and given a 6 month expiration date.

2.4.1 Primary Stock Standard Solution

Prepared individual stock solutions of GA, NAG, MPP, and MPA at ~100 µg/mL. Standards used to prepare initial stock solutions were weighed on an analytical balance capable of accurately weighing samples to ± 0.01 mg. The

standards were quantitatively transferred to a 100 mL volumetric flask using water, and diluted to volume with water.

A mixed stock solution containing a 10 µg/mL of GA, NAG, MPP and MPA was prepared by measuring an appropriate volume (~10 mL) of each of the primary stock solutions and diluting to 100 mL with water.

2.4.2 Fortification Standard Solutions

1 µg/mL mixed solution of GA, NAG, MPP, and MPA

Transferred 10 mL of the 10 µg/mL mixed stock standard solution into a 100mL volumetric flask. Diluted to volume with water.

Transferred 5 mL of the 10µg/mL mixed stock standard solution into a 50mL volumetric flask. Diluted to volume with water and mixed well.

100 ng/mL mixed solution of GA, NAG, MPP, and MPA

Transferred 5 mL of the 1 µg/mL mixed stock standard solution into a 50mL volumetric flask. Diluted to volume with water.

2.4.3 Isotopic Internal Standard Solutions

Prepared individual stock solutions of GA-d3, NAG-d3, and MPP-d3 at approximately 100µg/mL. Standards used to prepare initial stock solutions were weighed on an analytical balance capable of accurately weighing samples to ± 0.01 mg. The standards were quantitatively transferred to a 50 mL volumetric flask using water, and diluted to volume with water.

A mixed stock solution containing a 1 µg/mL of GA-d3, NAG-d3, and MPP-d3 was prepared by measuring an appropriate volume (~1 mL) of each of the primary stock solutions and diluting to 100 mL with water.

2.5 Calibration Standard Solutions

Soil Calibration Standard Solutions

Prepared working calibration solutions consisting of 5, 10, 25, 50, 100 and 150 ppb of GA, NAG, MPP, and MPA by diluting to 10mL with acetonitrile and water to bring the final concentration to 80/20 acetonitrile/water. Before bringing the calibration solutions to volume, added by pipette 0.2 mL of the 1 µg/mL internal standard solution to each of the calibration standard solutions.

Concentration of Standard Solution used for dilution (µg/mL)	Concentration of Internal Standard Solution used for dilution (µg/mL)	Aliquot Native mix Taken (mL)	Aliquot Internal Standard Taken (mL)	ACN volume (mL)	Dilution Volume (mL)	Concentration of Calibration Solution (ppb)	Concentration of Internal Standard (ppb)
10	1	0.150	0.2	8	10	150	20
10	1	0.100	0.2	8	10	100	20
1	1	0.500	0.2	8	10	50	20
1	1	0.250	0.2	8	10	25	20
1	1	0.100	0.2	8	10	10	20
0.1	1	0.500	0.2	8	10	5	20

Water Calibration Standard Solutions

Prepared working calibration solutions consisting of 5, 10, 25, 50, 100 and 150 ppb of GA, NAG, MPP, and MPA by diluting to 10 mL with acetonitrile and water to bring the final concentration to 80/20 acetonitrile/water. Before bringing the calibration solutions to volume, added by pipette 0.25 mL of the 1 µg/mL internal standard solution to each of the calibration solutions

Concentration of Standard Solution used for dilution (µg/mL)	Concentration of Internal Standard Solution used for dilution (µg/mL)	Aliquot Native mix Taken (mL)	Aliquot Internal Standard Taken (mL)	ACN volume (mL)	Dilution Volume (mL)	Concentration of Calibration Solution (ppb)	Concentration of Internal Standard (ppb)
10	1	0.150	0.25	8	10	150	25
10	1	0.100	0.25	8	10	100	25
1	1	0.500	0.25	8	10	50	25
1	1	0.250	0.25	8	10	25	25
1	1	0.100	0.25	8	10	10	25
0.1	1	0.500	0.25	8	10	5	25

3. PROCEDURE

3.1 Soil Extraction

1. Weigh 5 ± 0.05 grams of soil/sediment into a 125 mL glass jar.

2. Fortify the recovery samples at the desired fortification level with the appropriate mixed standard solution. Let the fortified samples sit for a minimum of 5 minutes.

