Doc No. FAD 27(27080)WC
September 2025

BUREAU OF INDIAN STANDARDS
DRAFT FOR COMMENTS ONLY
(Not to be reproduced without the permission of BIS or used as an Indian Standard)

भारतीय मानक मसौदा

एलसी–एमएस/एमएस का उपयोग कर अम्लीकृत मेथनॉल द्वारा पौध-जनित उत्पादों में पैराक्वाट और डाइक्वाट अवशेषों का निर्धारण — परीक्षण विधि

Draft Indian Standard

ESTIMATION OF PARAQUAT AND DIQUAT RESIDUES IN PLANT ORIGIN PRODUCTS WITH ACIDIFIED METHANOL USING LC-MS/MS – METHOD OF TEST

ICS 65.100.10

Pesticide Residues Analysis Sectional Committee, FAD 27	Last Date of Comments – 07 November 2025

FOREWORD

(*Formal clause would be added later*)

Quaternary ammonium herbicides, often called "quats," are a challenging group to analyse. Paraquat (PQ) and Diquat (DQ) are bipyridyl, non-selective herbicides used worldwide to control weeds and grasses in plantation crops, pasture, as well as defoliants on several agricultural crops. These compounds by virtue of their polarity and low volatility, are typically analysed using ion-pair high-performance liquid chromatography (HPLC) combined with mass spectrometry (LC-MS) ensuring improved sensitivity and accuracy. Therefore, a specific standardized method for analyzing PQ and DQ herbicides is needed for consistent testing across India.

This draft Indian Standard provides a method for extracting and analyzing cationic polar pesticides, PQ and DQ herbicides in food and agricultural products using LC-MS/MS. This method involves using an acidified methanol for extraction, followed by LC-MS/MS analysis with a hydrophilic interaction liquid chromatography (HILIC) column. The implementation of this method would greatly support ensuring the safety of food products in India.

In reporting the result of a test or analysis made in accordance with this standard, is to be rounded off, it shall be done in accordance with IS 2 : 2022 ‘Rules for rounding off numerical values (*second revision*)’.

WARNING — Analyst applying this document should be familiar with laboratory practice for Pesticide residue analysis. This document does not purport to address all of the safety problems, if any, associated with its use. It is the responsibility of the user to establish appropriate safety and health practices.

IMPORTANT — It is essential that the laboratory ensures that personnel have the competence to perform laboratory activities for which they are responsible.

1 SCOPE

1.1 This standard prescribes LC-MS/MS (Liquid Chromatography Triple Quadrupole Mass Spectrometry) based residue analysis method for quantitative estimation of paraquat (PQ) and diquat (DQ) herbicide residues of cationic nature with acidified methanol as the extraction solvent.

1.2 The method is applicable to foods or raw commodities of plant origin such as fruits, vegetables, cereals and pulses, seeds, spices, oil-seeds, and nuts.

2 REFERENCES

The standard given below contain provisions which, through reference in this text, constitute provisions of this standard. At the time of publication, the editions indicated were valid. All standards are subject to revision, and parties to agreements based on this standard are encouraged to investigate the possibility of applying the most recent editions of these standards.

IS No.	*Title*
IS/ISO 17034 : 2016	General requirements for the competence of reference material producers
IS 11380:1985	Methods of sampling for determination of pesticide residues in agricultural and food commodities

3 PRINCIPLE

The residues of target analytes are extracted from the homogenized test portion of matrix by using acidified methanol (water adjustment is required for low moisture matrices). This whole mixture is centrifuged to separate out the solid portion and liquid layer. The upper liquid phase is diluted and directly analysed by LC-MS/MS. Based on the highly polar and ionic nature of the analytes, HILIC column chromatography (Hydrophilic Interaction Liquid Chromatography) is used. Quantification of the residues is performed by means of procedural calibration standard (Pre-extraction spike) linearity, which compensates for sample dilution, minimize matrix effect and losses in analyte recovery during extraction. Use of isotopically labeled internal standards (ILISs) for respective analytes is another approach for correct quantification, wherever practical and feasible to adapt, however this is not considered under purview of this standard. Standard addition technique is also preferred technique for correct quantification, when control matrix is not available for preparing procedural calibration standards. Sample comminution, sample homogeneity and maintaining about 80 percent to 90 percent water content in the sample homogenate are crucial in achieving appropriate extraction efficiency.

