Title: Glyphosate Analysis in Vegetation by LC/MS/MS

Table of Contents

Approval Signatures

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Revision Summary:

1. Added internal standard as standard procedure.
2. Clarified ball bearing size.
3. Added additional PMP volumetric flask sizes (50 and 100 mL).
4. Clarified preparation of intermediate standard mixes.
5. Added internal standard mix preparation instructions.

1. Synopsis

1.1 **Scope:** This is a liquid chromatographic/mass spectrometer/mass spectrometer (LC/MS/MS) method that has been optimized for the extraction of glyphosate and its degradation product, aminomethylphosphonic acid (AMPA), in vegetation.

1.2 **Principle:** A vegetation sample is mixed with water and centrifuged. An aliquot is diluted, acidified, enriched with internal standard and vortex mixed. After mixing, the sample is filtered through an IC-RP cartridge connected to a nylon syringe filter and placed into plastic autosampler vials for analysis by liquid chromatography/mass spectrometry/mass spectrometry.

2. Apparatus and Materials

2.1 Robot Coupe™ or equivalent sample processer

2.2 Top loading balance

2.3 GenoGrinder™ or equivalent sample processer and 9.5 mm ball bearings

2.4 Centrifuge

2.5 Vortex mixer

2.6 Digital pipettes and corresponding pipette tips

2.7 20 mL polypropylene scintillation vials

2.8 50 mL plastic centrifuge tubes

2.9 15 mL plastic centrifuge tubes

2.10 Plastic (PMP) volumetric flasks (10, 25, 50 and 100 mL), other sizes as necessary

2.11 Nalgene disposable droppers

2.12 Grace Maxi-Clean™ IC-RP SPE cartridge, 0.5 mL

2.13 Nylon Acrodisc syringe filter 13 mm * 0.2 um

2.14 Polypropylene screw top autosampler vials with preslit PTFE/Silicone septa

2.15 Disposable 3 mL syringe

2.16 Graduated cylinders (50 and 100 mL), additional sizes as necessary

3. Reagents and Standards or Media

3.1 Pesticide Grade (nanograde, distilled in glass) Solvents:

- 3.1.1 Acetonitrile
- 3.1.2 Pure Water, HPLC grade

3.2 Extran™ 300, cleaning solution, EM Science

3.3 Formic Acid, 88%

3.4 Phosphoric Acid, Reagent grade (85%):

3.4.1 30% phosphoric acid: Add 35.3 mL of concentrated phosphoric acid to HPLC grade water and bring to 100 mL final volume. Procedure may be adjusted to prepare different volumes of solution.

3.4.2 1% phosphoric acid: Add 1.67 mL of 30% phosphoric acid to HPLC grade water and bring to 50 mL final volume.

3.5 Stock Standard Solutions: Stock standard solutions can be prepared from pure standard materials or purchased as certified solutions. Internal standards; [13C], [15N][1] Glyphosate and (d2) 13C, 15N AMPA were obtained from Monsanto and prepared in addition to Glyphosate and AMPA.

3.5.1 Prepare separate 1.00 mg/mL stock standard solutions of each compound by weighing approximately 0.0100 g of a certified standard of known purity into a plastic scintillation vial with a screw top cap. Calculate the amount of pure standard by multiplying the amount weighed in milligrams (mg) and the purity. Add an appropriate amount of freshly opened/poured HPLC grade water to obtain 1.00 mg/mL.

3.5.2 The Monsanto (step 14.1) and DuPont (step 14.2) methods say that stock standards prepared in water should be stable for at least 1.3 years and up to 23 months, if stored in the refrigerator.

3.6 Intermediate Standards: Made with freshly opened/poured HPLC grade water in plastic volumetric flasks and stored in plastic 50 mL centrifuge tubes in the refrigerator. Suggested standard volumes are listed, but other volumes may be prepared if necessary.

