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Determination of 25 Pesticides By GC-ECD And MSD With Measurement of Uncertainty In Tomato Using Modified QuEChERS Technique

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ABSTRACT

A multi-residue method was developed by slight modification in QuEChERS method and subsequently validated for determination of 25 pesticide residues including organochlorine, organophosphates, synthetic pyrethroids and herbicides in tomato. Samples were extracted with acetonitrile and clean up was done by PSA and C_{18}. Recovery studies were carried out at three spiking concentration level namely 1 LOQ (Limit of quantification), 5 LOQ and 10 LOQ levels. Mean recovery varied from 74 % to 117 % with Relative Standard Deviation (RSD) below 20%. For the measurement of uncertainty (MU), three main independent sources viz. weighing, purity of the standard and repeatability were considered. MU for more than 80 percent of pesticides were below 5 percent and for rest 20 percent pesticides MU were in the range of 5 to 10 percent. The method developed can be used for the analysis of all 25 pesticides in one single determination step.

Keywords: Multi-residue analysis, Pesticides, LOQ, Tomato, QuEChERS.

INTRODUCTION

Plant protection products (more commonly known as pesticides) are widely used in agriculture to increase the yield, improve the quality, and extend the storage life of food crops [1]. The controlled pesticide uses in agriculture will not affect the environment whereas uncontrolled pesticide use will cause adverse impacts on the environment such as water, soil and air which cause unbalance in ecosystem[2]. Detailed investigation of pesticide residues began in mid-20th century. Numerous methods of analysis were developed, mostly using a traditional liquid-liquid extraction (LLE). Up-to-date methods should be cost-efficient and should considerably lower the risk of affecting the analyst's health and environmental contamination. The method of solid phase extraction (SPE) expanded significantly in early 1980s, while the innovative solid phase microextraction (SPME) was introduced in late 1990s [3]. The pesticides studied were chosen based on their having vapour pressure values high enough to allow analyte concentration in the gas phase and on their widespread use for crop[4].

However, improvements in the sample preparation techniques led to modification of the existing methods and development of new techniques, in order to save time and reduce use of chemicals and thus improve

the overall performance of analytical process. As a result, several rapid, low cost, environment friendly and readily automated methods of extraction are now available. Future developments in all areas of analytical sample preparation are expected to continue to be application-driven in a quest for improved recovery, higher sample throughput, and reduced consumption of organic solvent with capability to provide accurate results [5]. Traditional sample preparation methods (liquid-liquid extraction, Soxhlet extraction, etc.) are laborious, time consuming, expensive, requires large amounts of organic solvents and usually involve many steps, leading to loss of some analyte quantity and other consequences solvents use are ozone depletion and generation of considerable cancer waste, lead to reduction of not only their use but also their manufacture. As a result, modern sample preparation procedures, such as accelerated solvent extraction (ASE) , supercritical fluid extraction (SFE), microwave assisted extraction (MAE), solid phase extraction (SPE), solid phase micro extraction (SPME), matrix solid phase dispersion (MSPD), extraction and QuEChERS (quick, easy, cheap, effective, rugged and safe), have been developed to overcome the drawbacks of the traditional approaches[6-11].

Since most of the pesticides are toxic in nature, their continuous ingestion by man even in trace amounts, can result in their accumulation in body tissues with serious adverse effects on health [12]. Vegetables devour 14% of the total pesticides used in India, in which, the share of different types of pesticides in Indian agriculture market shows that organophosphorus (50%) ranked first, followed by pyrethroids (19%), organochlorines (18%), carbamates (4%) and biopesticides (1%)[13]. Many countries have established regular monitoring programs for quantitative determination of residues in food products as pesticide residues above the maximum residue limits (MRL) at harvest time are a subject of great concern both globally and nationally[14].

The determination of pesticide residues is a requirement to support the enforcement of legislation, ensure trading compliance, conduct monitoring residue programs in dietary components and in environmental samples, and study their mode of action and movement within the environment[15]. Analytical methodologies that are capable of residue measurement at very low levels, and that also provide unambiguous evidence of the identity and magnitude of any residues detected are strongly required[16-17]. In this paper, we report the single-laboratory validation and uncertainty measurement of multiresidue analysis for 12 organochlorine and 6 organophosphorus pesticides, 4 synthetic pyrethroids and 3 herbicides in tomato matrices with good selectivity, sensitivity, and cost effectiveness.

