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Zoology

Standardization and Validation of Dot ELISA, Finding Detection Limit of Chlorpyrifos in Field Sprayed Samples Comparatively To Indirect Plate ELISA and Gas Chromatography

Prabha Parmar*
Ph.D Student, Department of Zoology, Punjab Agriculture University Ludhiana, Punjab, India

Original Research Article

***Corresponding author**
Prabha Parmar

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Abstract: A Dot enzyme linked immonosrbent assay (Dot ELISA) was developed and compared with standard indiect plate ELISA and gas chromatography (GC) for chlorpyrifos detection in the field samples. Firstly Dot ELISA for detection of Antichlorpyrifos antibodies (ACA) with prepared antigen and pure chlorpyrifos was standardized with the dilution range of 1µg and 1:1000µg/ml considering its limit of detection (LOD) value. Further since Dot ELISA was evaluated for field samples (fruit fodder, vegetables) for which, known dose of pure chlorpyrifos were spiked into field samples and determined with indirect plate ELISA, Dot ELISA and compared with GC. During the study four other parameters viz. sensitivity, specificity, accuracy, and K value were also determined by using Kappa statatics. Kappa statatics was used to interpret the comparative result between both types of ELISA. Results obtained from the studies showed accuracy between (50 -60%) sensitivity (10-70%) and specificity (12-90%). Percentage of accuracy above 80 considered to be positive and vice versa. While the K value i.e. % agreement between both tests lied between 0.96-0.1.0 (agreement above 0.81 showed perfect agreements). These results showed K value greater than 0.81 in all the field samples indicating perfect agreement between two techniques followed i.e. plate ELISA and Dot ELISA. This showed that both of the techniques were comparable for detection of pesticide residue detection at 75-125µg/ml dilution of antigen for Dot ELISA and 75-125ng/ml dilution of antigen among the field sprayed samples. As all the test samples showed more than 0.81 % agreement which interprets that both the test (Dot and plate ELISA) are comparable with each other. Same sample were tested with GC also which showed absence of chlorpyrifos in the field samples which supports the results obtained from the immunoassay studies. Major finding of the present studies has shown that Dot ELISA is a best tool for the qualitative detection of pesticides in the field samples with the LOD of 100µg /ml of antigen in case of chlorpyrifos.

Keywords: LOD, ACA, Kappa statastics, K- value, Chlorpyrifos.

INTRODUCTION

Among the widespread use of pesticide/insecticide for upliftment of agricultural output organophosphate (OP) insecticides, no doubt are being used successfully for controlling a number of pests. But on onothe hand presence of pesticide residues in food and environment has posed a serious threat to human health and caused a great concern. In order to keep human from being affected from pestides, analytical and monitoring system of pesticide residues in food and environment must be developed. Environmental contamination (inorganic and organic) is recognized as a worldwide problem..In this concern present pesticide detection method such as GC, HPLC, have been available for decades but due to its high cost expenditure and skill analyst requirement had made them restrict to laboratory level detection only. As a consequence, attention has been directed to new methods like immunoassay which seems to be a good alternative, at least for screening purposes. The immunoassay is not new, because it has been used for many years in clinical chemistry as a reliable, sensitive, and selective method to determine low concentrations of organic compounds in, for example, blood, urine, tissue extracts, etc1. Immunoassay has a rather long history and has become a widely accepted technique, particularly in the clinical area.

Though ELISA's have been developed for the detection of various pesticides, a few attempts have been made for CPF [1, 2]. Even in those, the detection assays previously developed had low sensitivity limits. Moreover not all immunoassays are completely specific to one single pesticide. Although highly sensitive plate ELISA has been developed for the detection of chlopyrifos but its performance has not been compared with the most commonly used classical methods GC/HPLC. It has also not been evaluated with field samples. Further since plate ELISA cannot be applied at field level, so it needs to be extended to the format of Dot ELISA which is based on simple principle. The Dot ELISA is a qualitative ELISA test, which can be performed more quickly without the need of equipments or technical expertise has highly desirable. Dot ELISA is a micro ELISA utilizing antigen "dotted" onto nitrocellulose filter discs that had been used for more than 25 years. The easy availibilty of nitrocellulose membrane (NCM) and the fact that the paper strips can be retained as permanent record for reference purpose, make this test suitable for most laboratories in the tropics. Because of its relative speed and simplicity, the Dot ELISA is an attractive alternative to standard ELISA. This technique can even detect at nano-gram scale among targeted compounds in situ Pappas [3].

Different studies had been conducted on Dot ELISA method for the detection of various diseases like viral bacterial and parasitic. The qualitative and quantitative detection of aflatoxin B1 in poultry sera was done by ELISA Sekhon *et al*. [4]. Their positivity with Dot ELISA is confirmed by standard method of Romer [5] followed by TLC [6]. A few false positives are observed in sera collected from birds fed on a toxin free diet. Significant improvement of the assay is noted after incorporation of an additional step of preincubation of free standard AFB1 (10 µg-100 µg) with antibody prior to competition. In another study Dot dye immunoassay for the diagnosis of Schistomasis mansoni was done Xue *et al*. [7]. Although spots obtained in Dot ELISA are slightly more intense than in dot double immunodiffusion assay (DIA), but provides the preliminary substitute with advantage of serological diagnosis of S.mansoni. Present study was focused on Standardization and validation of Dot ELISA to find out detection limit of chlorpyrifos in field sprayed samples comparatively to indiect plate ELISA and gas chromatography and the procedure followed is previously described in [8].

