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RESEARCH ARTICLE

Screening and Quantitation of Pesticide Residues in Indian Honey Samples by LC-MS/MS and GC-MS/MS

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Abstract

Objectives: To quantify a broad spectrum of pesticides of various chemical classes such as neonicotinoids, organochlorines, organophosphates, triazoles, carbamates, dicarboximides, and dinitroaniline in honey at ppb level using Liquid chromatography-tandem mass spectroscopy (LC-MS/MS) and Gas chromatography-tandem mass spectroscopy (GC-MS/MS). **Methods:** QuEChERS based sample preparation followed by the Multiple Reaction Monitoring (MRM) method was developed for quantitation of pesticides in honey. This method was validated as per SANTE/12682/2019 guidelines. **Findings:** Acceptable values were obtained for matrix-matched linearity, the limit of detection (2 ng/g) and limit of quantification (5 ng/g), and an intraday precision of less than 7%. A recovery of 70-120% was obtained for more than 85% of the compounds. However, there were compounds such as Alanycarb, Propiconazole, Benzoximate, etc. showed recovery values between 60-70%, however, these values were found consistent among multiple batches. Twelve honey samples were analyzed employing the developed method, out of which 8 samples were sourced from apiculture farms located in five districts of Kerala state, India. Rest four samples were commercial honey brands in India. Many of the pesticide residues were identified below the limit of quantitation. Some of the pesticides were quantified above the LOQ levels in samples, however, Propoxur and deltamethrin were the only pesticides found above the maximum residue limit as per the India residue monitoring program to export honey samples to EU countries. **Novelty:** Studies on the presence of pesticide residues in Kerala honey samples have not yet been published. The proposed method was able to detect an extensive range of pesticides with remarkably high sensitivity, selectivity, and precision.

Keywords: Pesticide residues; Quantitation; Indian honey; LCMS/MS; GCMS/MS

1 Introduction

The presence of pesticide residues and other contaminants in honey can negatively affect consumers. Also, these residues decrease the quality of honey and devalue its beneficial properties. Typically, pesticide contamination in honey occurs when bees, visit crops that have been treated with various agrochemicals. Beekeepers use various chemicals such as pesticides to control bee pests and the associated diseases. So far, several studies have reported various residues of pesticides in honey. However, these results were based on studies either based on less sensitive analytical techniques or covering a lesser number of analytes. This confirms the need for a method for the trace level quantification of a broad spectrum of pesticide residues in honey. Monitoring pesticides at low levels helps to assess their quality, any potential health risks to their end-users. Furthermore, pesticides in freshly collected honey samples can be used as an indicator of pesticides applied in the nearby agricultural fields.

The numerous national and international regulations that come into force, concerning permissible levels of pesticide residues, are driving the development of new analytical techniques and the improvement of existing ones. According to European Union regulations, honey is considered a natural product and must be free from chemical residues and other contaminants. There is a growing demand for high-throughput multi-residue methods, which should be easy to perform, provide a high selectivity without intense sample preparation, and allow analyzing a broad range of analytes. The amount of organic contaminants in the environment (not just pesticides) is growing by the day, and modern sample treatments (such as QuEChERS) can extract all of them, even unknown or untargeted pollutants like metabolites of the targeted pollutants[(1–4)].

To simplify the sample preparation, QuEChERS as a quick, easy, cheap, effective, rugged, and safe multiclass, the multi-residue analytical approach gained popularity[(5)]. It replaces many complicated analytical steps commonly used in traditional sample preparation methods, entails a low amount of solvent, and incurs a low cost of analysis per sample providing high-quality results, besides the sample extraction and purification procedures, the choice of appropriate separation and detection techniques are also important. The determination of pesticide residues in food matrices is a challenge especially because of the low concentration of analytes and large amounts of interfering substances that were coextracted with analytes and, in most cases, adversely affect the analysis results. Technological advances in mass spectrometry techniques allow for meeting the criteria of sensitivity and selectivity. However, polar, semi-polar, and non-polar pesticides cannot be analyzed using a single analytical technique. Accordingly, the performance of liquid chromatography and gas chromatography coupled with tandem mass spectrometry (LC-MS/MS & GC-MS/MS) have shown great success in multi- pesticide classes in complex food matrices. More Polar pesticide classes were analyzed by LC-MS/MS whereas semipolar and non-polar pesticides can be analyzed by GC-MS/MS. These complementary techniques provide information regarding the characteristic ion of each analyte as well as two or more daughter ions, useful to quantify and confirm the analytes at concentrations consistent with maximum residue levels (MRLs) established with good signal to noise ratios (S/N) (He and Aga, 2019)[(6)]. Using gas chromatography (GC) - and liquid chromatography (LC) - quadruple-time-of-flight mass spectrometry (Q-TOFMS), Pang et al. developed a combination detection method for screening 733 pesticides and chemical contaminants simultaneously[(7)].

The present study aimed to develop a method for the quantification of more than 400 pesticides belonging to both polar, semi-polar, and non-polar categories. Different classes of pesticides in this study were neonicotinoids, organochlorines, organophosphates, triazoles, carbamates, dicarboximides, and dinitroaniline. Also, by analyzing honey samples collected from five different districts of Kerala state, India, this study tried to understand the type of pesticides applied in the Kerala state and exposure levels of the same in honey. This study focuses on pesticide analysis in honey samples collected from Kerala state, south India.

2 Methodology

2.1. Sample collection

Twelve honey samples were collected from various districts of Kerala. Out of these samples, six were collected from independent apiaries, two were wild honey samples and the rest were commercially available branded samples. Sample collection points were located in five districts of Kerala (Table 1). The wild honey samples were collected through the tribal cooperative societies. Samples were collected in February 2018, before the monsoon season. Honey samples were collected into 50 mL polypropylene tubes and stored in icepacks during transport to the analysis location, where they were kept frozen at -20°C until samples were prepared for instrumental analysis. The four were commercial branded honey samples, purchased from the grocery stores in Kerala (Table 2).

