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भारतीय मानक मसौदा

तरल क्रोमैटोग्राफी-ट्रिपल क्वाड्रुपोल मास स्पेक्ट्रोमेट्री द्वारा खाद्य पदार्थों या पौधे की उत्पत्ति की कच्ची वस्तुओं में ध्रुवीय कीटनाशकों का निर्धारण - परीक्षण पद्धति

Draft Indian Standard

Determination of Polar Pesticides in Foods or Raw Commodities of Plant Origin by Liquid Chromatography-Triple Quadrupole Mass Spectrometry – Method of Test

ICS 65.100.10

Pesticide Residues Analysis Sectional Committee, FAD 27	Last Date of Comments – 21 April 2024

FOREWORD

(*Formal clause would be added later*)

Multi-residue analysis of pesticides is inevitable in testing for regulatory compliance and ensuring food safety of food and row agricultural commodities. Acetonitrile and ethyl acetate-based QuEChERS extraction workflow followed by liquid chromatography tandem spectroscopy (LC-MS/MS) or gas chromatography tandem mass spectrometry (GC-MS/MS) analysis is the most widely adopted methodology across the world for wide range of pesticides in food commodities. However, this method restricts its application for analysis of polar pesticides (insecticides, herbicides, fungicides, plant growth regulators etc) due to non-amenability of these analytes to LC-MS/MS or their poor extractability in acetonitrile or ethyl acetate solvent which necessitate a specific method for polar pesticides for their harmonization of testing across the country.

This Indian Standard describes LC-MS/MS based analytical method for extraction and analysis of polar pesticides in food and agricultural commodities involving acidified methanol extraction followed by LC-MS/MS analysis by C-18 and HILIC column chemistry.

For the purpose of deciding whether a particular requirement of this standard is complied with, the final value, observed or calculated, expressing the result of a test or analysis, shall be rounded off in accordance with IS 2 : 2022 ‘Rules for rounding off numerical values (*second revision*)’. The

number of significant places retained in the rounded off value should be the same as that of the specified value in this standard.

1 SCOPE

1.1 This standard prescribes LC-MS/MS (Liquid Chromatography Triple Quadrupole Mass Spectrometry) based residue analysis method for quantitative estimation of pesticides residues of of polar nature (ionic or non-ionic).

1.2 This analytical approach is applicable for residues which are non-amenable to liquid-liquid partitioning techniques, like QuEChERS (acetonitrile and Ethyl acetate-based extraction methods).

NOTE – 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 standards 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

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 analyte nature, different chromatographic techniques are employed, viz. reverse phase for polar and mid-polar analytes and HILIC (Hydrophilic interaction liquid chromatography) for highly polar and ionic analytes. Quantification of the residues is performed by means of procedural calibration standard (Pre-extraction spike) linearity to 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 water content in the sample homogenate are crucial in achieving appropriate extraction efficiency.

4 SAMPLING

The representative laboratory samples for the purpose of estimating polar pesticide residues in various raw commodities shall be in accordance with the sampling procedures as prescribed in the relevant Indian Standards/ FSSAI/ CODEX guidelines or other relevant regulations, wherever available.

4.1 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 – 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 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.

5 REQUIREMENTS

5.1 Apparatus

Following apparatus shall be used –

a) Blender or vertical cutter-mixer

b) Ultra Turrax/ Vortex mixer of capacity ≥ 3000 rpm.

c) Centrifuges machine with a rotor having capacity of 50 ml and 2 ml centrifuge tubes.

d) Analytical Balance, accurate to 0.01 mg.

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

f) Liquid chromatography tandem mass spectrometry (LC-MS/MS) with adequate sensitivity.

g) Water purification system to generate HPLC grade water (resistivity >18mΩ).

h) Ultrasonic Bath.

j) Centrifuge Tubes – Polypropylene, 50 ml, 15 ml and 2 ml for sample extraction.

k) Auto sampler vials (polypropylene or PTFE).

m) Standard storage screw cap bottles (amber colored are preferred), for standards prepared in water, polypropylene or PTFE screw cap bottles are desirable to use.

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

p) Mobile phase solvent filtration assembly with suitable membrane filters and vacuum pump.

q) 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.

r) Suitable screw cap glass or PTFE bottles for storage of standard solutions preferably amber in colour.

s) Reverse phase (C18) chromatographic column: 150 mm x 3 mm x 3 µm particle size HILIC column: 150 mm x 4.6 mm x 5 µm particle size.

