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Validation of QuEChERS: Based analytical method for determination of chlorpyrifos residues in chicken meat using gas chromatography: Electron capture detector

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Abstract
A simple QuEChERS-based (Quick, Easy, Cheap, Effective, Rugged, and Safe) analytical method for the determination of chlorpyrifos residues in chicken meat samples by gas chromatography coupled with electron capture detector was validated. QuEChERS method involves sample extraction with acetonitrile, salting out phase separation followed by clean up with dispersive solid phase extraction using primary secondary amines. The method was validated by specificity, linearity, limit of detection (LOD), limit of quantification (LOQ), accuracy, precision and matrix effect. The method showed good linearity with the limit of detection and the limit of quantification of 0.02 and 0.05 µg/g, respectively. The per cent recovery of this method ranged from 86.52 – 94.25% with optimal intra-day and inter-day precision (Coefficient of variation < 8.0%). The matrix effect was negative and between -20% to + 20%, complying with the standard guidelines. The validated was applied for determination of chlorpyrifos residues in 50 chicken meat samples collected from Tamil Nadu and no chlorpyrifos residues were detected in the sample analyzed. The validated method was simple, sensitive and can be routinely employed for monitoring chlorpyrifos residues in chicken meat samples.

Keywords: chlorpyrifos, gas chromatography, electron capture detector, pesticide, residue, chicken

Introduction
Pesticides are widely used in agricultural and livestock sector for the control of pests. The increased use of pesticides in agricultural sector increased the contamination of soil, water and air (Grewal *et al*., 2017 and Mahugija *et al*, 2018) [1, 2] . Pesticide residues in animal food products may originate either from the contaminated feed and fodders or from the direct use pesticides for the control and treatment of external parasites (Zelelew *et al*., 2019) [3].
Chlorpyrifos (O, O-diethyl-O-(3,5,6-trichloro-2-pyridinyl) phosphorothioate), is an organophosphorus pesticide widely used in India used for the control of agricultural pests, household termites and control of pests in livestock and poultry sheds (Hongsibsong *et al*., 2020) [4]. The extensive use of the compound increases the possibility of occurrence of residues in food products including animal food products such as milk and meat. The continuous exposure of pesticides in human through food chain are known to cause adverse health effects such as immunosuppression, endocrine disruption, reproductive problems, and cancer (Jadhav and Waskar, 2011 and Mishra *et al*., 2019) [5, 6]. There has been a serious concern in the recent years about the presence of pesticide residues in livestock food products including chicken meat. In the present scenario, monitoring of pesticide residues in animal food products is required to minimize human health risk and to meet international standards of trade. The success of such monitoring programmes depends upon the sensitive analytical procedure for the detection of pesticide reissued.
Gas chromatography coupled with various detectors like mass spectrometry, Electron capture detector, Nitrogen phosphorus and flame ionization detectors are sensitive and commonly employed for the detection of volatile pesticides. The extraction of pesticide residues from complex matrices such as meat, milk, egg is a crucial step and it includes microwave assisted extraction, solid phase extraction, supercritical fluid extraction, accelerated solvent extraction, Matrix solid phase dispersion and dispersive liquid-liquid microextraction and QuEChERS (quick, easy, cheap, effective, rugged, and safe) method (Kiljanek *et al*., 2013 and LeDoux, 2011) [7, 8].
With this background in mind, the present study was undertaken with an objective to standardize gas chromatographic method for detection of chlorpyrifos residue after extraction

by QuEChERS method in chicken meat and to assay the residues of chlorpyrifos, if any, in retail broiler meat samples collected from the state of Tamil Nadu, India.

Materials and Methods

Chemicals and reagents

Chlorpyrifos analytical standard (100 µg/mL in acetonitrile) was purchased from M/s Sigma Aldrich Corporation, Bangalore. Ethyl acetate and acetonitrile used were of chromatography grade and purchased from M/s Thermo Fisher scientific, India. QuEChERS acetate extraction tubes and dispersive SPE cleanup tubes were purchased from M/s Agilent Technologies, USA. Working standards of chlorpyrifos was prepared in Ethyl acetate and stored at 4 °C.