MATERIALS AND METHODS

Certified reference material (CRM): Certified reference materials (CRM) were procured from Accustandard Inc. (USA) and Sigma Aldrich for all the pesticides under study i.e. 12-organochlorine pesticides, 6-organophosphorous pesticides, 4-synthetic pyrethroids and 3- herbicides. Percent purity of each CRM is given in table 1.

All the solvents used were of HPLC- grade and purchased from Merck. Primary Secondary Amine i.e. PSA (40 µm, Bondesil) sorbent was purchased from Agilent Technologies. C-18 silica sorbent used in this study was of Supelco and procured from sigma Aldrich. Anhydrous magnesium sulphate was procured from Merck, Germany.

Table 1. Percent purity of each pesticide

S.No	Pesticide	Percent Purity
	Organochlorine pesticides	
1	Alpha-HCH	99.8
2	Beta--HCH	99.2
3	Gamma--HCH	99.5

4	Delta--HCH	99.6
5	Endosulphan--I	99.5
6	Endosolphan--II	99.5
7	Endosulphan sulphate	98.8
8	p,p –DDE	99.5
9	p,p – DDT	99.7
10	o,p – DDE	99.5
11	o,p-- DDD	99.5
12	o,p-- DDT	99.3
	Organophosphorous pesticides	
1	Chlorpyrifos	99.6
2	Malathion	98.5
3	Dimethoate	99.6
5	Phorate	96.0
6	Quinolphos	99.4
7	Profenophos	96.0
	Synthetic pyrethroids	
1	Cypermethrin	97.2
2	Deltamethrin	98.9
3	Fenvalarate	99.0
4	Lamda--cyhalothrin	99.0
	Herbicides	
1	Alachlor	99.4
2	Butachlor	97.7
3	Pendimethlin	100

Instrument details and operating parameters: The present study was conducted using two instruments. Initially the samples were analysed by GC-ECD for the preliminary screening of the samples for presence of pesticides. Later on concentrated samples were analysed by GC-MS in full scan mode for further confirmation. Presence of pesticides 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 CRM gave unambiguous identification of the pesticides present in the sample. Final quantification was carried out on GC-ECD. Shimadzu make GC-ECD (GC-QP 2010 model) 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. Analysis was performed with oven temperature programming of 170°C as initial temperature for 5 min followed by a ramp rate of 5°C/min up to a final temperature of 280°C with a hold time of 10 min. The injector and detector temperature was set at 280°C, 300°C, respectively. The instrument was set in split mode of (10:1). Nitrogen was used as carrier gas at a flow rate of 1 mL min^{-1}.

GC-MSD (Mass Selective Detector, GC-QP 2010 plus MSD model) 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 confirmation of the target pesticides. Analysis was carried out using oven programming of initial temperature 50 ^{O}C for 2 min followed by a ramp rate of 20 ^{O}C min^{-1} up to a temperature of 130 ^{O}C followed by 12 ^{O}C min^{-1} ramp to a temperature of 180 ^{O}C with a hold time of 10 min. The injector was operated in split less mode at 280 ^{O}C temperature. The interface, ion source and quadruple temperatures were set at 280 ^{O}C and 250 ^{O}C and 150 ^{O}C, respectively. The instrument was operated in Electron Impact Mode (EI) with electron energy 70ev. Helium was used as carrier gas at a flow rate of 1 mL/min. Solvent delay time was given as 6.5 min.

Preparation of standard stock solution: The stock solution of each pesticide taken for the study was prepared by Certified Reference Materials (CRM) of pesticide having specific purity with uncertainty value. Sample weighed directly in clean and dried standard volumetric flask of 10 mL on analytical balance pan (Mettler, Toledo). CRM of individual pesticide weighed maximum up to 4mg, dissolved in few drops mL of HPLC grade acetone which is further make up to the mark with HPLC grade hexane. Further working standard was prepared by serial dilution of Stock solution by solvent n-hexane. Standard stock solution and working standards were stored in deep freezer at −20 ^{O}C. A working standard prepared for analysis having mixture all 25 pesticides, each at 1 ppm concentration are shown in figure **1**.