4 SAMPLING

The representative laboratory samples for the purpose of estimating PQ and DQ herbicide residues in various raw commodities shall be in accordance with the sampling procedures as prescribed in IS 11380.

5 REQUIREMENTS

5.1 Apparatus

5.1.1 Blender or vertical cutter-mixer;

5.1.2 Vortex mixer of capacity ≥ 3000 rpm;

5.1.3 Centrifuge machine with a rotor of 50 ml and 2 ml centrifuge tubes;

5.1.4 Analytical Balance, accurate to 0.01 mg;

5.1.5 Precision balance of the range 0.001 g to 400 g or more.

5.1.6 Liquid chromatography tandem mass spectrometry (LC-MS/MS) with adequate sensitivity;

5.1.7 Water purification system to generate LC-MS grade water (resistivity >18mΩ);

5.1.8 Ultrasonic Bath;

5.1.9 Centrifuge Tubes – Polypropylene, 50 ml, 15 ml and 2 ml for sample extraction;

5.1.10 Auto sampler vials (polypropylene or PTFE);

5.1.11 Standard storage screw cap bottles (amber colored are preferred), for standards prepared in water - Polypropylene or PTFE screw cap bottles are desirable to use;

5.1.12 Variable micro-pipettes (variable volumes) 10 µl, 100 µl, 1000 µl, 5000 µl capacity (alternatively, glass pipettes may be used for ≥1000 µl volume)

5.1.13 Mobile phase solvent filtration assembly with suitable membrane filters and vacuum pump (if required)

5.1.14 0.2 µm pore size membrane filters of Hydrophilized polytetrafluoroethylene (H-PTFE) or Cellulose Mixed Ester or Polyester of suitable dimension, e.g. 13 mm diameter.

5.1.15 Suitable screw cap PTFE bottles for storage of standard solutions preferably amber in color; and

5.1.16 HILIC/equivalent column: 150 mm x 4.6 mm x 5 µm particle size.

NOTES –
i) Any other suitable dimensions of columns may be used, while optimizing the chromatography
ii) Specific instructions by the manufacturer also needs to be considered as HILIC is highly specific column chemistries produced by different manufacturers.
iii) Use plastic vials/bottles are highly recommended throughout the sample preparation and standard handling since paraquat and diquat tend to interact with glass surfaces affecting recovery. Once opened, standard containers shall be stored in deep freezer (-18 °C) with appropriate temperature monitoring. The containers shall be closed tightly to avoid entering of moisture.
iv) Amber colored bottles are highly recommended for standard preparation and sample processing since DQ and PQ are light sensitive.

5.2 Chemicals

5.2.1 *Methanol LCMS Grade*.

5.2.2 *Acetonitrile***,** LCMS grade.

5.2.3 *Methanol High Purity*, AR grade or better.

5.2.4 *Deionized Water*.

5.2.5 *LC-MS Grade Water* .

5.2.6 *Ammonium Formate*, LCMS grade.

5.2.7 *Formic Acid*, LCMS grade.

5.2.8 0.1M *Aqueous HCL* – pipette 8.26 ml of concentrated HCl (37 percent) into approximately 100 ml of water, then dilute with additional water up to a final volume of 1000 ml.

5.2.9 *Mobile Phase* – For HILIC chromatography.

5.2.9.1 *Phase A* **–** 0.1 percent formic acid and 5 mM ammonium formate in water: Dissolve 1 ml formic acid and 315 mg ammonium format in 999 ml LCMS grade water. Filter through 0.2 µm Nylon 6,6 membrane filter using solvent filtration assembly. Sonicate for degassing the phase.

5.2.9.2 *Phase B* – 0.1 percent formic acid and 5 mM ammonium formate in acetonitrile: Dissolve 1 ml formic acid and 315 mg ammonium format in 999 ml LCMS grade acetonitrile. Filter through

0.2 µm Nylon 6,6 membrane filter using solvent filtration assembly. Sonicate for degassing the phase.

