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RESEARCH ARTICLE

Validation of methods for pesticide residue analysis in milk and milk products as per FSSAI regulation

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Abstract: Accurate analysis of 55 pesticides as per the regulatory requirement of FSSAI for milk and milk products is a challenging task as it requires performing six different types of extractions and detection techniques. Further, before putting to use methods has to be thoroughly validated or checked for its fitness of purpose as per the National or International guidelines to ensure the accuracy of test results. The objective of this study was to develop a validated test method for the determination of 55 pesticides in milk and milk products as per FSSAI regulation. QuEChERS based simultaneous extraction of multiclass pesticides involves different steps i.e. mixing of reagents, separation of phases, extraction of analytes, clean up followed by reconstitution in suitable solvents. For analysis of few pesticides of different chemical nature, certain analytical steps which are saponification, derivatisation, digestion of analyte to release CS_2, extractions using specific solvents, purification using specific cartridges at specific pH, temperature and flow conditions are required to be performed. For detection of pesticide residues, mass spectrometry either with gas or liquid chromatography is one of the best approach to meet method performance and validation criteria. In this study, the optimised extraction protocols were investigated for parameters like Linearity, Matrix Effect (ME), Limit of Quantification (LOQ), Specificity, Trueness, Precision, Ion Ratio and Retention Time (RT) to validate the fitness for purpose of the methods. The calibration curves for all the pesticides were linear over the tested range as the concentration of every analyte at each calibration level fall within the residual limit of ±20 %. The LOQ for most of pesticides is established at 5 µg/kg, whereas it is 10 µg/kg for Bifenthrin, Cypermethrin, Dichlorvos, Etofenprox, Phorate, Glufosinate Ammonium, and 25 µg/kg for Dithiocarbamtes as CS_2. The Trueness and Precision of the methods were evaluated by analysing control samples spiked at LOQ and 2 to 10x the RL/LOQ in 6 replicates as per SANTE/11321/2021 guideline. Results for Trueness and Precision meet validation criteria of recovery 70 to 120 % and % RSDr < 20 % respectively for all targeted pesticides. The methods fulfilled all other the requirements of SANTE/11321/2021 guidelines and can be extended for routine analysis of pesticide residues in milk and milk products.

Keywords: FSSAI, GC-MS/MS, Milk, Milk Products, LC-MS/MS, Pesticides, SANTE/11321/2021, Validation

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Introduction

The substances intended for preventing, destroying, and repelling any 'pest' are known as pesticides. Pesticides play an important role in sustainable agriculture by protecting crops and commodities from pests and diseases (Tripathy et al. 2019). The agro produce used as food and feed sources treated with pesticides may retain some amount of these residues (Muppalla et al. 2019). These pesticide residues get into the human body through the food chain and cause health and safety problems (Johansen and Muir 2004). Therefore, it is necessary to ascertain the levels of pesticides of food matrices, so that it remains within the limit *i.e.* Maximum Residue Limits (MRL) set by national and international regulatory body.

When animals are fed feed containing pesticide residues, it may lead to excretion of residues of pesticides in animal products including milk. FSSAI has set regulatory limits for various pesticides in milk and milk products. Therefore, to ascertain the compliance in milk and milk products, the use of accurate methodology of testing of pesticides residues is of paramount importance. FSSAI list of regulated pesticides has wide chemical nature due to which laboratory has to perform different protocols for extractions, chromatographic separations and mass spectrometric detections. Use of mass spectrometry with gas or liquid chromatography offers high selectivity and sensitivity at very low levels of MRL.

Milk is a complex biological matrix, its components (Lipids and proteins) interfere with extraction and quantification (Tripathy et al. 2019). Hence for optimum simultaneous extraction of multiclass pesticide residues, milk and milk products are required to be optimally extracted using solvents and salts followed by clean up with sorbents like C-18, PSA, and dehydrating agents like Na_2SO_4 and $MgSO_4$ before analysis. QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) technique has emerged as a method of choice for simple, fast, efficient and economical extraction of pesticides residues from food produce (Anastassiades et al. 2003; Wilkowska et al. 2011) and milk products (Singh et al. 2012; Golge et al. 2018). Few chemically different classes of pesticides requires to perform methods with different principles for their extraction which involves steps like saponification, derivatisation, digestion of analyte to release CS_2 and extraction using a specific solvents or purification or clean-up through specific cartridge at specific pH, temperature and flow conditions. All these methods need to be thoroughly validated at and around MRLs set by legislative bodies for analytical parameters like Linearity, Matrix Effect (ME), Limit of Quantification (LOQ), Specificity, Trueness, Precision, Ion Ratio and Retention Time (RT) for use in laboratory to meet regulatory requirement. In this context main objective of the present study is to provide a robust single laboratory validated methods meeting requirement of SANTE/11321/2021 for adoption to analyse milk and milk products for residues of pesticides as per FSSAI requirement in the country.

Materials and Methods

The method setup, development and validation work was conducted at CALF, NDDB, Anand which is an analytical laboratory, accredited by NABL as per ISO 17025:2017, recognised by Export Inspection Council (EIC), BIS and is a National Reference Laboratory (NRL) of FSSAI for milk and milk products.

Standards, Reagents, and Chemicals

Pesticides standards, acetonitrile, toluene, hexane, ethyl acetate, methanol, ammonium formate, formic acid, anhydrous $MgSO_4$, PSA, C18 and all other chemicals and reagents were purchased from authorised distributors of Sigma Aldrich, Dr. Ehrenstofer and Indian companies. All chemicals, reagents, and solvents used were of pesticide residue analysis or HPLC or MS grade and are supplied by major Indian chemical suppliers.

Milk samples

Milk samples collected from the local market were analysed to investigate the presence of pesticides for the selection of blank control sample to prepare calibration curve using the standard addition approach and other quality control checks.

Standard Preparation

Stock standards of pesticides were prepared from analytical grade Certified Reference Material (CRM) of purity more than 95 %. An amount of about 10 mg of pure CRM standards were accurately weighed (Sartorius CP225D) and were dissolved and diluted to 10 ml with a suitable solvent (toluene, methanol, acetonitrile, acetone, etc.) for better solubility. The standards were immediately labelled for a minimum requirement of identification (name, date of preparation, date of expiry, concentration) and stored at -20 ºC (Thermo Fischer Scientific Model 7320 U) in the amber coloured bottles and care was taken to prevent loss of solvent and entry of water. Stock standards need to be checked for solubility, there shall not be any precipitates if required, standards were sonicated (Cole Parmer 8894) for dissolution. The concentration of stock standard was calculated considering its purity and salts. Intermediate standards of 10 mg/litre in a group were prepared by pipetting (Eppendorf) required volume of individual stock standard and dissolving in a 10 ml volumetric flask. A mixture of working standards of 1 mg/litre from a mixture of all intermediate standards was prepared by dissolving the required volume of all intermediate standards in volumetric flask of 10 ml. All intermediate and working standards were labelled indelibly and stored at -20 ºC. In case, stability is an issue working standards needs to be prepared fresh. For preparation of standard for analysis of Dithiocarbamates as CS_2, the conversion factor (liberation of CS_2 from Thiram) of 0.633 was used.

Regulatory requirement of Pesticides for Milk and Milk Products as per FSSAI

The list of pesticides to be tested in milk and milk products as per FSSAI is given in Table No. 1. The table also contains information about the instrumental technique used for analysis of each pesticide. For a complete analysis of FSSAI listed pesticides for milk and milk products laboratory requires performing six different extraction protocols due to the different chemical and physicochemical nature of these pesticides.