3. Add 25 mL of water to each sample. Place samples on shaker for ~ 30 minutes.

4. Add 0.1 mL of the 1 ug/mL internal standard solution to each sample. Mix well.

5. Centrifuge for 5 minutes at 2000 rpm.

6. Remove a 10-mL aliquot and place into a culture tube.

7. Apply the aliquot to a preconditioned MCX cartridge that is placed above a MAX cartridge using an SPE adapter. Cartridges are preconditioned with 10 mL of water. A slight vacuum can be used to pull the sample through the cartridges.

8. Add 5 mL of water to the culture tube, rinse and apply to the MCX cartridge.

9. Remove the MCX cartridge and rinse the MAX cartridge with one column volume of methanol. Discard the effluent.

10. Apply 15 mL of 2% formic acid in methanol water (1:1) to the MAX cartridge and collect in a clean culture tube. Do not use vacuum to elute the cartridge.

11. Place the culture tube in a Turbovap at 60°C and evaporate to dryness.

12. Add 0.5 mL of 0.1 M ammonium formate to each sample tube, vortex and sonicate to dissolve all residues.

13. Add 1.5 mL of acetonitrile to each sample tube and vortex.

14. Place an aliquot of each sample into an HPLC vial for LC/MS analysis.

3.2 Water Extraction- Trial 1 (LOQ 0.05 ng/mL)

1. Place 400 mL of water into a 500 mL glass jar.

2. Fortify the recovery samples at the desired fortification level with the appropriate mixed standard solution.

3. Add 0.05 mL of the 1 ug/mL internal standard solution to each sample. Mix well.

4. Apply the sample to a preconditioned MAX cartridge (a SPE reservoir can be added above the cartridge using a SPE adapter). Cartridges are preconditioned with 10 mL of water. Vacuum can be used to pull the sample through the cartridge.

5. Add 5 mL of water to the jar, rinse and apply to the MAX cartridge.

6. Rinse the MAX cartridge with one column volume of methanol. Discard the effluent.

7. Apply 15 mL of 2% formic acid in methanol water (1:1) to the MAX cartridge and collect in a clean culture tube. Do not use vacuum to elute the cartridge.

8. Place the culture tube in a Turbovap at 60°C and evaporate to dryness.

9. Add 0.5 mL of 0.1 M ammonium formate to each sample tube, vortex and sonicate to dissolve all residues.

10. Add 1.5 mL of acetonitrile to each sample tube and vortex.

11. Place an aliquot of each sample into an HPLC vial for LC/MS analysis.

3.3 Water Extraction: modified- Trial 2 (LOQ 0.5 ng/mL)

1. Place 200 mL of water into a 100 mL graduated cylinder or a glass jar which can hold at least 100 mL.

2. Fortify the recovery samples at the desired fortification level with the appropriate mixed standard solution.

3. Add 0.025 mL of the 1 ug/mL internal standard solution to each sample. Dilute the sample to 100 mL with distilled or HPLC grade water. Mix well.

4. Apply the aliquot to a preconditioned MCX cartridge that is placed above a MAX cartridge using an SPE adapter. Cartridges are preconditioned with 10 mL of water. Vacuum can be used to pull the sample through the cartridges.

5. Add 5 mL of water to the jar, rinse and apply to the MCX cartridge.

6. Remove the MCX cartridge and rinse the MAX cartridge with one column volume of methanol. Discard the effluent.

7. Apply 15 mL of 2% formic acid in methanol water (1:1) to the MAX cartridge and collect in a clean culture tube. Do not use vacuum to elute the cartridge.

