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Method validation for analysis of pesticide residues in *Labeo rohita* fish through GC-MS/MS

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Abstract
The present study was undertaken to develop and validate multiresidue method (MRM) for twenty four pesticide residues in *Labeo rohita* fish using Gas Chromatography Triple Quadrupole Mass Spectrometer (GC-MS/MS). The method was based on QuEchERs method (quick, easy, cheap, efficient, rugged and safe) extraction with acetonitrile and dispersive solid phase extraction (d-SPE) cleanup with PSA and $MgSO_4$. At spiking level 0.01, 0.05 and 0.1 µg mL^{-1} recovery studies were conducted and percent recovery found to be within acceptable limit except synthetic pyrethrnoids at spiking level 0.01 µg mL^{-1}. All RSD values were below 20%. The coefficient of determination (R^2) for all pesticide ≥ 0.992. The developed method was validated and found to be fast, simple and reliable for analysis of pesticide residues in fish samples.

Keywords: GC-MS/MS, QuEchERs, Pesticide residue, Rohu fish, d-SPE

Introduction
As the human population is increasing there is demand for foods leads to substantial increase in the production and use of agro-chemicals such as pesticides and fertilizers. The use of pesticides such as organochlorines, organophosphates and synthetic pyrethronoids has increased the concern regarding for environmental contamination as well flora and fauna of universe (Das *et al*., 2002) [4].

Pesticide residues includes any derivatives of pesticides such as conversion products, metabolites, reaction products and impurities considered being of toxicological significance (Codex Alimentarius Commission, 2001) [3].

The accumulation of pesticides in the living organisms might pose potential hazards in the long run. The agriculture run off contaminates the aquatic biota which results in accumulation of pesticides in fish body which later comes to human body through food chain (Erhunmwunse *et al.*, 2012) [6].

Pesticide residues in human body have been reported to cause cancer, epilepsy, liver and kidney dysfunctions, somatic growth, depression, neuritis, testicular cancer, endocrine dysfunction, birth defects, carcinomas, neurological disorders and weakened immune system. Organochloride insecticides like DDT and PCB have been responsible for breast cancer and decreased fertility in human beings. Fat solubility of these compounds is responsible for their varied concentrations in the tissues and their accumulation in the lipoproteins of the cell membranes, thus changing their structures and permeability (Kiranmayi, 2012) [8].

Fishes are the source of proteins and easily acceptable for all age group of people in world. The presence of pesticides residues in water bodies make the fishes source of contamination to fish and human. In order to take precautionary measures for reducing health effects by these pesticides there is need of monitoring and analyzing of pesticide residues in fish samples (Mitema and Gitau, 1990) [10].

Pesticide residue analysis includes steps like extraction of analytes, clean up and subsequent determination of pesticide residues through GC or LC. Several extraction procedure can be applied in MRMs (multi residue methods) such as solid phase extraction (SPE), Solid liquid extraction, gel permeation chromatography (GPC), Pressurized liquid extraction (PLE) etc. (Singh, 2017) [15].

Method validation is the process of documenting an analytical method which provides analytical data acceptable for the intended use. Government and international agencies have issued guidelines for appropriate method validation particularly for methods for regulatory submission.

Generally steps used in this studies are selectivity and specificity, linearity (calibration), accuracy, precision, sensitivity, range, limit of detection (LOD), limit of quantification (LOQ), ruggedness or robustness (SANCO, 2013) [13].

The Gas Chromatography/Mass Spectrometry (GC/MS) instrument separates chemical mixtures in the GC component and identifies the components at a molecular level in the MS component. GC combined with MS, simultaneous determination and confirmation of pesticide residues can be obtained with one instrument in one analytical run (Karasek and Clement, 2012) [7].

The aim of study was to validate the MRM method in GC-MS/MS after extraction through QuEchERs method (quick, easy, cheap, efficient, rugged and safe) for twenty four pesticides (Organochlorines, Organophophorus compounds and synthetic pyrethronoids) in *Labeo rohita* fish.