Methods for analysis of Pesticides residues as per FSSAI for Milk and Milk products

Multiresidue (MR-1) analysis of multiclass pesticides (GC-MS/MS and LC-MS/MS)

Weigh 10.00 ± 0.01 g of homogenized sample for products containing approximately less than 30 % total solids (liquid milk, dahi, and buttermilk), 5 ±.0.01 g of homogenized sample for products containing approximately more than 30 % total solids (ice-cream, milk powder, paneer, cheese, khoa, and other traditional Indian dairy products) and 2 ±.0.01 g of homogenized sample for high fat products (cream, butter and ghee) in a 50 mL polypropylene centrifuge tube. Make slurry of milk products with more than 30% total solids by adding 5 ml water after weighing in a tube. Add 10 ml of Acetonitrile containing 1 % glacial acetic acid to weighed samples in 50 ml centrifuge tube and shake tubes

Table 1 Pesticide in Milk and Milk Products as per FSSR 2011(V-19.08.2020)

Sr. No.	Name of pesticides	MRL mg/kg	Techniques of analysis
1	Bifenthrin	0.20	GC-MS/MS MR1
2	Chlorothalonil	0.07	
3	Chlorpyriphos	0.02	
4	Cypermethrin (sum of isomers) (Fat soluble residue)	0.05	
5	Deltamethrin (Decamethrin)	0.05	
6	Dichlorvos (DDVP) (content of D.C.A. to be reported where possible)	0.01	
7	Ethofenprox (Etofenprox)	0.02	
8	Fenpropathrin	0.1	
9	Fenvalerate (Fat soluble residue)	0.01	
10	Fipronil	0.02	
11	Pirimiphos-methyl	0.05	
12	Phorate	0.05	
13	Acephate (expressed as mixture of Methamidophos and acephate).	0.02	LC-MS/MS MR1
14	Acetamiprid	0.02	
15	Azoxystrobin	0.01	
16	Sum of benomyl and carbendazim expressed as carbendazim	0.1	
17	Bitertanol	0.05	
18	Buprofezin	0.01	
19	Carbaryl	0.05	
20	Carbendazim	0.1	
21	Carbofuran (sum of carbofuran and 3-hydroxy carbofuran)	0.05	
22	Chlorantraniliprole	0.05	
23	Chlothianidin (Chlothianidin and its metabolites)	0.02	
24	Difenoconazole	0.02	
25	Dimethoate	0.05	
26	Dinotefuran	0.1	
27	Edifenphos	0.01	
28	Emamectin Benzoate	0.01	
29	Ethion (Residues to be determined as ethion and its oxygen analogue)	0.5	
30	Flubendiamide	0.1	
31	Flusilazole	0.05	
32	Imidacloprid	0.1	
33	Indoxacarb	0.1	
34	Kresoxim Methyl	0.01	
35	Methomyl	0.02	
36	Metolachlor	0.01	
37	Monocrotophos	0.02	
38	Oxydemeton-Methyl	0.01	
39	Penconazole	0.01	
40	Phenthoate	0.01	
41	Oxygen analogues of Phorate *i.e.* Phorate sulphoxides and Phorate sulphones, expressed as phorate)		0.05
42	Propiconazole	0.01	
43	Pyraclostrobin	0.03	
44	Tebuconazole	0.01	
45	Thiacloprid	0.05	
46	Thiamethoxam	0.05	
47	Thiophanate-Methyl	0.05	
48	Trichlorfon	0.05	
49	Triadimefon	0.01	
50	2,4-Dichlorophenoxy Acetic Acid (2, 4 D)	0.05	LC-MS/MS MR2
51	Methyl Chlorophenoxy Acetic Acid (MCPA)	0.04	
52	Glufosinate Ammonium	0.02	LC-MS/MS SR1
53	Paraquat dichloride (Determined as Paraquat cations)	0.01	LC-MS/MS SR2
54	Triacontanol	0.01	GC-MS/MS SR3
55	Dithiocarbamates (Mancozeb and metiram as CS2)	0.05	GC-MS SR4
	SR-Single Residue method, MR-Multi-Residue method, LC-Liquid Chromatography, GC-Gas Chromatography, MS-Mass Spectrometry, MRL-Maximum Residue Limit as per FSSAI		

vigorously for a minute and keep tubes aside in ice-cold water or a freezer at 4 °C for 15 minutes before extraction. Add 4 gm MgSO4 and 1.5 gm sodium acetate, shake tubes for 10 minutes (Tarsons Rotaspin tube shaker) and centrifuge (Thermo Scientific Sorvall Legend XTR) at 4000 RPM for 10 minutes, the supernatant would be used for further analysis.

For GC-MS/MS amenable pesticides (Table No. 1) take 2 ml of supernatant into 20 ml glass tube, dry the sample by using a nitrogen evaporator (Caliper Life Sciences TerboVap LV) at 40 °C. Reconstitute with 2 ml of ethyl acetate and vortex for 30 seconds and for cream, butter, and ghee samples reconstitute with 1 ml ethyl acetate. Transfer extract into a clean-up tube containing 150 mg $MgSO_4$, 50 mg PSA and 50 mg C18. Vortex (Abdos SWIRLEX) the clean-up tubes for 2 minutes and centrifuge at 4000 RPM for 10 minutes. Transfer the cleaned extract into a 2 ml auto-sampler GC vial through a 0.2 µm syringe filter (Nylon Agilent part No. 5190-5271 or equivalent).

For LC-MS/MS amenable pesticides (Table No. 1) transfer 2 ml of supernatant into a 5 ml clean-up tube containing 150 mg $MgSO_4$, 50 mg PSA and 50 mg C18. Vortex clean up tubes for 2 minutes and centrifuge at 4000 RPM for 10 minutes. Take 1 ml of supernatant into a 20 ml glass tube, dry the sample by using a nitrogen evaporator at 40 °C and reconstitute it with mobile phase A: B (80:20) as in Table No. 2. Transfer the cleaned extract into a 2 ml auto-sampler vial through a 0.2 µm Nylon syringe filter (Golge et al. 2018). Instrumental parameters and MRM method are summarised in Tables 2 and 5.

Multiresidue (MR-2) Analysis of 2, 4 D & MCPA - PGRs (LC-MS/MS)

Weigh 10.00 ± 0.01 g of homogenized sample for products containing approximately less than 30 % total solids (liquid milk, dahi, and buttermilk), 5 ±.0.01 g of homogenized sample for products containing approximately more than 30 % total solids (ice-cream, milk powder, paneer, cheese, khoa, and other traditional Indian dairy products) and 2 ±.0.01 g of homogenized sample for high fat products (cream, butter and ghee) in a 50 mL polypropylene centrifuge tube. Make slurry of milk products with more than 30% total solids by adding 5 ml water after weighing in a tube. Add 10 ml of Acetonitrile to weighed samples in 50 ml centrifuge tube and shake tubes vigorously for a minute and keep tubes aside for 15 minutes. Add 4 gm $MgSO_4$ and 1 gm NaCl shake tubes for a minute and centrifuge at 4000 RPM for 10 minutes. Transfer 2 ml supernatant into 5 ml centrifuge tube containing 500 mg $MgSO_4$, 50 mg PSA and 100 mg C18. Vortex tubes for a minute and centrifuge at 4000 rpm for 10 minutes. Take 1 ml supernatant and filter through Nylon syringe filter into LC-MS/MS vial (Chris S. 2015). Instrumental parameters and MRM method are summarised in Tables 2 and 5.

Single Residue (SR-1) Analysis of Glufosinate Ammonium (LC-MS/MS)

Weigh 1 gm of milk and milk products in 15 ml centrifuge tubes, add 3 ml extraction solvent containing 50mM acetic acid and 10mM Sodium EDTA (0.287 ml of acetic acid + 0.336 gm of sodium EDTA and make up the volume to 100 ml with water). Vortex for half a minute followed by centrifugation at 4000 RPM for 10 minutes at 4 °C. Take 2 ml supernatant into a 5 ml centrifuge tube containing 25 mg C18 for clean-up. Vortex the content and centrifuge at 4000 rpm for 5 minutes. Filter the supernatant through 0.2 µm Nylon syringe filter into vial for LC-MS/MS injection (Narong C. 2015). Instrumental parameters and MRM method are summarised in Tables 2 and 5.