3.6.1 50 ppm Glyphosate/AMPA mix to 50 mL volume

3.6.2 5.0 ppm Glyphosate/AMPA mix to 50 mL volume

3.6.3 0.5 ppm Glyphosate/AMPA mix to 50 mL volume

3.7 Internal Standard Mixes: Store in plastic containers in the refrigerator.

3.7.1 50 ppm mix prepared with HPLC grade water to 10 mL volume

3.7.2 0.5 ppm mix prepared with HPLC grade water to 50 mL volume

3.7.3 10 ppb mix (dilution solution), prepared to a 1% phosphoric acid concentration. Make 50 mL and prepare a fresh mix when the calibration standards are remade.

3.8 Calibration Standards: A minimum of three calibration standards (three if linear, more if not) for each parameter of interest should be prepared through dilution of Intermediate standards with HPLC grade water and 30% phosphoric acid to obtain a concentration of 1% phosphoric acid. The calibration range can be adjusted as necessary. Calibration solutions must be stored in a refrigerator in plastic containers until used. The Monsanto Water method (step 14.1) says the calibration standards prepared in aqueous acid should be stable for 99 days. Standard responses should be checked on the LC/MS/MS to assure standard/instrument integrity before proceeding.

Calibration Stnd.	Intermediate Stnd. (ppm)	Standard Volume (uL)	Internal Stnd (0.5 ppm) (uL)	30% phosphoric Acid Volume (uL)	Final Volume (mL)
2 ppb	0.5	40	200	333	10.0
4 ppb	0.5	80	200	333	10.0
10 ppb	0.5	200	200	333	10.0
50 ppb	5.0	100	200	333	10.0
100 ppb	5.0	200	200	333	10.0
200 ppb	5.0	400	200	333	10.0

4. Safety and Environmental Considerations

4.1 Each chemical should be treated as a potential health hazard and exposure to these chemicals should be minimized. Each laboratory is responsible for maintaining awareness of OSHA regulations regarding safe handling of chemicals used in this method.

4.2 Use mechanical pipetting aides.

4.3 Avoid skin contact or inhalation.

5. Sample Acceptance Criteria

5.1 The samples must be refrigerated at the time of collection, kept cold during transport and placed into a freezer at the time they are delivered to the laboratory, unless they are to be analyzed immediately.

6. Sample Preparation

6.1 Samples should be ground and homogenized using a sample processer such as a Robot Coupe™, however, professional judgment may be used regarding eliminating the sample grinding step if the sample size is exceptionally small or if the sample consists of other atypical sample types such as woody vegetation or debris. Samples shall be returned to the freezer unless they are analyzed immediately.

7. Procedure

Sample weights, extraction volumes and final solvent volumes may be adjusted at the discretion of the analyst if the sample size is limited, has an atypical consistency, low moisture content (see step 7.3) or if high concentrations are anticipated.

7.1 Weigh 5.0 g of sample into a 50 mL plastic centrifuge tube.

7.2 Fortify the matrix spikes. A suggested amount is 500 uL of the 5.0 ppm standard which will produce a sample concentration of 0.5 ppm and an on-column concentration of 30.5 ppb (including the spike volume in the calculation).

7.3 Add 20 mL of reagent water to each centrifuge tube and homogenize the sample briefly with a vortex mixer. If entire amount of vegetation does not thoroughly mix with the water, more water should be added. Record total volume of water used.

7.4 Add 2 large (9.5 mm) ball bearings to each centrifuge tube and place samples on the GenoGrinder™ for approximately 3 minutes with a speed of approximately 800 strokes/minute.

7.5 Centrifuge at about 5000 rpm, or higher, for 10 minutes to settle the sample.

7.6 Prepare a 15 mL plastic centrifuge tube for each sample by adding 1393 uL of HPLC grade water, 67 uL of 30% phosphoric acid, and 40 uL of the 0.5 ppm internal standard mix to the tubes. Add 500 uL of the centrifuged sample extract to each corresponding 15 mL tube. Clearly label all tubes with the corresponding sample numbers.

7.7 Vortex the 15 mL centrifuge tubes with the sample aliquots.

7.8 Attach an IC-RP cartridge to a 3 mL disposable syringe and a nylon syringe filter to the outlet of the IC-RP cartridge. Pass the sample through the filter into labeled polypropylene autosampler vials. Due to the small capacity of these vials, an extra vial or tube should be kept for backup or to prepare sample dilutions.