8. Place the culture tube in a Turbovap at 60°C and evaporate to dryness.

9. Add 0.4 mL of 0.1 M ammonium formate to each sample tube, vortex and sonicate to dissolve all residues.

10. Add 0.6 mL of acetonitrile to each sample tube and vortex.

11. Place an aliquot of each sample into an HPLC vial for LC/MS analysis.

4. ANALYSIS BY LC/MS/MS

Recommended LC/MS/MS analysis conditions listed in the Bayer Method GL-003-S11-01 (See Appendix 1) were used on a Thermo Finnigan Ultra instrument. Modifications to these LC/MS/MS conditions were made throughout the study to obtain satisfactory system response on available JRF America instrumentation. The Study Sponsor was informed of the changes. The following are examples of the conditions used on JRFA's AB Sciex API 4000 and 4000 QTRAP instruments. The specific LC/MS/MS instrument parameters used for each analytical set can be found in the raw data.

4.1 HPLC Conditions

Mobile Phase A: 100 mM ammonium formate
Mobile Phase B: Acetonitrile
HPLC column: SeQuant ZIC-HILIC, 150 mm X 4.6 mm, 5 µm particle size
Serial Numbers: 146160, 146143
Column Temp.: Ambient
Injection volume: 5-40 µL adjusted as necessary depending on instrument sensitivity

Time (min)	Mobile Phase A	Mobile Phase B	Flow rate µL/min
0.0	40	60	800
1.0	40	60	800
3.0	70	30	800
4.0	70	30	800
4.5	40	60	800
8.0	40	60	800

4.2 Mass Spectrometer Parameters

The following conditions were used on an API 4000 instrument and were adjusted as necessary depending on instrument sensitivity.

Positive ion mode for GA Analysis

CUR: Curtain Gas 35
CAD: Collision Gas 11
GS1: Ion Source Gas 1 70
GS2: Ion Source Gas 2 50
TEM: Source Temp. 550°C
IHE: Interface Heater ON
IS: Ion Transfer Voltage 5500
EP: Entrance Potential 10

Negative ion mode for NAG, MPP and MPA Analysis

CUR: Curtain Gas	35
CAD: Collision Gas	11
GS1: Ion Source Gas 1	50
GS2: Ion Source Gas 2	70
TEM: Source Temp.	550 °C
IHE: Interface Heater	ON
IS: Ion Transfer Voltage	-4500
EP: Entrance Potential	-10

4.3 Mass Spectrometer Data Collection

The analyst modified the mass spectrometer data collection conditions throughout the study to obtain satisfactory system response on available instrumentation. The daughter ions used in this method were chosen due to their optimum sensitivity on the AB Sciex API 4000 and 4000 QTRAP instruments used for this study. The values in the following table are taken from Soil Trial 2 analysis.

Primary ion

Analyte Name	Q1 Mass (amu)	Q3 Mass (amu)	Dwell Time (msec)	DP (volts)	CE (volts)	CXP (volts)	Res Q1	Res Q3	RT (min)
GA	181.96	136.0	150	51	19	8	unit	unit	~4.1
GA-d3 IS	185.0	139.0	150	26	27	28	unit	unit	~4.1
NAG	222.86	135.7	50	-65	-28	-19	unit	unit	~3.8
NAG-d3 IS	225.01	139.2	50	-55	-34	-9	unit	unit	~3.8
MPP	151.0	133.0	50	-45	-18	-7	unit	unit	~4.1
MPP-d3 IS[1]	154.0	136.0	50	-20	-20	-5	unit	unit	~4.1
MPA	136.98	78.1	50	-40	-28	-5	unit	unit	~4.1
Confirmatory ion									
Analyte Name	Q1 Mass (amu)	Q3 Mass (amu)	Dwell Time (msec)	DP (volts)	CE (volts)	CXP (volts)	Res Q1	Res Q3	RT (min)
C-GA	181.96	119.1	150	51	27	8	unit	unit	~4.1
C-GA-d3 IS	185.0	122.0	150	26	7	26	unit	unit	~4.1
C-NAG	222.03	180.0	50	-50	-24	-1	unit	unit	~3.8
C-NAG-d3 IS	225.01	183.0	50	-55	-20	-1	unit	unit	~3.8
C-MPP	151.0	107.0	50	-50	-22	-3	unit	unit	~4.1
C-MPP-d3 IS[1]	154.0	110.0	50	-20	-20	-3	unit	unit	~4.1
C-MPA	136.8	62.8	50	-20	-42	-9	unit	unit	~4.1

[1] Used as a surrogate internal standard for MPA.