NOTE –
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.

5.2 Analytes

Following reagents shall be used –

a) 1-naphthylacetamide;

b) 1-naphthylacetic acid;

c) 2,4-dichlorophenoxy acetic acid (2,4-D);

d) 6-benzylaminopurine (6-BA);

e) Chlormequat (CCC);

f) Mepiquat;

g) Nereistoxin;

h) Thiocyclam;

j) Ethephon;

k) Cartap;

m) 4-trifluoronicotinic acid (TFNA);

n) (N-(4-trifluoronicotinoyl) glycine) (TFNG);

p) Fosetyl aluminium;

q) Phosphonic acid;

r) 4-chlorophenoxy acetic acid (4-CPA);

s) Forchlorfenuron;

t) Homobrassinolide;

u) Validamycin;

v) Kasugamycin;

w) Streptomycin;

y) Tetracycline;

z) Isoprothiolane;

aa) Propamocarb; and

bb) Flonicamid.

Note – Apart from above list of analytes, the method may be applied to other analytes of polar nature not included in this standard, and also, to the analytes having difficulties in meeting method performance criteria in multi-residue analysis, but only after appropriate method validation performed in respective representative matrices to establish acceptable method performance.

5.3 Chemicals

5.3.1 *Methanol Gradient or LCMS Grade* (for mobile phase).

5.3.2 *Acetonitrile***,** LCMS grade (for mobile phase).

5.3.3 *Methanol High Purity*, AR grade (for sample extraction).

5.3.4 *Deionized Water* (for addition to sample).

5.3.5 *Type-I Water/ LCMS Grade Water* (for mobile phase preparation).

5.3.6 *Ammonium Formate*, LCMS grade.

5.3.7 *Formic Acid*, LCMS grade.

5.3.8 *Percent Formic Acid Solution in Methanol* – Dissolve 1 ml of formic acid in 99 ml HPLC/ gradient grade methanol.

5.3.9 *Mobile Phase* – For reverse phase chromatography (C_{18} column).

5.3.9.1 *Phase A* **–** 0.1 percent formic acid in water: Dissolve 1 ml formic acid in 999 ml Type I/ LCMS grade water. Filter through suitable membrane filter using solvent filtration assembly. Sonicate for 5 min for degassing the phase.

5.3.9.2 *Phase B* **–** 0.1 percent formic acid in methanol: Dissolve 1 mL formic acid in 999 ml Gradient/ LCMS grade methanol. Filter through suitable 0.2 µm membrane filter using solvent filtration assembly. Sonicate for 5 min for degassing the phase.

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

5.3.10 *Mobile Phase* – For HILIC chromatography.

5.3.10.1 *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 Type I/ LCMS grade water. Filter through 0.2 µm Nylon 6,6 membrane filter using solvent filtration assembly. Sonicate for degassing the phase.

5.3.10.2 *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 Gradient/ 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.

5.3.11 Certified reference standards of the test polar compounds shall be procured from reliable sources, preferably from ISO 17034 accredited manufacturers, with appropriate traceability, purity, measurement uncertainty, date of manufacture and date of expiry, lot/ batch no. mentioned on the certificate. 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.

6 STOCK AND WORKING STANDARD SOLUTION PREPARATION

6.1 Prepare the stock solutions of these compounds individually by dissolving ≥10 mg in 10 ml water or methanol according to their solubility (approximately 1000 µg/ml concentration).

NOTE – Some standards are available as salts or hydrates and thus appropriate conversion factor need to be applied to convert typical standard in salt form to the form in which it is analyzed.

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 methanol. Dilute this further in methanol to achieve working standard mixtures (WS) of 1 µg/ml and 0.1 µg/ml.

7 CALIBRATION STANDARD PREPARATION

7.1 Solvent Based

7.1.1 Using working standard (WS) mixture of appropriate concentration, solvent based calibration standards in the range of 0.001 µg/ml – 0.1 µg/ml are prepared in methanol and water (For analysis on HILIC column, methanol can be replaced with acetonitrile). The ratio of methanol 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.

NOTE –
i) Solvent based linearity can be used for quantification, only when suitable Isotopically labelled internal standards are being used is standards and added to samples prior to extraction.
ii) The calibration range may vary based on the instrument sensitivity and sample dilution.