Note: Glufosinate Ammonium tends to interact with glass surfaces. Such interactions are stronger in presence of aprotic solvents (e.g. acetonitrile). Therefore, use only plastic tubes and vials during this analysis. Always prepare fresh working standards from intermediate.

Single Residue (SR-2) Analysis of Paraquat Dichloride (LC-MS/MS)

Weigh 10.00 ± 0.01 g of homogenized sample for products containing approximately less than 30 % total solids (liquid milk, dahi, and buttermilk), 5 ±.0.01 g of homogenized sample for products containing approximately more than 30 % total solids (ice-cream, milk powder, paneer, cheese, khoa, and other traditional Indian dairy products) and 2 ±.0.01 g of homogenized sample for high fat products (cream, butter and ghee) in a 50 mL polypropylene centrifuge tube. Make slurry of milk products with more than 30 % total solids by adding 5 ml water after weighing in a tube. Add 10 ml methanol containing 1 % formic acid to weighed samples into a 50 ml centrifuge tube. Vortex the content for a minute and shake the tubes using a tube shaker for 15 minutes. Centrifuge the samples tubes at 4000 rpm for 10 minutes at 4 °C and collect the supernatant in another centrifuge tube and adjust the pH to 6-7 using disodium dihydrogen phosphate buffer (Prepare 400 mM buffer and adjust its pH to 7 using orthophosphoric acid). Condition the cartridge (Waters Oasis WCX 3 cc Vac Cartridge, 60 mg sorbent per cartridge, 30 µm or equivalent) with 3ml methanol containing 1 % formic acid followed by 3 ml water and 3 ml methanol containing 1 % formic acid. Pass the 9 ml of supernatant through cartridges at a flow rate of approximately 1 ml/minute. Allow air to pass through the cartridge for few minutes. Elute the analyte in 3 ml methanol containing 10 % formic acid. Evaporate the content to dryness under nitrogen at 50 °C and reconstitute the sample in 1 ml water containing 10 % acetonitrile and 0.1 % formic acid**.** Filter through a 0.2 µm Nylon syringe filter into vial (Ionara R. 2016). Instrumental parameters and MRM method are summarised in Tables 2 and 5.

Note: Paraquat Dichloride tends to interact with glass surfaces. Such interactions are stronger in presence of aprotic solvents (e.g. acetonitrile). Therefore, use only plastic tubes and vials during this analysis. Always prepare fresh working standards from intermediate.

Single Residue (SR-3) Analysis of Triacontanol (GC-MS/MS)

Weigh 2 gm of homogenized liquid milk and 1 gm of milk products in a 20 ml glass test tube. Make slurry of milk products with more than 30% total solids by adding 5 ml water after weighing in a tube. Add 10 ml of ethanolic NaOH (1N NaOH dissolved in 20 parts water/80 parts ethanol. Vortex the mixture for a minute and subject content to saponification at 80 ºC for 60 minutes. After saponification add 2 ml of 5N HCL and keep samples in oven at 70 ºC for 15 minutes. Extract the samples thrice with 3 ml of heptane and collect organic phase in another glass tubes after every extraction. Wash the heptane extract with 6 ml of ultrapure water and transfer solvent layer to another glass tube. Evaporate the organic solvent to dryness under stream of nitrogen at 40 °C. Reconstitute the residues in 500 ul of derivatizing agent N, O-Bis-trimethylsilyl-trifluoroacetamide (BSTFA product no. 15238 Merck make or equivalent), incubate tubes at 80 °C for 20 minutes in oven. Evaporate the derivatizing agent BSTFA at 40 °C under a stream of nitrogen to dryness and reconstitute with 1 ml of heptane. Filter it through a 0.2 um Nylon syringe filter and collect it in GC vial. Inject 1 µl of solution in GC-MS/MS. Prepare calibration standard using standard addition technique by spiking working standard to control samples (Daniela et al. 2009) Instrumental parameters and MRM method are summarised in Tables 2 and 5.

Note: For complete dissolution of triacontanol prepare stock standard of around 100 ppm in 100 ml heptane and further prepare intermediate standard of 10 ppm in 10 ml of heptane. Analyst may observe the loss of response after few injections which may be because of acidic BSTFA affecting source or filament assembly leading to carbonization of source or oxidation of filament. It is suggested to completely dry derivatized samples before reconstitution with heptane. Further, keeping solvent delay of 6 minutes would save heavy mist of heptane and residual BSTFA ionizing in source and affecting the source conditions. Upon continual injection of samples, it is suggested to clean the MS source part after every 20 injections. For continual performance of method on wide variety of milk products laboratory would require fine-tuning of instrument method and saponification process.

Single Residue (SR-4) Analysis of Dithiocarbamates as CS_2 (GC-MSMS)

Weigh 25.00 ± 0.10 g milk and milk products in 250 ml of stoppered conical bottle, add 75 ml of the reaction mixture (take one litre water in glass bottle of capacity of 2.5 to 3.0 litres, gradually add solution of Tin (II) Chloride (30 g of 98 % purity) dissolved in one litre of concentrated HCl with continual stirring to obtain clear solution). Add 25 ml of isooctane and immediately stopper the bottle with a screw cap. Place the bottle in a water bath at 80 (±5) °C. Mix the content of the bottle by inversion after approximately every 20 minutes. After the total reaction time of 60 minutes, remove the bottle from the water bath and mix the contents of the bottle. Transfer the bottle in ice water bath to cool down the temperature quickly. After cooling the reaction mixture to about 10-20 °C, transfer 1.8 ml of isooctane layer in a 2 ml centrifuge tube and centrifuge at 4000 rpm for 10 min at 10 °C. Transfer the supernatant (1 ml) to an auto-sampler vial (Sumaiyya et al. 2014). Instrumental parameters and MRM method are summarised in Tables 2 and 5. (See supplementary file online)

Note: Analyse the samples immediately on GC-MSMS, avoid storage of the prepared sample vials. For quantification prepare calibration standards by spiking respective/representative sample commodity using standard addition approach.

Results and Discussion

Validation of methods

Validation of method is required to be done to demonstrate that a method is fit-for-intended purpose. A validated test method ensures accurate, reliable, and consistent results and validation of in-house test methods is a mandatory requirement of accreditation as per ISO17025:2017. Parameters and criteria of validation for analysis of pesticides residues as per SANTE/11321/2021 are indicated in Table 3. These parameters were evaluated and results obtained are discussed as per criteria of SANTE/11321/2021.

