**INDIAN STANDARD**

Method for determination of Glyphosate, Glufosinate and their metabolites residues in fruits and vegetables products by LC-ESI-MS/MS

1. **Introduction**

Glyphosate (N-phosphonomethyl glycine) and glufosinate [ammonium (S)-2-amino-4-[hydroxyl (methyl)phosphonyl] butyrate] are non-selective post-emergence herbicides used for the control of thebroad spectrum of grasses and broadleaf weed species in agriculture. Aminomethylphosphonic acid (AMPA) is the major metabolite of glyphosate, whereas, glufosinate metabolizes to 3-methylphosphonicopropionic acid (3-MPPA), N-acetyl-glufosinate (NAG). These analysts are highly polar small molecules and incompatible with multiresidue methods. For these chemicals, a specific sample pretreatments and chromatographic conditions are required. Acidified water was used for an extraction from the plant matrix in presence of dichloromethane. For glyphosate, AMPA and glufosinate are derivatized with fluorenylmethyloxycarbonyl chloride (FMOC-Cl) and analysed by using reverse phase chromatography with LC-MS/MS. Because of derivatization, good retention was observed for these compounds. But the glufosinate metabolites NAG and MPPA are unable to derivatize due to absence of amine groups in their structure. An isotopically labelled internal standard were used for accurate quantitation.

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| --- | --- | --- | --- | --- |
| ChemSpider 2D Image | Glyphosate | C3H8NO5P  Glyphosate | ChemSpider 2D Image | Aminomethylphosphonic acid | CH6NO3P  AMPA | ChemSpider 2D Image | Glufosinate | C5H12NO4P  Glufosinate | ChemSpider 2D Image | N-acetylphosphinothricin | C7H14NO5P  NAG | 3-(Methylphosphinico)propionic acid PESTANALÂ®, analytical standard  3-MPPA |

1. **Scope**

This procedure is applicable for the glyphosate, AMPA, glufosinate, MPPA and NAG residue analysis in different plant origin commodities.

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| --- | --- | --- | --- |
| **Glyphosate** | **+ 20mM FMOC-Cl in ACN** | pH=9.5  5% Borate buffer | **Glyphosate-FMOC** |

Figure 1: Dearivatisation reaction of glyphosate with FMOC-Cl**.**

1. **Equipment**
   1. ***Apparatus (Note:*** *Equivalent equipment may be substituted.)*
2. Blender or vertical cutter-mixer
3. Vortex mixer
4. Centrifuges machine with a rotor having capacity of 250mL, 50mLand 2 mL centrifuge tubes.
5. Balance, accurate to 0.01 mg
6. Rough balance of the range 0.001 to 400g or more
7. Centrifuge tubes - Polypropylene, 250 mL, 50mL, 15mL and 2 mL for sample extraction.
8. Vacuum manifold
9. Low volume concentrator
10. Autosampler vials (polypropylene or PTFE)
11. Standard storage screw cap bottles (polypropylene or PTFE)
12. Volumetric flasks - 10, 25, 50 mL
13. Micro-pipettes (variable volumes) 10, 100, 1000, 5000 µL capacity
14. Mobile phase solvent filtration assembly.
15. Nylon membrane filter papers 13mm diameter with 0.2 µm pore size.
16. Hydrophilic-Lipophilic Balance(HLB) cartridge (200 mg, 6cc)

***Note:*** *These target analytes are highly polar, water soluble and having tendency to adsorb on glass as well as metallic parts so the polypropylene or PTFE bottles are preferred.*

1. ***Reagent and solutions (Note-*** *Equivalent reagents and solutions may be substituted).*
2. Methanol - HPLC/gradient grade.
3. Acetonitrile – HPLC/gradient grade
4. HPLC grade Water
5. Ammonium formate - AR grade (99%)
6. Formic acid AR grade/ higher (high purity).
7. Methylene Chloride (synonym Dichloromethane)-HPLC/gradient grade
8. Disodium tetra borate (Na2B4O7.10H2O)-AR grade (borate buffer)
9. 9-Flurenylmethylchloroforamte (FMOC-Cl).
10. 0.1% formic acid solution in water- Dissolve 1 mL of formic acid (> 99% purity) in 999 mL HPLC grade water.
11. 20% formic acid solution in water- Add 2 mL of formic acid (> 99% purity) to8 mL HPLC grade water.
12. 5% borate buffer: 5.0 g borate buffer dissolved in 100 mL HPLC grade water.
13. 20 mM FMOC-Cl: 0.522 g FMOC-Cl dissolved in 100 mL acetonitrile.