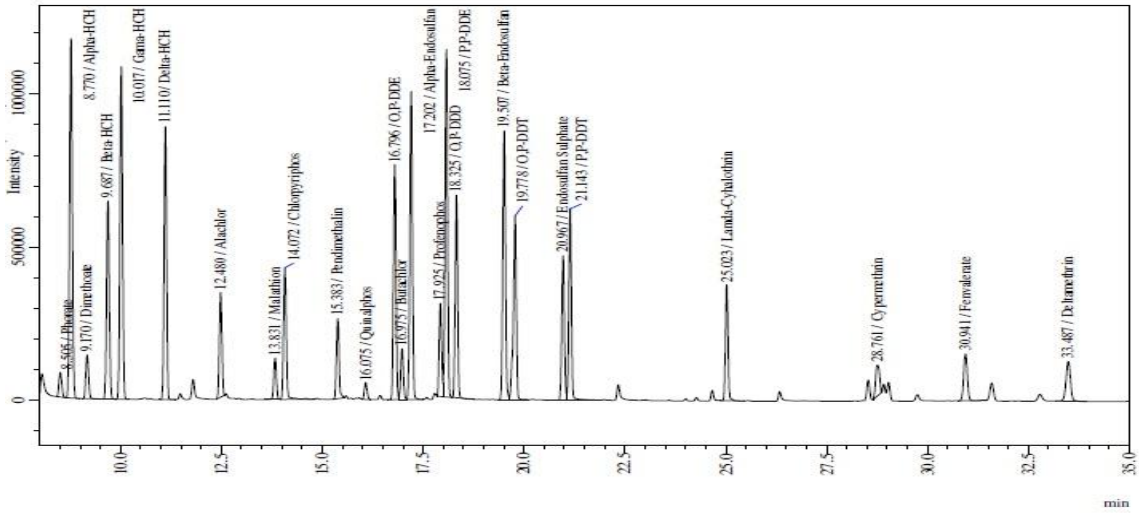

Fig. 1 Representative chromatogram of standard mixture (25 pesticides) at 1ppm concentration level.

Method validation: Justification and reliability of particular method depends upon validation of method. Data of Instrument linearity, Method recovery, Repeatability, Limit of Detection (LOD), Limit of Quantification (LOQ), Specificity and Selectivity were collected for validation studies. Linearity was based on a six-point calibration graph obtained by plotting the detector response (i.e. peak area) against concentration of the calibration standards were of 0.005, 0.010, 0.050, 0.100, 0.250 and 0.500 ppm level. Before calculation of LOD (Limit of detection) and LOQ (Limit of quantification) matrix extract was checked for the absence of test pesticides. LOD and LOQ were calculated by taking two equal portions of the same matrix blank extracts out of which one was spiked with pesticide mixture at 1 ppm level and

other remains untouched. Both matrix were processed following developed standard operating procedure and injected both aliquots under the same conditions in GC-ECD[18]. LOD was calculated by dividing matrix area upon standard area whole divided by 2, whereas three times of LOD is equal to LOQ.

Sample preparation and recovery study: Tomato was collected from local market Gurgaon, Haryana which was grinded homogenously. The samples weighed at (10 g ± 0.1 g) in triplicate, were fortified at three concentration levels- 1 LOQ, 5 LOQ, and 10 LOQ by pesticide standard mixture. Unfortified control matrix were also processed separately in triplicate. Extraction of sample/matrix was done by 10mL Acetonitrile HPLC grade solvent in 50 mL centrifuge tube (TARSON). 4 g $MgSO_4$ and 1 g NaCl were added to centrifuge tube and vortex on rotospin for 10-15 min. Samples were centrifuged (REMI) for 5 min at 4000-5000 rpm. 1.5 mL aliquot from supernatant layer was taken from centrifuged tubes to microcentrfuge tubes (TARSON, 2 ml) having preweighed PSA 37mg, 25mg MgSO4 and 37 mg C-18 in, for clean-up of extractants . Microcentrifuge tubes were vortex for 1 min and put in centrifuge for 3 min at 4000-5000 rpm. 1 mL of aliquot from supernatant layer was taken in glass tubes for evaporating solvent upto near dryness, using gentle nitrogen stream (Turbo Vap LV, Caliper Life Sciences). Finally 0.5 mL of HPLC grade n-Hexane was added to glass tubes stirred to completely dissolve and transferred to fresh GC vials for quantification of analytes by GC-ECD. Samples were evaporated again to near dryness and make up to 100µL in n-hexane for confirmation of analytes by GC-MS.

