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Method Validation for Analysis of 24 Pesticides in *Catla catla* fish through Gas Chromatography Triple Quadrupole Mass Spectrometer (GC-MS/MS)

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ABSTRACT

A multiresidue method was developed and validated for 24 pesticide (Organo chlorine, Organophophorus and Synthetic pyrethroids) residue in Catla fish sample 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 $MgSO_4$. The recovery studies were conducted at three spiking levels i.e. 0.01, 0.05 and 0.1 µg mL^{-1} 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 estimated LOD was in the range from 0.000-0.017 µg mL^{-1} and LOQ was in the range from 0.001- 0.057 µg mL^{-1} for all pesticides. The coefficient of determination (R^2) for all pesticide ≥ 0.992. The devlop method was found to be fast, simple and reliable for analysis of pesticide residue in fish samples.

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

Pesticide residues mean any specified substances in food, agricultural commodities or animal feed resulting from use of pesticides. The term includes any derivatives of pesticides such as conversion products, metabolites, reaction products and impurities considered being of toxicological significance (Codex Alimentarius Commission, 2001).

Pesticides differ in their mode of action, uptake by the body, metabolism and elimination from the body and toxicity potential. Because of these differences some pesticides show acute short term effects, while others tend to accumulate in the body and with time demonstrate sub lethal adverse health effects. Many of these compounds also persist in the environment and bioaccumulate in the animal and human tissues (El-Shahawi *et al*., 2010).

As usage of pesticides are inevitable to meet the demands of growing population and to secure the food grain production, there is need for regular screening of animal products for pesticide residues which is being felt in the trade and also consumers level (Singh, 2017). Several international and national organizations set the MRL (maximum reside level) for each pesticide to ensure the safety of foodstuffs (Jaggie *et al*., 2001).

Method validation is the process of documenting or proving that an analytical method 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).

Pesticide residue analysis includes several 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. (Kiranmayi, 2012). Some of these techniques are laborious and time consuming.

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 (Alder *et al*., 2006).

The aim of study was to validate the multi residue method in GC-MS/MS after extraction through QuEchERs method (quick, easy, cheap, efficient, rugged and safe) for 24 pesticides (Organochlorines, Organophophorus compounds and Synthetic pyrethroids) in *Catla catla* fish.

MATERIALS AND METHODS

Chemicals and reagents

Certified reference materials (CRMs) of organochlorine compounds (α HCH, β-HCH, ɣ-HCH, 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°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 × 250 µm × 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°C held for 1 min, then 40°C to 170°C for 0 min, then 10°C-275°C for 7 min. The column oven maximum temperature was 310°C with equilibration time 0.5 min. The total run time was 21.25 minutes and the post run temperature was 275°C for 1.5 minute. The MSD (Mass Selective Detector) transfer line was 280°C and ion source was set at 300°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°C and solvent delay was at 4 minute. 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 Catla fish samples were collected from local markets of Greater Hyderabad Municipal Corporation and kept in freeze at -20°C until analysis in laboratory. 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 2 minute 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°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 2 minutes 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°C- 45°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.

The sample extraction method was based on QuEchERs (quick, easy, cheap, efficient, rugged and safe) method (Anastassiades *et al*., 2003).

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 (standard deviation of multiple measurements).

RESULTS AND DISCUSSION

Solvents such as acetonitrile, acetone, hexane and reagents anhydrous magnesium sulfate were tested for imputies before analysis and found to be no interference. The total ion chromatogram (TIC) obtained is shown in Fig. 1.

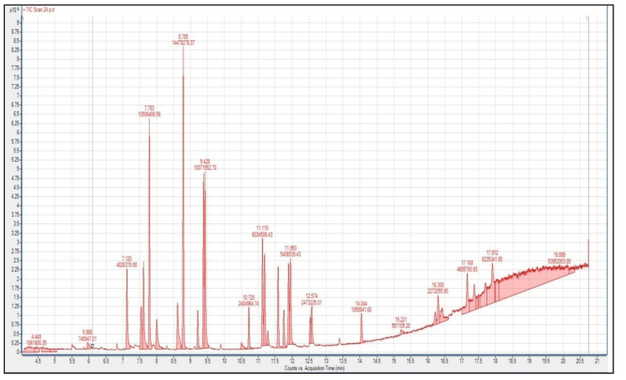

Fig. 1: Total ion Chromatogram (TIC) of 1 ppm standard mixture of 24 pesticides obtained by GC-MS/MS (ESI+)

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

Table 1: Multiple reaction monitoring (MRM) transitions, collision energy for each transition, average retention time (RT) of 24 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

peak response and retention time expressed in terms of standard deviation and relative standard deviation. The total run time was 21.25 min. The RSD for retention time (RT) for all pesticides was below 2%. 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 coefficient of determination value (R^2) for all pesticide was found ≥ 0.992. Accuracy was studied by spiking of analyte at 0.01, 0.05 and 0.1 µg mL^{-1} in the sample and recovery was calculated. Three replicate were maintained for each spiking with one control and reagent blank sample. The recovery percent for all pesticide is given in Table 3. In the present study recovery percentage of all pesticides were in the range of 70.33 -106.06 % except the SPs pesticides at concentration 0.01 µg mL^{-1}. The recovery percentages for cypermethrin, esfenvalerate, fenvalerate and deltamethrin at concentration 0.01 µg mL^{-1} were below 70%. The per cent relative standard deviation for all pesticide was below 20%. The limit of detection and limit of quantification was estimated from the slope of the calibration curve). In the present study the estimated LOD was in the range from 0.000-0.017 µg mL^{-1} for organochlorine, organophophates and synthetic pyrethroid pesticides where as the estimated LOQ was in the range from 0.001- 0.057 µg mL^{-1} for all pesticides. The ruggedness of this method was found to be resistance to change in the experimental conditions as well as minor deviation in the methods.