Collection of samples

The present study was carried out in the Department of Veterinary Pharmacology and Toxicology, Madras Veterinary College, Chennai, India. A total of 50 chicken meat samples were collected from broiler shops from districts of four districts of Tamil Nadu (Chennai, Tiruppur, Thiruvannamali and Vellore). The samples were stored at – 20 °C until analysis.

Chromatographic analysis

Gas chromatography (model 7890B GC, Agilent, USA) equipped with an electron capture detector, auto sampler (G4513) with split less injection mode was used. The chromatographic separation was performed on a HP-5 (5% Phenyl Methyl Siloxane) capillary column (30 m X 320 µmX 0.25 µm film thickness). Nitrogen was used as the carrier gas flow at 1 mL/min at a constant pressure of 4.9551 psi. The initial temperature of the column was adjusted to 50 ºC and held for 5 min, then the temperature was increased to 150 ºC at a rate of 20 ºC $min^{-1}$and then to 290 ºC at a rate 1ºC $min^{-1}$with a final hold time of 5 min. The total run time was 30 min. The injector and detector temperature were set at 290 ºC. The volume of injection was 2 µL.

Sample Extraction and clean up

The extraction and clean-up of pesticide residues from chicken meat samples was performed following QuEChERS method with slight modifications as given hereunder. About 10 g of homogenized meat sample was taken in a 50 mL polypropylene centrifuge tube and 10 ml of acetonitrile containing 1% acetic acid was added and mixed vigorously for 1 min. The contents of acetate extraction pouch (6g magnesium sulfate and 1.5g anhydrous sodium acetate) was further added and then vortexed immediately for 1 min. The mixture was then centrifuged at 3075 x g for 10 min to separate out the phases. The upper 5 mL of acetonitrile layer was transferred into 15 mL tube containing dispersive SPE tube followed by vortex mixing for 1 min and centrifugation at 3075 x g for 10 min. After centrifugation, supernatant was filtered using HNN membrane filter (0.4 µm) and then injected into GC. The total run time was 30 min.

Validation of the analytical method

The analytical method was validated in terms of specificity, linearity, accuracy, precision, linearity, limit of detection (LOD) and limit of quantification (LOQ)

Specificity

The specificity of the method was evaluated to ensure that there was no interference from the components present in the matrix / solvent. It was studied by injecting chlorpyrifos in solvent (ethyl acetate), solvent alone (ethyl acetate), blank matrix (chicken meat) and matrix spiked with chlorpyrifos.

Linearity

Chicken meat samples were spiked with chlorpyrifos at six different concentration (0.05, 0.08, 0.15, 0.25, 0.5, 1.0 µg/g) levels. The samples were then extracted and analysed. The calibration curves were obtained by plotting peak area against concentration of the corresponding calibration standards. The mean correlation coefficient (r^2) was calculated. Each point of calibration curve represented mean of three replicates.

Limit of detection (LOD) and Limit of quantification (LOQ)

LOD and LOQ were determined based on the slope of the regression line and the standard deviation (S.D.) of the calibration curve using the following formula:

$$LOD = 3.3 \times \frac{S.D}{Slope}$$

$$LOQ = 10 \times \frac{S.D}{Slope}$$

Accuracy / recovery of the method

The accuracy of the method is the measure of closeness of the experimental value to the actual amount of substance present in the matrix. The accuracy of the most was estimated by spike recovery method at three concentration levels (0.05, 0.5 and 1 µg/g). The analyses were performed in triplicate and per cent recovery was calculated (Conc. recovered / concentration spiked*100).

Precision of the method

The precision of this method was verified by repeatability and reproducibility of the instrumental response with respect to retention time and area of the analyte. Intra-day and Inter-day precision was determined at three concentration levels, 0.05, 0.5 and 1.0 mg/mL. Intra-day variations were determined by assaying three difference occasions at least 24 h apart between each assay.The precision of the method was expressed as the per cent of co-efficient of variation (CV).