MATERIALS AND METHODS

Collection of chlorpyrifos free water samples

First of all, chlorpyrifos free water samples were collected from the Department of Entomology, PAU, and Ludhiana. Gas chromatography (GC) was performed for the confirmation of free chlorpyrifos samples. These water samples were further detected with indirect plate ELISA following the procedure as mentioned in Parmar and Kocher, 2017 part 1.For positive control (1-1000µg/ml) pure chlorpyrifos was used with antibody dilution of 1:5000. In case of negative control no antigen was used, while in test chlorpyrifos free water sample were used as antigen with similar antibody dilution of 1:5000. Confirmation of CPF free water sample was done by taking absorbance at 492 nm on ELISA reader.

Spiking of pure chlorpyrifos into chlorpyrifos free water samples (free of chlorpyrifos) and its detection by (GC) and indirect plate ELISA

For spiked samples a known concentration of standard (pure chlorpyrifos) was added (spiked) into chlorpyrifos free water sample as detected by GC. Different concentrations of pure CPF ranging from 0.000001-100 µg/ml were added to detect the presence of CPF following the procedure mentioned. In Parmar and kocher, 2017 part 1.Percent inhibition in terms of binding inhibition (%) was calculated by indirect plate ELISA at 492 nm. In the next step stock solution for ELISA and gas chromatography (GC) were prepared. For ELISA a chlorpyrifos stock solution was prepared in methanol at the concentration of 1000 µg/ml and for GC a stock solution was prepared in acetonitrile at the concentration of 10µg/ml. The ELISA stock standard was diluted to yield working standards of 1000, 100, 10, 1, 0.1, 0.01, 0.001 and 0.0001 ng/ml.While the stock solution in case of GC was diluted to make working standards of 10, 1, 0.1, 0.01, 0.001 and 0.0001ng/ml.

Statistical performance for standard curve immunoassay for chlorpyrifos

- Accuracy =Measured value of sample replicates in each matrix to value of sample added or spiked.
- Cofficient of variance (CV): It is the ratio of standard deviation to its mean. Value of CV% should not exceeds ± 20% [9]
- Recovery%= Calculated concentration of spiked amount /estimated concentration of spiked amount. Recovery of each sample should fall within the range of 80%-120% showed accuracy of experimentation [10].

Standardization and development of Dot ELISA for detection of chlorpyrifos pesticide

Design of immunocomb for Dot ELISA

Nitrocellulose membrane (NCM) strips of 5 x 5 mm^2 was marked with lead pencil at 1 cm intervals for orientation of antigen Dots. After that (NCP) were coated separately with 2 to 3µl of field sample for the test and CPF spiked samples as (positive control). The negative controls coated with preparation from CPF water free samples. The

coated NCM strips were dried at 65°C for 2 h in an incubator and then blocked in PBS containing 0.05% Tween -20 (PBS-T). Then plate was incubated with 2 to 3µl of prepared antigen with the above concentrate (section 3.11). Thereafter presence of CPF pesticide in the field samples were qualitatively detected by following Dot ELISA procedure (Flow chart 5).

Development of Dot ELISA for detection of chlorpyrifos pesticide

For standardization and development of Dot ELISA first of all limit of detection (LOD) for pure chlorpyrifos and prepared antigen were calculated by following the procedure as given in Flow chart 5. After obtaining the cut of dose for chlorpyrifos detection free water samples (as detected by GC and indirect plate ELISA) were spiked with different concentrations (1000-0.1µg/ml) of pure chlorpyrifos. An un-spiked/negative control was also maintained for comparison. The limit of detection (LOD) was calculated by Dot ELISA method as described by Venkatesh *et al*. [11].

Collection and preparation of field samples for detection of chlorpyrifos

Different samples of agricultural produce were collected from three separate local markets of Ludhiana i.e Clock tower, Ghumar mandi and Agar nagar. Samples were divided into three groups: I, II and III i.e. fruits, vegetables and fodder respectively. All the samples were analysed for the detection of chlorpyrifos residue by three methods: gas chromatography, indirect plate ELISA and Dot ELISA.

Preparation of field samples for gas chromatography (GC)

All the samples of three groups i.e. I, II and III were weighed to 50g and homogenized with homogenizer for 2 minutes in the ratio of 1:5 in acetone. Then clear supernatant was filtrated and further spiked with CPF with different concentrations of 1, 10, 20, 50 and 100 ng/ml. Residue was concentrated by removing excess of solvent by using rotary evaporator. From concentrate of 10 ml, 1ml of residue was taken and made the volume of 10 ml with PBS (pH 7.6) i.e. in the ratio of 1:9. After that chlorpyrifos residue was detected with gas chromatography through its recovery calculation and peak area obtained from the chromatogram.

Preparation of field samples for indirect plate ELISA

All the samples of three groups i.e. I, II and III were weighed to 50g and homogenized with homogenizer for 2 minutes in the ratio of 1:5 in PBS. Then clear supernatant was filterated and further spiked with CPF with different concentrations of 0.01, 0.1, 1, 10, 20, 50 and 100 ng/ml. Residue was concentrated by removing excess of solvent by using rotary evaporator. From concentrate of 10 ml, 1ml of residue was taken and made the volume of 10 ml with PBS (pH 7.6) i.e. in the ratio of 1:9. After that chlorpyrifos residue was detected with indirect plate ELISA through its recovery calculation.

Preparation of field samples for Dot ELISA

All the samples of three groups i.e. I, II and III were weighed to 50g and was homogenized by using 100 ml of methanol with high speed homogenizer for 2 minutes. Residue was concentrated by removing excess of solvent and collected 10 ml of residue it by using rotary evaporator. From concentrate of 10 ml, 1ml of residue was taken and made the volume of 10 ml with PBS (pH 7.6) i.e. in the ratio of 1:9. Now samples were ready to utilize for coating of as antigen on Dot ELISA comb.