NOTE – If required, alternative mobile phase combination may be considered for optimum performance.

5.2.10 Certified reference standards shall be procured from reliable sources, preferably from IS / ISO 17034 accredited reference material producers, with appropriate traceability, purity, measurement uncertainty, date of manufacture and date of expiry, lot/ batch no. mentioned on the certificate.

6 STOCK AND WORKING STANDARD SOLUTION PREPARATION

6.1 Prepare the stock solutions of these analytes individually by dissolving ≥10 mg in 10 ml water (approximately 1 000 µg/ml concentration) in plastic standard preparation bottles.

6.2 Prepare intermediate working standard solution mixture of concentration 10 µg/ml (appropriate concentration may be prepared) by mixing appropriate quantities of stock solutions in acetonitrile. Dilute this further in acetonitrile to achieve working standard mixtures (WS) of 1 µg/ml and 0.1 µg/ml.

7 CALIBRATION STANDARD PREPARATION

7.1 Solvent Based Calibration Standards - Using working standard (WS) mixture of appropriate concentration, solvent based calibration standards in the range of 0.001 µg/ml to 0.1 µg/ml are prepared in acetonitrile and water. The ratio of acetonitrile and water is maintained to be 1:1 in all the calibration standards to maintain uniform solubility of analytes and avoid possible effects on peak shapes. An exemplary table for preparation of calibration standards is mentioned at Table 1.

NOTE -

i) Solvent based linearity can be used for quantification, only when suitable isotopically labelled internal standards are being used in standards and prior to extraction in sample.

ii) The calibration range may vary based on the instrument sensitivity and sample dilution during sample preparation.

Table 1 Preparation of Calibration Standards (Solvent-Based Standards)
(Clause 7.1.1)

SI. No.	WS Concentration µg/ml	Volume of WS taken µl	Acetonitrile µl	0.1% Aqueous formic acid	Calibration Standard Concentration µg/ml
(1)	(2)	(3)	(4)	(5)	(6)
1	1.0	100	500	400	0.10
2	1.0	50	500	450	0.05
3	0.1	100	500	400	0.01
4	0.1	50	500	450	0.005
5	0.1	20	500	480	0.002
6	0.1	10	500	490	0.001

7.2 *Procedural Calibration Standards (pre-extraction spiked matrix calibration standards)* – A set of control matrix samples are spiked at desired concentration levels (0.010 µg/g to 0.5 µg/g, minimum 5 points calibration) and extracted as per the protocol. The final extracts are injected to establish procedural calibration standard.

7.3 In absence of control matrix, standard addition technique can be used for quantification. In this, sample extracts (after initial judgement of concentration present in sample) are spiked at various concentrations using at least three higher concentrations. From the y-intercept and slope of calibration equation, concentration of analyte in the sample is calculated.

7.4 Use of Internal Standards (ISs) shall be done to assess the issues related to false positives or quantification errors. These ISs are added directly to the test portion at the beginning of the procedure to compensate for any factors having an influence on the recovery such as volume-deviations, recovery losses during sample preparation and matrix-effects.

8 Initial Sample Preparation

Obtain an appropriate portion of analytical sample by removing unwanted/ non-edible parts from the portion of the product to be analysed. To obtain a laboratory sample, 0.2 kg to 2.0 kg, (depending on the size, e.g. large size, medium or small size fruits, or vegetables), chop the fruits or vegetables in small pieces and homogenize using appropriate grinder to a fine homogenate. At this stage, appropriate quantity of water may be added to adjust moisture content to around 80 percent to 90 percent and achieve slurry-like consistency. This is done for improved homogeneity and residue

accessibility. Immediately take the homogenized sample for further processing to avoid any losses in residue concentration.

NOTE – Two step homogenization offers a uniform and fine particle size of the matrix.

9 EXTRACTION PROCEDURE

9.1 Weigh a representative portion of the sample homogenate into a 50 ml centrifuge tube.

9.1.1 In case of fresh fruits and vegetables as well as juices take 10 g $\pm$ 0.1 g of the homogenized sample.

9.1.2 In case of dried fruits, dried vegetables, cereals, pulses, nuts and oil seeds take 5 g $\pm$ 0.05 g of the homogenized sample.