Matrix effect

The matrix effect (ME) of the analytical technique was evaluated by matrix matched standard with that of solvent standard. It was calculated using the formula (Tripathy *et al.*, 2016) [9].

Matrix effect (%) = (analyte $area_{matrix}$ - analyte $area_{solvent}$) x 100 / analyte $area_{solvent}$

Results and Discussion

In the present study, gas chromatographic method for the determination of chlorpyrifos residues in chicken meat was standardized and validated. QuEChERS protocol for the extraction of chlorpyrifos residue from chicken meat was standardized. The QuEChERS method was more advantageous compared to other extraction protocols in terms of ease of use, speed of extraction, efficiency of extraction, requirement of solvent and suitability for complex matrices (Garcia and Gotah, 2017, Lozowicka *et al.*, 2017 and Paz *et al.*, 2017) [10, 11, 12].

The chromatogram of chlorpyrifos spiked in chicken meat is given in figure 1. The specificity of the method was evaluated by inspection of interfering peaks in the blank samples. The chromogram of blank solvent, chlorpyrifos in solvent, blank sample and samples fortified with chlorpyrifos were examined. There were no interfering peaks in blank sample at the retention time of chlorpyrifos which indicates that the method was highly specific. The retention time of chlorpyrifos was 18.7 min.

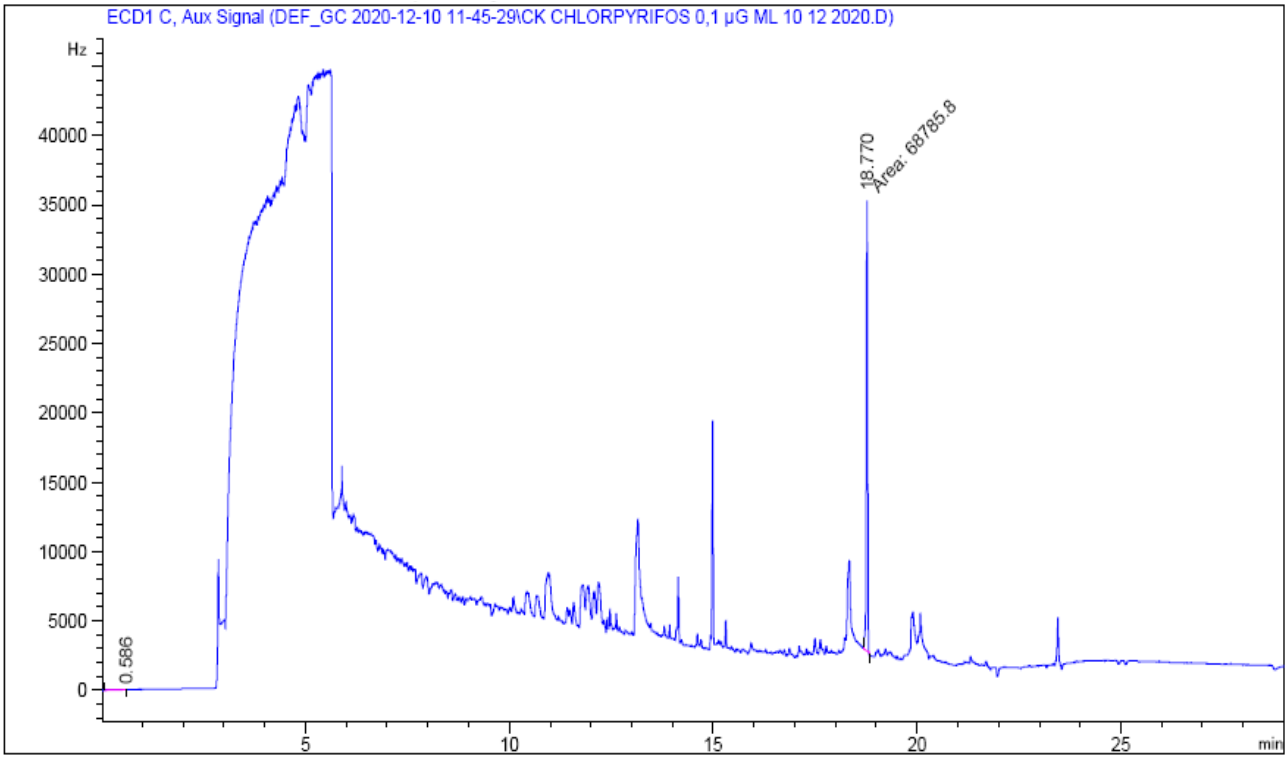

Fig 1: GC-ECD chromatogram of chlorpyrifos (1 ppm) spiked in chicken matrix

The linearity of the method was studied by plotting calibration curve at six concentrations levels and was found to be linear in the range of 0.05 to 1 µg/g (fig. 2). The regression equation was 86540.41 X -388.42 and the correlation co-efficient was 0.999.