Table 1 Preparation of Calibration Standards (Solvent-Based Standards)
(*Clause* 8.4**)**

Sl. No.	**WS Concentration µg/mL**	**Volume of WS taken µL**	**Methanol/ Acetonitrile µL**	**Water or 0.1%formic acid solution**	**Calibration Standard Concentration µg/mL**
(1)	**(2)**	**(3)**	**(4)**	**(5)**	**(6)**
1	1.0	100	400	500	0.10
2	1.0	50	450	500	0.05
3	0.1	100	400	500	0.01
4	0.1	50	450	500	0.005
5	0.1	20	480	500	0.002
	0.1	10	490	500	0.001

7.1.2 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 – 0.5 µg/g) and

extracted as per the protocol. The final extracts are injected to establish procedural calibration standard.

7.1.3 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.

8 EXTRACTION PROCEDURE

8.1 Weigh 10.00 g ± 0.10 g sample in a 50 ml polypropylene centrifuge tube (*see* **9**).

8.2 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.

8.3 Add 20 ml of 1 percent formic acid in methanol.

8.4 For high oil/ high protein containing matrices, additional 100 µl formic acid is required (Table 1).

8.5 Homogenize/ vortex the sample at high speed for 2 min. (The speed is 10000 rpm – 15000 rpm for homogenizer and 2000 rpm for vortex mixer)

8.6 Centrifuge at approximately 4000 rpm – 5000 rpm for 5 minutes.

8.7 For high oil containing matrices, clean-up of final extract is required. Cleanup is achieved using dispersive solid phase extraction by means of C18 adsorbent (50 mg C18 per ml of extract).

8.8 Take 0.5 ml of the supernatant into 2 ml centrifuge tube.

8.9 Dilute to 1 ml with water (for C18 chemistry) and with acetonitrile (for HILIC chemistry).

8.10 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.

8.11 Filter the supernatant and inject to the LC-MS/MS system. Flow chart of extraction procedure for polar pesticides 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 Indicating sample homogenization and sample extraction for various raw commodities under the scope of this Indian Standard

(*Clause* 8)

Sl. No.	Commodity group	Commodity category	Weight of sample	Water addition	Extraction solvent
1.	High water content	Pome fruit, stone fruits, legumes, alliums, cucurbits, fruit vegetables, fresh fungi, root and tuber vegetables	10 g	None	1 percent formic acid in methanol
2.	High acid content and high water content	Citrus fruits, berries, small fruits	10 g	None	1 percent formic acid in methanol
3.	High sugar and low water content1	Dried fruit	5 g	10 ml	1 percent formic acid in methanol
		Dried fruits	15 g, If sample is soaked in 1:2 proportion in water before comminution	None	1 percent formic acid in methanol
4.	High oil content and very low water content	Tree nut, oil seeds and their pastes	5 g	10 ml	1 percent formic acid in methanol + 100 µl FA
			15 g, If sample is soaked in 1:2 proportion in water before comminution	None	
5.	High oil content and intermediate water content	Oily fruits and products	10 g	None	1 percent formic acid in methanol + 100 µl FA
6.	High starch and/or protein content and low water and fat content	Dry legume vegetables/pulses	5 g	10 ml	1 percent formic acid in methanol + 100 µl FA
			15 g, If sample is soaked in 1:2 proportion in water before comminution	None	

Sl. No.	Commodity group	Commodity category	Weight of sample	Water addition	Extraction solvent
		Cereal grain and products thereof	5 g	10 ml	1 percent formic acid in methanol + 100 µl FA
			15 g, If sample is soaked in 1:2 proportion in water before comminution	None	
7.	"Difficult or unique commodities"	Spices, herbs, coffee beans	2 g to 5 g	10 ml	1 percent formic acid in methanol + 100 µl FA

9 CHROMATOGRAPHIC AND MASS SPECTROMETRIC METHOD

9.1 Analysis by LC-MS/MS:

9.1.1 *LC-MS/MS Conditions (Reverse Phase Chromatography)*

a. LC column – (*see* **5**)
b. Mobile phase (A) – (*see* **5.3.9.1**)
c. Mobile phase (B) – (*see* **5.3.9.2**)
d. Flow rate – 0.4 ml/min (or appropriate).

Time Interval (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

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

9.1.2 *LC-MS Conditions (HILIC Chromatography)*

a. LC column – (*see* **5**)
b. Mobile phase (A) – (*see* **5.3.10.1**)
c. Mobile phase (B) – (*see* **5.3.10.2**)
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.

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

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.