Experimental

Chemicals and reagents

Certified reference materials (CRMs) of organochlorine compounds (α HCH, β-HCH, ɣ-HCH, *δ*HCH, aldrin, dieldrin, endrin, endosulfan-α & β, endosulfan sulphate, heptachlor, p,p'DDE, p,p'DDD, p,p DDT and o,p DDT), oragnophophorus compounds (dichlorvos, diazinon, chlorpyriphos methyl,chlorpyrifos and methyl Parathion) and synthetic pyrethroid compounds (cypermethrin, deltamethrin, esfenvalerate and fenvalerate) were procured from Dr. Erhenstorfer, Germany. Pesticide quality solvents (Acetone, acetonitrile and n-hexane) were from HPLC grade, Merck Pvt. Limited. Chemicals such as Primary secondary amine (Agilent Technologies), Magnesium sulphate Anhydrous (Merck Pvt. Limited) and Sodium chloride (Qualigens) were used.

The stock solution of individual pesticide approximatele 1000µg mL^{-1}(OCs, OPs and SPs) were prepared in 25 mL volumetric flask using acetone and hexane as solvents. Each standard stock solution was diluted to 100 µg $mL^{-1}$with solvent hexane. Then, 5 µg mL^{-1}mixture of organochlorine compounds standard solution was prepared from 100µg mL^{-1}standard solution in 25 mL volumetric flask with solvent hexane. Silimarly, mixture of 5µg mL^{-1}standard solution of oragnophophorus and synthetic pyrethnoids compounds were prepared.

For working standard, 1µg mL^{-1} mixture of 24 pesticides (organochlorine, oragnophophorus, synthetic pyrethnoids) was prepared from 5 µg mL^{-1}mixture of organochlorines and 5µg mL^{-1} mixture of organophophates plus synthetic pyrethnoids in 10 mL volumetric flask with solvent n-hexane. Similarly, 10 and 1 µg mL^{-1}mixture of 24 pesticides standard solution was prepared in 10 mL using acetone for spiking. All the standards were stored in deep freezer maintained at -20 ^{0}C.

Chromatographic conditions

The mass of each pesticide was scanned in between 50-700 Dalton. The detection and quantification of the analytes was performed by using Agilent 7000 triple quadrupole mass spectrometer (GC-MS/MS) with electron ionization (EI) interfaced to an Agilent 7890A gas chromatograph and auto-sampler was 7693 Agilent Technologies. The separation was achieved on a 30m x 250 µm x 0.25 µm thickness HP-5MSUI GC column. Ultrahigh purity helium was used as carrier gas at 1.8148 $mLmin^{-1}$ constant flow rate. The GC oven temperature was optimized for the best separation of the target analytes and was follows: 60 ^{0}C held for 1 min, then 40 ^{0}C to 170 ^{0}C for 0 min, then 10 ^{0}C- 275 ^{0}C for 7 min. The column oven maximum temperature was 310 ^{0}C with equilibration time 0.5 min. The total run time was 21.25 minutes and the post run temperature was 275 ^{0}C for 1.5 minute. The MSD **(**Mass Selective Detector**)** transfer line was 280 ^{0}C and ion source was set at 300 ^{0}C. The QQQ **(**triple quadrupole mass spectrometer**)** collision gas nitrogen at 1.5 $mLmin^{-1}$and quench gas was He at 2.25 mL min^{-1}. EI energy was -70ev, quadrupole temperature was set at 250 ^{0}C and solvent delay was at 4minute.The injection volume was 2µL. Each analyte of concentration ranging from 1 to 2 ppm was injected in scan mode to determine the most intense ions. Product ion and collision energy determination were performed to optimize two products ions, collision energies and ratios between quantifier and qualifier ions.

