WORLD JOURNAL OF PHARMACEUTICAL RESEARCH
SJIF Impact Factor 7.523
Volume 6, Issue 4, 1174-1185. Research Article ISSN 2277– 7105

DEVELOPMENT OF METHOD AND VALIDATION FOR DETERMINATION OF PESTICIDE RESIDUES IN CHILLI BY GC-ECD (ELECTRON CAPTURE DETECTOR) AND GC-MS (MASS SPECTROMETER)

Sudeep Mishra[1,*] Neelam Richhariya[2], Samsul Alam[1], Lalitesh Kumar Thakur[1] and Phool Kumar Patanjali[1]

[1]Institute of Pesticide Formulation Technology, Sector-20, Udyog Vihar, Opp. Ambience Mall, NH-8, Gurgaon, Haryana-122016, India.
[2]Govt. Indra College Satna Madhya Pradesh-485001, India.

Article Received on
10 Feb. 2017,
Revised on 02 March. 2017,
Accepted on 22 March 2017
DOI: 10.20959/wjpr20174-8174

***Corresponding Author**
Neelam Richhariya
Institute of Pesticide
Formulation Technology,
Sector-20, Udyog Vihar,
Opp. Ambience Mall, NH-8,
Gurgaon, Haryana-122016,
India.

ABSTRACT

Study was conducted to validate QuEChERS method for qualitative and quantitative determination of some most frequently used organochlorine pesticides residue levels on chilli by Gas Chromatography. Solid Phase Extraction (SPE) using ethylacetate, anhy. sodium sulphate, magnesium sulphate was used to extract pesticides present in a sample and clean up of interfering pigments was done by Primary Secondary Amine (PSA), Activated charcoal and C18. Instruments GC-ECD (Agilent Technologies) and GC Model 7890A (Agilent Technologies) with mass **(**5975C inert XL EI/CI MSD) triple axis detector was taken for study. Limit of Detection (LOD) and LOQ (Limit of Quantification) were calculated for specific pesticides against chilli matrix and was found that values are below MRL (Maximum Residue Limit) set by PFA/Codex/EPA and FDA. Recovery studies were carried out at 0.05 mg/kg concentration level with mean recovery falls from 82.67 % to 92.0 % with Relative Standard Deviation (RSD) below 10%. Repeatability and reproducibility of the instrument taken for study as per Codex, EPA and FDA guidelines. Linearity of the instrument were checked and found linear response when calibration curve was plotted between an area of standard mixture against different concentration levels of 0.001, 0.005, 0.05, 0.01, 0.5 and 1.0 mg/kg with regression co-efficient (r^2) lies in the range 0.996 to 0.999.

KEYWORDS: Chilli, Pesticides, LOD, LOQ, MRL, QuEChERS.

INTRODUCTION

Chilli is one of the higher consumption vegetable in the Indian Market. It is unfortunate that all farmers do not follow legal pesticide or having little or no knowledge of pesticides. Method for pesticide residue analysis was introduced (Anastassiades et al.,2003), which provides quality results in a fast, easy an inexpensive approach. The QuEchERS approach has been extensively validated for hundreds of pesticides residues in many types of foods and has become Association of Analytical Communities (AOAC) official method 2007.01 (Lehotayet al., 2007) and CEN (2008).

Persistent organic pollutants (POPs) such as organochlorines have been of great concern due to their occurrence in high concentration even in remote ecosystems, despite bans on production and usage (Iwata et al., 1994; Guruge and Tanabe, 2001). Organochlorine pesticides (OCPs) such as hexachlorocyclohexane (HCH) and 1,1,1- trichloro-2,2′-bis(p-chlorophenylethane (DDT) are persistant and broad-spectrum toxicants that accumulate in the food web cause high risks to the ecosystem, human health and environment (Willett et al., 1998; Nakata et al., 1998). Many of persistant compounds act as hazardous environmental hormones, which disrupt reproductive cycles of humans and wildlife (Colborn and Smolen, 1996). It is reported that approximately three million people are poisoned and 200,000 died each year around the world from pesticide poisoning, and a majority of them belongs to the developing countries (WHO, 1990; FAO, 2000).

