FDA Foods Program Compendium of Analytical Laboratory Methods: Chemical Analytical Manual (CAM)

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METHOD TITLE: Glyphosate and Related Residues in Food – Harmonized Method for Detection and Quantitation

VALIDATION STATUS: Multi-laboratory validation per the Guidelines for the Validation of Chemical Methods for the FDA Foods Program 3rd Edition

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METHOD SUMMARY/SCOPE:

Analyte(s): Glyphosate, Glufosinate, N-acetyl-glyphosate

Matrices: High-moisture foods, low-moisture foods and high lipid foods

REVISION HISTORY: None

OTHER NOTES: None

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Version 2021.1

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Table of Contents

2021.1 METHOD TITLE: Glyphosate and Related Residues in Food – Harmonized Method for Detection and Quantitation

2021.2 SCOPE OF APPLICATION

This method applies to the determination of glyphosate, glufosinate, and N-acetyl glyphosate in food and feed using a water-based extraction procedure followed by HPLC-MS/MS.

2021.3 PRINCIPLE

Glyphosate is a very widely used herbicide with thousands of tons applied to food crops every year. It is a very polar molecule and cannot be extracted from samples using standard organic solvent methodologies. This procedure is based on using water as the extraction solvent followed by use of a solid phase extraction (SPE) cartridge to remove most of the non-polar compounds in the sample extract.

Being very polar also makes the instrumental analysis of these compounds very difficult. This procedure uses a C8 HPLC column with an ion-pairing reagent in the mobile phase to provide the necessary chromatographic separation.

The use of water as the extraction solvent with high moisture commodities makes it impossible to know the final volume of the extract. This procedure uses isotopically labeled compounds as internal standards to correct for any volume changes. The labeled standards also correct for any matrix suppression or enhancement caused by the matrix compounds in the extract.

2021.4 REAGENTS and SUPPLIES

A. Acetonitrile, HPLC grade
B. Petroleum ether (PE)
C. Dichloromethane (DCM, optional)
D. Water, commercial HPLC grade, or in-house purified de-ionized water (18.2 MΩ)
E. Formic acid, 98%
F. Acetic acid, glacial, ACS grade
G. Ethylenediaminetetraacetic acid disodium salt (Na_2EDTA)
H. Tetrabutylammonium hydroxide – optional forms:
    1. Tetrabutylammonium hydroxide 1.0 M (TBAOH solution) – Sigma-Aldrich No. 230189 – 100 mL
    2. Tetrabutylammonium acetate (TBAA) –Sigma-Aldrich No. 335991 – 10 grams
    3. Tetrabutylammonium acetate 1.0 M (TBAA solution) - Sigma-Aldrich No.401803 – 50 mL
I. 50 and 15 mL polypropylene centrifuge tubes
J. Waters Oasis HLB SPE cartridges, 60 mg, 3 cc, 30 µm, or equivalent
K. Syringe filters: 25 mm, 0.45 or 0.2 µm, nylon (optional: centrifugal filter)
L. Polypropylene autosampler vials or micro-inserts (optional: Thomson Standard Filter Vials, 0.2 µm, nylon)

M. Molecular weight cut-off (MWCO) filter (optional)-
   1. Amicon Ultra-4 Centrifugal Filter Unit, MilliporeSigma, P/N#UFC800396, or equivalent
   2. Amicon Ultra-0.5 Centrifugal Filter Unit, MilliporeSigma, P/N#UFC5003BK, or equivalent (low volume, optional)

N. 50 mL centrifuge tube containing built-in 0.45 or 0.2 µm nylon filter(optional)

O. Extraction solvent fortified with isotopically labeled Internal standards (50 ng/mL) and if applicable surrogate analyte (50 ng/mL) :
   1. Mix 2.9 mL of acetic acid and a 3.7 gm of Na_2EDTA in 1L DI water (50 mM acetic acid/10 mM Na2EDTA).
   2. Add 5 mL of 10 µg/mL mixed internal standards solution.

P. Mobile Phase A - 4mM Aqueous Tetrabutylammonium formate, pH 2.8±0.05. For example, mix 4.0 mL of 1.0 M TBAA aqueous solution to 1 L DI Water. Adjust to 2.8±0.05 with formic acid.