Table 1. Honey sample ID and information (natural sources)

Sl. No.	Sample source	Sample information
S1	SulthanBatheri, Wayanadu	Small honey
S2	Kalady, Ernakulam	Small honey
S3	Piravom, Ernakulam	Small honey
S4	Marayoor, Idukki	Small honey
S5	SulthanBatheri, Wayanadu	Big honey
S6	Kalavoor, Alappuzha	Big honey
S7	Athirampuzha, Kottayam	Big honey
S8	Kalady Ernakulam	Big honey

Table 2. Honey sample ID and information (branded)

Sl. No.	Sample source
1	Brand 1
2	Brand 2
3	Brand 3
4	Brand 4

2.2. Chemicals and Reagents

Solvents used for the mobile phase preparation and extraction such as Water, MS grade- Procured from Biosolve (P/N,232141), Methanol, MS grade- Procured from Biosolve (P/N,136841), and Acetonitrile, MS grade-Procured from Biosolve (P/N,012041). Modifier additive for mobile phase preparation, Formic acid, LCMS grade was Procured from Sigma Aldrich (P/N, 5330020050). Sample preparation kits such as Agilent QuEChERS extraction kit - procured from Agilent Technologies (P/N, 5982-5650) sand the cleanup kit, Agilent Bond Elut Dispersive SPE 15 ml- procured from Agilent Technologies (P/N, 5982-5058). LCMS/MS comprehensive pesticide test mix was procured from Agilent Technologies (P/N, 5190-0551) whereas GC multi-residue pesticide kit of various standards was procured from Restek.

2.3. Sample Preparation

Samples were brought to room temperature and extracted by the modified QuEChERS method. 2.0 g honey sample in 50 mL falcon tube. Added 5mL of Millipore water to it to reduce the viscosity of honey samples. The sample was vortexed for 1 min to get a homogenized mixture of honey and water, after which 10 mL of acetonitrile (ACN) was added. The mixture was then vortexed for 1 minute and cooled the contents at -20°C for 30 minutes. Agilent Bond Elut QuEChERS extraction kit contents were added (P/N, 5982-5650) and shaken vigorously for 1 min. Contents of Tarson tubes were centrifuged at 6000 rpm for 6 minutes. 6 mL of the supernatant was pipetted out to an Agilent Bond Elut Dispersive SPE 15 ml (P/N, 5982-5058). Contents were thoroughly vortexed for 1 minute, followed by centrifugation at 10000 rpm for 2 min. Pipetted out 1mL solution in HPLC vial. Each of the samples was prepared in triplicates (AOAC Official Method 2007.01).

2.4. Instrumentation

2.4.1. LC-MS/MS method conditions

2.4.1.1. Chromatographic parameters. A 6470A LCMS (G6470AA) Triple Quadrupole (Agilent Technologies Inc., Santa Clara, USA) operated in ESI Positive Ionization mode coupled with a 1290 Infinity II UHPLC system (Agilent Technologies Inc., Santa Clara, USA) consists of a high-speed binary pump (G7120A) having a maximum pressure limit of 1300 bar, Multi sampler (G7167B) and Multi-column Thermostat (G7116B) was used for the sample analysis. MRM transitions were set and optimization of the compound-related voltages such as Fragmentor voltage and the Collision energy was performed by automatic optimization tool and by pesticide MRM database available with the instrument. Source parameters were optimized based on the flow rate and the composition ratio of the mobile phase. Mobile Phase A used was 0.1 % Formic acid and 5 mM ammonium Formate in Water. 0.1 % Formic acid and 5 mM ammonium Formate in Methanol are used as mobile phase B. Injection volume used was 2ul. The analytical column responsible for the separation of nonvolatile pesticides was Agilent Zorbax Eclipse Plus (150 X 2.1 mm, 1.8 μm, P/N, 959759-902). The column was kept at a constant temperature of 35°C. Twenty-minute-long Gradient elution was employed to chromatographically separate the 253 pesticides. The initial composition of the

mobile phase was 95.5 (Mobile phase A, B) which was constant for 0.5 minutes. From 0.5 minutes to 3.5 minutes, the mobile phase B composition gradually increased to 50%. At 17.0 minutes, the % B becomes 95%. This ratio was maintained for twenty minutes. A post-run of 2 minutes was also included in the time program for column washing and the stabilization of the column with the initial mobile phase conditions to make it ready for the next injection. 30-Second-long washing of the injection needle and the needle seat was also employed using Methanol, and Water (60,40, V/V) as wash solvent to minimize the carryover.

2.4.1.2. MS parameters. ESI positive ionization mode was used to acquire the samples and the standards. A retention time-based MRM method was developed which provide more cycle time for each of the analyte resulting in improved data quality in terms of more number data points. Also, standards and samples were acquired in spectral acquisition mode to enable library spectrum matching. Nebulizer pressure was kept at 40 pSi. The heated gas temperature was 200°C with a flow of 8L/min. Thermal confinement of the expanded spray is obtained with the help of Sheath gas of temperature 325°C has a flow rate of 11L/min. The capillary voltage was kept at 3500V and the nozzle voltage for the optimized method was 500V. Fragmentor Voltages and Collision energies of individual pesticides were obtained from the pesticide MRM database. Wherever database data was unavailable, an automatic optimizer tool was used to do optimization of voltages. The resolution setting was kept as a unit resolution for both quadrupole 1 and quadrupole 3 mass analyzers where the mass spectral peak width is maintained between 0.6- 0.8 Da. Before data acquisition, the status of the MS instrument is verified by the check tune option. Electro Spray Ionization (ESI) low tuning mix provided by Agilent Technologies was introduced by the calibrant delivery system (CDS) Spectral peak width and intensity are measured during this process and warn the user in case it is not meeting the above criteria.

