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Method Validation and Uncertainity measurement for Determination of 25 Pesticides by GC-ECD and MSD in Banana using modified QuEChERS Technique

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Abstract

Method for the determination of multi-residue was developed by slight modification in QuEChERS method and validated for 25 pesticides of different class in banana. Extraction of samples was done with acetonitrile and cleaning by PSA and C_{18} using Solid Phase Extraction technique. Recovery studies at three spiking concentration level 1 LOQ (Limit of quantification), 5 LOQ and 10 LOQ varied from 74 % to 117 % with Relative Standard Deviation (RSD) below 20%. Measurement of uncertainity (MU), for three main independent sources viz. weighing, purity of the standard and repeatability were considered. The method developed can be used as one single determination step for the analysis of all 25 pesticides by GC-ECD and their confirmation by GC-MS.

Keywords: Multi-residue analysis, pesticides, banana, QuEChERS.

Introduction

Pesticides are extensively used in agricultural production to check or control pests, diseases weeds and other plant pathogens in an effort to reduce or eliminate yield losses, extend the storage life of food crops and preserve high product quality[1-2]. The uncontrolled pesticide use will cause adverse impacts on the environment such as water, soil and air which cause unbalance in ecosystem[3]. Pesticides are toxic in nature, their continuous exposure can result in their accumulation in body tissues with serious adverse effects on health[4]. Methods of analysis were developed, using a traditional liquid-liquid extraction (LLE), Solid phase extraction (SPE) in early 1980s, Solid phase microextraction (SPME) in late 1990s[5]. 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 which result in the development of up-to-date methods which is cost-efficient and should considerably lower the risk of affecting the analyst's health and environmental contamination[6]. For studies pesticides chosen having vapour pressure values high enough to allow analyte concentration in the gas phase and on their widespread use for crop[7].

The current trend in pesticide residues analysis is developing multi-residual methods used to determine large number of pesticides and also applicable for large numbers of samples of different origin involves several stages (sample preparation, analyte separation, quantification and data analysis). Traditional methods of extractions (liquid-liquid extraction, Soxhlet extraction, etc.) are time consuming, laborious, expensive, requires large amounts of organic solvents and involve many steps, leading to loss of some analyte quantity. Modern extraction procedures, are 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[8-13].

Regular monitoring programs for qualitative and quantitative determination of pesticide residues in food commodities 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]. Developed analytical methods are required to identify and measure pesticide residue at very low levels[16-17]. In this paper, we report the single-laboratory validation and uncertainity measurement of multiresidue analysis for 12 organochlorine and 6 organophosphorus pesticides, 4 synthetic pyrethroids and 3 herbicides in banana matrices with good selectivity, sensitivity, and cost effectiveness.

Material 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 (Alpha—HCH, 99.8%; Beta HCH,

99.2%; Gamma-HCH, 99.5%; Delta-HCH, 99.6%; Endosulphan-I, 99.5%; Endosolphan-II, 99.5%; Endosulphan sulphate, 98.8%; p,p–DDE, 99.5%; p,p–DDT, 99.7%, o,p–DDE, 99.5%; o,p-DDD, 99.5%; o,p-DDT, 99.3%), 6-organophosphorous pesticides i.e (Chlorpyrifos, 99.6%; Malathion, 98.5%; Dimethoate, 99.6%; Phorate, 96.0%; Quinolphos, 99.4%; Profenophos, 96.0%), 4-synthetic pyrethroids (Cypermethrin, 97.2%; Deltamethrin, 98.9%; Fenvalarate, 99.0%; Lamda—cyhalothrin, 99.0%) and 3-herbicides (Alachlor, 99.4%; Butachlor, 97.7%; Pendimethlin, 100%). All the solvents, used for the study, 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.