2021.5 STANDARDS

A. Glyphosate
B. Glufosinate hydrochloride
C. N-acetyl-glyphosate
D. Glyphosate-$^{13}C_2$,^{15}N, isotopically labeled internal standard
E. Glufosinate-D_3 hydrochloride, isotopically labeled internal standard
F. N-Acetyl glyphosate-$^{13}C_2$,^{15}N
G. Other forms of these standards, e.g. salts and prepared solutions, are acceptable.

2021.5.1 Standard Solutions

A. General Instructions

1. Unless otherwise indicated prepare standards in DI water.

2. Store standard solutions in plastic containers. Glass can adsorb glyphosate from solution. Glass volumetric flasks may be used for standards preparation if the solution is promptly transferred to a plastic container.

3. Store standards in a refrigerator and not in a freezer because the water will freeze and may break/warp the container.

4. Alternative appropriate dilution schemes are acceptable.

B. Prepare Standard Solutions

1. Prepare individual stock standard for each compound at 1 mg/mL. For the ICV, prepare a separate set of individual stock standards, excluding internal standards.

2. IS 20 µg/mL mixed isotope internal standard: Combine isotopes glyphosate-$^{13}C_2$,^{15}N and glufosinate-D_3. Dilute 1 mg/mL stock isotope internal standards 1:50.

3. Intermediate mixed native standard: for instance, combine native 1 mg/mL stock standards, including glyphosate, glufosinate, and N-acetyl-glyphosate. Dilute 1:20 for 50 µg/mL.

4. 5.0 µg/mL working mixed standard: Dilute mixed intermediate standard 1:10.

5. LC-MS/MS calibration standard: Dilute working mixed standard 1:100, e.g. 20 µL to 2.0mL final volume, using 50 ng/mL IS fortified extraction solvent.

2021.6 PREPARATION OF SAMPLES OR TEST PORTIONS

A. High moisture samples: weigh 5.0 grams of well comminuted sample in a 50 mL polypropylene centrifuge tube. Add 25.0 mL of the IS fortified extraction solvent.

B. Low moisture samples: weigh 2.0 grams of well comminuted sample in a 50 mL polypropylene centrifuge tube. Add 10.0 mL of the IS fortified extraction solvent.

C. High lipid samples (Matrices with >2% fat), weigh 2.0 gram of well comminuted sample in a 50 mL polypropylene centrifuge tube. Add a 10.0 mL of the fortified extraction solvent. Vortex for 10 seconds.

Add 10.0 mL of PE for lipid removal. Alternatively, DCM may be used. (NOTE: When using PE to remove lipid co-extractants from high fat matrices, PE will be the top layer. When using DCM, it will be the bottom layer.)

D. Shake the prepared tubes with sample for 10 minutes mechanically using Genogrinder at 1000 RPM.

E. Centrifuge for 5 minutes at >3000 RPM.

F. Transfer approximately 2 to 3 mL of the aqueous layer to an unconditioned Oasis HLB SPE cartridge. Place the cartridge in a centrifuge tube; elute the extract at <3000 RPM centrifugation.

G. High protein matrices (>10% protein)

1. Filter the extract with MWCO filter. Centrifuge for 10 minutes at >5000 RPM. Transfer into injection vial. The sample is ready for analysis.

2. Alternatively, denature the protein at 80°C for 20 minutes in a 15 mL centrifuge tube. Centrifuge for 2 minutes and filter as below.

H. If the extract appears cloudy, filter through a 0.45 or 0.2 µm nylon filter. Plastic injection vials containing built-in nylon filters may be used.

2021.7 APPARATUS/INSTRUMENTATION

A. Rapid Vap Heater/Evaporator (optional)
B. Genogrinder
C. Electronic pipettes
D. HPLC-MS/MS
   1. Shimadzu HPLC System. Two LC-20AD pumps. Sil-20A auto sampler, CTO-20AC Column Oven
   2. Sciex Model 5500 QTrap or 6500 QTrap Mass Spectrometer

2021.8 METHOD

2020.8.1 LC parameters and gradient

1. Column: Phenomenex Luna C8 (2 columns), 150 x 4.6 mm, 5 µm OR Phenomenex Luna C8, 150 x 4.6 mm, 5 µm with Phenomenex KrudKatcher Guard column
2. Mobile Phase A: 4 mM tetrabutylammonium formate + 0.1% formic acid in water (pH 2.8 ± 0.05
3. Mobile Phase B: Acetonitrile
4. Flow rates: 0.45 mL/min (4.6 mm column); 0.3 mL/min (2.0 column)
5. Injection volume: 10 µL
6. Temperature: 40 ° C
7. Gradient