7.9 The sample should be stored in the refrigerator or chilled autosampler if analysis is delayed.

7.10 Run samples under LC/MS/MS methods “GlyphosateIS”.

7.11 Sample dilutions should be prepared in the Dilution Solution (10 ppb internal standard mix prepared in 1% phosphoric acid).

8. LC/MS/MS Procedure

8.1 The general procedure for operating the LC/MS/MS can be found in EA-WI-002, “LC/MS/MS Start-up and General Instrument Operation for the Environmental Analysis Unit”.

8.2 Equipment/Hardware:

- 8.2.1 Waters Alliance™, or equivalent, Liquid Chromatograph.
- 8.2.2 Waters Quattro LC™, or equivalent, Mass Spectrometer/Mass Spectrometer.
- 8.2.3 Bio-Rad Cation H Micro-guard cartridge or Bio-Rad Industrial H Micro-guard cartridge. Method development was performed on the Cation H Micro-Guard.
- 8.2.4 Bio-Rad Cartridge holder.
- 8.2.5 Opti-Solv™ mini filter, 0.2 micron.

8.3 External Standard Calibration Procedure:

- 8.3.1 Inject each calibration standard using the same technique that will be used to introduce the actual samples into the LC/MS/MS. The instrument calibration must be checked or recalibrated each day of use.
- 8.3.2 If the instrument was running this method on the previous day/batch or a sample dilution needs to be made, a calibration check may be used in place of running a new calibration curve. If the response of the analyte of

interest varies by more than ± 25 percent, a new calibration curve must be prepared for that compound.

8.3.3 Calibration checks or a complete second curve should be incorporated at the end of each run.

8.3.4 For every target analyte found in the sample, there should be a minimum of 3 calibration levels within the linear range ($r^2 > 0.990$). If the curve is nonlinear, and the analyte in question is present in the sample, additional levels should be added. One of the external standards should be at a concentration 2.0 ng/mL, and the high level should be approximately 200 ng/mL. The other levels should be between 2.0 and 200 ng/mL. The calibration range may be extended or shortened depending on linearity.

8.3.5 Use the acquisition and processing methods (GlyphosateIS) that incorporate the internal standards.

8.4 Recommended Acquisition Parameters:

8.4.1 Acquisition mode = MRM

8.4.2 Photomultiplier voltage = 750

8.4.3 Quad temperature = 120˚ C

8.4.4 Flow rate = 0.5 mL/min

8.4.5 Oven temp = 50˚ C

8.4.6 Autosampler temperature = 5˚ C

8.5 LC Mobile phase: Isocratic

Mobile Phase B: Acetonitrile – 20%
Mobile Phase C: 0.1% formic acid – 80%

8.6 LC/MS/MS Conditions (masses, times and voltages are instrument dependant and may need to be slightly modified/adjusted over time):

Glyphosate and AMPA Mass Spec Method Voltages
ES negative

Function 1: Time 0.0 – 7.0 min.

Analyte	RT	Prnt(Da)	Dau(Da)	Cone(V)	Coll(eV)
Glyphosate	1.7	167.8	62.8	20	22
		167.8	**149.9**	20	11
Internal standard	1.7	**171.8**	**62.8**	20	22

Function 2: Time 3.5 – 15.0 min.

Analyte	RT	Prnt(Da)	Dau(Da)	Cone(V)	Coll(eV)
AMPA	12.0	109.9	62.8	30	20
		109.9	**80.9**	30	13
Internal standard	12.0	**113.9**	**80.8**	30	13

Note: Quantitation ions are in boldface.

9. Calculations

9.1 Determine the concentration of individual compounds according to the formula:

$$PPM = LC/MS conc\,(ppb) * \frac{(2.0\ mL)}{(5\ g)} * \frac{(20.0\ mL)}{(0.5\ mL)} \div 1000$$

Or:

$$PPM = LC/MS\ conc\,(ppb) * 0.016$$

Where: LC/MS conc. = value from calibration curve in ng/mL (ppb).