Linearity

Linearity can be tested by examination of a plot of residuals produced by linear regression of the responses verses concentrations. In general, the use of weighted-linear regression is recommended for low part per billion (µg/kg) concentrations. Ideally, the value of the intercept should be close to zero to reduce errors in the calculation of concentrations at lower levels, at the same time calibration curve should not be forced through the origin/zero without justification. Formula for linear equation is as given below,

$y = mx \pm c$

Where, y is the instrument response (plotted on Y axis)

m represents the slope (sensitivity),

c is a constant that describes the background (intercept on Y axis)

x is the analyte concentration (plotted on X axis) of unknown samples,

Linearity of a test method was studied by injecting standards at five concentration levels as showed in Table 4 and was considered

Table 2 Instrumental parameters

Multiclass: MR-1 Multiresidue method GC-MS/MS	Multiclass: MR-1 Multiresidue method LC-MS/MS	2, 4 D and MCPA: MR-2 (PGR) Multiresidue method
Instrument Conditions (Agilent 7010B)	Instrument conditions (Waters Xevo TQS)	LC-MS/MS
GC Oven conditions	Instrument Settings	Instrument conditions (Waters Xevo TQS)
Oven Temperature Program	Mobile Phase A: 0.1 % formic acid and 5	Instrument Settings
60 ºC for 1 minutes,	mM ammonium formate in water:	Mobile Phase A: 0.1 % acetic acid in water
40 ºC per minute to 170 ºC,	methanol (90:10)	Mobile Phase B: 0.1 % acetic acid in
10 ºC per minutes to 310 ºC,	Mobile Phase B: 0.1 % formic acid and 5	acetonitrile
3 minutes hold	mM ammonium formate in methanol:	Flow rate: 0.4 ml/min
Run time- 20.75 minutes	water (90:10)	BEHC18 1.7µm, 2.1 X 100mm Column
GC injection conditions	Flow rate: 0.4 ml/min	Temperature: 40 ºC
Inlet Type: Multi-Mode Inlet (MMI)	Column Temperature: 40 ºC	Injection Volume: 5 µL
Liner: 2 mm id Agilent's part no. 5190-2293	Injection Volume: 5 µL	Run Time: 11 minutes
Injection Volume: 1 µl (Syringe 10 µl)	Column:	UPLC Gradient:
Injection Mode: Split less	BEHC18 1.7µm, 2.1 X 100mm	Time / Mobile Phase (%)
Inlet Temperature (ºC): 280	Run Time: 22 minutes	Min. / A / B
Septum Purge (ml/min): 3	UPLC Gradient:	0.0 / 90 / 10
GC Column Flow Conditions	Time / Mobile Phase (%)	0.5 / 90 / 10
Carrier Gas: Helium	Min. / A / B	4.0 / 10 / 90
Column 1 and 2 connected through union	0.0 / 98 / 2	4.5 / 10 / 90
DB5MS 15m x 250µm x0.25µm	0.5 / 98 / 2	5.0 / 90 / 10
Column 1 flow: 1.197 ml /min	15 / 2 / 98	8.0 / 90 / 10
Column 2 flow: 1.397 ml/min	17 / 2 / 98	MS conditions
MS conditions	17.5 / 98 / 2	Mode: ESI (Negative mode)
MS Source (eV): 70	22 / 98 / 2	Capillary (kV): 2
Source temperature (^{0}C): 280	MS conditions	Cone (V): 20
Quadruple Temp (^{0}C).: 150 ºC	Mode: ESI (Positive mode)	Source offset (V): 80
Transfer Line Temp.: 280	Capillary (kV): 1.00	Source temperature (^{0}C): 150
Helium Quench (ml/min): 2.25	Source offset (V): 80.0	Desolvation Temp. (^{0}C): 550
N2 Collision (ml/min): 1.5	Source temperature (^{0}C): 150	Cone Gas Flow (L/Hr): 150
	Desolvation Temp. (^{0}C): 500	Desolvation Gas flow (ml/Min): 1000
	Cone Gas Flow (L/Hr): 150	Collision Gas (ml/Min): 0.15
	Desolvation Gas flow (ml/Min): 1000	
	Collision Gas (Bar): 0.15	

Glufosinate Ammonium: SR-1 Single Residue method LC-MS/MS
Instrument Settings
Mobile Phase A: 50 mM ammonium formate in water (pH adjusted to 2.9 using formic acid)
Mobile Phase B: Acetonitrile containing 0.5% formic acid
Flow rate: 0.5 ml/min
Column Temperature: 40 ºC
Injection Volume: 10 µL
Column: Torus DEA 1.7 µm, 2.1 X 100mm
Run Time: 10 minutes
UPLC Gradient

Time (Min.)	Mobile Phase (%) A	B
0.0	10	90
0.2	10	90
4.5	60	40
5	10	90
10	10	90

MS conditions
Mode: ESI (Negative mode)
Capillary (kV): 1.00
Source offset (V): 80.0
Source temperature (^{0}C): 150
Desolvation Temperature (^{0}C): 550
Cone Gas Flow (L/Hr): 150
Desolvation Gas flow (ml/Min): 1100
Collision Gas Flow (Bar): 0.15

Paraquat Dichloride: SR-2 Single Residue method LC-MS/MS
Instrument Settings
Mobile Phase A : 50 mM ammonium formate in water (pH adjusted to 2.9 using formic acid)
Mobile Phase B: Acetonitrile containing 0.5% formic acid
Flow rate: 0.6 ml/min
Column Temperature: 40 ºC
Injection Volume: 5 µL
Column: X-Bridge HILIC 2.5 µm, 2.1 X 100mm
Run Time: 10 minutes
UPLC Gradient

Time (Min.)	Mobile Phase (%) A	B
0.0	00	100
0.5	00	100
3.5	60	40
4.5	60	40
5.0	00	100
10	00	100

MS conditions
Mode: ESI (Positive mode)
Capillary (kV): 1
Cone (V): 20
Source offset (V): 80
Source temperature (^{0}C): 150
Desolvation Temperature (^{0}C): 550
Cone Gas Flow (L/Hr): 150
DesolvationGasflow (ml/Min): 1100
Collision Gas Flow (ml/Min): 0.15

Triacontanol: SR-3 Single Residue method GC-MS/MS
Instrument conditions (Agilent 7010B)
GC Oven conditions: 200 ºC hold for 1 min.
8 ºC per minute to 270 ºC hold 2 minutes,
20 ºC per minutes to 310 ºC hold 8 minutes
Run time- 20.75 minutes,
GC injection conditions
Inlet Type: Multi-Mode (MMI)
Injection Volume: 1µl
Injection Mode: Split less
Inlet Temperature: 320 ºC
Septum Purge: 3 ml/min
GC Column Flow Conditions
Carrier Gas: Helium
Column 1 and 2 connected through multi union
DB5MS, 15 m x 250 µm x 0.25 µm back flush.
Column 1 flow: 1.063 ml /min
Column 2 flow: 1.263 ml/min
MS conditions
MS Source (eV): 70
Source temperature (ºC): 300
Quadruple Temperature (ºC): 150
Transfer Line Temperature (ºC): 320
Solvent Delay (min): 6
Helium Quench gas (ml/min): 2.25
N2 Collision Gas (ml/min): 1.5
Acquisition mode: MRM
MRM transitions: 494.9>75(Q), 97.1(q1),

DTC (Mancozeb and metiram as CS2): SR-4 Single Residue method
GC-MS/MS-Instrument conditions (Agilent 7010B)
GC Oven conditions: 40 ºC for 5 minutes,
40 ºC per minute to 200 ºC hold for 3 minutes
Run time 12 minutes
GC injection conditions
Inlet Type: Multi-Mode Inlet (MMI)
Liner: 2 mm id
Injection Volume: 1 µl
Injection Mode: Splitless
Inlet Temperature: 100 ºC
Septum Purge: 3 ml/min
GC Column Flow Conditions
Carrier Gas: Helium
Column 1 and 2 connected through multi union
DB5MS, 15 m x 250 µm x 0.25 µm
Column 1 flow: 1.063 ml /min
Column 2 flow: 1.264 ml/min
MS conditions
MS Source (eV): 70
Source temperature (ºC): 250
Quadruple Temperature (ºC): 150
Transfer Line Temperature (ºC): 280
Solvent Delay (min): 1
Helium Quench gas (ml/min): 2.25
N2 Collision Gas (ml/min): 1.5
Acquisition mode: SIM, Ions: 76 (Q) and 78 (q)

Table 3 Pesticides Method Validation Parameters and Criteria as per SANTE/11321/2021