Determination of uncertainties: Calculation of uncertainty is important step for method development process. Combined uncertainty (U) was determined at 5 LOQ level for all the pesticides taken under study as per the statistical procedure of the EURACHEM/CITAC Guide CG 4. [19] For the determination of uncertainty three individual sources of uncertainty were taken. First relative standard uncertainty (U1) due to purity of analytical standards, second uncertainty due to weighing (U2) and at last uncertainty associated with precision (U4).

Uncertainty due to purity of analytical standards (U1)

As confidence level was not mentioned in purity certificate of Certified Reference Material (CRM) therefore for calculation of uncertainity for purity by rectangular distribution was assumed equation 1.

$$SU1 = (u(x)/\sqrt{3}) \quad (1)$$

where u(x) is the uncertainty value given in the certificate for purity of CRM whereas relative standard uncertainty (U1) was derived according to the Eq. (2).

$$U1 = (SU1 \times 100)/\%\ \text{purity} \quad (2)$$

Uncertainty of weighing (U2)

To estimate relative standard uncertainty due to weighing (U2) normal distribution was assumed by Eq. (3)

$$U2 = (0.0001/2)/Wi \quad (3)$$

where Wi is the weight of the pesticide standard weighed using precision analytical balance, 0.0001 is the value of uncertainty at 95% confidence level taken from the valid calibration certificate of balance. Considering normal distribution the uncertainty of the balance was divided by taking two.

Uncertainty associated with precision (U3)

During sample processing steps, errors caused at extraction, clean up, and GC analyses steps were approximated by Standard Deviations (s), calculated from triplicate determinations of analytes expressed as repeatability in Eq. 4.

$$U4 = s/(\sqrt{n} \times x) \quad (4)$$

where standard deviation (s) is obtained from the recovery study, n is the number of replications and x is the mean value of the concentration recovered.

The combined uncertainty (U) was calculated by Eq. 5.

$$U = x\,[(U1)^2+(U2)^2+(U4)^2]^{1/2}$$

Expanded uncertainty (2U) was twice of combined uncertainty (U) at 95% confidence level.

RESULTS AND DISCUSSION

Pesticide Selection: The main purpose of this study was to develop multiresidue method incorporating the pesticides which are commonly used in India for the selected food matrices. Among chosen pesticides (endosulphan, lindane, isomers of DDT and BHC etc) many of them are highly persistent to environmental degradation and usually found in environmental matrices like soil and water, though Government of India banned them several years before. All these pesticides need to be identified by multi-residue method developed for identification and evaluation. By preliminary experiments best chromatographic technique was carried out for selected pesticides which were analyzed by GC-ECD and GC-MS in terms of peak shape, response and LOD/LOQ. All twenty five pesticides gave good response GC-ECD thus quantified in GC-ECD and confirmation were done by using GC-MSD (FULL SCAN mode).

Validation of the method: Linearity of calibration curve, LOD and LOQ
For method validation, linearity curve for each pesticides was drawn between GC response area versus concentration. All pesticides shows linear behaviour for the conc range of 0.5-0.005 ppm standard mixture analyzed by GC-ECD) and their correlation coefficient (R^2) was found to be in range of 0.98 to 0.99 for each pesticide in each matrix are given in following figure **2,3**.