2.4.2. GC-MS/MS method conditions

2.4.2.1. Chromatographic parameters. For GC-MS/MS analysis, Agilent 7010 pesticide analyzer coupled to GC 7890B ((Agilent Technologies Inc., Santa Clara, USA) was used. Agilent J and W HP-5ms UI 15 m × 0.25 mm × 0.25 μm (P/N, 19091S-431 UI) GC column was used for the separation of analytes. From the inlet, two Agilent J and W DB-5ms Ultra Inert columns (15 m × 0.25 mm, 0.25 μm, p/n 19091S-431 UI) were coupled to each other through a purged ultimate union (PUU) for the use of mid-column/post-run backflushing. The carrier gas used for GC was Helium with a hot and cold splitless injection. Collision and quenching gases were Nitrogen. Purged Ultimate Union was used for backflush. 1ul was injected to analyze the samples. The total run time per sample was 40 minutes. The retention time locking option using chlorpyrifos-methyl was utilized to maintain the retention time of various analytes. From the inlet, two Agilent J and W DB-5ms Ultra Inert columns (15 m × 0.25 mm, 0.25 μm, p/n 19091S-431 UI) were coupled to each other through a purged ultimate union (PUU) for the use of mid-column/post-run backflushing.

2.4.2.2. MS parameters. The GC was configured with a Multimode Inlet (MMI) equipped with a 4 mm ultra-inert, splitless, single taper, glass wool liner (p/n 5190-2293). Electron Impact (EI) ionization source with 70ev was used for analysis. The source and the transfer lines were kept at 280°C. Time scheduled MRM (dMRM) method was used for acquisition to get the optimized cycle time for the collection of the maximum number of data points across the chromatographic peaks. EM gain used was 10 with MS1 and MS2 resolutions were kept wide. Both the quadrupole analyzers were kept at heated conditions of 150°C so that the fluctuations in outside temperature were not affecting the performance in mass accuracy of the system.

For both LC-MS/MS and GCMS/MS at least two MRM transitions consisting of one parent ion and two fragment ions are selected per analyte to satisfy the criteria of four identification points for confirmation of a compound in a sample as per the SANTE guidelines. The MRM transition having more intensity, Quantifier ion, and the second high intense MRM transition Qualifier ion were selected from the production spectra of the pesticides. MRM ratios (Qualifier to Quantifier ratio) were compared between matrix-matched standard and sample of each analyte for confirming any positive sample [8].

3 Results and Discussion

3.1. Validation of the developed MRM method

The developed method was validated by the European Union SANTE/12682/2019 guidelines. An extracted sample was injected into both the analytical instruments to check the selectivity of the method. Finding out matrix blank was a real challenge for most of the samples were having traces of pesticides. The absence of any signal above a signal-to-noise ratio of 3 or more at the retention time of the target analyte was evaluated. The majority of the pesticides showed no interference in the blank matrix. In case of interference in blank material, criteria mentioned in the SANTE guidance document were followed. The spiking level for recovery should be $\geq$3 times the level present in the blank material. In those cases, recoveries can be calculated using blank subtracted calibration [8].

The matrix-matched calibration curve was plotted by spiking different concentration levels of pesticide standard mix in the extracted honey blank matrix. However, here to select the blank, the organic labeled honey sample was checked for the presence of pesticides. 253 pesticides were analyzed using LC-MS/MS method whereas 222 pesticide methods were used for GC-MS/MS. Representative matrix-matched chromatograms of LC-MS/MS amenable pesticides at 5 ng/ml are given in Figure 1 . Some of the pesticides such as Malathion, Chlorpyriphos, Chlorpyriphos methyl, Dichlorvos, Dimethoate, Diazinon, Chlorothalonil, fenitrothion, Penconazole, triadimenol, Bupirimate, Flusilazol, Myclobutanil, Buprofezin, Benalaxyl Propargite, Tebuconazole, Bifenthrin, Cyfluthrin, Coumaphos, propoxur, Methyl parathion and many more pesticides could be analyzed by both the techniques. The final concentration of the matrix-matched calibration standards was typically ranging from 0.1 ng/ml to 50 ng/ml, however, this range varies with various compounds depending on the sensitivity of different analytes. At least 5 concentration levels are included in the matrix-matched calibration curve plotted. A minimum regression coefficient of 0.995 was observed for all the pesticides involved in the study with linear regression and 1/X weighing. Fit quality, the significance of the regression model, and the corresponding error were evaluated by using the fit curve assistant functionality of the MassHunter Quantitative analysis software version B. 07.00. Representative matrix-matched calibration curves of 10 pesticides are given in figure 2 .