Instrument details and operating parameters: In this study mainly two instruments were involved. Samples were analyzed by GC-ECD for identification and conc. of pesticides, whereas the detected pesticides were confirmed by GC-MS in full scan mode. Presence of pesticides was confirmed with the help of two parameters, Retention Time (RT) and Mass Spectrum (MS). Matching of RT and MS data of the sample peak with that of the standard gave confirmation for presence of pesticides in the sample. Analysis was performed using GC-ECD (Shimadzu make GC-QP 2010 model) equipped with DB-5MS fused silica capillary column (Agilent J and W GC column, 5% Phenylated methyl siloxane, 30 m length × 0.25 mm i.d. × 0.25 µm film thickness) with split mode of (10:1), Nitrogen was used as carrier gas with 1 mL/min flow rate. Oven temperature programming was set at 170°C as initial temperature for 5 min with ramp rate of 5°C/min up to a final temperature of 280°C with a hold time of 10 min. Injector was set at 280°C and detector at 300°C. Above mentioned instrumental conditions was used for preliminary screening and final quantification of pesticide residues.

Confirmation of pesticides was performed by GC-MSD (Mass Selective Detector, GC-QP 2010 plus MSD model) equipped with DB-5MS fused silica capillary column (Agilent J and W GC column, 5% Phenylated methyl siloxane, 30 m length × 0.25 mm i.d. × 0.25 µm film thickness) with oven programming of initial temperature 50^{o}C for 2 min followed by a ramp rate of 20 ^{o}C /min up to a temperature of 130^{o}C followed by 12^{o}C /min ramp to a temperature of 180^{o}C with a hold time of 10 min, injector temperature at 280^{o}C, splitless mode, interface at 280^{o}C, ion source at 250^{o}C and quadruple temperatures were set at 150^{o}C. 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 set at 6.5 min.

Preparation of standard stock solution: Standard stock solution of each pesticide, taken for the study, was prepared by Certified Reference Materials (CRM) of pesticide having specific purity with uncertainity value. Sample weighed directly in clean and dried standard volumetric flask of 10 ml on analytical balance pan (Mettler, Toledo). CRM of individual pesticide was weighed maximum up to 4mg, dissolved in few drops/ml of HPLC grade acetone which was further made up to the mark of standard volumetric flask with HPLC grade n-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 laboratory refrigerator at 4°C. A working standard, having mixture all 25 pesticides at 1 ppm concentration level, was prepared and used for the analysis.

Method validation: Method validation was performed by establishing the Instrument linearity, Method recovery, Repeatability, Limit of Detection (LOD), Limit of Quantification (LOQ), Specificity and Selectivity. For linearity, six-point calibration curve was obtained by plotting the detector response (i.e. peak area) against concentration of the calibration standards at 0.005, 0.010, 0.050, 0.100, 0.250 and 0.500 ppm level. 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 left untouched. Both matrix were processed following developed method for sample processing 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 value had been considered as LOQ value.

Sample preparation and recovery study: Banana was collected from local mandi of Gurgaon, Haryana and grinded homogenously. Grinded samples were weighed at (10 g ± 0.1 g) in triplicate, fortified at three concentration levels- 1 LOQ, 5 LOQ, and 10 LOQ by pesticide standard mixture. Unfortified control matrixes were also processed separately in triplicate. Sample/matrix was extracted using 10ml Acetonitrile HPLC grade solvent in 50 ml centrifuge tube (TARSON). Later on 4 g $MgSO_4$ and 1 g NaCl were added, vortex on rotospin for 10-15 min and 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 pre-weighed PSA 37mg, 25mg MgSO4 and 37 mg C-18 in, for clean-up of extractants . Microcentrifuge tubes were shaken on vortex shaker 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 and evaporated to near dryness using gentle nitrogen stream (Turbo Vap LV, Caliper Life Sciences). 0.5 ml of n-Hexane was added to glass tubes and stirred to dissolve analyte completely and transferred to fresh GC vials for quantification by GC-ECD. Samples were evaporated again to near dryness and reconstituted with 100µl of n-hexane for confirmation of analytes by GC-MS.