*Note:*

*Mol.* [*Formula*](https://www.google.co.in/search?rlz=1C1GCEA_enIN789IN789&q=fmoc+formula&stick=H4sIAAAAAAAAAOPgE-LSz9U3ME4uyjCr0FLPTrbST85Izc0sLimqhLCSE3Pik_NzC_JL81Ks0vKLcktzEgH0ABJrNwAAAA&sa=X&ved=0ahUKEwiEnuGGwubaAhWLuI8KHSMnCTgQ6BMIswEoADAO)*: C15H11ClO2*

[*Molar mass*](https://www.google.co.in/search?rlz=1C1GCEA_enIN789IN789&q=fmoc+molar+mass&stick=H4sIAAAAAAAAAOPgE-LSz9U3ME4uyjCr0NLKTrbST85Izc0sLimqhLCSE3Pik_NzC_JL81KscvNzEosUchOLiwEGxr5zOgAAAA&sa=X&ved=0ahUKEwiEnuGGwubaAhWLuI8KHSMnCTgQ6BMItgEoADAP)*: 258.7 g/mol*

*For 20mM FMOC-Cl,*

*= 0.02 x 258.7*

*= 5.174 g in 1000mL (1L)*

*For 20mM FMOC-Cl in 100 mL = 0.5174g (100% purity)*

*For 20mM FMOC-Cl in 100 mL = 0.522g (99% purity)*

1. Phase A: 5 mM ammonium formate in methanol & water (20:80): Dissolve 0.317 g ammonium formate in800 mL HPLC water and 200 mL methanol. Filter through 0.2 µ Nylon 6,6 membrane filter using solvent filtration assembly. Sonicate for 5 min for degassing the phase.
2. Phase B: 5 mM ammonium formate in methanol & water (90:10): Dissolve 0.317 g ammonium formate in 100 mL HPLC water and 900 mL methanol. Filter through 0.2 µ Nylon 6,6 membranefilter using solvent filtration assembly. Sonicate for 5 min for degassing the phase.

*Note: How to prepare 5mM ammonium formate?*

*e.g.* [*Molar mass*](https://www.google.co.in/search?newwindow=1&safe=strict&q=ammonium+formate+molar+mass&stick=H4sIAAAAAAAAAOPgE-LSz9U3MCqvNDer0tLKTrbST85Izc0sLimqhLCSE3Pik_NzC_JL81KscvNzEosUchOLiwGI2jVOOgAAAA&sa=X&ved=0ahUKEwi5jcuNj9DaAhVJtI8KHXddDyYQ6BMI5QEoADAR)*: 63.0559 g/mol*

*Mol.* [*Formula*](https://www.google.co.in/search?newwindow=1&safe=strict&q=ammonium+formate+formula&stick=H4sIAAAAAAAAAOPgE-LSz9U3MCqvNDer0lLPTrbST85Izc0sLimqhLCSE3Pik_NzC_JL81Ks0vKLcktzEgGVPBYUNwAAAA&sa=X&ved=0ahUKEwi5jcuNj9DaAhVJtI8KHXddDyYQ6BMI6AEoADAS)*: NH4HCO*

*1000mM = 1M*

*5mM = 0.005M*

*For 5 mM ammoniumformate concentration,*

*= 63.0559 x 0.005*

*= 0.315 g (for 100% pure)*

*(If purity is 99% then 0.318 g ammonium formate required for 1000 mL volume)*

1. ***Reference standards***
2. Glyphosate
3. Glyphosate internal standard (IS) (D213C215N)
4. AMPA (aminomethylphosphonic acid)
5. AMPA IS (D213C215N)
6. Glufosinate
7. 3-methylphosphonicopropionic acid (3-MPPA)
8. N-acetyl glufosinate(NAG)
9. The stock solution of glyphosate, glyphosate IS, AMPA, AMPA-IS, NAG, 3-MPPA and glufosinate were prepared separately by dissolving 10mg in 10mL water (approximately 1000 µg/mL concentration).

Example:

11.4 mg Glyphosate (Purity 99.2%) dissolved in 10 mL of water.