Development and standardization of field spray residual method for detection of chlorpyrifos in field samples by GC, plate ELISA and Dot ELISA methods

Chlorpyrifos residue in the sprayed samples (one from each group i.e. wheat leaves, orange and cabbage) was detected by three different techniques i.e Gas chromatography, Indirect plate ELISA and Dot ELISA Different concentrations of pure chlorpyrifos were sprayed on individual plants. One unspiked plant was kept as control for each case. After ten days of spraying the sample were collected and were further processed by following the preparation procedure mentioned above. Different concentration of antigen 500, 300, 200, 150, 100 and 75 ng /ml with antibody dilution (1:1000 µl/ml) and 1:1500 HRPO conjugate dilution were used for indirect plate ELISA and 100, 50,10 , and 1, 0.1 and 0.01 ng /ml concentration of antigen were used for gas chromatography. While in case of Dot ELISA antigen concentration were used: 1000, 500,250, 125,75, and 50µg/ml with antibody dilution (1:1000 µl/ml) and 1:1500 HRPO conjugate dilution. Sensitivity of all the methods were compared with each other by calculating their limit of detection (LOD). All the three methods were compared each other by calculating their limit of detection. Higher the LOD value was directly proportional to the sensitivity of the test/method.

Statistical analysis

Statistical analysis for immunological parameters

Comparisons of immunological parameters were made between control, vehicle and treated group on computer using "Analysis of Variance (ANOVA)" as a Statgraphics statistical package. A "P" value of 0.05 was selected as a criterion for statistically significant differences.

Statistical analysis for Dot ELISA

The sensitivity, specificity and accuracy of Dot ELISA were compared with indirect plate ELISA by (neutralization test) agreement between antigen and antibody as described by Venkatesh et al. (2006). Where, Sensitivity:

a / (a+c)

Specificity: d / (b+d)

Accuracy: a+d / (a+b+c+d)

K = (a+d – P) / 1- P,

Where P= (a+b) (a+c) + (c+d) (b+d) and P is the probability,

a: is the number of samples positive by both i.e., test to be compared and gold standard test.

b: is the number of samples positive by standard test whereas negative by test to be compared.

c: is the number of samples negative by standard test and positive by test to be compared.

d: is the number of samples negative by both.

k value > 0.81 indicates perfect agreement.

RESULTS AND DISCUSSION

Standardization of Dot ELISA for chlorpyrifos LOD at laboratory levelProcedure followed as mention in section 4.2 Parmar and kocher 2017 part 2.

Collection and detection of chlorpyrifos free water samples

Water samples collected from department of Entomology, PAU Ludhiana were tested for their confirmation as CPF free water samples by gas chromatography and indirect plate ELISA and the results are given below:

Detection of chlorpyrifos free water samples through gas chromatography (GC)

When water samples were run through GC, no peak with reference to the basic area peak of chlorpyrifos standard was obtained from the chromatogram thereby indicating that the solvent water sample run for the analysis was free of chlorpyrifos (chromatogram.no.1).

Detection of chlorpyrifos free water sample through indirect plate ELISA

The chlorpyrifos free water samples confirmed through gas chromatography (GC) in previous section were further detected by using indirect plate ELISA, so as to determine the efficacy of this technique in comparison to GC. Results showed colour development in the first two rows i.e. A and B in 1-12 wells) table no.1 because of the positive control, as it was having pure CPF as antigen. Rows C and D were kept as negative control, while in E and F rows (wells 1-12) free water samples were used instead of antigen. As there is no CPF in the water samples, thus no reaction for antibodies occurred; therefore no colour development was seen in E-F lines. Thus it was proved from the present experiment that these water samples do not contain chlorpyrifos as also observed by GC and can be used as negative control for further experimentation.

Spiking of chlorpyrifos free water samples with pure chlorpyrifos and its detection

Known amount of pure CPF was spiked into CPF free water samples and detection of CPF was done by following two methods:

Through gas chromatography (GC)

Results revealed from the chromatogram no.2 that one peak has observed with reference to standard chlorpyrifos basic area peak thereby indicating the presence of chlorpyrifos in the solvent run through GC. On the other hand in all sets of different concentrations the % recovery lied within the range of 93.5-106 % (Table 13). It determined that the CPF pesticides detected in the free water samples showed consistency in the result with respect to % recovery. Therefore it can be inferred from the results that spiking range was not affected by the difference between the dilutions.

Through indirect plate ELISA (i ELISA)

Similar concentrations were spiked to detect the CPF by indirect plate ELISA for comparison with GC method. Results in table no. 2 showed that almost total spiked amount was recovered/ estimated from each concentration with % recovery of 89.9- 106.2. On the other hand maximum % inhibition i.e. 98.24% was observed at 100 ng/ml and above that range no colour was developed thereby indicating that sensitivity remained 100 ng or 10 fg. Therefore this LOD can be considered as cut of dose for immunoassay experiment. Estimated amount of spiked samples was calculated to determine the effect of consistency of the samples on the results. In the present study it was determined that for each set of spiked samples amount corresponded to total amount estimated by ELISA. The recovery is considered to determine that whether the antigen- antibody detection is affected by the difference between the dilutions used to prepare the sets of

concentration. Chen *et al*. [12] observed that recovery for the spiked concentration in the samples calculated out is identical to the result for the analyte prepared in standard diluents, and then the experimental procedure followed is considered to be valid for assay.