Uncertainity Calculation: Calculation of uncertainity is

important step for method development process. Combined uncertainity (U) was determined at 5 LOQ level for all the pesticides taken under study as per the statistical procedure of the EURACHEM/CITAC Guide[19]. Three main sources of uncertainity first relative standard uncertainity (U1) due to purity of analytical standards, second uncertainity due to weighing (U2) and at last uncertainity associated with precision (U4) were taken for determination of uncertainity. Uncertainity due to purity of analytical standards (U1)

Calculation of uncertainity for purity by rectangular distribution was assumed as SU1 = (u (x) /√3),

Where: u(x) is the uncertainity value given in the certificate for purity of CRM whereas relative standard uncertainity (U1) was derived as U1 = (SU1 × 100)/% purity, *Uncertainity of weighing (U2)*

Relative standard uncertainity due to weighing (U2) normal distribution was assumed U2 = (0.0001/2)/Wi

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

Uncertainity associated with precision (U3): 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

$U4 = s/(\sqrt{n} \times x)$
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 uncertainity (U) was calculated by equation-5.
$U = x\ [(U1)^2+(U2)^2+(U4)^2]^{1/2}$
Expanded uncertainity (2U) was twice of combined uncertainity (U) at 95% confidence level.

Results and Discussion

Pesticide Selection: Purpose of the study was to develop multiresidue method for the commonly used pesticides in India. Among selected pesticides endosulphan, lindane, isomers of DDT and BHC etc are highly persistent and usually found in environmental matrices like soil, water, food commodities. 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 showed good response while quantified by GC-ECD and further confirmation by GC-MSD.

Validation of the method: Linearity of calibration curve, LOD and LOQ: For method validation, linearity curve for each pesticide was plotted between GC response area versus concentration. All pesticides show linear behaviour for the concentration range of 0.5-0.005 ppm in GC-ECD figure 1 and 2. The correlation coefficient (R^2) was found to be in range of 0.98 to 0.99 for each pesticide in the substrate matrix. The LOD and LOQ for the test pesticides in banana have been presented in table-1. LOD and LOQ values were in the range of 0.001 to 0.01 and 0.004 to 0.01 respectively. Matrix-matched standards were prepared with specific matrix blanks to overcome the matrix effect.