In India, organochlorine pesticides (OCPs) especially DDT and HCH were used extensively till recently both for agricultural and sanitary purposes (Pandit et al., 2001; Kumar et al., 2006). It is estimated that about 25,000 MT of chlorinated pesticides was used annually in India and DDT accounted over 40% of this group (Mathur, 1993). Although substantial portions of applied pesticide are dissipated at the site of application through chemical and biological degradation processes. Still, a reasonable fraction of the OCPs residues reaches the oceans through agricultural run-off, atmospheric transport and sewerage discharge (GESAMP, 1989). In the present work a chromatographic methodology was validated for use to identify pesticide residue in different samples of chilli produced in various locations of Satna district. This paper describes the use of a quick, easy, cheap, effective, rugged, and safe (QuEChERS) method for extraction and cleanup of 28 pesticide residues of interest in chilli. Gas chromatography (GC) equipped with an electron-capture detector (ECD) or mass spectrometer (MS) has been the analytical technique more frequently applied to the analysis of pesticides in food for many years (Anastassiades et al. 2003; Hernandez-Borges et al. 2009; Lehotay et al. 2005; Nguyen et al. 2008; Paya et al. 2007).

MATERIALS AND METHODS

Sampling

Samples of chilli was purchased from local vegetable mandi of Satna District. Purchased sample were transported in ice pack to the laboratory in cool-box, stored at 4°C and extracting within 48 h.

Reagents and materials

Pesticide reference standards were purchased from Sigma-Aldrich (Germany). Ethyl Acetate (EtAc), n-Hexane of HPLC quality (Merck Germany), Sodium Sulphate (anhy. Na_2SO_4), Magnesium Sulphate (anhy. $MgSO_4$) were purchased from Merck (Germany), Primary Secondary Amine (PSA) sorbent was obtained from Varian (Varian Incorporated, Harbor City, CA, USA), and graphitized carbon black sorbent was obtained from Supelco (Bellefonte, PA, USA). Fluoroethylenepropylene (FEP) centrifugation tube of 50ml, 15 ml (Tarson make) was taken for sample preparation. Mixer grinder (Bajaj make), Vortox shaker (Tarson make), Centrifuge mechine (Remi, India).

Sample preparation

QuEChERS (quick, easy, cheap, effective, rugged and safe) method (Anastassiades et al. 2003) was followed for extraction of pesticides from chilli samples. Sample preparation has the following steps: chilli was finely chopped and homogenized in a mixer grinder. Fifteen gram of thoroughly homogenized sample weighed into a 50 ml centrifugation tube and 30 ml of ethyl acetate was added and shaken for 1 min vortex shaker. Ten gram anhydrous Na_2SO_4 was added and shaken vigorously for 30 min by rotospin (Tarson make) at 120 rpm and were centrifuged at 3,450 relative centrifugal force at 5,000 to 6000 rpm on the centrifuge mechine (Remi, India) at about 5 minutes. Cleanup was performed according to Lehotay (2007). 6 ml extract was transferred from the upper layer into a 15 ml FEP tube, and 0.9 g anhydrous $MgSO_4$, 0.25 g PSA and 0.25 g activated charcoal were added and shaken vigorously for 1 min by vortex shaker (Tarson Make). The tubes were centrifuged at 5,000 rpm (Remi, India) for 5 min. The supernatant 4 ml was dried and finally make up to 1 ml for injection in GC-ECD and GC-MS.

Instrument operating parameters

Validation of method and residue screening of chilli samples were conducted using two instruments. First samples were analysed by GC-ECD for the initial screening of the samples for presence of pesticides. Secondly concentrated samples were analysed by GC-MS in full scan mode for further confirmation of pesticides. Presence of pesticides in samples was confirmed with the help of two parameters, namely Retention Time (RT) and Mass Spectrum (MS). Matching of RT and MS data of the sample peak with that of the standard confirms identification of the pesticides present in the sample. Final quantification was carried out on GC-ECD.

GC-ECD operating parameters

GC-ECD (Agilent) equipped with DB-5MS fused silica capillary column (Agilent J&W GC column, 5% Phenylated methyl siloxane, 30 m length × 0.25 mm i.d. × 0.25 µm film thickness) was used for preliminary screening and final quantification of pesticide residues.