9.2 Use actual weights and volumes used if adjustments were made due to moisture content etc.

9.3 Report results in mg/kg without correction for recovery data.

10. Critical Control Points

10.1 The estimated detection level for Glyphosate is 0.05 mg/kg and 0.08 mg/kg for AMPA.

10.2 Glyphosate adsorbs to glass surfaces, especially in the absence of matrix, so plastic ware is used in this procedure to minimize this problem (see step 14.4).

10.3 A dirty cone will have a negative impact on method performance. See step 11.2.

10.4 Solvents, reagents, water, glassware and other sample processing hardware may yield discrete artifacts and/or elevated baselines causing misinterpretation of chromatograms. Due to the trace levels of analysis, all of these materials must be demonstrated to be free from interference's under the conditions of the analysis by analyzing at least one method blank for each sample batch.

10.5 Sample preparation hardware, especially the Robot Coupe™, is a probable source of carry over contamination. All stainless steel pans, blades, spatulas, and any other objects that came into contact with the sample must be washed and solvent rinsed prior to being used for another sample.

11. System Maintenance

11.1 The LC chromatography column is only a guard cartridge and as such, Bio-Rad (see step 14.3) has seen a typical lifetime with standard test mixes of over 300 injections. Change the cartridge when resolution begins to degrade. The Industrial H cartridge may be tested and if resolution is suitable, may be substituted to provide a longer life expectancy.

11.2 The cone, cone/gas nozzle assembly and spray baffle will need to be cleaned frequently with this method, possibly before each batch.

12. Precision and Accuracy

12.1 Standard quality assurance practices must be used with this method. Spiked samples may be used to validate the accuracy of the analysis, however, positive

samples may make this approach less effective if the spikes need to be diluted to remain on the calibration curve.

12.2 To monitor continuing laboratory performance, spike and analyze at least one matrix or laboratory control spike and a spike duplicate for each sample batch processed. If a batch contains more than 10 samples, use professional judgment to determine the number of spikes needed.

12.3 Extract and analyze at least one laboratory control or reagent blank as explained in step 10.4.

12.4 Use calibration standards to evaluate overall instrument performance, including evaluation of column performance and peak shape, area and retention time reproducibility, cleanliness and other factors which affect performance. Only after the standard mix demonstrates that all parameters are acceptable can the instrument be used to analyze samples.

13. Responsibilities

13.1 The division director and the assistant director are responsible for ensuring that analysts have the training, facilities, equipment and supplies required for conducting this procedure.

13.2 The quality assurance officer is responsible for monitoring process data, and has the authority to initiate corrective action if monitoring reveals inconsistencies between these instructions and actual or best laboratory practice.

13.3 The supervisor shares responsibility with the division director and assistant director for ensuring that analysts have the training, equipment and supplies necessary to conduct this procedure. The supervisor is also responsible for resolving any technical problems associated with this method. The supervisor has the authority to discontinue or correct this method, and the authority to modify in the Laboratory Information Management System (LIMS) any data associated with this procedure that quality assurance monitoring reveals to have been incorrectly transcribed.

13.4 The analyst is responsible for analyzing samples and recording data according to these instructions, and for recording any deviations from this procedure. The analyst is also responsible for notifying the supervisor of compromised sample condition, and of technical or other difficulties with the procedure. The analyst has the authority to stop analysis if he or she identifies conditions that will compromise the integrity of the results.

14. References

14.1 “Determination of Glyphosate in Ground, Surface, and Finished Drinking Waters Using LC/MS/MS”, Monsanto.

14.2 “Analytical Method for the Determination of Glyphosate and Degradate Residues in Various Crop Matrices Using LC/MS/MS”, DuPont-15444, Revision No. 2.

14.3 Bio-Rad, “Micro-Guard® Cartridges Instruction Manual”.

14.4 "Determination of Glyphosate and (Aminomethyl)phosphonoic Acid in Crops by Capillary Gas Chromatography with Mass-Selective Detection: Collaborative Study", Philip L. Alferness and Lawrence A. Wiebe, Journal of AOAC International, 2001, Vol. 84, No. 3.

14.5 Communications with chemist/supervisor at Oklahoma Department of Agriculture regarding LC/MS FMOC procedures, 2007-2010.

14.6 EA-WI-002, "LC/MS/MS Start-Up and General Instrument Operation for the Environmental Analysis Unit".