Weight of standard= 11.4mg

Purity = 99.2%

Volume of water = 10 mL

Conc. of stock solution (mg/mL) = (Weight of std. x Purity)/ (volume of water x 100)

= (11.4x99.2)/ (10x 100)

= 1.1308

Conc. of stock solution (µg/mL) = 1.1308x 1000

= 1130.8

*Note:*

*These solutions should be prepared in polypropylene/ PTFE bottles with HPLC grade water.*

*If the reference standards are received in solution forms then further dilutions are made accordingly.*

* 1. ***Working standard mixture***

1. Working standard mixture of 50µg/mL
2. Working standard mixture of 20µg/mL
3. Working standard mixture of 10µg/mL
4. Working standard mixture of 5µg/mL
5. Working standard mixture of 2µg/mL
6. Working standard mixture of 1µg/mL
7. Working standard mixture of 0.2µg/mL
8. Working standard mixture of IS (1µg/mL)
   1. **External calibration solvent standards**:
9. A calibration range of 0.01-1.0μg/mL was prepared in solvent by using working standard.(*with Internal standard*)

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| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Working std. mixture** | **Vol to be taken from working std. (µL)** | **IS**  **mix (µL)** | **5% Borate buffer (µL)** | **20mM**  **FMOC–Cl (µL)** | **Water (µL)** | **20% formic acid (µL)** | **Final conc. (µg/mL)** | **Total Volume (µL)** |
| 10µg/mL | 100 | 50 | 350 | 150 | 300 | 50 | 1.00 | 1000 |
| 10 µg/mL | 50 | 50 | 350 | 150 | 350 | 50 | 0.50 | 1000 |
| 1µg/mL | 250 | 50 | 350 | 150 | 150 | 50 | 0.25 | 1000 |
| 1 µg/mL | 100 | 50 | 350 | 150 | 300 | 50 | 0.10 | 1000 |
| 1 µg/mL | 50 | 50 | 350 | 150 | 350 | 50 | 0.05 | 1000 |
| 1 µg/mL | 10 | 50 | 350 | 150 | 390 | 50 | 0.01 | 1000 |

1. External calibration solvent standards for LC-MS: A calibration range of 0.01-1.0μg/mL was prepared in solvent by using working standard (*without internal standard****).***

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| --- | --- | --- | --- | --- | --- | --- | --- |
| **Working std. mixture** | **Vol to be taken from working std. (µL** | **5% Borate buffer (µL)** | **20mM**  **FMOC–Cl (µL)** | **Water (µL)** | **20% formic acid (µL)** | **Final conc. (µg/mL)** | **Total Volume (µL)** |
| 10µg/mL | 100 | 350 | 150 | 350 | 50 | 1.00 | 1000 |
| 10 µg/mL | 50 | 350 | 150 | 400 | 50 | 0.50 | 1000 |
| 1µg/mL | 250 | 350 | 150 | 200 | 50 | 0.25 | 1000 |
| 1 µg/mL | 100 | 350 | 150 | 350 | 50 | 0.10 | 1000 |
| 1 µg/mL | 50 | 350 | 150 | 400 | 50 | 0.05 | 1000 |
| 1 µg/mL | 10 | 350 | 150 | 440 | 50 | 0.01 | 1000 |

1. Preparation of matrix matched standards

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| --- | --- | --- | --- | --- | --- | --- | --- |
| **Working std. mixture** | **Vol. to be taken from working std. (µL)** | **5% Borate buffer (µL)** | **20mM**  **FMOC–Cl (µL)** | **Aqueous layer of extracted Matrix (µL)** | **20% formic acid (µL)** | **Final conc. (µg/mL)** | **Total**  **Volume (µL)** |
| 20µg/mL | 50 | 350 | 150 | 400 | 50 | 1.00 | 1000 |
| 10µg/mL | 50 | 350 | 150 | 400 | 50 | 0.50 | 1000 |
| 5µg/mL | 50 | 350 | 150 | 400 | 50 | 0.25 | 1000 |
| 2µg/mL | 50 | 350 | 150 | 400 | 50 | 0.10 | 1000 |
| 1µg/mL | 50 | 350 | 150 | 400 | 50 | 0.05 | 1000 |
| 0.2µg/mL | 50 | 350 | 150 | 400 | 50 | 0.01 | 1000 |

1. ***Extraction***
2. Homogenise the laboratory sample in a blender or grinder.
3. Take approximately 200 g of the crushed sample and homogenize it for one minute.