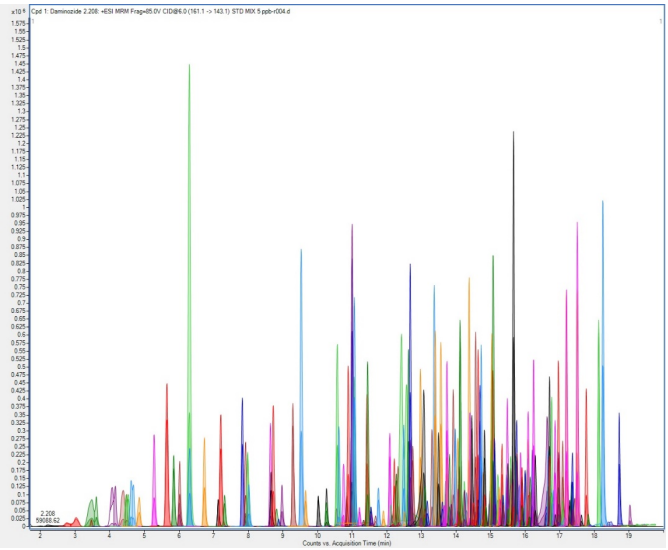

Fig 1. EIC of LCMS amenable pesticides at 5 ppb

Limit of detection (LOD) and limit of quantitation (LOQ) were calculated by considering the signal to noise ratio (S/N) observed at lower concentration levels. As per the regulations, the S/N ratio should be at least 3:1 to consider that concentration as the limit of detection. A limit of quantitation, S/N should be at least 10:1. 95% of the analytes showed a good response at 0.2 ppb of pesticide standard prepared in solvent standard. However, by considering the overall analytes, the limit of detection (LOD) is fixed as 2 ng/ml. Pesticide concentration of 5 ng/g in the honey sample could be quantified confidently satisfying the criteria of selection of limit of quantification as per SANTE/12682/2019. Therefore, the limit of quantification of this method was found to be 5 ng/g of honey sample. The initial concentration of 5 ng/g of pesticide in the sample results in 1 ppb of absolute concentration in the vial considering five times dilution of pesticide content during the extraction process. All Signals to noise calculations were made using the raw data with the help of MassHunter Qualitative Software Version B. 07.00. Noise calculation was made using the Root Mean Square (RMS) method with defined noise ranges without applying any smoothing. The linearity range of representative pesticides and their regression coefficients are given in Table 3. Intraday repeatability was evaluated by analyzing six injections at the LOQ level in the matrix. The relative standard deviation of the response of these analytes found to be less than 7% is given in Table 4.

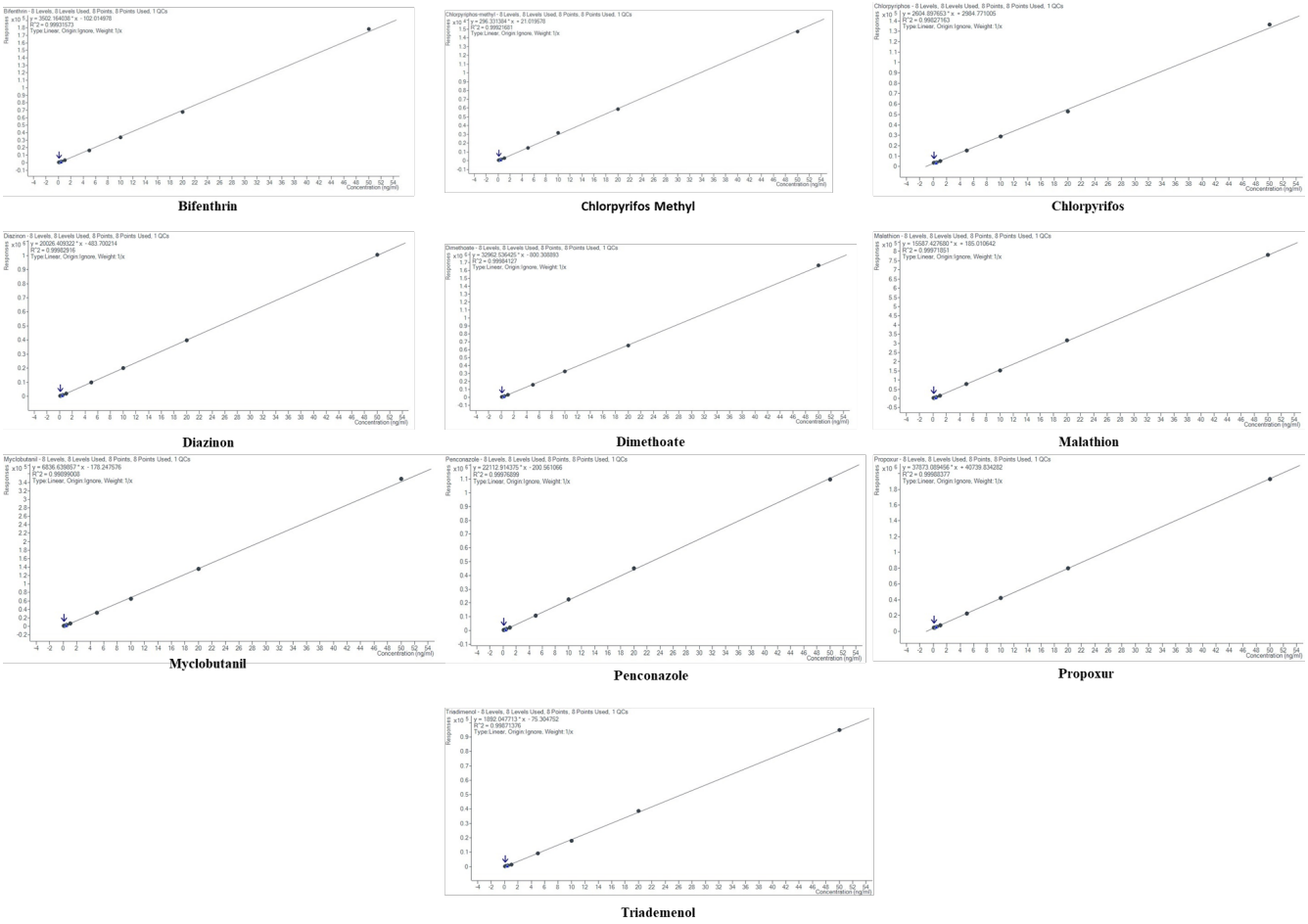

Fig 2. Representative matrix-matched calibration curves of 20 pesticides

Table 3. Reproducibility data of representative pesticides at 5 ng/ml matrix matched calibration point