Sample preparation and extraction of fish samples

The *Labeo rohita* (Rohu) fish samples were collected from local markets of Greater Hyderabad Municipal Corporation and kept in freeze at -20 ^{0}C until analysis in laboratory. The sample extraction method was based on QuEchERs (quick, easy, cheap, efficient, rugged and safe) method (Anastassiades *et al*., 2003) [1]. Ten gram of crushed fish muscle sample was weighed in 50mL polypropylene centrifuge tube in triplicate. The samples were spiked with standard pesticide mixture at 0.01, 0.05 and 0.1 µg mL^{-1} and kept for 45 minutes to make proper interactions of pesticides with fish matrix. Along with this one control (without pesticide) and one reagent blank (without fish and pesticide) were maintained. The sample was extracted with 10 mL of acetonitrile and tubes were vigorously shaken by hands for 30 sec and thoroughly homogenized by Heidolph homogenizer for 2minute at 1300- 1400 rpm. Then, 4 g anhydrous $MgSO_4$ and 1 g NaCl was added and shaken vigorously by hand for 1 minute and samples were centrifuged for 5 minutes at 5000 rpm at 10 ^{0}C. After centrifugation, dispersive solid phase (d-SPE) clean up was carried out in which 6 mL supernatant was transferred to 15 mL centrifuge tube containing 900 mg anhydrous $MgSO_4$ and 300 mg PSA (150 $mgmL^{-1}$ $MgSO_4$and 50 mg mL^{-1} PSA). The samples were shaken vigorously with help of vortex shaker and then centrifuged for 2minutes at 5000 rpm. After centrifugation, an aliquot of 2 mL extract was transferred into 3 mL glass vials and evaporated to near dryness by using nitrogen evaporator at 35 ^{0}C- 45 ^{0}C. The extract was reconstituted with n-hexane and filtered into 2mL GC vials by using syringe filter (13mm, 0.22µm PTFE). The 2 µL filtrate was injected in GC-MS/MS for analysis.

Method validation

In this study matrix blank, matrix match standard and standard solutions 0.01 µg mL^{-1} were injected (six replicate) to compare the response & interferences and retention time of each analyte. A minimum of five standard solutions of 0.01, 0.04, 0.06, 0.08 and 0.1µg mL^{-1} concentration were prepared to study the linearity. Similarly, five different concentration of standard in matrix at 0.01, 0.04, 0.06, 0.08 and 0.1 µg mL^{-1} were injected for the calibration study. Linearity data were often judged from the coefficient of determination (r^2) and y intercept of the linear regression line. High correlation coefficient linear, $r^2 \geq 0.99$ are considered as evidence of goodness of fit of the data to the regression line. For a linear calibration curve, it is assumed that the instrument response *y* is linearly related to the standard concentration *x* for a limited range of concentration. It can be expressed in a model such as $y=a+bx$. Three replicate were maintained for each spiking concentration i.e. 0.01, 0.05 and 0.1 µg mL^{-1} along with one control/blank (fish with no pesticide) and one reagent bank

(without sample and pesticide) respectively. Recovery percentage were calculated for each analyte at three spiking level.The precision was determined by analyzing replicate sample into GC-MS/MS or multiple injection of same sample.The acceptance criteria for relative standard deviation must be less than 20%. The limit of detection can be calculated from the slope of the calibration curve (y = bx + c) and is generally defined as LOD = 3*S.D/b. The limit of quantification can be calculated from the slope of the calibration curve (y = bx + c) and is generally defined as LOQ = 10*S.D/b, where b - slope of the curve and S.D - average standard deviation of the response.