Table-1: Detection of chlorpyrifos free water samples through indirect plate ELISA

Antibody concentrations (wells 1-12)				
Type of antigen in rows	1:500	1:100	1:100	1:10
	1-3	4-6	7-9	10-12
% Inhibition				
Row –A (pure CPF)	2.85±0.55	2.74±0.52	2.37±0.47	1.73±0.43
Row –B (pure CPF)	2.57±0.56	2.63±0.58	2.32±0.48	1.75±0.48
Row –C (-ve)control	93.41±0.54	77.57±0.57	58.43±0.54	35.57±0.57
Row –D (-ve)control	95.47±0.54	73.54±0.57	55.49±0.54	32.53±0.57
Row-E (free water sample)	98.76±0.57	95.76±0.38	92.78±0.51	91.78±0.42
Row-F (free water sample)	98.45±0.54	98.85±0.42	97.84±0.49	99.84±0.52

All values are mean ±S.E

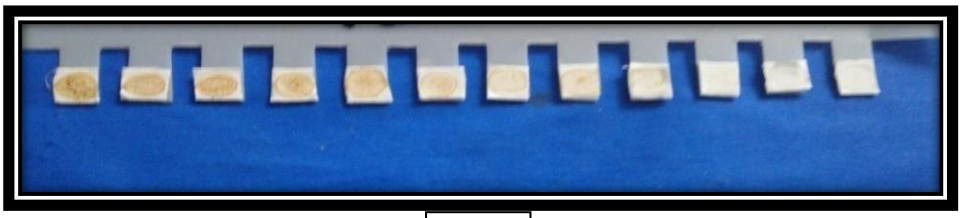
Table-2: Detection of spiked concentration of pure chlorpyrifos through indirect plate ELISA

S. No.	Spiked concentration of CPF ng/ml	Estimated concentration of CPF ng/ml	%Inhibition	% Recovery
1	100	100	98.24	89.9
2	50	52.9	60.56	94.2
3	20	20.2	32.89	106.2
4	10	11.1	10.54	96.4
5	1	1	1	93.5

Table-3: Detection of spiked concentrations of pure chlorpyrifos in water samples through Dot ELISA

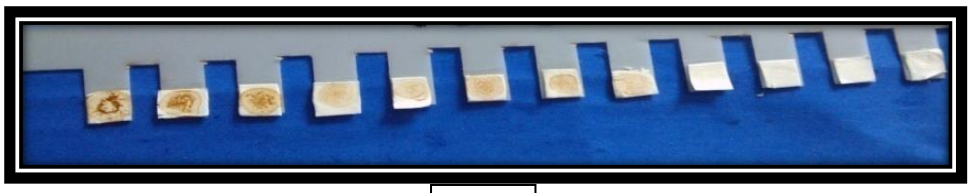
Type of antigen (CPF)	Positive control	Antigen dilution µg /ml (comb 1-7)					Negative control
		1000	100	10	1	0.1	
Replicate-1	+	+	+	-	-	-	-
	+	+	+	-	-	-	-
Replicate-2	+	+	+	-	-	-	-
	+	+	+	-	-	-	-

+ sign represents colour development
-sign represents no colour
Antibody dilution 1:1000 in all