Column	:	DB-5 (length: 30 m, I.D.: 0.25 mm, film thickness: 0.25 µm)
Oven temperature program	:	170 °C (2 min), @ 10 °C, 280 °C (10 min)
Detector temperature	:	300 °C
Injector temperature	:	250 °C
Carrier gas (N_2) flow rate	:	1.35 ml/min
Retention time	:	9.86 min
Injection volume for standard & sample	:	1 µl

GC-MS

GC Model 7890A (Agilent Technologies) with mass **(**5975C inert XL EI/CI MSD) triple axis detector was used for pesticide residue analysis. Injector port temperature was set at 280 ^{o}C. DB-5MS fused silica capillary column (Agilent J&W GC column, 5% Phenylated methyl siloxane, 30 m length × 0.25 mm i.d. × 0.25 µm film thickness) was used with linear flow at 1ml/min. 1 micro litre of concentrated extract was injected in splitless mode with carrier gas helium (grade-1). The pesticides were separated with 40.75 min oven programming of initial temperature 50 ^{o}C for 2 min followed by a ramp rate of 8 ^{o}C /min up to a temperature of 280°C with a hold time of 10 min. The injector was operated in splitless mode at 280 ^{o}C temperature. The interface, ion source and quadruple temperatures were set at 280^{o}C and 230 ^{o}C and 150 ^{o}C respectively. The mass spectrometer was operated in electron impact (70ev), selected ion monitoring (SIM) and Scan mode with solvent delay time 3 min. The scanning mode offer enhances selectivity over either full scan or selected ion monitoring (SIM).

RESULTS AND DISCUSSION

In-house validation of the method was carried out on chilli matrix and target 28 pesticides. European guidelines for single laboratory validation and pesticide residue analysis were used for establishing method performance criteria.

Pesticides taken for study

28 pesticides (Organochlorine, Synthetic Pyrethroids and Herbicides) which are commonly used in India viz Alpha-HCH, Beta-HCH, Gamma HCH, Delta HCH, Alachlor, Aldrin, Dicofol, Pendimethlin, O,P DDE, Alpha-Endosulphan, Heptachlor, P,P DDE, Endosulphan Sulphate, Dialdrin, O,P DDD, Beta- Endosulphan, P,P DDD, O,P DDT, P,P DDT, Bifenthrin, Fenpropathrin, Lambda Cyhalothrin, Beta Cyfluthrin, Cypermethrin, Fenvalarate, Fluvalinate, Deltamethrin are considered for study. Out of 28 pesticides taken, Endosulphan, BHC and DDT isomers are highly persistant in environment. Premilinary scrrening was done to find out the best chromatographic techniques can be used in terms of peak shape, response and LOD/LOQ. All 28 pesticides gave good resposnse for ECD detector which can be used for quantification and confirmation by GC-Mass.

Method Development and Validation

Before any analytical method can be used routinely in the laboratory, it must be validated. Validation is the test for the suitability of a particular method for collecting analytical data. There are several key parameters for ensuring a robust method, they are - Recovery, Selectivity (Specificity), Calibration, Repeatability, Reproducibility, Limit of Detection (LOD), Limit of quantitation (LOQ), (European Commission, Directorate General Health and Consumer Protection, SANCO/2007/3131(Supersedes Document No. SANCO/10232/2006).

Limit of Detection and Limit of Quantification

LOD & LOQ will be measured by using EPA method as it is simple, easy and practical to implement (Johannes Corley Oct 24, 2002). To measure the LOD, peak to peak noise of blank matrix (chilli) at or around the R.T. of individual pesticides chromatogram of standard mix is noted and averaged for three replicates. Concentration of the individual pesticide is calculated (in mg/kg) from the matrix spiked chromatogram which could produce the signal equal to three times of blank matrix..LOQ is calculated by multiplying the LOD value by factor 3. Measured method LOD, LOQ and the relevant legislative limits (MRLs) and ADI (K.K. Sharma, 2013) are listed in Table 1.

Table 1- Shows Class, Retention Time (RT), Limit of detection (LOD), limit of quantification (LOQ), Maximum residues limits (MRL) and acceptable daily intake (ADI) of organochlorine, synthetic pesticide and herbicides from spiked chilli matrix by GC-ECD.