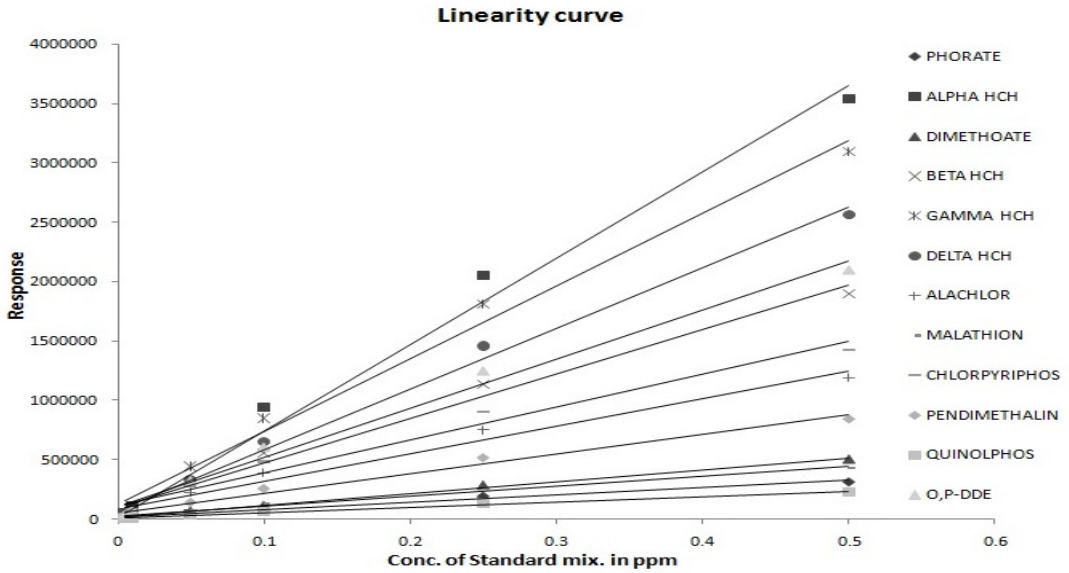

Figure-1
Linearity curve of different pesticides

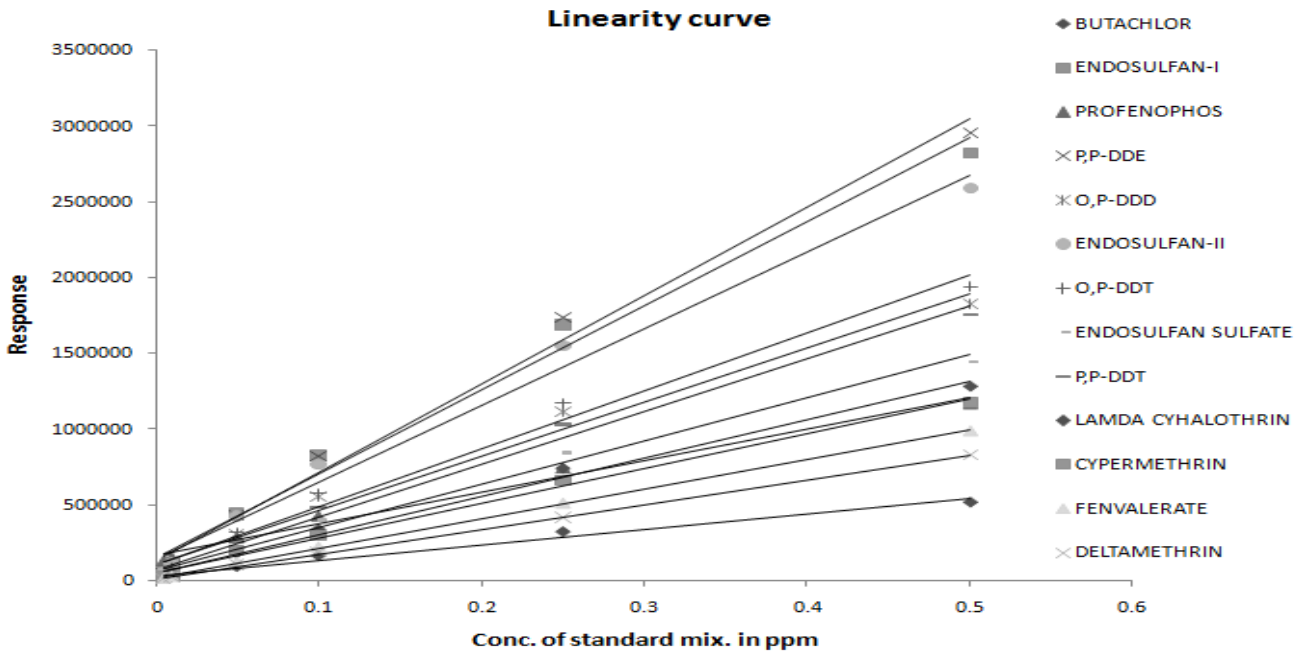

Figure-2
Linearity curve of different pesticides

Table-1
Correlation coefficient, LOD and LOQ values of OC's, OP's, synthetic pyrethroids and herbicides in banana

S. No.	Pesticide	R^2	Banana	
			LOD (µg/g)	LOQ (µg/g)
1.	Phorate	0.98	0.010	0.040
2.	Alpha HCH	0.99	0.001	0.003
3.	Dimethoate	0.99	0.010	0.040
4.	Beta HCH	0.99	0.004	0.010
5.	Gama HCH	0.99	0.009	0.030
6.	Delta HCH	0.99	0.006	0.020
7.	Alachlor	0.98	0.010	0.040
8.	Malathion	0.98	0.020	0.070
9.	Chlorpyriphos	0.98	0.002	0.006
10.	Pendimethlin	0.98	0.030	0.080
11.	Quinalphos	0.99	0.020	0.050
12.	o,p DDE	0.99	0.008	0.030
13.	Butachlor	0.98	0.004	0.010
14.	Endosulfan-I	0.99	0.002	0.007
15.	Profenophos	0.99	0.020	0.060
16.	p,p DDE	0.99	0.002	0.007
17.	o,p DDD	0.98	0.003	0.008
18.	Endosulfan-II	0.99	0.001	0.004
19.	o,p DDT	0.99	0.002	0.006
20.	Endosulfan sulphate	0.99	0.004	0.010
21.	p,p DDT	0.99	0.003	0.008
22.	Lamda cyhalothrin	0.99	0.004	0.010
23.	Cypermethrin	0.99	0.007	0.020
24.	Fenvalarate	0.99	0.007	0.020
25.	Deltamethrin	0.99	0.005	0.020

Recovery and precision: Dispersive solid phase extraction technique was used for extraction of banana samples. Three replicates of banana matrix were taken along with control at each fortification level e.g. 1, 5 and 10 LOQ in table-2. For all 25 pesticides taken, high recovery percentages were obtained at all the three spiking concentration levels viz. 1 LOQ, 5 LOQ and 10 LOQ. Recovery percentage values were in the range of 74-117.