Sr. No.	Parameter	Details of the study	Criterion
1	Linearity	Linearity check from five levels	Deviation of back calculated concentration =±20 %
2	Matrix Effect (ME)	Comparison of response from solvent standards and matrix-matched standards	< 20 % signal suppression or enhancement, if ME >20 %, use procedural calibration.
3	LOQ	Lowest spike level meeting criteria for trueness and precision	≦ MRL Mean Recovery 70 to 120% Mean Precision RSD <20%
4	Specificity	Response in reagent blank and blank control samples	≦30 % of RL/LOQ
5	Recovery (Trueness)	Average recovery at spike level tested	70 to 120%
6	Precision (RSDr)	RSDr for each spike level tested	RSD <20%
7	Precision (RSDwR)	RSDwR from on-going method validation / verification	RSD <20%
8	Robustness	Average recovery and RSDwR, derived from on-going method validation	Mean Recovery 70 to 120% Mean Precision RSD <20%
9	Ion ratio	Shall comply requirements for MS	Within ±30% (relative)
10	Retention time	Repeatability throughout the batch	±0.1 Minute

acceptable when residuals (Deviation or difference of back-calculated concentration from calibration curve verses actual concentration) were ≤±20 %. Standard addition approach was used for checking the linearity. As evident from the data in Table 4, calibration curves were linear over the tested range as the residuals/deviation from back calculations were ≤±20 % for all pesticides and regression coefficients (r^2) were higher than 0.99 except for Edifenphos (0.984).

Matrix Effect

Matrix effect is an influence of the one or more undetected matrix components from the sample on the measurement of the analyte

concentration. The matrix effect of milk constituents at a retention time of the analyte of interest is determined by comparing the response of an analyte with and without matrix component. Matrix effect occurs frequently in both gas and liquid chromatographic techniques and should be assessed at the initial method validation stage. Majorly matrix effect in GC analysis is attributed to shielding of active sites in GC liner and column by matrix components which reduce the interaction of the analytes on these active sites and lead to enhanced analytes response. Matrix effect in LC analysis is attributed to the ionisation behaviour of the analytes in source in presence and absence of the matrix components. Percentage matrix effect (ME) can be calculated using following equation,

$$\% \text{ ME} = 100 - (100 \times A_{\text{m extract}} / A_{\text{s standard}})$$

Table 4 Results for Linearity Study

Multiresidue (MR-1) analysis of multiclass pesticides (GC-MS/MS)							
Sr. No.	Name of the Parameter	r^2	% Deviation of from calculation (µg/kg)				
			5.0	10.0	20.0	40.0	80.0
1	Bifenthrin	0.9985	-5.7	0.7	4.3	3.0	-2.3
2	Chlorothalonil	0.9950	-9.5	4.9	1.0	7.8	-4.1
3	Chlorpyriphos	0.9997	-2.4	0.7	2.9	-1.0	-0.2
4	Cypermethrin (Total)	0.9984	0.0	0.0	-0.7	1.8	-1.2
5	Deltamethrin	0.9996	0.4	1.8	-3.5	1.4	-0.1
6	Dichlorvos	0.9985	0.3	-1.3	1.3	1.0	-1.3
7	Etofenprox	0.9970	-7.8	2.1	3.7	5.3	-3.4
8	Fenproprathrin	0.9990	-4.9	0.6	4.3	1.8	-1.7
9	Fenvalerate (Total)	0.9996	1.1	-2.7	0.4	2.0	-0.8
10	Fipronil	0.9987	1.4	-6.5	4.6	1.9	-1.4
11	Phorate	0.9985	0.2	-0.6	-0.4	2.0	-1.2
12	Pirimiphos Methyl	0.9992	-0.8	-2.8	2.3	2.9	-1.6
Multiresidue (MR-1) analysis of multiclass pesticides (LC-MS/MS)							
Sr. No.	Name of the Parameter	r^2	% Deviation of from calculation (µg/kg)				
			5	10	20	35	50
13	Acephate	0.9957	-4.0	-3.8	1.0	5.6	-2.3
14	Acetamiprid	0.9986	0.0	-2.0	-0.5	-4.5	3.6
15	Azoxystrobin	0.9980	-2.0	2.0	2.5	-9.4	5.4
16	Bitertanol	0.9979	10.0	0.0	-0.5	0.6	-0.6
17	Buprofezin	0.9996	0.0	-3.0	-4.0	1.7	0.8
18	Carbaryl	0.9985	0.0	-7.0	-7.5	-14.3	13.6
19	Carbendazim	0.9975	-6.0	14.0	19.5	4.9	-12.2
20	Carbofuran	0.9996	-8.0	9.0	11.0	0.0	-5.0
21	Carbofuran Hydroxy	0.9992	0.0	-2.0	-1.0	-5.7	4.6
22	Chlorantraniliprole	0.9992	-4.0	-2.0	1.5	-12.9	8.6
23	Clothianidin	0.9961	0.0	-1.0	1.5	-8.3	5.2
24	Difenoconazole	0.9997	-4.0	-4.0	-2.0	1.1	0.8
25	Dimethoate	0.9993	-2.0	-3.0	-2.0	-7.7	6.6
26	Dinotefuran	0.9895	-2.0	-15.0	-6.5	-16.3	-4.8
27	Edifenphos	0.9843	-2.0	3.0	1.0	0.5	-1.2
28	Emamectin Benzoate	0.9991	-2.0	-5.0	-4.0	-2.6	4.0
29	Ethion	0.9982	0.0	3.0	5.0	3.7	-4.8
30	Flubendiamde	0.9991	-15.6	-10.4	-3.4	2.0	8.0
31	Flusilazole	0.9971	-2.0	1.0	-2.5	-1.4	2.0
32	Imidacloprid	0.9989	0.0	-2.0	-3.5	-8.0	7.0
33	Indoxacarb	0.9998	0.0	-1.0	-4.5	-3.1	4.0
34	Kresoxim Methyl	0.9964	-6.0	3.0	7.5	-1.7	-1.8
35	Methamidophos	0.9932	-1.0	0.6	1.3	4.9	-9.1
36	Methomyl	0.9987	2.0	-2.0	2.0	-0.3	-0.4
37	Metolachlor	0.9995	-2.0	-2.0	-3.5	0.6	1.4
38	Monocrotophos	0.9989	-2.0	-2.0	-1.0	-5.1	4.4
39	Oxydemeton methyl	0.9994	-2.0	-6.0	-5.0	-9.7	9.4
40	Penconazole	0.9992	-4.0	-2.0	-2.5	-1.7	2.6
41	Phenthoate	0.9871	-4.0	2.0	3.5	-0.6	-1.0
42	Phorate-sulfone	0.9936	-6.9	-5.8	3.0	1.0	2.6
43	Phorate-sulfoxide	0.9971	-12.4	-5.6	-2.6	4.0	6.5
44	Propiconazole	0.9992	-4.0	-4.0	1.0	-5.1	4.0
45	Pyraclostrobin	0.9998	-4.0	-4.0	-3.0	-0.3	2.2
46	Tebuconazole	0.9988	-2.0	1.0	-0.5	1.1	-0.4
47	Thiacloprid	0.9962	14.0	2.0	2.0	-2.9	-15.8
48	Thiamethoxam	0.9968	-2.0	1.0	1.5	-5.4	3.4
49	Thiophanate methyl	0.9994	-2.0	-10.0	-6.0	-4.6	6.8
50	Triademefon	0.9989	-2.0	-1.0	0.5	1.4	-1.0
51	Trichlorfon	0.9992	-2.0	-2.0	-2.0	-7.7	6.2