*(note:Two step homogenization offers a uniform and fine particle size of the matrix)*

1. Weigh 10.00 ± 0.10 g samples in a 250 mL polypropylene centrifuge tube.

[For recovery experiment: Controls samples should be spiked before extraction. (Choose spiking level based on instrument sensitivity)]

*Example Calculations:*

We calculate volume to be taken from stock by V1C1= V2C2

Where

V1 = Weight of sample = 10g

C1 = Spiking level (conc.) = 0.05µg/g (mg/kg)

V2 = Volume of standard to be taken from stock

C2 = Available concentration of stock solution = 1µg/mL

V2 (mL) = (weight of sample x spike level)/ conc. Std.

= (10 x 0.05) / 1

= 0.5 mL.

So for spiking at 0.05 µg/g, a volume of 0.5 mL of 1 µg/mL working standards mixture of target analyst has to be added to 10g sample.

1. Add 40 mL of 0.1% formic acid in water and 40 mL methylene chloride (dichloromethane).
2. Homogenize the sample at high speed for 2 min.

*(If homogenizer is not available, keep the whole mixture on orbital shaker for 30 min at 200 rpm).*

1. Centrifuge at approximately at 4000-5000 rpm for 5 minutes.
2. For direct analysis (without cleanup):
   1. Take 0.4 mLof the aqueous upper layer into a 2 mL centrifuge tube.

*Note: If the centrifuge tube volume is less than 2mL capacity then derivatisation reaction will not be completed within time.*

* 1. Add 50 µL of IS mixture of 1µg/mL.
  2. Add 0.35 mL of 5% borate buffer.
  3. Add 0.15mLof 20mM FMOC-Cl solution.

*Note: after addition of FMOC-Cl, the sample extract became whitish milky solution. if the matrix has more amino acid content, then accordingly the FMOC-Cl concentration needs to be adjusted.*

* 1. Vortex for 10 minutes with 2000 rpm. Derivatization occurs during this period.

*Note: if the vortex mixture is not available in lab, then keep the same mixture on orbital shaker for 45 min at 200 rpm speed.*

* 1. After derivatisation, add 0.05mL of 20 % formic acid in water and then centrifuge the tube for 5 minutes at 5000 rpm.
  2. Filter the supernatant and inject to the LC-MS/MS system.

1. Cleanup:
   1. Take 4 mL of the aqueous upper layer into a 50 mL centrifuge tube.
   2. Add 50 µL of IS mixture of 1µg/mL.
   3. Add 4 mL of 5% borate buffer.
   4. Add 2mLof 20 mM FMOC-Cl solution.

*Note: after addition of FMOC-Cl, the sample extract became whitish milky solution. if the matrix has more amino acid content, then accordingly the FMOC-Cl concentration needs to be adjusted.*

* 1. Vortex for 10 minutes with 2000 rpm. Derivatization occurs during this period.

*Note: if the vortex mixture is not available in lab, then keep the same mixture on orbital shaker for 45 min at 200 rpm speed.*

* 1. Dilute the derivatized extract with 1% formic acid upto 50 mL.
  2. Condition the HLB cartridge (or equivalent) with 10 mL methanol followed by 1% formic acid in water.
  3. Load the diluted extract (step h6) with the flow rate of 2-3mL min.
  4. Wash the cartridge with 10 mL water (to remove borate buffer)
  5. Wash the cartridge with 5mL dichloromethane (to remove FMOC-OH).
  6. Apply vacuum and remove traces of dichloromethane from cartridge.
  7. Elute with 2.5 mL methanol (2X) and evaporate to dryness.
  8. Reconstitute with 0.5 mL methanol and 0.5 mL water.
  9. Inject to LC-MS/MS

1. **LC-MS conditions**
2. HPLC column: C18 (100 x 2.1mm x 5 um)
3. Mobile phase (A): 5mM ammonium formate in methanol (20%) + Water (80%)
4. Mobile phase (B): 5mM ammonium formate in methanol (45%)+acetonitrile (45%) + Water (10%)
5. Flow rate: 0.4 mL/min.
6. Gradient condition.

|  |  |  |
| --- | --- | --- |
| Total time (min) | A % | B% |
| 0.0 | 85 | 15 |
| 0.5 | 85 | 15 |
| 5.0 | 2 | 98 |
| 8.0 | 2 | 98 |
| 9.0 | 85 | 15 |
| 15.0 | 85 | 15 |