Table-2
Recovery (%) of the pesticides from banana at three fortification levels

S. No.	Pesticide	10 LOQ		5 LOQ		1 LOQ	
		Mean	RSD	Mean	RSD	Mean	RSD
1	Phorate	95.79	0.53	93.38	0.55	93.89	0.27
2	Alpha HCH	92.96	0.53	91.71	2.88	89.55	1.58
3	Dimethoate	87.24	4.08	89.67	1.27	95.21	3.01
4	Beta HCH	94.41	1.39	93.38	0.24	94.16	1.23
5	Gamma HCH	91.16	1.06	91.93	1.35	91.55	1.46
6	Delta HCH	90.76	5.97	92.43	4.86	97.74	4.47
7	Alachlor	94.80	0.60	94.13	0.12	95.38	3.92
8	Malathion	93.39	1.90	94.33	3.20	90.33	2.13
9	Chlorpyriphos	93.22	0.24	93.15	0.65	95.67	1.29
10	Pendimethalin	93.33	0.97	93.27	2.38	94.01	0.25
11	Quinalphos	91.55	1.59	94.84	3.60	96.91	7.06
12	o,p-DDE	95.24	1.83	93.44	0.65	94.56	0.52
13	Butachlor	98.55	0.69	93.81	0.45	95.11	0.45
14	Endosulfan-I	96.99	1.68	92.70	0.96	94.07	1.31
15	Profenophos	95.63	4.44	96.07	3.36	86.63	5.98
16	p,p-DDE	98.24	2.35	93.83	0.85	88.40	1.38
17	o,p-DDD	96.75	1.84	92.28	1.34	93.85	0.77
18	Endosulfan-II	101.43	3.62	92.76	5.73	85.67	3.59
19	o,p-DDT	95.25	1.34	90.05	1.55	97.56	2.33
20	Endosulfan sulfate	94.33	8.82	95.87	2.58	91.30	2.80
21	p,p-DDT	93.55	4.94	95.97	1.20	95.03	2.03
22	Lamda cyhalothrin	99.13	13.78	90.93	11.94	77.52	11.35
23	Cypermethrin	95.20	13.81	86.03	18.70	77.60	7.87
24	Fenvalerate	95.28	18.45	90.16	9.80	84.19	11.89
25	Deltamethrin	90.36	17.05	97.34	8.37	75.58	4.52