Time (minutes)	% Mobile Phase B
0	5
1	5
5	90
7	90
8	5

2020.8.2 MS parameters

1. Instrument parameters

Parameter	Setting
CUR	35
CAD	9 (med)
IS	-4000
Gas 1 & 2	65
Temperature	650 °C (6500); 600 °C, (5500)
Q1 Resolution	Unit
Q3 Resolution	Unit

2. Divert Valve Timing

Setting	Time (minutes)
Waste	1.0
Mass Spectrometer	2.5
Waste	7.0

2020.8.3MS/MS Parameters

Q1	Q3	RT[1]	Transition	DP[2]	EP	CE	CXP
180	63	3.0	Glufosinate 1	-60	-11	-66	-9
180	95	3.0	Glufosinate 2	-40	-11	-19	-5 (-9)
180	85	3.0	Glufosinate 3	-60	-11	-25	-9
183	63	3.0	Glufosinate IS	-60	-11	-40 (-60)	-9
168	63	4.4	Glyphosate 1	-30	-11	-28	-9
168	79	4.4	Glyphosate 2	-30	-11	-56	-9
168	150	4.4	Glyphosate 3	-30	-11	-16	-9
171	63	4.4	Glyphosate IS	-30	-11	-28	-9
210	150	5.3	N-acetyl glyphosate 1	-20	-11	-20 (-28)	-13 (-9)
210	63	5.3	N-acetyl glyphosate 2	-20	-11	-40	-13 (-9)
210	168	5.3	N-acetyl glyphosate 3	-20	-11	-18 (-17)	-13 (-9)

[1]RT listed is based on 2.0 mm column. If using 4.6 mm column, retention order will be consistent, but retention time will be later.
[2]Instrument parameters other than the ions selected are tunable and may vary with the different instruments. Values in parentheses are for the 5500 instrument.

2021.8.4 QUALITY CONTROL

A. Analytical Batch: an analytical batch will consist of all regulatory samples analyzed in a day along with all required quality controls, consistent with current SOP QA/QC Processes for the ORS Regulatory Pesticide Program definitions and requirements.

B. System Suitability (SS): The instrument will be determined to be suitable for analysis with each batch. The SS determination will evaluate retention time (RT) reproducibility and MS sensitivity. Three (3) injections or more of a standard solution of glyphosate at 2 ng/mL will be injected, monitoring glyphosate RT and signal-to-noise (SN) of transition 1. Passing Range: the relative standard deviation (RSD) of the RT should not be more than 5% for the replicate injections and SN must be ≥ 10.

C. Calibration: a single point standard at 50 ng/mL, prepared in fortified extraction solvent, will be used to calibrate the instrument with each batch. Method validation reports indicate acceptable linearity, and consequently uniform instrument response, for glufosinate, glyphosate, and N-acetyl glyphosate on both MS platforms, with demonstrated linearity for AB Sciex 5500 to 500 ng/mL and 200 ng/mL for AB Sciex 6500.

D. Independent Calibration Verification (ICV): an independently prepared solution will be prepared at 50 ng/mL and injected once per batch, after the calibration standard, to

verify the calibration is accurate. Glyphosate will be monitored and must be within 70-130% of calibration solution.

E. Reagent Blank (RB): Used to verify reagents are uncontaminated by interfering components, the reagent blank is an extract that contains no sample matrix. Carried thorough the extraction as if it was a sample, one must be extracted with each batch, at a rate of not less than 1 for 20 samples and display no interference peaks at the reference times of interest.

F. Continuing Calibration Verification (CCV): a calibration standard will be injected to assure that the instrument calibration has not changed. The CCV should be injected at a rate not less than 1 for each 20 samples. Passing Range: 70-130%.

G. Matrix spike and Matrix spike duplicate: a matrix is fortified in duplicate with N-Acetyl glyphosate-$^{13}C_2$,^{15}N at 50 ng/mL once for every 20 samples to establish the accuracy and precision of the method. Recovery will be reported as a percentage. Both recovery and relative percent difference (RPD) of spike pairs will be trended with control charts. Initial acceptance criteria are 70-130% recovery and 30% RPD, until sufficient points are collected to establish statistical control limits.