Multiresidue (MR-2) analysis of PGR pesticides (LC-MS/MS)							
Sr. No.	Name of the Parameter	r^2	% Deviation of from calculation (µg/kg)				
			5	10	20	50	100
52	2, 4 D	0.9921	-3.6	-1.5	-1.5	-2.8	1.8
53	MCPA	0.9919	-0.3	0.9	-2.0	6.2	-4.7
Single Residue (SR-1) analysis of Glufosinate Ammonium (LC-MS/MS)							
Sr. No.	Name of the Parameter	r^2	% Deviation of from calculation (µg/kg)				
			5	10	20	40	60
54	Glufosinate ammonium	0.9970	0.2	-0.8	0.9	-0.5	0.2
Single Residue (SR-2) analysis of paraquat dichloride (LC-MS/MS)							
Sr. No.	Name of the Parameter	r^2	% Deviation of from calculation (µg/kg)				
			5	10	20	50	100
55	Paraquat	0.9960	-0.6	0.5	4.4	0.4	-4.4
Single Residue (SR-4) analysis of Dithiocarbamates as CS2 (GC-MS)							
Sr. No.	Name of the Parameter	r^2	% Deviation of from calculation (µg/kg)				
			25	50	100	200	400
56	Dithiocarbamates as CS2	0.9989	-1.8	-5.3	4.2	6.4	-3.5

Where, % ME is the Percentage Matrix Effect

$A_{m\ extract}$ is the peak area of analyte of interest with matrix

$A_{s\ standard}$ is the peak area of the analyte without matrix

ME value less than zero (i.e. negative value) indicates matrix suppression, while greater than zero (i.e. positive value) is a sign of matrix enhancement. SANTE/11321/2021 has set criteria of matrix effect <20 % signal suppression or enhancement. In the present study, both signal suppression and enhancement were observed for most of the pesticides. To compensate for the matrix effect SANTE/11321/2021 has also suggested an approach of standard addition technique or use of isotopically labelled internal standards for quantification. Therefore, in this work standards addition technique was adopted for plotting calibration curves and quantitation.

Limit of Quantification (LOQ)

LOQ is commonly defined as the minimum concentration of the analyte in sample that can be quantified with acceptable precision (repeatability) and accuracy. As per the theoretical definition by analytical chemists, the LOQ is the concentration at which the signal/noise ratio (S/N) equals 10 in the analysis. Due to the the latest instrumental developments and improved detection capabilities of the LC-MS/MS or GC-MS/MS, the significance of this theoretical definition of LOQ is limited. Thus, in case of pesticides residue analysis spiking at the target LOQ (must be well below target regulatory MRL and within the linear dynamic range of the instrument) is the more descriptive and practical approach. In essence, the point of the establishing a LOQ is not to determine how low the instrument can detect analytes of interest, but to demonstrate that the lowest reported concentration is meeting the requirement of the need for the analysis at and around or below the target regulatory MRL.

In the current study LOQ for all the compounds is determined by checking mean relative standards deviation (RSD) and percentage average recovery of six replicates at target LOQ. To meet accuracy criteria average % recovery at target LOQ shall be between 70 to 120 % and to meet precision criteria for mean relative standard deviation shall be less than 20 % of data from a minimum of 6 replicate injections. As showed in Table 5 and 6 the target LOQ for most of pesticides is established at 5 µg/kg, whereas it is 10 µg/kg for Bifenthrin, Cypermethrin, Dichlorvos, Etofenprox, Phorate and Glufosinate Ammonium and 25 µg/kg for Dithiocarbamtes as CS_2.

Specificity/Selectivity

Specificity/Selectivity is the extent to which a method can determine a particular analyte in a mixture or matrix without interferences from other components of similar properties. Ideally, specificity/selectivity should be evaluated to demonstrate that interferences are not significantly influencing the results. It is impractical to test the method against every potential interferant, but it is required that common interferences are checked by analyzing a reagent (process) blank for every batch of reagents. When reagents and solvents are changed between analytical batches, additional process blank evaluations must be performed. Background levels of plasticizers, bleed of septa, cleaning agents, impurities of reagent, laboratory contamination, last run carry-over, etc. tend to appear in process blank which must be recognized by the analyst when they occur. Formula for calculation of specificity/selectivity in terms of interference at retention time (RT) of analytes of interests is as given below,

$$\text{Specificity / selectivity}_{(\%\ \text{interference at RT of analyte})} = (A_c / A_{LOQ})*100$$

Where, A_c is area of control samples at RT of analyte of interest

A_{LOQ} is area of standards at reporting level or LOQ at RT of analyte of interest

The specificity/selectivity was evaluated for each of the MRM transitions by analyzing the control sample, process blank, and solvent blank. Chromatograms did not show any response of

interfering peaks at the analyte retention time for any of the pesticides investigated in this work. SANTE/11321/2021 has given criteria for specificity/selectivity response of interfering compounds at a retention time of analytes of interest shall be less than 30 % of reporting limit or LOQ.

Trueness (bias)/Accuracy

The closeness of agreement between test results and the accepted reference value of the property being measured is termed as trueness. Quantitatively trueness is stated in terms of bias, smaller the bias greater the trueness. Typically bias is tested by evaluating the response of the method to a CRM with an assigned known value. Trueness (bias) can also be determined by calculating the recovery percentage of spiked samples at different levels and comparing them with acceptance criteria of 70 to 120 %. Recovery of an analyte is termed as the amount of analyte determined in the final result compared with the amount added to a control sample before extraction. The trueness for multiresidue method was evaluated by extracting 6 replicates of blank samples spiked at LOQ and at tested levels as in Table 5 and 6. Results showed in Table 5 and 6 shows that trueness (bias) meets validation criteria for all pesticides at all tested levels. Minimum and maximum acceptable % recovery of all pesticides among all tested levels were credited to Triademefon (73.4±1.2 % @ 40 µg/kg) and Fenproprathrin (118.5±6.4 % @ 5 µg/kg) respectively.

Table 5 and 6

Precision (RSDr)

Precision is the closeness of agreement between the results of replicate tests obtained under stipulated conditions and it is usually specified in terms of standard deviation (SD) or relative standard deviation (RSD) or the coefficient of variation (CV). It is expressed as repeatability in terms of relative standard deviation (RSD %). SANTE/11321/2021 has given criteria for precision in terms of repeatability (RSDr) at each spike level tested, shall be less than equal to ±20 %. The precision was evaluated by calculating % RSDr upon analysis of 6 replicates of spiked control samples at LOQ and tested level as in Table 5 and 6. It is evident from Table 5 and 6 that results for precision meet validation criteria for all pesticides at all tested levels. Minimum and maximum acceptable precision expressed as % RSDr of all pesticides among all tested levels were attributed to Tebuconazole and 2, 4 D (1.0 % @ 5 and 50 µg/kg respectively) and Paraquate Dichloride (19.5 % @ 10 µg/kg) respectively.

Precession (RSDwr) and Robustness

Both parameters can be carried out during on-going method validation and can be performed during routine testing and are required to be evaluated statistically as evaluated in the above parameters. Precession (RSDwr) is a within lab reproducibility which can be evaluated by checking the precision of six replicates injection at LOQ, and at levels as in trueness or precision trials on three different days. Robustness (often synonymous with ruggedness) of an analytical method is the resistance to change in the results produced by the analytical method. The aspects of the method should be identified that are likely to affect results, and their influence on method performance evaluated by using ruggedness tests. Examples of the factors that a robustness test could address are small changes in the instrument, brand/lot of reagent or operator or analyst, the concentration of a reagent, pH of a solution, the temperature of a reaction, the time allowed for completion of a process, and/or other pertinent factors.