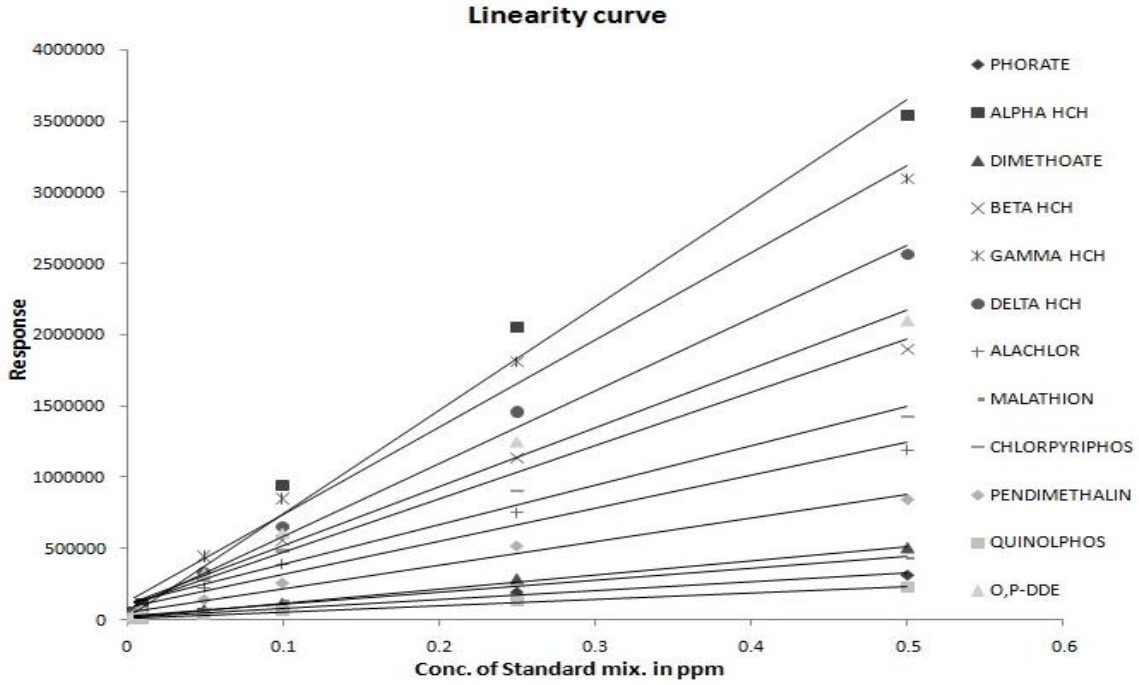

Fig.2 Linearity curve of different pesticides.

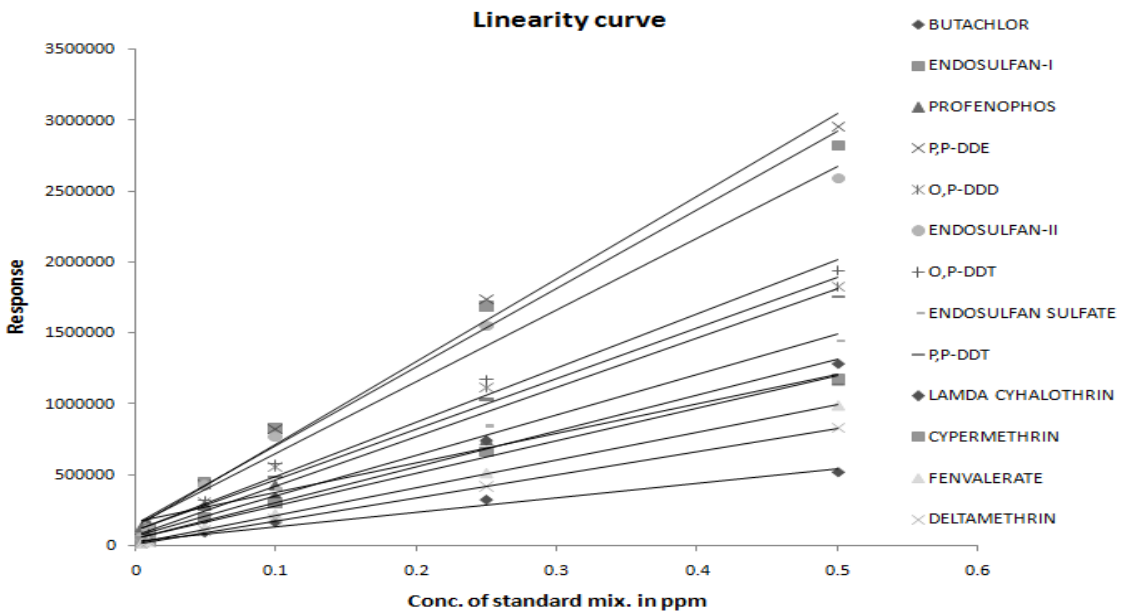

Figure. 3 Linearity curve of different pesticides.

The LOD and LOQ for the test pesticides in tomato are presented in below table **2**. LOD and LOQ lies in the range 0.001 to 0.03 and 0.004 to 0.03 in case of tomato. Effect due to matrix was overcome by quantifying with matrix-matched standards prepared in specific matrix blanks.

Table-2 Linearity, LOD and LOQ levels of OC's, OP's, synthetic pyrethroids and herbicides in tomato.