Results and discussion

The method validation was performed according to the recommendation of SANTE/11945/2015 (supersedes SANCO/12527/2013). The total ion chromatogram (TIC) obtained is shown in Figure-1. Product ion, collision energy for each transition and retention time (RT) are presented in Table-1.The multiple reaction monitoring (MRM) transitions, method was divided into 17 segments, each containing different MRM transitions. Neat standard and matrix match standard solutions 0.01µg mL^{-1} injected in six replicates to compare peak response and retention time expressed in terms of standard deviation and relative standard deviation. The RSD for retention time (RT) for all pesticides was below 2%. The findings of RSD values for RT was supported by the statement of Stout *et al*. (2009) [16]. Similar findings were also reported earlier (Perez *et al*., 2016) [12]. Five different concentration of standard in matrix at 0.01, 0.04, 0.06, 0.08 and 0.1 ppm were injected for the calibration study. Coefficient of determination (R^2) of all the pesticide in matrix standard and neat standard is shown in Table-2. The R^2 for all pesticide was found to be ≥ 0.992 in the present study. Earlier Yang *et al*. (2012) [17] reported R^2 not below 0.99, where as ≥0.997 was reported by Molina Ruiz *et al*., (2014) [11]. The accuracy and recovery percentage were calculated at 0.01, 0.05 and 0.1 µg mL^{-1} spiking level. The recovery percentages for all pesticides are given in Table-3. In the present study recovery percentage of all pesticides were within the acceptable limit except the SPs pesticides at concentration 0.01 µg mL $^{-1}$. In the present study the recovery percentages for OCs and Ops were within the acceptable limit at spiking concentration 0.01, 0.05 and 0.1 µg mL^{-1}. The recoveries for OCs and OPs were in the range 70.33- 106.06%, where as for SP compounds recovery study at concentration 0.01 µg mL $^{-1}$ it was below 70%. All the RSD values for all pesticides were below 20%. Earlier 78-91% recoveries were reported by Malhat and Nasr (2011) [9] in organochlorine and organophosphorus compounds with RSD lower than 15%. Like wise 50.9-142.2% recoveries with RSD 2.3-24.9% in the fish samples was also reported by Yang *et al*. (2012) [17]. Several researchers also reported the similar results as per present study findings (Molina –Ruiz *et al*., 2014; Caldas *et al*., 1998) [11, 2]. Sankar *et al*. (2006) reported the recovery percentages within 80-102% for OCPs in fish samples [14]. The LOD is the smallest concentration of analyte that can be *detected* with no bias or imprecision of the result by an assay and the LOQ is the concentration at which analyte can be quantitated with a *linear* response (David and Armbruster, 2008) [5]. The limit of detection and limit of quantification was calculated from the slope of the calibration curve. The recovery studies were conducted below the 0.01 µg mL $^{-1}$ i.e. at estimated LOQ for OCs and OPs but the recoveries were not found to be in acceptable limit. Therefore LOQ for OCs and Ops were set at 0.01 µg mL $^{-1}$. Similarly the recovery studies were also conducted below 0.05 µg mL $^{-1}$ for SPs compounds. As the recoveries were not coming within acceptable limit the LOQ for SPs was set at 0.05 µg mL $^{-1}$. Singh, (2017) reported LOD and LOQ level for organochlorine and organophosphorus compounds 0.01 and 0.05 ppm respectively where as LOQ for both compounds was 0.05 ppm in fish samples [15]. The LOD range 0.001 - 0.025 mg Kg^{-1} for 49 organophosphorus compounds and 0.001-0.003 mg Kg^{-1} were reported by Yang *et al*. (2012) and Molina -Ruiz *et al*. (2014) respectively [17, 11]. Similarly Malhat and Nasr (2011) reported the LOD level 0.02- 0.07 ng g^{-1} for organophophorus compounds in tilapia fish samples collected from Egypt [9]. In this study validation parameters obtained for fish matrix demonstrate that the developed analytical method meets the method performance acceptability criteria (mean recoveries in the range 70%-120%, precision with RSD < 20% (SANCO, 2013) [13].

Table 1: Showing MRM transitions, collision energy for each transition, average retention time (RT) of various pesticides

Sl. No.	Pesticide	RT	PI	Q_1	CE (V_1)	Q_2	CE (V_2)	Dwell (ms)
1.	Dichlorovos	4.64	108.9	79.0	5	47.0	22	10
2.	Alpha HCH	7.12	180.8	145.0	12	109.0	32	10
3.	Beta HCH	7.20	180.8	145.0	18	109.0	32	10
4.	Lindane	7.61	180.8	145.0	18	109.0	32	10
5.	Diazinon	7.78	178.9	164.0	22	136.0	22	10
6.	Delta HCH	8.06	180.8	145.0	18	109.0	32	10
7.	Chlorpyrifos methyl	8.64	285.8	270.9	22	93.0	22	10
8.	Parathion methyl	8.74	263.0	246.0	2	79.0	32	10
9.	Heptachlor	8.77	271.1	236.8	22	234.8	22	10
10.	Aldrin	9.37	262.8	227.8	22	192.9	32	10
11.	Chlorpyrifos	9.44	313.7	257.8	12	190.0	22	10
12.	Endosulfan alpha	10.71	240.7	205.9	15	169.9	25	10
13.	4,4 DDE	11.13	245.8	211.0	22	176.0	32	10
14.	Dialdrin	11.17	79.0	77.0	18	51.0	32	10
15.	Endrin	11.57	263.0	193.0	32	192.0	32	10
16.	Endosulfan beta	11.75	194.8	159.0	2	125.0	32	10
17.	2,4 DDT	11.91	234.9	165.1	32	164.9	22	10
18.	4,4 DDD	11.93	234.9	199.0	22	165.1	32	10
19.	Endosulfan sulfate	12.53	271.7	236.8	10	234.8	16	10
20.	4,4 DDT	12.57	234.9	199.0	22	164.9	22	10
21.	Cypermethrin	15.20	180.7	152.0	32	77.0	32	10
22.	Esfenvalearte	18.34	166.9	125.0	2	89.0	32	10
23.	Fenvalerate	18.77	166.9	125.0	2	89.0	32	10
24.	Deltamethrin	19.78	252.8	174.0	2	93.0	22	10