S.No.	Pesticides	Class	RT	LOD (mg/kg)	LOQ (mg/kg)	MRL (mg/kg)	ADI (mg/kg/day)
1.	Alpha-HCH	Organochlorine	9.80	0.002	0.006	1.00	0.005
2.	Dicofol	Organochlorine	10.80	0.05	0.15	1.00	0.002
3.	Beta-HCH	Organochlorine	11.25	0.002	0.006	1.00	0.005
4.	Gamma HCH	Organochlorine	11.57	0.001	0.003	1.00	0.005
5.	Delta HCH	Organochlorine	12.86	0.003	0.009	1.00	0.005
6.	Heptachlor	Herbicide	15.78	0.01	0.03	0.05	0.0001
7.	Alachlor	Herbicide	15.87	0.01	0.030	NA	0.0005
8.	Aldrin	Organochlorine	18.05	0.003	0.009	0.1	0.0001
9.	Pendimethlin	Herbicide	21.06	0.005	0.015	NA	NA
10.	O,P DDE	Organochlorine	23.21	0.001	0.003	3.50	0.01
11.	Alpha-Endosulphan	Organochlorine	23.52	0.002	0.006	2	0.006
12.	Butachlor	Herbicide	24.22	0.005	0.015	NA	0.003
13.	Dialdrin	Organochlorine	25.53	0.002	0.006	NA	0.0001
14.	P,P DDE	Organochlorine	25.68	0.001	0.003	3.50	0.01
15.	O,P DDD	Organochlorine	26.30	0.003	0.009	3.50	0.01
16.	P,P DDT	Organochlorine	26.40	0.004	0.012	3.50	0.01
17.	Beta-Endosulphan	Organochlorine	28.14	0.005	0.015	2	0.006
18.	P,P DDD	Organochlorine	29.47	0.002	0.006	3.50	0.01
19.	O,P DDT	Organochlorine	29.72	0.006	0.018	3.50	0.01
20.	Endosulphan Sulphate	Organochlorine	32.60	0.004	0.012	NA	NA
21.	Bifenthrin	Synthetic Pyrethroids	41.72	0.007	0.021	NA	0.02
22.	Fenpropathrin	Synthetic Pyrethroids	42.19	0.002	0.006	0.2	0.03
23.	Lambda Cyhalothrin	Synthetic Pyrethroids	47.44	0.01	0.03	NA	0.002
24.	Beta Cyfluthrin	Synthetic Pyrethroids	52.7-52.9	0.01	0.03	NA	0.02
25.	Cypermethrin	Synthetic Pyrethroids	53.03-53.44	0.005	0.015	NA	0.05

26.	Fenvalarate	Synthetic Pyrethroids	56.23	0.005	0.015	2	0.02
27.	Fluvalinate	Synthetic Pyrethroids	56.9-57.2	0.01	0.03	NA	NA
28.	Deltamethrin	Synthetic Pyrethroids	58.63	0.006	0.018	NA	0.01

With the present method, all pesticides taken for study, their LOD and LOQ in the range of below MRL for that specific pesticide in chilli. It always happens that matrix effect was predominant for some pesticides. To over come variable matrix effect, the quantification was done using matrix- matched standards prepared in matrix blank.

Linearity

The calibration curves of GC-ECD instrument were plotted between an area pesticide mixture against various concentration levels i.e 0.001, 0.005, 0.01, 0.05, 0.1, 0.5 and 1 mg/Kg with regression co-efficient (r^2) range between 0.999- 0.996. Figure 1&2 shows calibration curve of pesticides mixture taken for study.