S. No.	Pesticide	R^2	Tomato	
			LOD (µg/g)	LOQ (µg/g)
1.	Phorate	0.98	0.020	0.060
2.	Alpha-HCH	0.99	0.002	0.006
3.	Dimethoate	0.99	0.030	0.090
4.	Beta HCH	0.99	0.006	0.020
5.	Gama HCH	0.99	0.004	0.010
6.	Delta HCH	0.99	0.002	0.006
7.	Alachlor	0.98	0.020	0.050
8.	Malathion	0.98	0.030	0.090
9.	Chlorpyriphos	0.98	0.007	0.020
10.	Pendimethlin	0.98	0.020	0.060
11.	Quinolphos	0.99	0.030	0.080
12.	o,p DDE	0.99	0.002	0.005
13.	Butachlor	0.98	0.007	0.020
14.	Endosulfan--I	0.99	0.001	0.004
15.	Profenophos	0.99	0.003	0.009
16.	p,p DDE	0.99	0.010	0.030
17.	o,p DDD	0.98	0.002	0.006
18.	Endosulfan--II	0.99	0.001	0.004
19.	o,p DDT	0.99	0.002	0.004
20.	Endosulfan sulphate	0.99	0.003	0.009

21.	p,p DDT	0.99	0.004	0.010
22.	Lamda cyhalothrin	0.99	0.004	0.010
23.	Cypermethrin	0.99	0.008	0.030
24.	Fenvalarate	0.99	0.005	0.010
25.	Deltamethrin	0.99	0.003	0.009

Recovery and precision: Tomato samples were extracted by single step extraction using dispersive solid phase extraction technique. Three replicates of tomato matrix were taken along with control for each level at fortification 1, 5 and 10 LOQ given in following table **3**.

Table-3 Recovery (%) of the pesticides from tomato three fortification levels

S.No.	Pesticide	10 LOQ		5 LOQ		1 LOQ	
		Mean	RSD	Mean	RSD	Mean	RSD
1	Phorate	88.51	0.40	87.62	1.15	86.48	4.39
2	Alpha-HCH	86.50	0.73	90.46	1.85	86.76	3.22
3	Dimethoate	88.37	1.38	100.98	1.22	85.05	4.23
4	Beta HCH	91.45	1.95	93.85	0.72	89.22	0.14
5	Gamma HCH	87.96	0.49	91.21	1.50	85.94	2.32
6	Delta HCH	101.84	9.98	108.46	3.61	88.45	12.18
7	Alachlor	92.57	1.51	95.05	2.25	87.97	15.72
8	Malathion	79.92	0.25	88.60	1.17	81.14	0.60
9	Chlorpyriphos	85.35	0.33	90.95	0.38	88.69	2.71
10	Pendimethalin	87.24	1.05	88.97	1.56	88.01	2.07
11	Quinolphos	89.37	1.16	91.84	0.28	94.38	3.38
12	o,p--DDE	95.91	0.83	94.12	1.73	94.11	2.66
13	Butachlor	88.69	0.20	90.37	1.09	88.46	1.53
14	Endosulfan--I	91.82	2.28	97.00	1.02	75.78	11.39
15	Profenophos	74.37	6.95	94.36	10.45	93.15	4.54
16	p,p--DDE	94.00	0.57	93.90	0.94	91.30	0.79
17	o,p--DDD	98.00	1.91	93.88	1.23	110.30	17.24
18	Endosulfan--II	96.52	1.03	91.35	7.36	86.92	10.72
19	o,p--DDT	88.35	3.05	88.13	3.40	85.42	2.30
20	Endosulfan sulphate	87.74	3.29	96.48	7.42	83.19	1.56
21	p,p--DDT	89.74	3.24	89.87	2.56	92.21	5.29
22	Lamda cyhalothrin	89.26	5.03	92.07	8.41	80.46	10.70
23	Cypermethrin	103.74	6.65	115.27	11.57	76.11	2.96
24	Fenvalerate	110.34	1.43	101.98	18.05	95.53	4.06
25	Deltamethrin	116.79	3.31	104.94	0.67	87.07	4.80

RSD--Relative Standard Deviation

At 5 LOQ and 10 LOQ fortification levels high recovery lies between 74-117% were recorded for all 25 pesticides taken for study. Recoveries of all pesticides at all level were analyzed using GC-ECD detector.

Uncertainty measurement: Uncertainties arising from three major steps were measured viz. - weighing, purity of the standard and repeatability. The expanded uncertainty of the pesticides falls under three ranges viz., (a) ≤10% (b) 11–15% and (c) 15–20% shown in table **4**. In case of tomato, 24 out of 25 pesticides fall in the range (a) and 1 in the (c) range respectively, therefore multi-residue method adopted for the pesticides falling in the range (a) is suitable and efficient in determining these pesticides from the matrices.