A

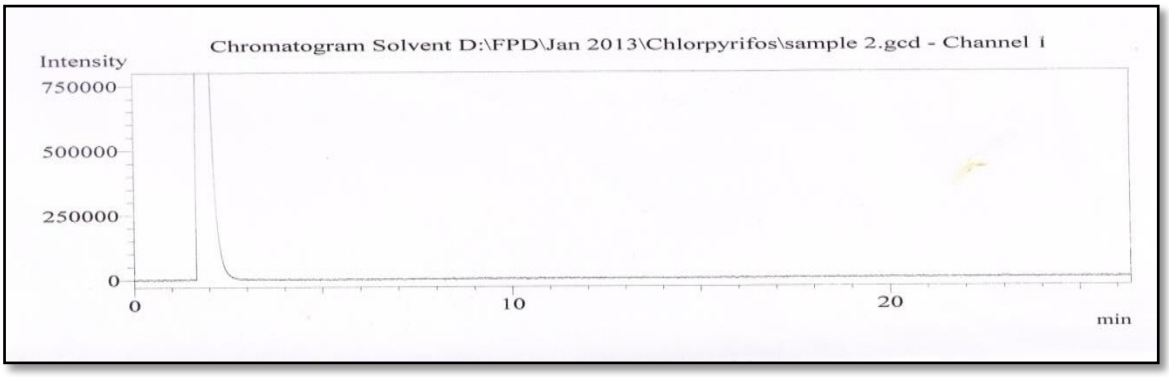
Replicate-1



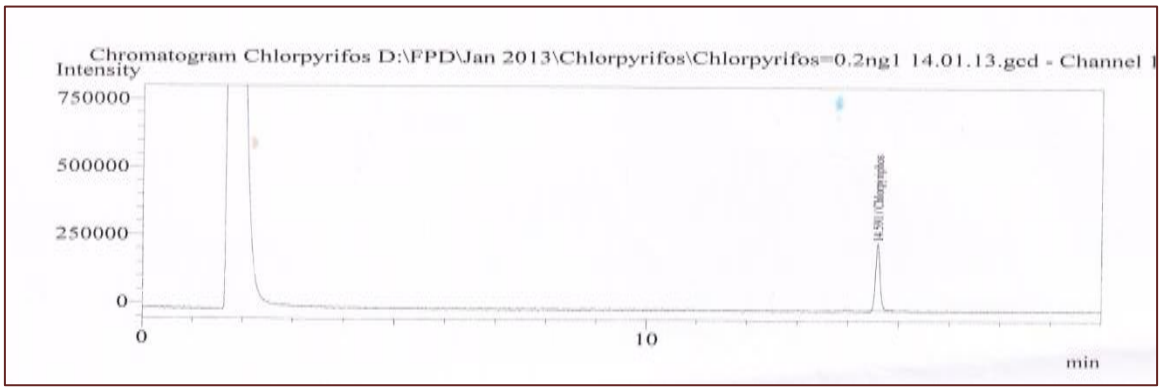
B

Replicate-2

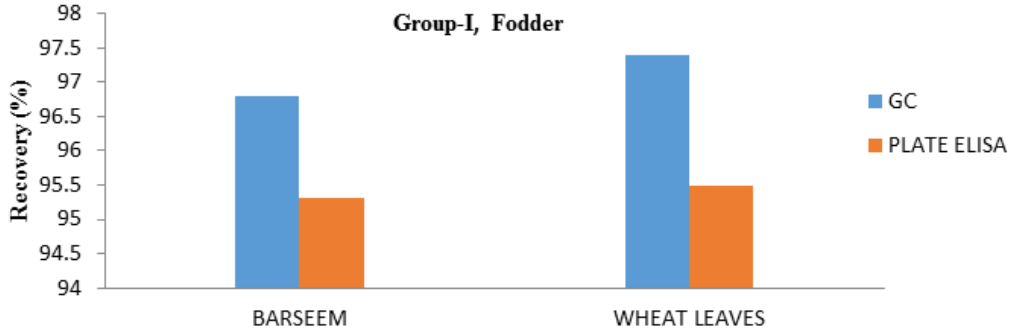
Plate 1



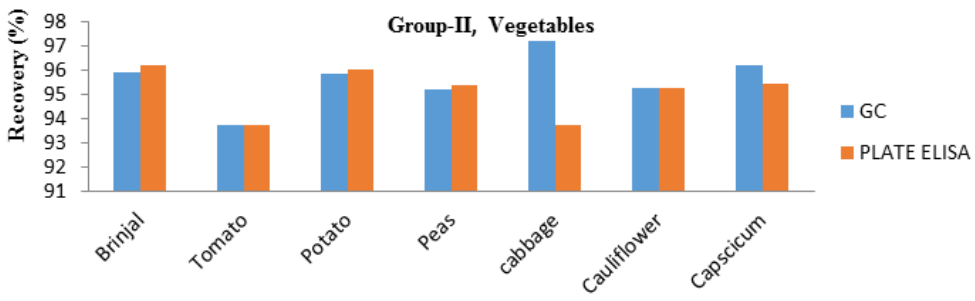
Chromatogram- 1: Chromatogram showing basic area peak of chlorpyrifos standard with no peak of water sample tested



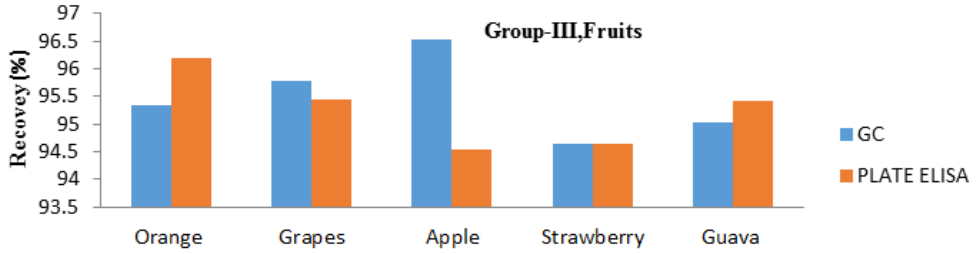
Chromatogram-2: Chromatogram showing basic area peak of chlorpyrifos standard with one more peak correspond to it



(A)



(B)



(C)

Fig-1: Comparison of recovery (%) of spiked chlorpyrifos by gas chromatography and plate ELISA in fleld samples