RT- Retention time, PI - Precursor ion, Q_1 - Quantifier ion, Q_2 – Qualifier

Table 2: Showing recovery percentage with RSD for various fortification level

Sl. No.	Pesticide	Fortification level 0.01 µg mL^{-1}		Fortification level 0.05 µg mL^{-1}		Fortification level 0.1 µg mL^{-1}	
		Average Recovery (%)	% RSD	Average Recovery (%)	% RSD	Average Recovery (%)	% RSD
1.	Dichlorovos	81.7	4.93	87.44	1.86	93.67	4.29
2.	Alpha HCH	80.77	1.36	88.24	1.92	91.69	7.42
3.	Beta HCH	77.63	6.73	82.18	1.67	106.06	7.36
4.	Lindane	71.40	2.52	82.13	7.02	77.27	8.52
5.	Diazinon	70.63	3.28	70.73	1.92	79.15	3.88
6.	Delta HCH	79.03	2.71	91.05	4.18	87.45	6.12
7.	Chlorpyrifos methyl	71.13	2.55	70.39	1.71	99.54	5.03
8.	Parathion methyl	76.70	6.06	77.87	3.22	95.40	5.51
9.	Heptachlor	70.60	1.95	83.69	10.42	92.26	3.24
10.	Aldrin	70.33	2.47	70.42	1.61	71.19	4.98
11.	Chlorpyrifos	71.80	5.04	70.79	2.76	93.67	1.30
12.	Endosulfan alpha	71.10	2.44	72.40	6.11	82.75	2.30
13.	4,4 DDE	71.83	7.63	70.46	3.42	84.03	1.50
14.	Dialdrin	76.90	7.71	70.60	7.47	90.19	7.76
15.	Endrin	75.50	5.30	72.18	1.80	80.82	4.07
16.	Endosulfan beta	76.7	8.21	80.96	5.54	83.47	3.10
17.	2,4 DDT	75.10	3.27	76.04	3.41	92.23	1.72
18.	4,4 DDD	76.17	12.57	75.19	9.04	89.81	9.21
19.	Endosulfan sulfate	73.07	6.50	80.19	10.54	83.60	3.26
20.	4,4 DDT	79.00	10.82	76.07	7.23	89.36	7.32
21.	Cypermethrin	63.00	4.20	80.68	2.99	85.44	2.06
22.	Esfenvalearte	61.33	1.88	86.45	12.99	95.97	5.78
23.	Fenvalerate	62.00	5.82	88.24	11.15	92.26	7.29
24.	Deltamethrin	62.33	6.68	82.97	2.92	79.65	4.12

Table 3: Coefficient of determination, (R^2) for 24 pesticides in Neat standard and Matrix match standard