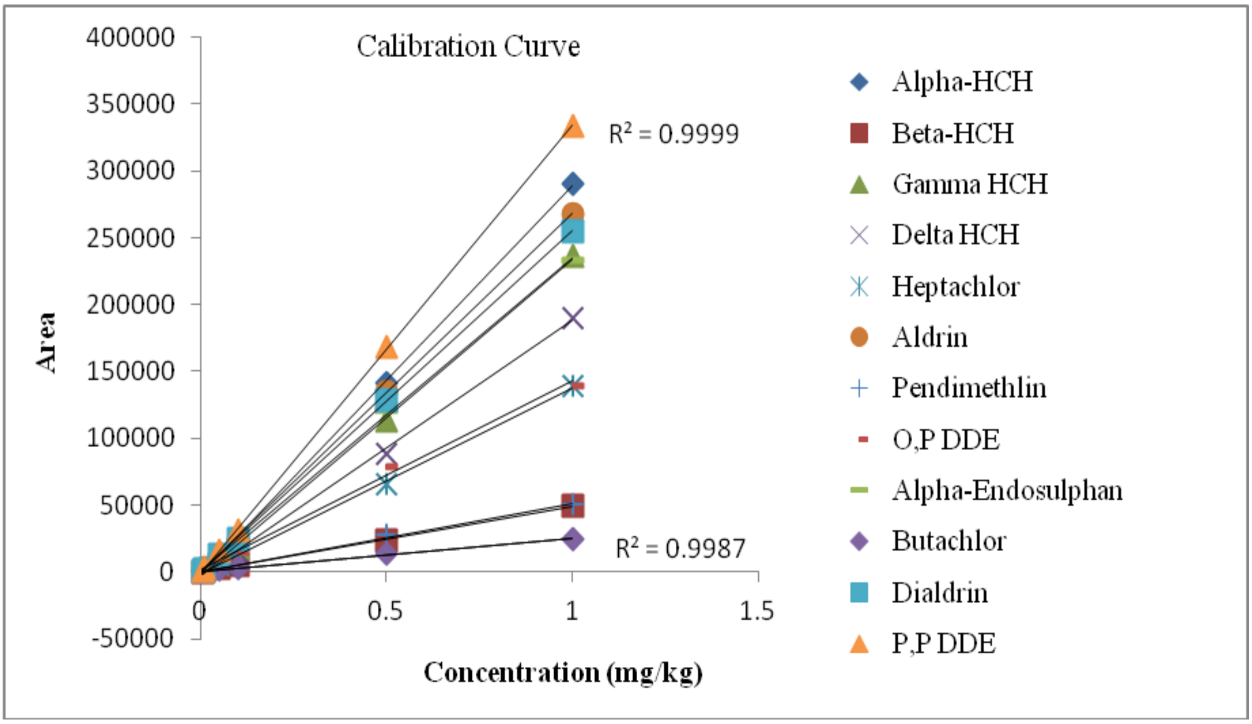

Fig.-1 Linearity Curve of Pesticides mixture

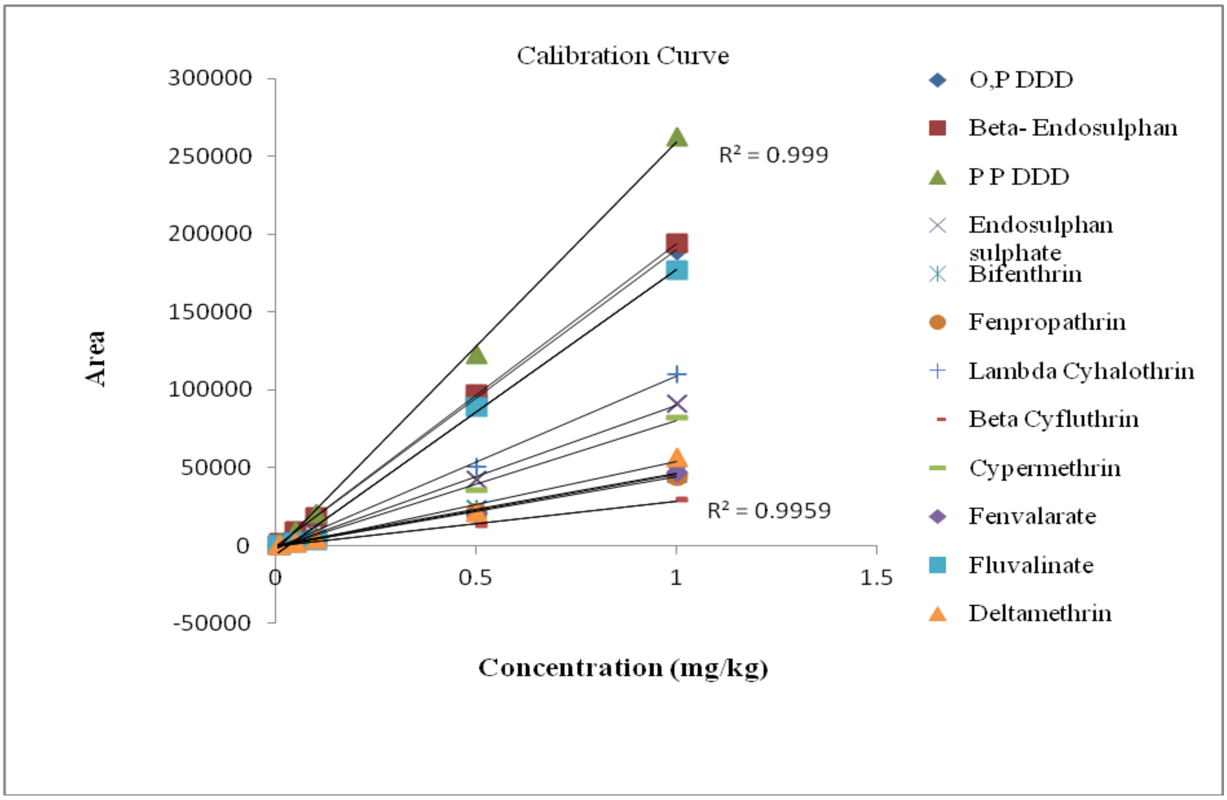

Fig.-2 Linearity Curve of Pesticides mixture

Recovery

Method trueness was confirmed by recovery studies using blank chilli matrices spiked at 0.05 ppm concentration level and injected in three individually prepared replicates. Spiking of samples occurred prior to sample preparation. Found concentrations, recovery, standard deviation and relative standard deviation (% RSD) were calculated (Table 2). According to SANCO requirements recovery values lies within acceptable range between 70–120%. Relative Standard Deviation (RSD) for three replication (R1, R2, R3) was calculated and found below 8%.

Table-2. Shows Recovery, Mean Recovery, Standard Deviation (S.D), Relative Standard Deviation (RSD) of organochlorine, synthetic pyrithroids and herbicides pesticides from spiked chilli matrix at 0.05 ppm.

Pesticide	RT	**Spiking Conc (Ppm)**	**Recovery Percent R1**	**Recovery Percent R2**	**Recovery Percent R3**	**Mean Recovery Percent**	**S.D.**	**R.S.D**
Alpha-HCH	9.8	0.05	82	86	92	86.67	4.11	4.74
Dicofol	10.8	0.05	92	86	84	87.33	3.40	3.89
Beta-HCH	11.25	0.05	82	98	96	92.00	7.12	7.74
Gamma HCH	11.57	0.05	92	80	82	84.67	5.25	6.20
Delta HCH	12.86	0.05	82	92	90	88.00	4.32	4.91
Heptachlor	15.78	0.05	92	86	90	89.33	2.49	2.79