9.1.3 In case of unique and difficult commodities like spices, herbs, tea, coffee beans etc take 2 g $\pm$ 0.02 g of the homogenate. Details of the sample size in representative commodities are specified in Table 2.

9.2 For matrix-based linearity and recovery experiment, controls samples (control samples are same or similar test matrix without any significant levels of test analytes) should be spiked before extraction. Selection of spiking levels are based on required Limit of quantification (LOQ)/ reporting limit/ regulatory limit.

9.3 Add 10 ml of a 1:1 mixture of methanol plus aqueous HCl (0.1M) to the water adjusted analytical portion.

9.4 For high oil/ high protein containing matrices, additional of 100 µl formic acid is required.

9.5 Homogenize/vortex the sample at high speed for 1 min. (The speed is 10 000 rpm to 15 000 rpm for homogenizer and 2 000 rpm for vortex mixer).

9.6 Then thermal treatment of 15 minutes at 80 °C in a water bath. Then shake again for 1 minute and wait for the sample to cool down to room temperature before centrifuging.

9.7 Centrifuge at approximately 4 000 rpm to 5 000 rpm for 5 minutes.

9.8 Take 0.5 ml of the supernatant into 2 ml vial.

9.9 Dilute to 1 ml with acetonitrile solvent.

9.10 Dilution of control matrix extract is performed to reduce matrix interferences and to make the extract compatible with initial chromatographic conditions. This step also enables better chromatographic peak shapes.

9.11 Filter the supernatant and inject to the LC-MS/MS system. Flow chart of extraction procedure for paraquat and diquat herbicides residues in foods or raw commodities is placed in Annex A.

NOTE – To avoid the blockage in LC tubing, nebulizer capillaries, dilution of extracts, filtration or centrifugation steps are important during extraction.

Table 2 Commodity – wise homogenization, water addition and extraction solvent during extraction for various raw commodities
(*Clause* 9.1.3)

Sl. No.	Commodity group	Commodity category (Examples)	Weight of the sample homogenate (g)	Volume of Water added (ml)	Volume of extraction solvent (ml)
1.	Fruits and vegetables	*Fruits and vegetables with 85-90% water content* Fruits: Grape fruit, peach, goose berry, straw berry, papaya etc. Vegetables: beetroot, carrot, radish, onion, cucumber, lettuce, melons, tomato, broccoli, cauliflower, cabbage, spinach, okra, brinjal, bitter gourd, etc.	10 (no water addition during homogenization)	1	10
			10 (1:1 water addition for homogenization)	0.5	10
			10 (1:2 water addition for homogenization)	0	10
		Fruit and vegetables with 80-85% Fruits- Lemon, lime, oranges, apple, Litchi, Sauces, pear, apricot, plum, grapes, black berry, blue berry, pine apple, fig, mango, kiwi etc. Vegetables: Chilli, potato, leek, shallots etc.	10 (no water addition during homogenization)	2	10
			10 (1:1 water addition for homogenization)	1	10
			10 (1:2 water addition for homogenization)	0.5	10
		Fruit and vegetables with 75-80% (Banana, Garlic, drumstick, curry	10 (no water addition during homogenization)	3	10
			10 (1:1 water addition for homogenization)	1.5	10

Sl. No.	Commodity group	Commodity category (Examples)	Weight of the sample homogenate (g)	Volume of Water added (ml)	Volume of extraction solvent (ml)
		leaves, pomegranate etc.)	10 (1:2 water addition for homogenization)	1	10
2.	Dehydrated fruits	Dried fruits with 15-20% moisture (Apple, apricot, fig, mango, prunes, raisins etc.)	5	9	10
3.	Cereals	Rice, wheat, Barley, Corn, flour etc containing <10% water.	5	9	10
4.	Pulses	Dried Beans, Peas, Lentils	5	9	10
5.	Nuts and oil seeds	Almonds, Cashew nuts, Dried Hazel-nuts, Pecans, Pistachios, Peanuts, Poppy seeds, Pumpkin seeds, Sesame seeds, Soybeans, Sunflower seeds etc.) containing <10% water.	5	9	10
6.	Difficult or unique commodities	Spices, herbs, coffee beans/powder, tea, dried mushrooms etc.	2	10	10
7.	Liquid matrices	Wine, fruits juices, beverages etc.	10	1	10

NOTES –

i)The water content in the analytical portion of the sample (2g, 5g or 10 g) should be adjusted to 10 ml including the water present in the homogenate or externally added before extraction.

ii) If water is added during homogenization, the actual sample present in the analytical portion should be considered for calculating the dilution factor in to 20 ml of the extract.