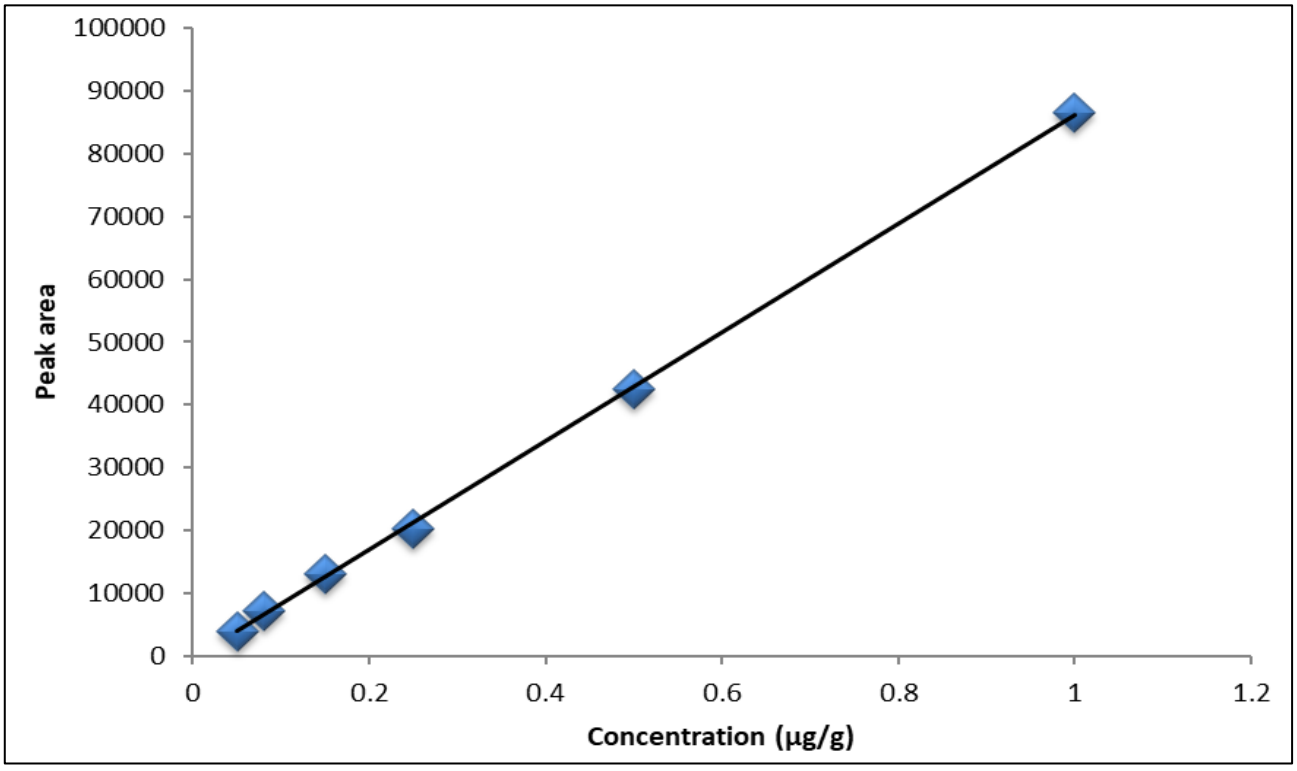

Fig 2: Calibration curve of chlorpyrifos in chicken meat

The LOD and LOQ of the method, as determined using slope and standard deviation of the calibration curve, was found to be 0.02 and 0.05 µg/g, respectively. The LOQ of the analytical method was less than the MRL value recommended by Food Safety Standards Authority of India (FSSAI) [13] in poultry meat (0.1 mg/kg in meat and meat products). It is important that the LOQ is comfortably lower than the MRLs prescribed that provide information on residue status unequivocally.

The recovery studies are carried out to rule out any significant loss of analyte during sample preparation and matrix interferences. The accuracy of the method was evaluated at three different concentration levels and the values are expressed at per cent recovery in table 1. The mean % recovery was in the range of 86.52 – 94.25% and it is well within the acceptable limits recommended (70-120%) (EC SANTE, 2017) [14].

Table 1: Accuracy, Precision, and matrix effect of the analytical method

Concentration spiked (µg/g)	Accuracy		Precision (CV, %)		Matrix effect (%)
	Mean recovery (µg/g)	Mean % recovery	Intra-day	Inter-day	
0.05	0.044	87.40	7.262	4.034	-12.60
0.5	0.471	94.25	6.114	2.909	-5.75
1.0	0.865	86.52	1.989	3.359	-13.48

The precision of an analytical method is the study of closeness of agreement between results obtained from multiple measurements of the same sample under prescribed conditions. In the present study, the precision of the analytical method was determined at two levels, repeatability (intra-day precision) and intermediate precision (inter-day precision), at three different concentrations (0.05, 0.5 and 1.0 µg/g) and the results are given in the table 1. The co-efficient of variation was less than 8%, indicating excellent precision and the performance of the method meets the international acceptance criteria of CV ≤ 20% (EC SANTE, 2017) [14].