Analyte	Response 1	Response 2	Response 3	Response 4	Response 5	Response 6	Mean	SD	% CV
Aldicarb fragment	32510	31900	31856	31759	32115	32354	32082.3	298.9	0.9
Avermectin B1A	357	375	369	348	395	384	371.3	17.3	4.6
Beflubutamide	157316	157935	156649	156987	157315	157865	157344.5	496.6	0.3
Bispyribac	15222	15398	15456	15530	14983	14897	15247.7	260.5	1.7
Carbaryl	55352	54564	54963	55645	55852	55496	55312.0	473.1	0.9
Carbendazim	401919	405061	398533	395055	414629	403119	403052.4	6689.4	1.7
Carfentrazone ethyl	32563	32545	33195	32845	32658	33862	32944.7	510.2	1.5
Chlorpyriphos	14982	15324	15590	14135	14835	15782	15108.0	595.0	3.9
Chlorpyriphos methyl	1452	1467	1385	1322	1478	1545	1441.5	77.8	5.4
Clethodim	17975	17845	17730	17824	17945	17811	17855.0	90.7	0.5
Clofentazine	34356	35125	35645	34895	34587	35648	35042.7	536.1	1.5
Cycloate	4310	4454	4615	4585	4389	4387	4456.7	120.4	2.7
Cymiazol	43251	43265	44879	43548	43849	44569	43893.5	686.5	1.6

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Table 3 continued

DEET	354951	355456	355879	354986	355741	345954	353827.8	3876.0	1.1
Dimethoate	161816	162461	174041	172306	169174	167683	167913.3	5007.9	3.0
Ethidimuron	62157	62154	63984	62458	63548	64545	63141.0	1025.3	1.6
Ethofumesat	1245	1189	1354	1284	1356	1374	1300.3	73.7	5.7
Fenarimol	4510	4467	4470	4497	4452	4508	4484.1	24.2	0.5
Flonicamid	497	456	511	548	465	489	494.3	33.2	6.7
Flumioxazin	1689	1756	1784	1598	1645	1578	1675.0	83.5	5.0
Fluquinconazole	8547	8698	8954	8754	8457	8576	8664.3	177.6	2.0
Foramsulfuron	5846	5482	5642	5589	5548	5642	5624.8	124.2	2.2
Furathiocarb	232800	224850	229800	223350	215400	217200	223900.0	6817.1	3.0
Imazalil	34251	35456	35487	34652	34259	35489	34932.3	614.5	1.8
Isocarbophos	42517	42875	41548	43251	42159	43127	42579.5	645.4	1.5
Isoprothiolane	296772	296491	296203	295400	295395	294946	295867.8	722.7	0.2
Isoxaflutole	7586	7521	7558	7642	7345	7748	7566.7	134.3	1.8
Ivermectin B1A	352	385	389	349	389	363	371.2	18.7	5.0
Lufenuron	6210	5845	5896	6125	6178	6254	6084.7	171.9	2.8
Malathion	78730	77800	78519	78211	76990	77938	78031.3	617.2	0.8
Metaflumizone	2347	2495	2411	2503	2354	2548	2443.0	84.2	3.4
Methacriphos	1154	1245	1274	1134	1168	1310	1214.2	72.0	5.9
Methamidophos	80950	80820	80560	80410	80036	79468	80374.0	548.0	0.7
Molinate	2254	2178	2354	2248	2279	2365	2279.7	70.4	3.1
Monocrotophos	44075	39946	42438	43711	40737	45558	42744.1	2124.7	5.0
Moxidectin	1645	1689	1642	1589	1532	1754	1641.8	77.0	4.7
Nicosulfuron	584	549	647	625	596	609	601.7	34.0	5.6
Phosalone	33617	32259	33427	32653	32006	34762	33120.7	1023.5	3.1
Pirimicarb	416384	432890	461309	441609	424681	423185	433342.8	16226.3	3.7
Procymidon	3775	3856	3715	3890	3824	3782	3807.0	62.8	1.6
Pyrazon	67456	68125	68645	67948	67542	67254	67828.3	513.8	0.8
Pyridat	7654	7545	7689	7701	7519	7580	7614.7	77.1	1.0
Rimsulfuron	1001	958	972	905	1032	985	975.5	42.9	4.4
Tebuconazole	131955	130212.5	134941.5	128893	127511	125925	129906.3	3232.9	2.5
Thiamethoxam	66500	66742	66204	66112	66322	66312	66365.3	225.8	0.3
Tralkoxydim	6142	6278	6134	6378	6105	6203	6206.7	104.2	1.7
Triazophos	435915	438037	436178	433595	432921	429243	434314.7	3100.8	0.7
Tribenuron methyl	1152	1348	1257	1306	1178	1233	1245.7	74.5	6.0
Tricyclazole	196519	195934	195918	195746	195527	195499	195857.0	373.4	0.2
Trietazin	42501	43011	43247	42654	43861	42357	42938.5	558.9	1.3
Vamidothion	322800	321975	322657	323598	324956	324578	323427.3	1165.0	0.4
Zoxamide	114978	114906	114857	115003	115155	114726	114937.5	145.2	0.1

Table 4. Reproducibility data of representative pesticides at 5 ng/ml matrix matched calibration point