The uncertainty associated with repeatability for the pesticides belonging to the range (c) has contributed to their relatively higher values of expanded uncertainties. The two major steps for the cause of uncertainty was repeatability (52%) and purity of the pesticide standard 41% of the total uncertainty. Repeatability of determination of analytes in spiked samples and also uncertainty associated with the preparation of the calibration standard solutions (weighing, diluting) were identified as the most significant sources of combined uncertainty.

APPLICATIONS

From the above study we came to know that, the method developed by slight change in QuEChER method for multiresidue analysis is more convenient, cost effective, less chances of contamination, less steps involve in extraction as compared to old QuEChER method. One foremost thing was that pesticides of different groups are analysed by a single detector i.e. ECD detector with single analysis step and was confirmed by mass detector.

Table-4 Results of individual and combined uncertainties with expanded uncertainty for each pesticide calculated at 5 LOQ level in tomato.

S.No.	Pesticide	U1	U2	U3	U	2U
1	Phorate	0.0030	4.03226E--05	0.0066	0.0019	0.0038
2	Alpha-HCH	0.0029	3.33333E--05	0.0107	0.0003	0.0006
3	Dimethoate	0.0029	3.64964E--05	0.0070	0.0035	0.0069
4	Beta HCH	0.0029	2.80899E--05	0.0042	0.0005	0.0010
5	Gamma HCH	0.0029	1.35870E--05	0.0087	0.0004	0.0008
6	Delta HCH	0.0029	4.34783E--05	0.0208	0.0007	0.0014
7	Alachlor	0.0029	4.13223E--05	0.0130	0.0032	0.0063
8	Malathion	0.0029	3.90625E--05	0.0068	0.0029	0.0059
9	Chlorpyriphos	0.0029	4.46429E--05	0.0022	0.0003	0.0007
10	Pendimethalin	0.0029	4.54545E--05	0.0090	0.0025	0.0051
11	Quinolphos	0.0029	1.37363E--05	0.0016	0.0012	0.0024
12	o,p--DDE	0.0029	2.50000E--05	0.0100	0.0002	0.0005
13	Butachlor	0.0030	3.90625E--05	0.0063	0.0006	0.0013
14	Endosulfan--I	0.0029	4.03226E--05	0.0059	0.0001	0.0003
15	Profenophos	0.0030	2.82486E--05	0.0603	0.0026	0.0051
16	p,p--DDE	0.0029	1.69492E--05	0.0054	0.0009	0.0017
17	o,p--DDD	0.0029	3.18471E--05	0.0071	0.0002	0.0004
18	Endosulfan--II	0.0029	3.33333E--05	0.0425	0.0008	0.0016
19	o,p--DDT	0.0029	2.13675E--05	0.0197	0.0004	0.0007
20	Endosulfan sulfate	0.0029	3.90625E--05	0.0428	0.0019	0.0037
21	p,p--DDT	0.0029	4.09836E--05	0.0148	0.0007	0.0014
22	Lamda cyhalothrin	0.0029	2.82486E--05	0.0485	0.0022	0.0045
23	Cypermethrin	0.0030	2.57732E--05	0.0668	0.0116	0.0231
24	Fenvalerate	0.0029	4.62963E--05	0.1042	0.0053	0.0106
25	Deltamethrin	0.0029	3.37838E--05	0.0039	0.0002	0.0005

U1 = Relative Standard Uncertainity of analytical standards, U2 = Relative Standard Uncertainity of weighing, U3 = Uncertainity associated with precision, U = Combined Uncertainity, 2U = Expanded Uncertainity

CONCLUSIONS

Pesticides taken for study belong to different class, which were widely used in India and persistent in environment for long time. The results obtained indicate that the developed method is rapid, accurate, selective, and reproducible. All pesticides shows linear behaviour for the conc. range of 0.5-0.005 ppm standard mixture analyzed by GC-ECD and their correlation coefficient (R^2) was found to be in range of 0.98 to 0.99 for each pesticide in each matrix. The method has been successfully applied for the analysis of vegetables samples. It can be used for the routine analysis of multiresidue in different vegetables matrix. Validation of this method was accomplished, getting results meeting all requirements. The method is simple, reproducible, with a good accuracy and linearity.

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