Table 2: Recovery percent and relative standard deviation

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	81.87	3.64	87.44	1.86	93.83	4.51
2.	Alpha HCH	82.27	4.60	88.24	1.92	91.48	7.27
3.	Beta HCH	78.90	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	70.57	3.32	70.73	1.92	79.15	3.88
6.	Delta HCH	79.43	2.47	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	71.33	4.05
11.	Chlorpyrifos	71.80	5.04	70.79	2.76	93.58	1.16
12.	Endosulfan alpha	70.63	3.23	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	77.50	7.97	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	76.47	7.79	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	62.33	3.34	80.45	3.33	85.20	2.21
22.	Esfenvalearte	60.00	3.33	86.44	13.02	95.97	5.78
23.	Fenvalerate	60.33	10.66	88.02	11.20	92.26	7.29
24.	Deltamethrin	62.00	5.82	82.76	2.80	78.81	4.40

The validation was performed with the recommendations of the document SANTE/11945/2015 (supersedes SANCO/12527/2013). This guidance document describes the method validation and analytical quality control requirements to support the validity of data used for checking compliance with maximum residue limits, enforcement actions, or assessment of consumer exposure to pesticides. Validation parameters obtained for muscle matrix demonstrate that the developed analytical method meets the method performance acceptability criteria (mean recoveries in the range 70%-120%, precision with RSD < 20%).

Selectivity/specificity is the capability of the analytical procedure to identify the target analytes in the presence of other compounds that may be expected to be present (Craig *et al*. 2014). The RSD of retention time in case of neat standard and matrix match standard were below 2% in the present study. The similar findings were also reported by Perez *et al*. (2016). Stout *et al*. (2009) stated that the relative standard deviation for retention time should be within 2% of the average of calibrators which is accordance with present findings.

In the present study the coefficient of determination (R^2) for neat standard and matrix match standard were ≥ 0.992. Earlier Yang *et al*. (2012) reported the linearity correlation not below 0.99 using only standard solution for OPs. Calibration curves were prepared at six levels of fortification 0 to 200 ng mL^{-1} and correlation coefficients

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

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

were higher than 0.997 in all instants for all analytes (Molina -Ruiz *et al*., 2014). Good linearity with coefficient ≥ 0.99 between 0.05 and 1.5 µg mL^{-1} was reported by Gang *et al*. (2011).

The recovery percentages for cypermethrin, esfenvalerate, fenvalerate and deltamethrin at concentration 0.01 µg mL^{-1} were below 70%. All the RSD values for all pesticides were below 20%. Acceptable mean recoveries should be within the range 70-120% with associated RSD≤ 20% (Sanco, 2013). Malhat and Nasr (2011) reported the recovery percentages of organochlorine and organophosphorus compounds in the range 78-91% where as RSD value reported by them was below 15. Yang *et al*. (2012) reported the recovery percentages for 49 pesticides in the range of 50.9-142.2% and they also reported the RSD value in the range 2.3-24.9 in the fish samples. In the present study the recovery percentages for OCs and OPs in three concentration such as 0.01 µg mL^{-1}, 0.05 µg mL^{-1} and 0.1 µg mL^{-1} were not below 70.33 %. The similar findings were also reported by several researchers from fish samples (Molina –Ruiz *et al*. (2014); Caldas *et al.* (1998); Yang *et al*. (2006); Sankar *et al*. (2006)).

Limit of detection (LOD) and limit of quantification (LOQ) parameters are related but have distinct definitions and should not be confused. The LOD is the smallest concentration of analyte that can be *detected* with no guarantee about the bias or imprecision of the result by an assay and the LOQ, concentration at which *quantification* as defined by bias and precision goals is feasible, and finally the concentration at which the analyte can be quantitated with a *linear* response (David and Armbruster, 2008). The 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 (Procedures, 2000). In

the present study the estimated LOD was in the range from 0.000-0.017 µg mL^{-1} for organochlorine, organophophates and synthetic pyrethrnoid pesticides where as the estimated LOQ was in the range from 0.001- 0.057 µg mL^{-1} for all pesticides. 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} (Sanco, 2013). Earlier Singh (2017) reported the LOD level for organochlorine and organophosphorus compounds 0.01 and 0.05 ppm where as LOQ for both compounds was 0.05 ppm. Ealier Yang *et al*. (2012) reported LOD range 0.001 -0.025 mg Kg^{-1} for 49 organophosphorus compounds in fish sample where as Molina -Ruiz *et al*. (2014) reported the LOD and LOQ ranged from 0.001-0.003 mg Kg^{-1}, 0.004-0.009 mg Kg^{-1} respectively. The LOD was ranged from 0.019-0.055 ng Kg^{-1} for organochlorine pesticides in fish reported by Yang *et al*. (2006).

CONCLUSION

The method validated in this study 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. As in this study the detection limit is lower, this method can be applied for analysis of pesticide residue in fish samples for future work.

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