Analyte	**Response 1**	**Response 2**	**Response 3**	**Response 4**	**Response 5**	**Response 6**	**Mean**	**SD**	**% CV**
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Cymiazol	43251	43265	44879	43548	43849	44569	43893.5	686.5	1.6
DEET	354951	355456	355879	354986	355741	345954	353827.8	3876.0	1.1
Dimethoate	161816	162461	174041	172306	169174	167683	167913.3	5007.9	3.0
Ethidimuron	62157	62154	63984	62458	63548	64545	63141.0	1025.3	1.6
Ethofumesat	1245	1189	1354	1284	1356	1374	1300.3	73.7	5.7
Fenarimol	4510	4467	4470	4497	4452	4508	4484.1	24.2	0.5
Flonicamid	497	456	511	548	465	489	494.3	33.2	6.7
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Fluquinconazole	8547	8698	8954	8754	8457	8576	8664.3	177.6	2.0
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Imazalil	34251	35456	35487	34652	34259	35489	34932.3	614.5	1.8
Isocarbophos	42517	42875	41548	43251	42159	43127	42579.5	645.4	1.5
Isoprothiolane	296772	296491	296203	295400	295395	294946	295867.8	722.7	0.2
Isoxaflutole	7586	7521	7558	7642	7345	7748	7566.7	134.3	1.8
Ivermectin B1A	352	385	389	349	389	363	371.2	18.7	5.0
Lufenuron	6210	5845	5896	6125	6178	6254	6084.7	171.9	2.8
Malathion	78730	77800	78519	78211	76990	77938	78031.3	617.2	0.8
Metaflumizone	2347	2495	2411	2503	2354	2548	2443.0	84.2	3.4
Methacriphos	1154	1245	1274	1134	1168	1310	1214.2	72.0	5.9
Methamidophos	80950	80820	80560	80410	80036	79468	80374.0	548.0	0.7
Molinate	2254	2178	2354	2248	2279	2365	2279.7	70.4	3.1
Monocrotophos	44075	39946	42438	43711	40737	45558	42744.1	2124.7	5.0
Moxidectin	1645	1689	1642	1589	1532	1754	1641.8	77.0	4.7
Nicosulfuron	584	549	647	625	596	609	601.7	34.0	5.6
Phosalone	33617	32259	33427	32653	32006	34762	33120.7	1023.5	3.1
Pirimicarb	416384	432890	461309	441609	424681	423185	433342.8	16226.3	3.7
Procymidon	3775	3856	3715	3890	3824	3782	3807.0	62.8	1.6
Pyrazon	67456	68125	68645	67948	67542	67254	67828.3	513.8	0.8
Pyridat	7654	7545	7689	7701	7519	7580	7614.7	77.1	1.0
Rimsulfuron	1001	958	972	905	1032	985	975.5	42.9	4.4
Tebuconazole	131955	130212.5	134941.5	128893	127511	125925	129906.3	3232.9	2.5
Thiamethoxam	66500	66742	66204	66112	66322	66312	66365.3	225.8	0.3
Tralkoxydim	6142	6278	6134	6378	6105	6203	6206.7	104.2	1.7
Triazophos	435915	438037	436178	433595	432921	429243	434314.7	3100.8	0.7
Tribenuron methyl	1152	1348	1257	1306	1178	1233	1245.7	74.5	6.0
Tricyclazole	196519	195934	195918	195746	195527	195499	195857.0	373.4	0.2
Trietazin	42501	43011	43247	42654	43861	42357	42938.5	558.9	1.3
Vamidothion	322800	321975	322657	323598	324956	324578	323427.3	1165.0	0.4
Zoxamide	114978	114906	114857	115003	115155	114726	114937.5	145.2	0.1

To evaluate the recovery of the pesticides from the honey sample matrix, a spike recovery study was conducted at the LOQ level. A recovery study was conducted at spiking levels at 5 ng/g to check the extraction efficiency of pesticides from the honey matrix by the sample preparation. The spiking procedure carried out to get required concentrations is illustrated by taking a spiking level of 5 ng/g as an example. 10 ul of 1 ppm (ug/ml) pesticide standard mix was spiked to 2 g of honey sample. The absolute quantity of pesticides present in 10 ul of 1 ppm pesticide mix is 10.0 ng. Therefore when 10 ng is spiked to 2 g honey sample, spike level concentration becomes 10ng/2g= 5 ng/g. This is extracted with 10 ml of extraction solvent. The resultant concentration of pesticide residues was diluted five times after extraction. Therefore, the dilution factor is 5. Effective final concentration injected into the system would become 5 ppb/5= 2 ppb. The majority of the pesticides in this study were showing