Sl. 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)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)
1.	Naphthyl acetic acid (NAA)	187>141	16	187>115	54	40	C18	Positive
	NAA-OH adduct	203.1>126.57	18	203.1> 127.09	24	36	C18	Positive
2.	Naphthylacetamide (NAD)	186>141	19	186>115	49	35	C18	Positive
3.	2,4-dichlorophenoxy acetic acid (2,4-D)	218.9>160.8	-22	218.9>124.9	-39	-84	C18	Negative
4.	6-benzylaminopurine (6-BA)	226>91	42	226>148	24	22	C18	Positive
5.	Chlormequat (CCC)	122.1>58.1	40	122.1>63.1	32	42	HILIC	Positive
6.	Mepiquat	114.1>98.1	37	114.1>58.3	41	61	HILIC	Positive
7.	Ethephon	143>107	-12	143>79	-24	-45	HILIC	Negative
8.	Cartap	238.1>73.1	39	238.1>150	23	46	HILIC	Positive
9.	Nereistoxin	150.2>105	23	150.2>61.1	39	41	HILIC	Positive
10.	Thiocyclam	182.2>137.2	23	182.2>73.2	35	36	HILIC	Positive
11.	4-trifluoronicotinic acid (TFNA)	189.9>68.9	-46	189.9>125.9	-34	-35	C18	Negative
12.	(N-(4-trifluoronicotinoyl) glycine) (TFNG)	249>202.9	28	249>148.2	44	65	C18	Positive
13.	Fosetyl aluminium	108.9>80.9	-18	108.9>78.8	-34	-35	HILIC	Negative
14.	Phosponic acid	80.9>63	-38	80.9>78.9	-22	-55	HILIC	Negative
15.	4-chlorophenoxy acetic acid (4-CPA)	185>126.9	-18	1857>128.9	-18	-30	C18	Negative
16.	Forchlorfenuron	248>129.1	25	248>93.1	48	52	C18	Positive
17.	Homobrassinolide	495.3>109.1	43	495.3>127.4	25	81	C18	Positive
18.	Validamycin	498.1>142	45	498.1>178.1	37	131	HILIC/ C18	Positive
19.	Kasugamycin	380.1>112.1	25	380.1>70.1	51	100	HILIC/ C18	Positive
20.	Streptomycin	614>582	30	614>263	47	181	HILIC	Positive
21.	Tetracycline	445>410	26	445>154	39	71	C18	Positive
22.	Isoprothiolane	291>231	19	291>189	34	58	C18	Positive
23.	Propamocarb	189>144	19	189>102	25	81	C18	Positive
24.	Flonicamid	230>203	35	230>174	35	75	C18	Positive

10 SEQUENCE OF INJECTION

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

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

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

10.4 Inject reagent blank and quality control sample.

10.5 Inject samples.

10.6 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.

11 DATA INTERPRETATION (QUALITATIVE AND QUANTITATIVE ANALYSIS)

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

11.2 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 reference standard ion ratio. Once confirm this identification criterion then start the quantification.

11.3 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 weighting factor.

11.4 Check linearity providing correlation coefficient >0.99 and residuals within ± 20 percent for all the target analytes.

12 REPORTING OF THE RESULTS

12.1 Apply a suitable conversion factor for the analytes as per the residue definitions given in the regulatory guidelines.

12.2 Convert the residue concentration to their parent component and then add the residue values where the residue definition is expressed as sum of residues.

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

ANNEX A
(*Clause* 8.11)

EXTRACTION PROCEDURE FOR POLAR PESTICIDES IN FOODS OR RAW COMMODITIES

Weigh 2.00/5.00/10.00/15.00 ± 0.10 g homogenized sample (Table 2) in a 50 ml polypropylene centrifuge tube

⇩

Add 20 ml of 1% formic acid in methanol

(Additionally, add 100 µL of formic acid for high oil/protein containing matrices (Table 2))

⇩

Vortex for 2 min at 2000 rpm

⇩

Centrifuge at approximately 4000 rpm – 5000 rpm for 5 minutes

(For high oil containing matrices, perform clean-up using dispersive solid phase extraction with C_{18} adsorbent (50 mg C_{18} per ml of extract)

⇩

Take 0.5 ml of the supernatant into a 2 ml centrifuge tube

⇩

Dilute to 1 ml with water (for C18 chemistry) or with acetonitrile (for HILIC chemistry)

⇩

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

⇩

Quantify the residue concentration against “Matrix match” calibration

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