Alachlor	15.87	0.05	90	86	84	86.67	2.49	2.88
Aldrin	18.05	0.05	88	80	92	86.67	4.99	5.76
Pendimethlin	21.06	0.05	90	84	86	86.67	2.49	2.88
O,P DDE	23.21	0.05	90	84	90	88.00	2.83	3.21
Alpha-Endosulphan	23.52	0.05	88	84	86	86.00	1.63	1.90
Butachlor	24.22	0.05	90	88	86	88.00	1.63	1.86
Dialdrin	25.53	0.05	82	84	92	86.00	4.32	5.02
P,P DDE	25.68	0.05	90	84	78	84.00	4.90	5.83
O,P DDD	26.3	0.05	90	78	86	84.67	4.99	5.89
P,P DDT	26.4	0.05	78	84	86	82.67	3.40	4.11
Beta-Endosulphan	28.14	0.05	86	82	88	85.33	2.49	2.92
P,P DDD	29.47	0.05	80	84	84	82.67	1.89	2.28
O,P DDT	29.72	0.05	92	84	82	86.00	4.32	5.02
Endosulphan Sulphate	32.6	0.05	88	86	92	88.67	2.49	2.81
Bifenthrin	41.72	0.05	90	86	92	89.33	2.49	2.79
Fenpropathrin	42.19	0.05	90	86	92	89.33	2.49	2.79
Lambda Cyhalothrin	47.44	0.05	84	86	84	84.66	0.94	1.11
Beta Cyfluthrin	52.7-52.9	0.05	88	92	86	88.66	2.49	2.81
Cypermethrin	53.03-53.44	0.05	92	82	84	86.00	4.32	5.02
Fenvalarate	56.23	0.05	88	86	92	88.66	2.49	2.81
Fluvalinate	56.9-57.2	0.05	86	90	92	89.33	2.49	2.79
Deltamethrin	58.63	0.05	92	82	86	86.66	4.11	4.74

Selectivity

NIST library of Gas chromatography mass spectrometry was used to confirm the presence of pesticides taken for study. Spectra match with reference spectra of NIST. Presence of pesticides was confirmed by their fragmentation pattern matches with reference spectra of NIST.