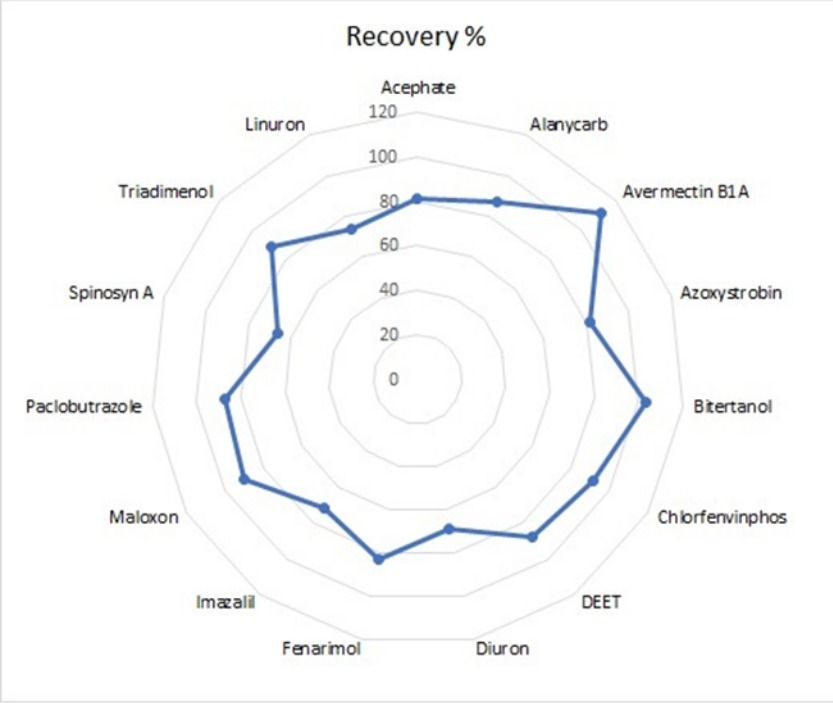

Fig 3. RADAR Plot showing Recovery % of selected pesticides

a recovery of between 70-120%. However, very few pesticides such as Febuconazole, Alanycarb, Benzoximate, Chloridazon, Epoxyconazole, Ethirimol, Fenpyroximate, Propiconazole, Spinosyn A, etc. were showing a slightly lower recovery. The lowest recovery reported was for Febuconazole (61%) whereas triazophos showed the highest recovery (119%).

However, recovery values obtained were consistent with repeated sample preparation. But, to increase the recovery of these analytes, procedural standard calibration curve as mentioned in SANTE/12682/2019 guidelines [3]. This method is an alternative to the use of internal standards for compensating the recovery loss. RADAR plot showing the recovery % of representative analytes in the honey matrix is given in Figure 3.

3.2 Sample analysis

Some of the pesticides such as Dioxathion, Chlorpyrifos, carbendazim, imidacloprid, Metalaxyl, Quinalphos, etc. were found in trace amounts in many of the samples, well below the limit of quantitation of the method. Chlorpyrifos was reported in the entire samples tested. The concentration of chlorpyrifos was at 1.62 ppb in sample S1. Chlorpyrifos, a broad-spectrum organophosphate pesticide was widely used in agriculture and residential pest control throughout the world. Also, pesticides such as Amitraz, Parathion, Parathion methyl, Deltamethrin, Anthraquinone, and 2 phenyl phenol were quantified above LOQ levels, however, not included in the monitoring program by regulatory. Parathion was found in sample S1 at a concentration of 221.36 ng/g, whereas Parathion methyl was found in five samples, (S1, S2, S7, B1, and B4) at an approximate concentration of 40 ng/g. The maximum residue limits mentioned by the residue monitoring program for honey by the Government of India to export honey to the EU (2019) were considered. As per the monitoring program, Deltamethrin was quantified in three samples above its MRL of 17.0 ppb. Deltamethrin was found in S2, S5, and B1. Pesticide, Propoxur was found in all 12 honey samples above its MRL level of 10 ppb. From the quantification result of 12 honey samples, it could be concluded that commercial honey (B1) is more contaminated than the other samples. Deltamethrin was found in B1 at 52.60 ppb level. Other than deltamethrin, Parathion methyl was detected in B1 at a concentration of 48.42 ppb. B1 was also reported to have Dioxathion at 3.2 ppb. The presence/absence of the above compounds was matched by comparing the MRM ratio between the Qualifier ion and Quantifier ion of standard and the sample. The maximum allowed variation of MRM ratios between standard and samples was +30% according to SANTE/12682/2019 guidelines.

Similar studies across the world were evaluated in terms of the analytical technique used, the pesticide classes covered, and the results obtained. Codling et al reported the presence of neonicotinoid insecticides in honey samples collected from central

Saskatchewan, Canada. More than 50% of the samples analyzed reported the presence of clothianidin with a mean concentration of 8.2 ng/g. Besides clothianidin, thiamethoxam was also found at a mean concentration of 17.2 ng/g.[9].

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) based screening revealed the presence of Acaricides such as Amitraz, Coumaphos, and Endosulfan were detected from the honey samples from Saudi Arabia. Typical residue concentration ranged from 1 ng/g to 43 ng/g in various samples which indicated the direct contamination of the honey samples collected from apiaries close to farms.[10].

Gas chromatography based analysis of honey samples from Egypt found Dicofol and other acaricides used by beekeepers against Varroa destructor were also detected (i.e., bromopropylate, tetradifon, malathion), indicating that the chemicals used by apiculturists inside the hives to control the disease are the main pollutants of the produced honey[11]. Irungu et al conduct a preliminary study of pesticide residues in commercial honey samples obtained from Kenya and Ethiopia which are among the major producers of honey in Africa. Accordingly, only malathion was detected at a level higher than the set MRL levels[12].