1. MRM parameters

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| --- | --- | --- | --- | --- | --- | --- | --- |
| **Sr.**  **No.** | **Name of analyte** | **Rt (min)** | **Quantitative MRM** | | **CE**  **(V)** | **Qualitative MRM** | **CE**  **(V)** |
| 1 | Glyphosate-FMOC | 3.91 | 390 | 168 | -18, | 150, 63 | -18,-38 |
| 2 | AMPA-FMOC | 5.67 | 332 | 110 | -12, | 136,63 | -22, -66 |
| 3 | Glufosinate-FMOC | 4.60 | 402 | 180 | -18 | 206, 63 | -22, -42 |
| 4 | IS Glyphosate-FMOC | 3.91 | 394 | 172 | -18 | 154 | -18 |
| 5 | IS AMPA-FMOC | 5.67 | 336 | 114 | -12 | 140, 63 | -24,-73 |
| 6 | NAG | 1.25 | 222 | 136 | -35 | 63 | -73 |
| 7 | MPPA | 1.20 | 151 | 63 | -45 | 107 | -22 |

**7.1 Sequence of injection**

1. Inject one blank as well as a standard mixture to ensure that the system is ready for the sample analysis.
2. Inject the solvent blank before and after standards to check the system free from carry over.
3. Inject mixture of solvent standards / matrix standards at least 5 levels including LOQ level.
4. Inject reagent blank and quality control sample.
5. Inject samples.
6. If there are more than ten samples in a batch, after every 10 sample inject one reagent blank and one calibration standard to check carry over and overall performance of the analytical instrument.

**7.2 Data interpretation (qualitative and quantitative analysis)**

1. Check the acquired data for standards as well as samples.
2. After data processing, check the two transitions per analyte are present at same (expected) retention time. Then calculate the ion ratio for two transitions. The ion ratio values should match within 30% of the reference standard ion ratio. Once confirm these identification criteria then start the quantification.
3. For quantitation, check the retention times (±0.1 min) and response of calibration standards is proportionally increased with respect to concentration. Prepare a quantitation method using an optimum level of concentration. By applying the quantitation method, prepare the calibration curve for the standards by using the linear equation with 1/x weighing factor.
4. Check linearity providing correlation coefficient >0.99 and residuals within ± 20% for all the target analytes.
5. **Example calculation:**

Sample was spiked with Glyphosate @ 0.05 µg/g level

Area of sample = 4.375e5

Area of IS = 5.76e6

Sample response = Area of sample/ Area of IS

= 4.375e5/ 5.76e6

= 0.0767

IS concentration = 0.05 µg/mL

Dilution factor (DF) = [(40/10) x (1.0/0.4)] = 10

Linear Regression Equation Y = 0.743x +0.0335

Unknown conc. (X µg/g) = [(Sample response-Intercept) / slope] x IS conc. x DF.

= [(0.0767-0.0335)/ 0.743] x 0.5 x 10

= 0.054

Reported value from the analyst software = 0.0543 µg/g

Recovery = (Cal. Conc. x 100)/ Spiked conc.

= (0.054 x 100)/ 0.05

= 108%

1. **Reference**

[Oulkar DP](https://www.ncbi.nlm.nih.gov/pubmed/?term=Oulkar%20DP%5BAuthor%5D&cauthor=true&cauthor_uid=28300023), [Hingmire S](https://www.ncbi.nlm.nih.gov/pubmed/?term=Hingmire%20S%5BAuthor%5D&cauthor=true&cauthor_uid=28300023), [Goon A](https://www.ncbi.nlm.nih.gov/pubmed/?term=Goon%20A%5BAuthor%5D&cauthor=true&cauthor_uid=28300023), [Jadhav M](https://www.ncbi.nlm.nih.gov/pubmed/?term=Jadhav%20M%5BAuthor%5D&cauthor=true&cauthor_uid=28300023), [Ugare B](https://www.ncbi.nlm.nih.gov/pubmed/?term=Ugare%20B%5BAuthor%5D&cauthor=true&cauthor_uid=28300023), [Thekkumpurath AS](https://www.ncbi.nlm.nih.gov/pubmed/?term=Thekkumpurath%20AS%5BAuthor%5D&cauthor=true&cauthor_uid=28300023), [Banerjee K](https://www.ncbi.nlm.nih.gov/pubmed/?term=Banerjee%20K%5BAuthor%5D&cauthor=true&cauthor_uid=28300023), Optimization and Validation of a Residue Analysis Method for Glyphosate, Glufosinate, and Their Metabolites in Plant Matrixes by Liquid Chromatography with Tandem Mass Spectrometry.[*J AOAC Int.*](https://www.ncbi.nlm.nih.gov/pubmed/28300023)*2017 May 1; 100(3):631-639. doi: 10.5740/jaoacint.17-0046. Epub 2017 Mar 23.*