Repeatability and reproducibility

Repeatability of the method is justified by analysing samples which are repeated for three times i.e R1, R2 and R3 and their Mean (M), Standard Deviation (SD), and Relative Standard Deviation (RSD) of each pesticide are calculated are shown in Table 3. Instrument injection precision was tested for both retention time and peak area for all target compounds by subsequent three injections of low concentration level (0.005ppm) standard solutions. Table shows repeatability with relative standard deviation lies within permissible limit < or = 5.

Table-3. Repeatability R1, R2 and R3 and their Mean (M), Standard Deviation (SD), and Relative Standard Deviation (RSD) of each pesticide.

Pesticide Mixture	RT	Area R1	Area R2	Area R3	Mean	SD	RSD
		Concentration 0.005 ppm					
Alpha-HCH	9.8	1090	1125.2	1075	1096.3	25.7	2.3442488
Beta-HCH	11.14	270.85	271.98	280.3	258.0433	5.16077837	1.999966
Gamma HCH	11.47	819.93	792.87	810.23	807.6767	13.7095052	1.6974001
Delta HCH	12.731	628.25	611.55	610	616.6	10.1189179	1.641083
Heptachlor	15.66	490.37	482.33	478.16	483.62	6.20637575	1.2833166
Aldrin	17.93	1284.85	1286.24	1231.44	1267.51	31.2452668	2.4650904
Pendimethlin	20.93	322.83	311.35	315.43	316.5367	5.8194616	1.8384793
O,P DDE	23.05	735.76	741.08	717.74	731.5267	12.2323233	1.6721636
Alpha-Endosulphan	23.37	1244.93	1235.34	1225.77	1235.347	9.58000174	0.7754908
Butachlor	24.09	198.05	182.26	147.56	618.6233	25.8284539	4.1751505
Dialdrin	25.35	1242.78	1263.37	1162.04	1149.397	53.5578575	4.6596483
P,P DDD	25.5	1506.13	1472.24	1559.09	1312.487	43.7725489	3.3350844
O,P DDD	26.17	972.55	955.52	942	956.69	12.0420285	1.2587179
Beta-Endosulphan	27.94	985.66	976.88	959	973.84667	6.20839754	0.6375128
P P DDD	29.25	867.15	850.89	820.4	846.1467	23.7332053	2.804857
Endosulphan sulphate	32.35	359.64	338.59	325.76	341.33	17.105388	5.0113931
Bifenthrin	41.46	328.68	310.41	303.61	314.2333	12.9649386	4.1258958
Fenpropathrin	41.95	260.95	250.71	241.65	253.77	9.65601022	3.8050243
Lambda Cyhalothrin	47.27	422.38	404.22	420.96	399.8533	10.0997492	2.5258637
Beta Cyfluthrin	52.68-52.9	90.77	91.83	88.88	90.4933	1.47715965	1.6323415
Cypermethrin	53.08-53.90	330.3	319.49	320.82	330.12	5.89484803	1.7856683
Fenvalarate	56.1	208.13	195.8	200.73	195.22	6.20609647	3.179027
Fluvalinate	56.76-56.88	178.16	166.26	170	165.4733	6.08527184	3.6774947
Deltamethrin	58.5	204.03	198.71	198.67	181.8033	3.08311531	1.6958522

CONCLUSION

The authors suggest that a regular monitoring, assessment and reporting machineries should be implemented in accordance with appropriate environmental policies, laws and regulations. The Government and other related agencies should educate farmers and agriculture managers on Good Agricultural Practices (GAP). The authors also recommend for Good Laboratory Practices (GLP) and Laboratory Standard Operating Procedures (SOPS) for reliable and dependable analytical systems and standardization of facilities for analysis of products with high lipid content, which can adversely affect pesticide recoveries and harm traditional chromatographic systems. For extraction, the main factors (oil and water content) were studied and optimized in experiments to maximize pesticide recoveries. Dispersive SPE with different sorbents was also investigated to minimize matrix coextractives and interferences. The objective of this research work is to prove a complete workflow solution that can be implemented for routine multi-residue pesticide analysis in representative chilli matrices and study the persistence of pesticide in chilli.

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