5. CALCULATIONS

In general, calculations were performed as described in analytical method GL-003-S11-01. Calculations for instrumental analysis were conducted using GLP validated software.

5.1 Calibration Curve

Linearity was assessed from the calibration curve using linear regression with 1/x weighting. The linear response range extended over the same concentrations as described in the method (5-150 ng/mL). All standards were used in constructing the linear curve. The correlation coefficient (r2) should be ≥ 0.98. Recoveries were determined using calibration curves generated with each analysis using Analyst software (Version 1.5.1) using linear regression with 1/x weighting. See Appendix 5 for example calibration curves.

The standards were fit to the linear equation:

$$Y = MX + B \text{ with } 1/x \text{ weighting}$$

Where: X is the reference standard concentration
M is the calibration line slope
B is the calibration line intercept
Y is the native peak area: isotopic peak area ratio

5.2 Fortification Recovery

After regression coefficients were calculated, the residue found in ng/g for soil samples or ng/mL for water samples was determined using the following equation:

$$\text{Residue found (ng/mL)} = \frac{(Y-B)}{M}$$

The % recovery was calculated using the following equation:

$$\text{Recovery (\%)} = \frac{(R-S)}{T} \times 100$$

Where: R = ppb of target analyte found in fortified sample
S = ppb of target analyte found in control sample, real or apparent
T = theoretical ppb in fortified sample

An example calculation for GA from Soil Trial 1 sample LOQ-1 is shown below. This sample was fortified at 10 ng/g for GA and at 20 ng/g for GA-d3. The chromatogram used in this example is presented in Appendix 6 (Chromatogram 5). The example shown below is for the calculation of GA. The metabolite residues NAG, MPP, and MPA are calculated in the same fashion.

The following data was obtained from the analyst software for the sample. The slope and intercept were obtained from the calibration curve generated by Analyst Software, and is presented in Appendix 5 (Figure 1).

Native Peak Area (GA)	IS Peak Area (GA-d3)	Y	M	B
11700	14800	0.788[2]	0.0703	-0.00617

$$\text{GA found in soil sample extract} = \frac{(0.788 + 0.00617)}{0.0703} = 11.3 \text{ ng/g}$$

No residues of GA were found in the control. Therefore, the % recovery was calculated using the following equation:

$$\% \text{ Recovery} = \frac{(11.3 \text{ ng/g} - 0.0 \text{ ng/g})}{10 \text{ ng/g}} \times 100 = 113\%$$

[2] Analyst Software calculates Y (native peak area: isotopic peak area ratio) using more significant figures than shown in the peak area fields of the quantitation results tables.

Method GL-003-S11-01 entitled: "*An Analytical Method for the Determination of Residues of Glufosinate Ammonium, N-acetylglufosinate, Glufosinate-MPP and Glufosinate-MPA in Soil, Sediment and Water Using LC/MS/MS*" has been shown through two trials to produce acceptable recoveries of GA, NAG and MPP in soil and NAG and MPP in river water.

MPA recoveries were somewhat erratic in both matrices and generally low in soil and high in river water. Using a surrogate internal standard (MPP-d3) for MPA quantification may be the root of this issue as the suppression/enhancement of the surrogate IS peak is different than the native peak. Using a different surrogate internal standard or an external standard calculation approach may resolve this problem.

The method was not sufficient to determine GA residues in river water. There is significant breakthrough of the analyte during the SPE clean-up step. This may be due to the presence of salts interacting with the ion exchange sorbent in the SPE cartridges (MCX and MAX). River water from two sources and cartridges containing different lots of SPE sorbent were used throughout this study with similar results. Using a more sensitive LC/MS/MS system may allow for the SPE clean-up step to be bypassed.