Sl. No.	Pesticide	Neat Standard	Matrix match standard
		Coefficient of Determination (R^2)	Coefficient of Determination (R^2)
1.	Dichlorovos	0.995	0.999
2.	Alpha HCH	0.995	0.999
3.	Beta HCH	0.995	0.999
4.	Lindane	0.996	0.999
5.	Diazinon	0.998	0.999
6.	Delta HCH	0.998	0.998
7.	Chlorpyrifos methyl	0.997	0.999
8.	Parathion methyl	0.998	0.999
9.	Heptachlor	0.995	0.999
10.	Aldrin	0.997	0.999
11.	Chlorpyrifos	0.995	0.999
12.	Endosulfan alpha	0.998	0.999
13.	4,4 DDE	0.999	0.998
14.	Dialdrin	0.999	0.999
15.	Endrin	0.999	0.997
16.	Endosulfan beta	0.999	0.999
17.	2,4 DDT	0.995	0.997
18.	4,4 DDD	0.999	0.999
19.	Endosulfan sulfate	0.999	0.999
20.	4,4 DDT	0.992	0.996
21.	Cypermethrin	0.995	0.998
22.	Esfenvalearte	0.995	0.999
23.	Fenvalerate	0.996	0.999
24.	Deltamethrin	0.998	0.998

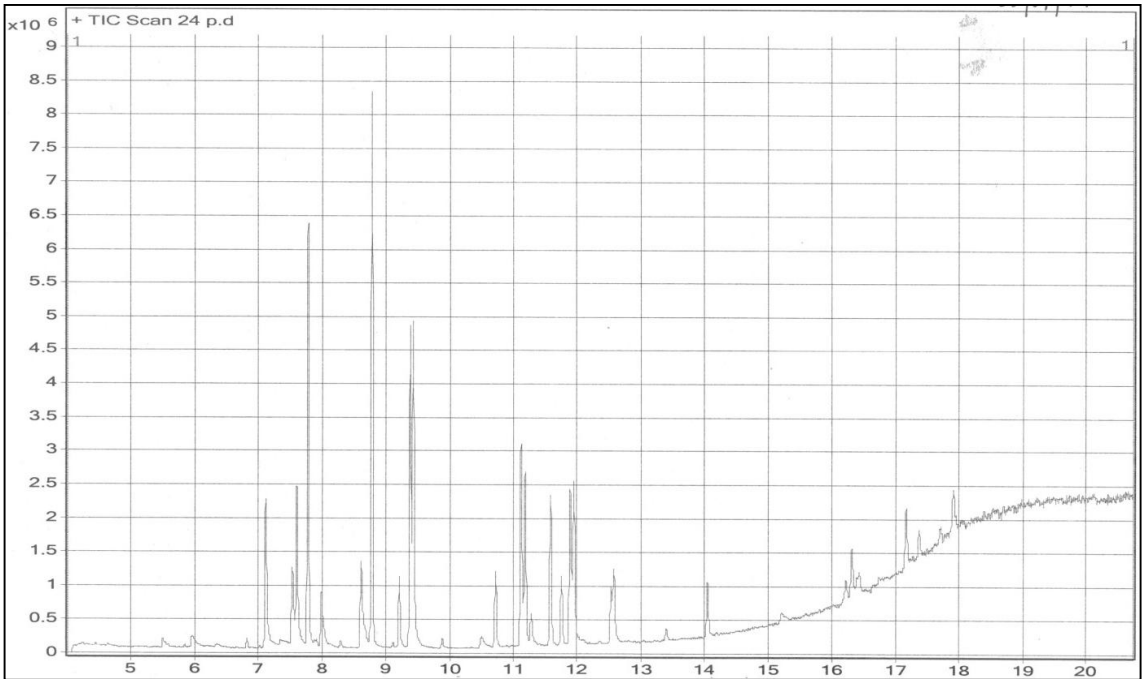

Fig 1: Total ion Chromatogram (TIC) of 1 ppm standard mixture of 24 pesticides obtained by GC-MS/MS (ESI^+)

Conclusion

This method validation was found to be reliable, simple and time shaving. The modified QuEchERs method (quick, easy, cheap, efficient, rugged and safe) with acetonitrile extraction was found to be fast and efficient. Clean up with PSA in d-SPE provided significant removal of co-extractive materials and there was excellent recoveries except at concentration 0.01 µg mL^{-1} for synthetic pyrethronoids. All RSD values were below 20% i.e. acceptable limit. For pesticide residue analysis this method can be applied as the detection limit is very less and future researchers can adopt this method.

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