2021.9 CALCULATIONS

A. Analyte results are calculated using a single point calibration standard based off the response ratio of the analyte and internal standard.
B. Calibrate using internal standard calibration for glyphosate and glufosinate.
C. Assign internal standard calibration standards
    a. Glyphosate: glyphosate-$^{13}C_2$,^{15}N
    b. Glufosinate: glufosinate-D_3
    c. Calibrate using external calibration for N-acetyl glyphosate (no internal standard correction).
D. Reportable residues must meet the identification criteria provided in CVM GFI #118 (Mass Spectrometry for Confirmation of Identity of Animal Drug Residues).
E. Quantitate residues using standard addition with minimum of 3 standard additions; OR a calibration curve of standards prepared in the same matrix (r^2>0.99 for 5 calibration points). Give preference to quantitation using the primary MS/MS transition, e.g. 'Glyphosate 1', however, use of secondary transitions for quantitation may be advisable if/when matrix co-extractants interfere with the primary transition response.

$$Standard\ Response\ Factor\ =\ (Concentration_{standard})/(Response\ Ratio_{standard}) \quad (1)$$

$$Sample\ Concentration\ \left(\frac{\mu g}{g}\right) =$$
$$Standard\ Response\ Factor\ x\ Response\ Ratio_{sample}\ x\ Dilution\ Factor \quad (2)$$

Where,

$Response\ Ratio_{standard} = Area\ response\ of\ the\ analyte\ in\ the\ standard/Area\ response\ of\ the\ internal\ standard$

Response Ratio$_{sample}$ = Area response of the analyte in the sample/Area response of the internal standard

2021.10 VALIDATION INFORMATION/STATUS

A multi-laboratory validation (MLV) was conducted of a method for the determination of residue levels of glyphosate, glufosinate, and a degradant of glyphosate, N-acetylglyphosate. Five FDA pesticide laboratories participated in the MLV. The MLV demonstrated that the method is suitable for quantitative determination for residues of glyphosate, glufosinate and N-acetylglyphosate in the three primary matrix types analyzed in the FDA pesticide program, i.e., high moisture, low moisture, and high fat items. The MLV meets all the requirements of a level three multi-laboratory validation as per the Guidelines for the Validation of Chemical Methods in Food, Feed, Cosmetics, and Veterinary Products, 3rd Edition.

The following summarizes some of the MLV results. For interested parties outside the FDA, more information on the MLV results can be obtained from the method author upon a specific request placed through the FDA Food and Cosmetics Information Center.

Summary Table. Summary data includes the average spike recovery for each lab, overall average recovery, RSD, method uncertainty (MU) of the spike recoveries and the average coefficient of determination (R^2) of the spike concentrations.

	Mean Spike Recoveries Per Lab					Accuracy, Precision, and Linearity - All Labs			
Matrix	**Lab 1**	**Lab 2**	**Lab 3**	**Lab 4**	**Lab 5**	**Mean**	**RSD**	**MU**	**R^2**
Glyphosate									
Avocado	96.6	87.2	85.3	88.5	83.5	88.2	6.1	12.6	0.9979
Carrot	83.7	85.9	80.0	83.1	80.4	82.6	5.3	10.8	0.9968
Corn	101.8	95.1	91.4	97.4	96.4	96.4	5.0	10.3	0.9986
Glufosinate									
Avocado	94.4	87.0	82.9	88.3	83.2	87.2	6.0	12.3	0.9958
Carrot	84.6	90.4	81.0	83.7	80.4	84.0	5.6	11.4	0.9956
Corn	102.0	101.4	98.4	98.0	99.5	99.9	2.3	4.7	0.9994
N-acetylglyphosate									
Avocado	106.3	90.3	85.7	89.4	80.9	90.5	12.0	24.6	0.9924
Carrot	97.7	86.7	79.7	85.6	83.7	86.7	9.8	20.0	0.9941
Corn	117.9	94.4	93.1	97.9	95.1	99.7	10.4	21.2	0.9986

2021.11 REFERENCES

(1) Single laboratory validation and multilaboratory validation information can be obtained by inquiry through the FDA Food and Cosmetics Information Center.