10 CHROMATOGRAPHIC AND MASS SPECTROMETRIC OPERATING CONDITIONS

The suggestive LC-MS/MS operating conditions are given below. However, these operating conditions are likely to change with change in equipment employed and allowed provided standardization is done.

10.1 Analysis by LC-MS/MS: *(HILIC Chromatography)*

a) LC column (*see* ***5.1.16***)
b) Mobile phase (A) (*see* ***5.2.9.1***)
c) Mobile phase (B) (*see* ***5.2.9.2***)
d) Flow rate – 0.4 ml/min.

Time Interval (min)	A%	B%
0.0	5	95
1.0	5	95
7.0	70	30
10.0	70	30
11.0	5	95
16.0	5	95

NOTE – The method parameters given are exemplary and suitable conditions may be optimized and used for optimum method performance.

10.1.3 *MRM Parameters for LC-MS/MS Analysis (For Reference Only)*

SI. No.	Name of analyte	Quantifier MRM Q1>Q3 (m/z) Da	CE (V)	Qualifier MRM Q1>Q3	CE (V)	Cone voltage (V)	LC column	Ionization mode
1	Paraquat	171>155	41	171>77	36	140	HILIC	Positive
2	Diquat	184>156	30	184>128	55	50	HILIC	Positive

NOTE – Ion (m/z) selection and source and mass parameters may vary depending on the instrument used and optimization may be performed for better stability and sensitivity of ions.

11 SEQUENCES OF INJECTION

11.1 Inject one blank as well as a standard mixture to ensure that the system is ready for the sample analysis.

11.2 Inject the solvent blank before and after standards to check the system free from carry over.

11.3 Inject mixture of solvent standards / matrix standards at least 5 levels including LOQ level.

11.4 Inject reagent blank and quality control sample.

11.5 Inject samples to LC-MS/MS.

11.6 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 drift.

12 DATA INTERPRETATION (QUALITATIVE AND QUANTITATIVE ANALYSIS)

12.1 Check the acquired data for standards as well as samples.

12.2 After data processing, check the two transition 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 percent of the average reference standard ion ratio. Once confirmed for this identification criterion, start the quantification.

12.3 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 quantification method, prepare the calibration curve for the standards by using the linear equation .

12.4 Check linearity providing correlation coefficient >0.99. Deviation of back- calculated concentration from true concentration ≤± 20 % .

13 REPORTING OF THE RESULTS

Appropriate dilution factor associated with the sample preparation should be applied in final residue calculation/ quantification.

ANNEX A
(*Clause* 9.11)

EXTRACTION PROCEDURE FOR PARAQUAT AND DIQUAT IN FOODS OR RAW COMMODITIES

Weigh required quantity (g) of the homogenized sample (Table 2) in a 50 ml polypropylene centrifuge tube

↓

Add the required quantity of water (Table 2)

↓

Add 10 ml of a 1:1 mixture of methanol plus aqueous HCl (0.1M)

↓

Vortex for 1 min at 2000 rpm and perform temperature treatment of 15 minutes at 80 °C in water bath, mix the contents by shaking at 1 minute interval and allow the sample to cool down to room temperature

↓

Centrifuge at approximately 4000 rpm at room temperature

↓

Take 0.5 ml of the supernatant into a 2 ml vial and dilute to 1 ml with acetonitrile

↓

Filter the supernatant (0.2 µm) and inject to the LC-MS/MS system

NOTES

i) Quantify the residues of PQ and DQ concentration against calibration curve (matrix based procedural calibration)
ii) Appropriate dilution factor associated with the sample preparation should be applied in final residue calculation/ quantification.