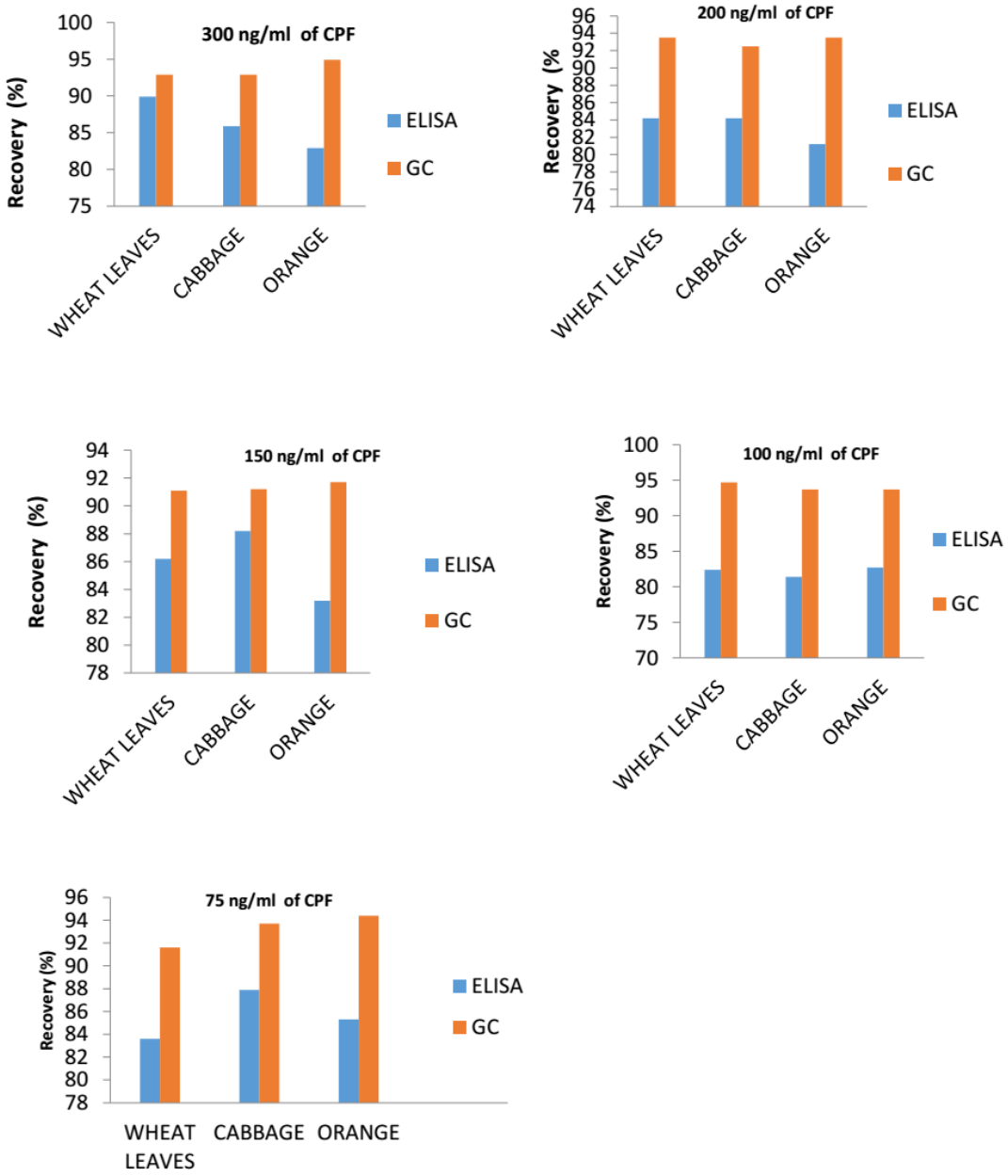


Fig-2: Comparison of recovery (%) of different concentrations of chlorpyrifos in sprayed field samples through gas chromatography and plate ELISA

Table-4: Limit of detection (LOD) for chlorpyrifos sprayed field sample of wheat leaves through Dot ELISA

Fig-A: Limit of detection (LOD) for chlorpyrifos sprayed field sample of wheat leaves through Dot ELISA

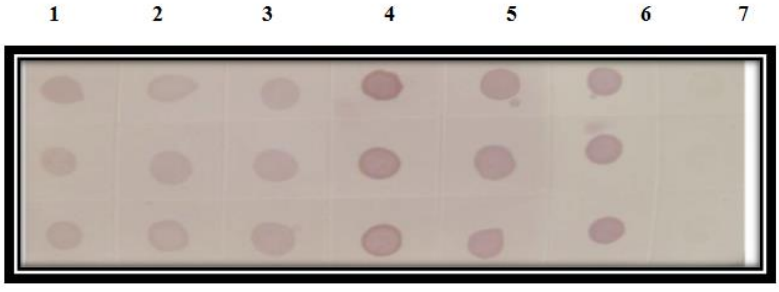
Rows	Sample tested-Wheat leaves						
	Positive control	Antigen concentration in µg/ml (blocks 1-7) Antibody dilution 1:100 in all					
A	+	1000	500	250	125	75	50
B	+	1000	500	250	125	75	50
C	+	1000	500	250	125	75	50

Fig-B: Limit of detection (LOD) for chlorpyrifos sprayed field sample of cabbage through Dot ELISA

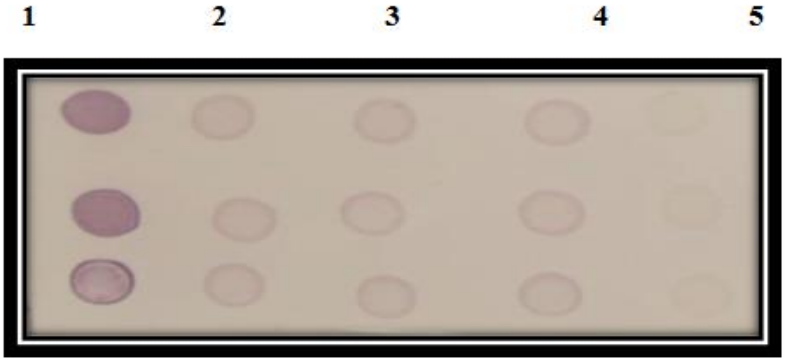
Rows	Sample-Cabbage				
	Antigen concentration in µg/ml (blocks 1-5) Antibody dilution 1:100 in all				
A,B and C	1000	500	250	125	75
	1000	500	250	125	75
	1000	500	250	125	75

Fig-C: Limit of detection (LOD) for chlorpyrifos sprayed field sample of orange through Dot ELISA

Rows	Sample tested-Orange						
	Positive control	Antigen concentration in µg/ml (blocks 1-7) Antibody dilution 1:100 in all					
A	+	1000	500	250	125	75	50
B	+	1000	500	250	125	75	50
C	+	1000	500	250	125	75	50