Ion ratio

It is the % relative abundance of qualifier (q) transition with the quantifier (Q) transition. Based on the relative abundance of two transitions, the ion ratio in samples, it should be within 30 % of the average reference value from the standard calibration curve. The average ion ratio obtained from the calibration curve was used as a reference ion ratio. The ion ratios for different concentrations of the standards for all pesticides were consistent and were within 30 % of the average reference value from the standard calibration curve. Calculation of Ion ratio with the example of Phorate is as given below,

% Ion Ratio= (Area of qualifier ion/Area of quantifier ion)*100

Example: When, Area of qualifier ion of phorate *i.e.,* 121.0 > 47.0 (q1) is 28003 at 5 µg/kg

Area of Quantifier ion of phorate *i.e. 260* > 75 (Q) is 93346 at 5 µg/kg

% Ion Ratio for phorate @ 5 µg/kg = (28003/93346)*100 = 29.99%

For positive confirmation of an analyte in the unknown sample average of ion ratio from the multi-level calibration curve should be taken as reference value to check ion ratio confirmation criteria of ±30 % for positive determination.

Retention Time (RT)

The retention time shift of the analyte in the extract and calibration standard is evaluated for acceptable tolerance of ±0.1 minute shift. The retention time of each pesticide was evaluated for a possible shift, which was found satisfactorily meeting the criteria of ±0.1 minute as per SANTE/11321/2021. Calculation of retention time shift with the example of Phorate is as given below,

Example: Retention time of phorate in 5-point calibration curve is consistently 7.51 minute. For confirm positive determination of phorate in an unknown sample, RT of a peak shall fall within criteria of acceptable tolerance i.e. ±0.1, For constant retention time instrument conditions like flow of mobile phase, temperature,

Table 5 Results for Validation Study (Multiresidue GC-MSMS)

Sr. No.	Name of the Parameter	Optimised conditions Quantifier (Q) qualifier (q) transitions			Method validation results at different levels of spiking (µg/kg)			
		RT	MRM Transitions	CE (Ev)	% Mean Recovery (± RSD, n = 6) (RSDr)			Target LOQ µg/kg
					5	10	40	
1	Bifenthrin	13.94	181.2 > 165.2 (Q)	25	120.7 ±7.8*	109.8 ±4.9	99.5 ±3.3	10
			181.2 > 166.2 (q1)	10				
			166.2 > 165.2 (q2)	20				
2	Chlorothalonil	8.42	265.9 >133 (Q)	45	113.7 ±17.9	100.7 ±6.9	92.3 ±8.8	5
			265.9 > 230.9 (q1)	20				
			265.9 >168 (q2)	30				
3	Chlorpyriphos	9.85	196.9 > 169 (Q)	15	113.1 ±11.5	108.1 ±6.9	101.9 ±5.3	5
			198.9 >171 (q1)	15				
			313.8 > 257.8 (q2)	15				
4	Cypermethrin (sum of Four Isomers)	16.62	181.2 > 152.1 (Q)	25	200.0 ±76.2*	114.5 ±11.1	107.3 ±4.4	10
			181 > 152.1 (q1)	25				
			165.1 > 91.1 (q2)	15				
5	Deltamethrin	18.2	252.9 > 93 (Q)	15	112.8 ±7.8	115.3 ±4.6	109.0 ±3.6	5
			181 > 152.1 (q1)	25				
			250.7 > 172 (q2)	5				
6	Dichlorvos (DDVP)	4.65	184.9 > 93 (Q)	10	128.8 ±22.4*	74.2 ±11.5	77.3 ±16.2	10
			144.9 > 109 (q1)	10				
			109 > 79 (q2)	5				
7	Etofenprox	16.89	163 > 107.1 (Q)	20	120.6 ±7.9*	114.3 ±5.5	103.1 ±4.2	10
			163 > 135.1 (q1)	10				
			107 > 77 (q2)	15				
8	Fenproprathrin	14.12	181.1 > 152.1 (Q)	25	118.5 ±6.4	109.7 ±7.6	100.7 ±3.8	5
			125 > 55.1 (q1)	10				
			207.9 > 181 (q2)	5				
9	Fenvalerate (sum of Two Isomers)	17.46 (I) 17.66 (II)	167 > 125.1 (Q)	5	110.7 ±9.2	107.4 ±4.2	102.4 ±5.3	5
			167 > 88.9 (q1)	40				
			224.9 > 119 (q2)	15				
10	Fipronil	10.46	366.8 > 212.8 (Q)	25	118.2 ±13.0	112.6 ±6.6	101.7 ±3.0	5
			254.9 > 228 (q1)	15				
			350.8 > 254.8 (q2)	15				
11	Phorate	7.5	260 > 75 (Q)	5	123.2 ±13.1*	106.2 ±9.8	95.1 ±7.6	10
			121 > 47 (q1)	15				
			128.9 > 65 (q1)	10				
12	Pirimiphos Methyl	9.5	290 > 125 (Q)	20	113.6 ±8.8	107.0 ±6.7	97.1 ±5.1	5
			232.9 > 151 (q1)	5				
			232.9 > 125 (q2)	5				

*Due to % recovery outside the accepted range, target LOQ for these compounds was established at 10 µg/kg

Table 6 Results for Validation Study (Multi and Single Residue LC and GC-MSMS)