RSD- Relative Standard Deviation

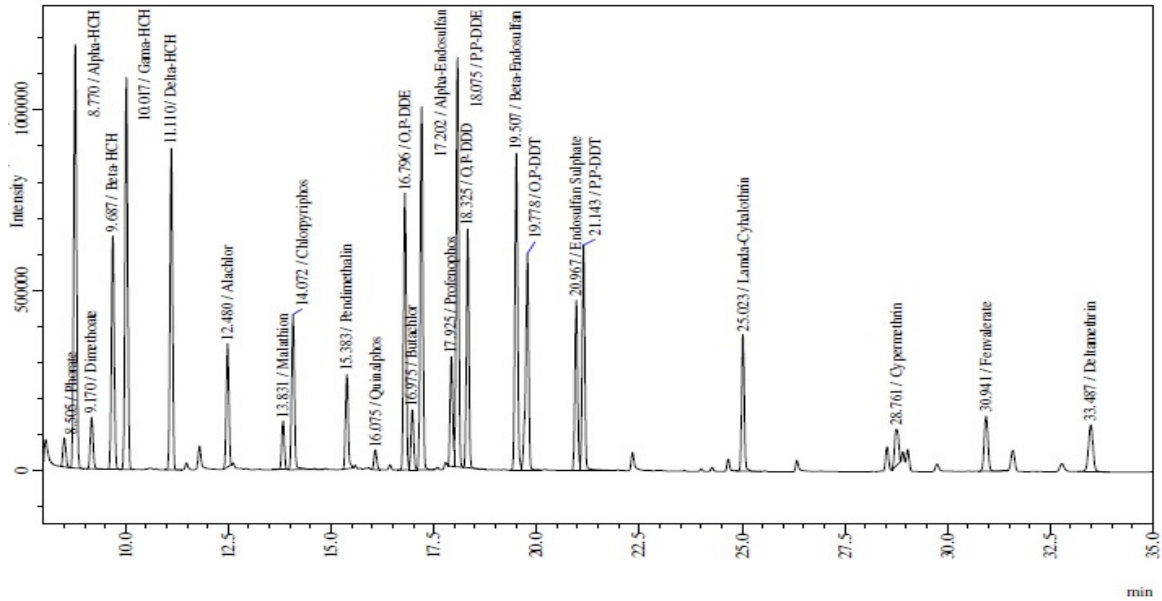

Figure-3
Representative chromatogram of standard mixture (25 pesticides) at 1ppm concentration level

Table-3
Results of measurement of uncertainity for the analysed pesticides in banana

S. No.	Pesticide	U1	U2	U3	U	2U
1	Phorate	0.0030	4.03226E-05	0.0031	0.0008	0.0016
2	Alpha HCH	0.0029	3.33333E-05	0.0166	0.0002	0.0005
3	Dimethoate	0.0029	3.64964E-05	0.0073	0.0014	0.0028
4	Beta HCH	0.0029	2.80899E-05	0.0014	0.0002	0.0003
5	Gamma HCH	0.0029	1.35870E-05	0.0078	0.0011	0.0023
6	Delta HCH	0.0029	4.34783E-05	0.0281	0.0026	0.0052
7	Alachlor	0.0029	4.13223E-05	0.0007	0.0006	0.0011
8	Malathion	0.0029	3.90625E-05	0.0185	0.0062	0.0123
9	Chlorpyriphos	0.0029	4.46429E-05	0.0038	0.0001	0.0003
10	Pendimethalin	0.0029	4.54545E-05	0.0137	0.0052	0.0105
11	Quinalphos	0.0029	1.37363E-05	0.0208	0.0050	0.0099
12	o,p-DDE	0.0029	2.50000E-05	0.0037	0.0007	0.0013
13	Butachlor	0.0030	3.90625E-05	0.0026	0.0002	0.0004
14	Endosulfan-I	0.0029	4.03226E-05	0.0056	0.0002	0.0004
15	Profenophos	0.0030	2.82486E-05	0.0194	0.0057	0.0113
16	p,p-DDE	0.0029	1.69492E-05	0.0049	0.0002	0.0004
17	o,p-DDD	0.0029	3.18471E-05	0.0078	0.0003	0.0006
18	Endosulfan-II	0.0029	3.33333E-05	0.0331	0.0006	0.0012
19	o,p-DDT	0.0029	2.13675E-05	0.0089	0.0003	0.0005
20	Endosulfan Sulfate	0.0029	3.90625E-05	0.0149	0.0007	0.0015
21	p,p-DDT	0.0029	4.09836E-05	0.0069	0.0003	0.0006
22	Lamda cyhalothrin	0.0029	2.82486E-05	0.0689	0.0031	0.0063
23	Cypermethrin	0.0030	2.57732E-05	0.1080	0.0093	0.0186
24	Fenvalerate	0.0029	4.62963E-05	0.0566	0.0051	0.0102
25	Deltamethrin	0.0029	3.37838E-05	0.0483	0.0047	0.0094

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

Uncertainity measurement: Weighing, purity of the standard and repeatability were identified as three major sources of uncertainity. The detailed uncertainity values for all the pesticides have been presented in table 3. Among the three major sources of uncertainity, contribution from repeatability and purity of the pesticide standards were maximum. Repeatability contributed 52% of the total uncertainity while the contribution from purity of the pesticide standards was 41%. In all the cases, expanded uncertainity values were found acceptable for the purpose of the study.

Conclusion

The above study reveals 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. Pesticides taken for study belong to different class, which were widely used in India and persistent in environment for long time. One foremost thing was that pesticides of different groups were analysed by a single detector i.e. ECD detector with single analysis step and confirmed by mass detector. Method is repeatable, reproducible and gives good recovery with uncertainity range within permissible limit.

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