A



B

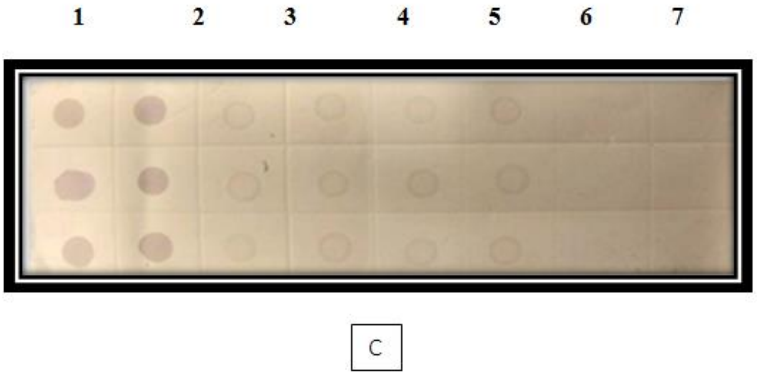


PLATE-2

Detection of anti chlorpyrifos antibodies (ACAb) through Dot ELISA technique

After determining the sensitivity limits and LOD for ACAb by two methods in the previous sections i.e. indirect plate ELISA and GC, the study was further extended to standardize and to develop Dot ELISA method for qualitative detection of CPF pesticide in the spiked samples as well as in the field samples following the method given in flow chart 1[13]. Later on the results obtained through Dot ELISA was compared with that of indirect plate ELISA and these results are shown below:

Detection of chlorpyrifos spiked samples by DOT ELISA

Chlorpyrifos free samples spiked with different concentrations of pure CPF were analysed for its detection by following the technique of Dot ELISA for comparison of results obtained from indirect plate ELISA. Different spiked concentrations of pure CPF in duplicates showed that Dot ELISA was found to have LOD with value of 100 µg/ml for CPF with 1:100 of antibody dilution i.e. above this range no colour development was observed (Plate- 1 Figs. A and B and Table 3). While the results obtained with indirect plate ELISA for spiked water samples showed LOD for CPF 100 ng/ml of antigen with 1:1000 of antibody dilution given by [8] part 1.Therefore, the comparative studies of LOD between Dot ELISA and indirect plate ELISA interpreted that even though Dot ELISA has lower LOD as compared to indirect plate ELISA, but it is able to detect CPF at the level of certain amount therefore, it can be adopted as a tool for pesticide detection.

Fodder samples from PAU fields and vegetable and fruit samples from local markets of Ludhiana were collected and divided into three groups (group I-fodder, group II-vegetables and group III-fruits). All samples were processed for the detection of CPF by gas chromatography. When field samples of barseem and wheat (under group-I), brinjal, tomato, potato, peas, cabbage, cauliflower and capsicum (under group-II) and orange, grapes, apple, strawberry and guava (under group-III) were run through GC along with standard solution of chlorpyrifos (0.1ng/ml), only one peak of standard was obtained in all the samples. Results thus indicated no CPF residue or very low amount of chlorpyrifos (not with in the limit of detection of GC) in the processed field samples. Therefore the detection of chlorpyrifos in field samples was not carried out with plate ELISA. Ramesh *et al*. [14] have also observed no CPF residue from the tomato fruit samples analysed by GC.

Spiking of field samples with chlorpyrifos and its recovery estimation

As no residue in the field samples was detected through GC, so the spiking of these samples was carried out with known concentrations of CPF and the % recovery was determined by GC and plate ELISA.

Through gas chromatography

In fodder samples

The group-I samples i.e. barseem and wheat leaves spiked with different concentrations of CPF (100-1ng/ml) after processing were run through GC and the results obtained thus indicated the average recovery of 96.8% in case of barseem sample and 97.4% in case of wheat leaves .

In vegetable samples

When the group-II samples i.e. different vegetables after processing were run through GC indicated the average recovery range of chlorpyrifos from 93.72 to 97.24%. Spiking of 1-100 ng /ml in different vegetable samples resulted in average recovery of 95.92% in brinjal, 93.72% in tomato, 95.84 % in potato, 95.2%1 in peas, 97.24% in cabbage, 95.30% in cauliflower and 98.24% in capsicum samples.

In fruit samples

When the group-III samples i.e. fruit samples after processing were run through GC indicated the average recovery range of chlorpyrifos from 94.64 to 96.54%. Spiking of 1-100 ng /ml in different fruit samples resulted in average recovery of 95.34 % in orange, 95.78 % in grapes 96.53 % in apple, 94.64 % in strawberry and 95.02 % in guava samples.

Through plate ELISA

In fodder samples

The group-I samples i.e. barseem and wheat leaves spiked with different concentration of CPF (100-1 ng/ml) after processing were run through plate ELISA and the results have been presented in table 21.These results indicated that the average recovery of 95.38% in case of barseem sample and 95.48% in case of wheat leaves was obtained.

In vegetable samples

When the group-II samples i.e. different vegetables after processing were run through plate ELISA indicated the average recovery range of chlorpyrifos from 93.72 to 96.24%. Spiking of 1-100 ng /ml in different vegetable samples resulted in average recovery of 96.24 % in brinjal, 93.72% in tomato 96.04% in potato, 95.4% in peas, 93.72% in cabbage, 95.30% in cauliflower and 95.48% in capsicum samples.

In fruit samples

When the group-III samples i.e. different fruit samples after processing were run through plate ELISA indicated the average recovery range of chlorpyrifos from 94.50 to 96.18. Spiking of 1-100 ng /ml in different fruit samples resulted in average % recovery of 96.18 in orange, 95.44 in grapes 94.53% in apple, 94.64 %in strawberry% and 95.42% in guava samples.