Matrix effect is one the major problems in the analysis of compounds embedded in complex matrices such as biological fluids and tissues. It is likely that other compounds are co-extracted with the analyte of interest causing inaccuracies in the result. A suitable extraction technique which can completely eliminate matrix effect is still elusive. The matrix effect may be manifested either as chromatographic response enhancement or chromatographic response suppression and hence, the calculated % ME could be either negative or positive. Based on the % ME, it could be classified into three categories, viz.: No matrix effect (-20% to + 20%), medium matrix effect (- 20-50% to + 20-50%) and strong matrix effect (> 50% and < -50%) (Dominguez *et al*., 2014, Rutkowska *et al*., 2018) [15, 16]. In the present study, the matrix effect was evaluated at three concentration levels and the results are given in table 1. The results suggest that the method had no matrix effect since % ME values were between -20% and +20%.

A total of 50 chicken meat samples collected from four districts of Tamil Nadu (Chennai, Tiruppur, Thiruvannamali, Vellore) were analyzed for chlorpyrifos residues using the validated method and no chlorpyrifos residue was detected. Mishra *et al* [6] analyzed chicken meat samples collected from Haryana and Rajasthan states of India and reported chlorpyrifos residues in 5% of chicken meat sample. Though the results are encouraging, studying more number of samples from more number of districts will be required to strengthen our results. Owing to greater awareness among stakeholders, these studies have to be conducted regularly for monitoring purpose.

Conclusion

A simple, sensitive analytical method for the detection of chlorpyrifos residues using gas chromatography coupled with electron capture detector was validated. The extraction of chlorpyrifos residues from chicken meat based on QuEChERS protocol was optimized. The validated method was applied successfully for the determination of chlorpyrifos residues in fifty chicken meat samples collected from Tamil Nadu, India. None of the samples tested were found positive for chlorpyrifos.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgement

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