Honey samples from Ghana were analyzed for organochlorine, organic phosphorous, and synthetic pyrethroids. But none of the analyzed compounds were detected above EU MRL. A three-year survey conducted in France studied the honeybee colony health related to the presence of pesticide residues in those colonies. A high concentration of imidacloprid and 6-chloronicotinic acid was detected in pollen loads, honey, and honey bee matrices. Vargas-Valeroet al established a relation between the presence of pesticides with colony collapse[13]. 24 pesticides were detected in honey analyzed by LC-QTOF and GC- MS/MS. Acetamiprid was found in all samples. The chemicals carbendazim, thiabendazole, azoxystrobin, chlorpyrifos, and imidacloprid were the most frequently detected in the honey samples collected by apiaries in six Brazilian states[14]. A study by Ruiz-Toledo et al. distinguished between the presence of organochlorine pesticides in honey and pollen collected from managed colonies of the honey bee, Apis mellifera L. as well as from colonies of the stingless bee, Scaptotrigona mexicana Guérin. The most prevalent pesticides identified were Heptachlor, HCH, DDT, and DDE[15]. Based on results of LC/MS-MS and GC/MS-MS in combination with modified QuEChERS, Oymen et al. quantified 4 pesticide residues (coumaphos, thiamethoxam, N-(2,4-dimethyl phenyl)formamide, piperonyl butoxide)[16]. Using Daikenchuto (DKT) as the subject, Saegusa et al. separated pesticides from DKT using acetone, then performed a simultaneous analysis in GC–MS/MS on the extract[17]. In their study, Kasiotis et al. used a complementary GC-EI-QqQ-MS method to identify metabolites of imidacloprid, chlorpyrifos, coumaphos, acetamiprid, fenthion, and amitraz[18]. Pesticide analysis supported LC-MS/MS and GC-MS/MS instrumentation that permits the determination of widespread agricultural pesticides worldwide such as chlorpyrifos, imidacloprid, dimethoate, and tebuconazole[19]. The QuEChERS extraction strategy with slight alterations, taken after by fluid and gas chromatography-tandem mass spectrometry, was connected for the assurance of pesticide buildups in crude nectar tests from northeastern Spain was performed by Lasheras et al. and identified Chlorfenvinphos and coumaphos buildups[20]. Xiao et al. detected carbendazim and pyrethroids among the honey samples of China using a modified version of the QuEChERS multi-residue method[21]. Mukiibi et al. investigated the quality of honey collected near an abandoned pesticide store in western Uganda's Masindi District. DDT, OCPs, lindane, endosulfans, and dieldrin were identified[22]. Pesticides in captured pollen from three commercial decorative plant nurseries in Connecticut were investigated by Stoner et al.[23]. The nitroguanidine neonicotinoids (imidacloprid, thiamethoxam, and its metabolite clothianidin) were the most common pesticides that increased Pollen Hazard Quotients, followed by the organophosphate acephate and its metabolite methamidophos. Prasanth et al. studied the photo-degradation of Sulfamerazin in honey samples from Kerala by Accurate Mass LC-MS/MS[24].

Concentrations of pesticides residues in honey sampled from the key honey-producing forest belts in Gold Coast were determined by Darko et al.[25]. Samples were purposively collected and extracted victimization the QuEChERS methodology and analyzed for synthetic pyrethroids, organochlorine, and insecticide chemical residues. All the chemical residues detected were extremely low and below their respective maximum residue limits set by the European Union. Protocol for the determination of pesticides residues in honey samples was standardized by Mukherjee[26], employing a straightforward technique of liquid-liquid extraction. The strategy was sensitive to finding low levels of pesticides in honey. The honey sample was fortified with pesticides, namely, cypermethrin, fenvalerate, alphamethrin, lamba–cyhalothrin, endosulfan (α, β and sulfate), and chlorpyrifos. Rissato et al. analyzed honey samples of Bauru (State of São Paulo, Brazil). Organohalogen and organophosphorus groups and lower levels of residues of some organonitrogen and pyrethroids were detected employing gas chromatography with electron impact mass spectrometric detection in the selected ion monitoring mode (GC–MS-SIM). Malathion residues were detected in all the samples, in a high concentration[27]. Solid-phase extraction with octadecyl sorbent followed by gas chromatography-mass-spectrometry (GC-MS) for organochlorines, and by liquid chromatography-atmospheric pressure chemical ionization-mass spectrometry (LC-APCI-MS), for organophosphorus & carbamates were employed by Blasco et al. for analyzing pesticide residues in honey samples of Portugal & Spain. Organochlorines made up the majority of the pesticides identified in honey. γ-HCH was found in 50% of the samples, followed by HCB in 32 percent, and

the other HCH isomers (α-HCH and β-HCH) in 28 and 26% of the samples, respectively. DDT residues and their metabolites were discovered in 20% of the samples. Methiocarb and carbofuran were found in ten percent of the samples, pirimicarb in 4%, and carbaryl in 2% of the samples. Heptenophos, methidathion, and parathion methyl were the only organophosphorus pesticides discovered in 16%, 4%, and 2% of honey samples, respectively[28].

Most of the pesticides reported in this study and concentrations were inconsistent with the available literature. One of the reasons for this difference could be the difference in the scope of these studies. Much research has focused on organochlorines and pyrethroids. This shows the difference in the availability and use of pesticides in different countries. Insecticides that control insect populations are available in both broad and narrow spectrums. Deltamethrin and propoxur found in samples in this study are classified as broad-spectrum insecticides that will kill insects indiscriminately, regardless of species. It is important to consider the impact of broad-spectrum insecticide use on beneficial insects.

4 Conclusion

A method for the analysis of more than 400 pesticide residues from honey samples has been developed using triple quadrupole LCMS/MS and GCMS/MS instruments. A simple extraction and cleaning procedure based on QuEChERS was applied for sample preparation. The method showed good sensitivity with the method's overall detection limit of 5 ng/g honey sample. All analytes in the study showed good linearity and the LOQ recovery experiment showed a recovery of 70-20% with few exceptions. The lowest reported recovery was for febuconazole, while triazophos had the highest recovery. In addition, this method shows good reproducibility. Deltamethrin and propoxur are two pesticides detected on MRLs in the studied samples. (In line with India's honey residue monitoring plan for exports to the EU). However, other pesticides detected on the LOQ were amitraz, parathion, parathion methyl, deltamethrin, anthraquinone, and 2-phenyl phenol. Deltamethrin is one of the most effective broad-spectrum insecticides in the synthetic pyrethroid class. It is commonly used in agriculture due to its stability. It is highly toxic to bees, parasites, and egg predators. Propoxur is a carbamate insecticide that is highly toxic to bees. The synergistic interactions of various chemical residues in the honey sample can be further investigated. The government's agriculture department should take initiatives to advise farmers to reduce the use of broad-spectrum pesticides. In addition, clear suggestions should be given to farmers about the responsible use of chemicals in agricultural practices. According to the available literature, the results of the present study do not correspond to studies conducted in other parts of the world. This shows differences in the availability and use of pesticides between different countries.

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