Multiresidue (MR-1) analysis of multiclass pesticides (LC-MS/MS)									
Sr. No.	Name of the Parameter	Optimised conditions Quantifier (Q) qualifier (q) transitions				Method validation results at different levels of spiking (µg/kg)			
		RT	MRM Transitions	Cone (V)	CE (Ev)	% Mean Recovery (± RSD, n = 6) (RSDr)			Target LOQ µg/kg
						5	10	40	
13	Acephate	1.54	183.9 > 142.8 (Q)	20	10	106.5	107.4	97.7	5
			183.9 > 124.9 (q)	20	12	±13.9	±10.8	±12.9	
14	Methamidophos	1.22	141.9 > 124.8 (Q)	30	14	97.3	114.9	103.2	5
			141.9 > 93.9 (q)	30	12	±9.2	±8.1	±7.3	
15	Acetamiprid	6.02	223.0 > 126.00 (Q)	30	20	99.0	95.5	76	5
			223.00 > 56.1 (q)	30	15	±1.6	±8.9	±5.9	
16	Azoxystrobin	11.6	404.1 > 372.0 (Q)	15	8	98.3	86.8	85.3	5
			404.1 > 328.9 (q)	15	30	±2.3	±14.1	±2.6	
17	Carbendazim	3.4	192.1 > 160.1 (Q)	10	15	94.7	85.7	96.1	5
			192.1 > 132.1 (q)	10	30	±2.0	±17.0	±9.2	
18	Bitertanol	13.9	338.1 > 98.9 (Q)	30	16	100.5	97.2	109.2	5
			338.1 > 70.1 (q)	30	8	±2.6	±13.7	±3.5	
19	Buprofezin	14.84	306.1 > 201.0 (Q)	31	12	95.9	101.1	114.2	5
			306.1 > 57.4 (q)	31	20	±3.3	±4.9	±3.9	
20	Carbaryl	9.13	202.1 > 145.1 (Q)	25	10	91.7	92	79.1	5
			202.1 > 127.1 (q)	25	25	±10.5	±12.2	±4	
21	Carbofuran	8.58	222.11 > 165.1 (Q)	5	10	114.50	106.3	113.8	5
			222.11 > 123.0(q)	5	20	±8.3	±5.7	±4.2	
22	Carbofuran hydroxy	5.75	238.0 > 163.00 (Q)	34	16	99.3	91.1	77	5
			238.00 > 107.0 (q)	34	16	±1.9	±10.9	±5.7	
23	Chlorantraniliprole	11.01	484.0 > 453.0 (Q)	18	17	92.9	90.6	73.4	5
			484.0 > 286.0 (q)	18	12	±2.7	±4.9	±4.4	
24	Chlothianidin	5.04	250.0 > 169.0 (Q)	25	10	99.0	90.9	76.4	5
			250.0 > 132.0 (q)	25	15	±4.0	±15.1	±5	
25	Difenoconazole	14.33	406.1 > 337.2 (Q)	35	12	94.9	91.6	81.6	5
			406.1 > 250.9 (q)	35	25	±3	±8.4	±4.8	
26	Dimethoate	5.4	230.0 > 198.8 (Q)	20	10	95.5	92.9	78.6	5
			230.0 > 124.8 (q)	20	22	±1.5	±7	±4.7	
27	Dinotefuran	2.53	203.2 > 129.1 (Q)	10	10	100.4	107.1	87.9	5
			203.2 > 114.1 (q)	10	15	±6.5	±9.2	±6.2	
28	Edifenphos	13.5	311.0 > 111.0 (Q)	23	26	98.3	101.4	93.7	5
			311.0 > 109.0 (q)	23	32	±1.5	±9.5	±2.3	
29	Emamectin Benzoate	15.68	886.6 > 158.0 (Q)	45	37	90.9	85.1	75.4	5
			886.6 > 82.0 (q)	45	35	±3.1	±8.2	±3.4	
30	Ethion	15.22	385.0 > 199.0 (Q)	30	10	94.5	94.4	92.3	5
			385.0 > 142.9 (q)	30	25	±3.2	±11.5	±4.1	
31	Flubendiamide	13.33	683.3 > 408.2 (Q)	5	5	98.5	103.9	103.1	5
			683.3 > 274.2 (q)	5	16	±10.7	±7.9	±10.8	
32	Flusilazole	13.02	316.0 > 247.0 (Q)	5	20	96.2	84.3	80.2	5
			316.0 > 165.0 (q)	5	25	±6.3	±9.1	±4.2	
33	Imidacloprid	5.14	256.1 > 209.0 (Q)	25	12	98.7	85.2	77.3	5
			256.1 > 174.9 (q)	25	20	±3.7	±27.8	±8.2	
34	Indoxacarb	14.4	528.1 > 217.9 (Q)	30	25	92.3	92.4	83.3	5
			528.1 > 202.9 (q)	30	40	±6.0	±6.2	±4.6	
35	Kresoxim Methyl	13.21	314.2 > 131.0 (Q)	30	25	105.5	110.9	112.6	5
			314.2 > 115.9 (q)	30	12	±5.4	±2.9	±1.3	
36	Methomyl	3.58	162.9 > 105.9 (Q)	15	10	97.0	91.3	103.3	5
			162.9 > 88.0 (q)	15	10	±3.2	±13.1	±5.9	
37	Metolachlor	12.83	284.1 > 252.1 (Q)	17	15	93.7	88.3	75.9	5
			284.1 > 176.10 (q)	17	25	±4.3	±11.1	±5.4	
38	Monocrotophos	4.31	224.10 > 127.1 (Q)	26	15	95.5	82.9	77.9	5
			224.1 > 98.0 (q)	26	12	±2.3	±3.2	±7.5	
39	Oxydemeton Methyl	5.41	263.0 > 169.0 (Q)	20	13	94.7	82.6	77.5	5
			263.0 > 120.99 (q)	20	14	±1.3	±2.7	±8.6	
40	Penconazole	13.32	284.0 > 159.0 (Q)	15	25	97.3	87.8	77.4	5
			284.0 > 70.1 (q)	15	15	±1.1	±16.3	±4.2	
42	Phenthoate	13.12	321.0 > 135.0 (Q)	9	20	112.1	118.9	110.4	5
			321.0 > 79.1 (q)	9	40	±6.1	±3	±1.5	
42	Phorate sulphones	9.9	293.2 > 171.2 (Q)	20	10	104.2	97.6	98.7	5
			293.2 > 97.1 (q)	20	10	±11.5	±9.5	±11.4	
43	Phorate sulphoxides	9.79	277.0 > 143.0 (Q)	24	20	105.3	110.3	99.3	5
			277.0 > 96.9 (q)	24	32	±10.9	±9.1	±8.5	

44	Propiconazole	13.67	342.1 > 158.9 (Q)	35	20	95.4	90.7	80.1	5
			342.1 > 69.1 (q)	35	30	±3.7	±13.8	±3.6	
45	Pyraclostrobin	13.85	388.1 > 193.9 (Q)	25	12	99.5	90.5	85.1	5
			388.1 > 163.0 (q)	25	25	±2.7	±13.4	±2.7	
46	Tebuconazole	13.35	308.2 > 125.1 (Q)	20	40	96.8	92.2	85.5	5
			308.2 > 70.1 (q)	20	24	±1.0	±11.4	±2.1	
47	Thiacloprid	6.88	253.0 > 125.8 (Q)	35	20	100.3	85	98.1	5
			253.0 > 90.0 (q)	35	40	±12.7	±4.7	±13.6	
48	Thiamethoxam	3.88	292.0 > 211.2 (Q)	25	10	97.1	90.2	73.4	5
			292.0 > 132.0 (q)	25	20	±2.3	±12.2	±1.2	
49	Thiophanate Methyl	8.56	343.0 > 151.0 (Q)	28	22	91.2	88..3	72.8	5
			343.0 > 93.0 (q)	28	40	±11.4	±7.1	±6.9	
50	Trichlorfon	5.23	257.0 > 109.0 (Q)	28	18	95.4	90.3	77.4	5
			257.0 > 79.0 (q)	28	30	±1.6	±7.8	±3.7	
51	Triadimefon	12.14	294.1 > 196.9 (Q)	30	16	95.8	89.2	73.4	5
			294.1 > 69.1 (q)	30	20	±2.2	±39.5	±1.2	
52	2, 4 D	3.46	218.9 > 125.0 (Q)	20	25	5*	10*	50*	5
						107.0	110.0	106.5	
						±2.4	±2.1	±1	
			218.9 > 160.9 (q)	20	15				
53	MCPA	3.46	199 > 140.9 (Q)	30	18	96.8	113.1	99.3	5
			199 > 154.9 (q)	30	18	±5.3	±1.6	±7.9	
54	Glufosinate Ammonium	2.92	180 > 85 (Q)	30	16	5*	10*	40*	10
						NA	112.4	99.8	
							±3.6	±2.8	
			180 >95 (q)	30	16				
55	Paraquat dichloride	3.46	171.1 > 155.0 (Q)	80	30	5*	10*	50*	5
						115.8	100.3	105.6	
						±7.7	±19.5	±5.9	
			185.1 > 170.1 (q)	10	18				
56	Dithiocarbamate as CS_2	1.89	76 (Q)	NA	NA	25*	50*	200*	25
						114.8	114.4	99.0	
						±14.7	±17.9	±15.6	
			78 (q)	NA	NA				

NA-Not Applicable as not validated at the concentration
*Concentration (µg/kg) for particular method validation study

condition of column, liner, injector port and any change in mobile phase plays an important role.

Conclusions

For complete confirmatory and accurate analysis of pesticide residues as per FSSAI in milk and milk products, a single laboratory validated method using liquid and gas chromatography with a mass spectrometer is presented. Out of all regulatory pesticides about 49 can be extracted using multi-residue extraction protocol followed by their detection on LC-MS/MS and GC-MS/MS. Because of the different chemical nature of the remaining pesticides, laboratory needs to perform another 5 different extraction and detection protocols. Further to ensure accuracy of testing, laboratory has to follow good laboratory practices (GLP). Standards should be prepared with accuracy and stored at recommended condition. Care must be taken to avoid cross-contamination of standards with test samples especially while reusing volumetric equipment, glassware, vials, and other chemicals. Each step of sample preparation that is extraction, clean-up, and evaporation should be fit for the purpose. During routine analysis quality control tools such as replicate determination, analysis of positive and negative blank, CRM analysis, Proficiency Testing (PT) participation, and monitoring of data through control chart should be used for continual performance verification of method.

All targeted methods of analysis meet the validation criteria of SANTE/11321/2021. The method validation study will help dairy industry and other laboratories to adopt methodology for regulatory compliances and ensure safety of consumers.

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