Comparative account of recovery estimation of spiked chlorpyrifos in field samples by gas chromatography and plate ELISA

In fodder samples

When the results of % recovery of spiked CPF through GC and plate ELISA were compared it showed the mean recovery of chlorpyrifos in fodder samples i.e. barseem was found to be 95.30% by plate ELISA and 96.80 % with GC, while 97.40 % and 95.48% in case of wheat leaves respectively (Fig.1, A).

In vegetable samples

When the results of % recovery of spiked CPF through GC and plate ELISA were compared and it showed that the mean recovery of chlorpyrifos in vegetable samples to lie in between the range of 93.72- 96.24 % both by GC and plate ELISA (Fig.1, B)

In fruit samples

When the results of % recovery of spiked CPF through GC and plate ELISA (Table 23) were compared, it showed the mean recovery of chlorpyrifos in fruit samples in the range from 94.64-96.50 % through GC and 94.64-96.18 plate ELISA (Fig.1, C).

In the light of above comparative results (Fig.1) it can be interpreted that as the % recovery range of spiked concentration of pure CPF was found to be almost similar whether it was run through GC or through plate ELISA, in another way indicating that both these techniques can be equally effective for pesticide residue detection in field samples. The recovery of thiamethoxam in water and tomato samples by GC was found to lie in the range of 92-98% and the results were comparable with HPLC. However in another study, chlorpyrifos from oil matrix was determined using GC with a flame photometric (FPD) and electron capture (ECD) detectors. In the first experiment, the detector (FPD) used gave recoveries ranging from 89 to 100% and in the second experiment, ECD showed recoveries of greater than 97% for chlorpyrifos in the test samples Halimah *et al*. [15]. Robert *et al*. [16] optimized the sweep codistillation apparatus to quantitatively analyse coumaphos and organophosphorus pesticide residues in animal fat and recovery of caumaphos obtained was 91%. Other organophosphorus pesticides (diazinon, chlorpyrifos, ethion and bromophosethyl) showed recovery ranging from 90% to 96%. Ana *et al*. [17] investigated a GC method for determination of 25 organophosphorus pesticides applied to horticultural crops involving GC with FPD for quantification further confirmed by gas chromatography- mass spectrometry, (GCMS) and found the recovery range from 68%-100%. Sullivan *et al*. [18] compared a specific and precise commercial magnetic particle-based enzyme-linked immunosorbent assay (ELISA) with gas chromatographic/flame photometric (GC/FPD) method and they found that ELISA was more consistent over GC/FPD method. From the reviewed literature and results obtained in the present study it can be interpreted that plate ELISA could also be used as effective tool for immunoassay experimentation, in comparisons to classically used tests like GC.

Detection of chlorpyrifos spiked field samples by Dot ELISA and its comparison with plate ELISA

Homogenates prepared separately from all the field samples (three replicates each) were detected through Dot ELISA (Table.3) by using only two dilution of antigen i.e spiked field sample (1000 and 100 µg/ml for Dot ELISA and 1000 and 100 ng/ml for indirect plate ELISA) with single antibody dilution (1:1000 µl/ml) and 1:1500 HRPO dilution**.** The results obtained from the tests (indirect plate ELISA and Dot ELISA) were analyzed for the percentage of agreement between antibodies and antigen (field samples) by using Kappa statistics Venkatesh et al. (2006) as described statistical analysis part 2 and To find out the efficacy of both the methods i.e Dot ELISA and indirect plate ELISA, the LOD found from Dot ELISA test was taken into consideration for comparison.

Development and standardization of field spray residual method for detection of chlorpyrifos in field samples by different techniques

Chlorpyrifos residue in the sprayed samples from fodder (wheat leaves), vegeteable (cabbage) and fruits (orange) was detected by three different techniques i.e Gas chromatography, Indirect plate ELISA and Dot ELISA. Different concentrations of antigen viz. 500, 300, 200, 150, 100 and 75 ng /ml with antibody dilution (1:1000 µl/ml) and 1:1500 HRPO conjugate dilution were used for indirect plate ELISA and 100, 50, 10, 1 and 0.1 ng /ml concentration of antigen were used for gas chromatography. While in case of Dot ELISA antigen concentration used were 1000, 500, 250, 125, 75 and 50 µg/ml with antibody dilution (1:1000 µl/ml) and 1:1500 HRPO conjugate dilution.

Comparative account on recovery estimation of chlorpyrifos sprayed field samples by gas chromatography and plate ELISA

When the results of % recovery of sprayed CPF through GC and plate ELISA (Fig.2) were compared and it showed the (%) recovery chlorpyrifos in wheat leaves, cabbage and orange was found lie between the ranges of 81-89% % in case of plate ELISA with sprayed concentration of 300-75 ng/ml. On the other hand result showed the % recovery of chlorpyrifos in these field samples was found to lie in between the range of 91-94% through gas chromatography with sprayed concentration of 100-0.1 ng/ml. As all the recoveries value falled into the acceptable range of 80-120% [10] So it can be interpreted from the results that both techniques are highly correlated with each other proving that immunoassay technique is equally sufficient for pesticide residue detection and could be used as effective tool for daily routine test as compared to GC.

Detection of chlorpyrifos sprayed field samples by Dot ELISA and its comparison with plate ELISA

To find out the efficacy of both the methods i.e Dot ELISA and indirect plate ELISA, the LOD found from both the tests were taken into consideration for comparision at the same level (Table 39). In case of wheat leaves it was observed that out of 20 samples, 9 were found to be positive by Dot ELISA and 14 were positive by indirect plate ELISA, while 6 negative both by Dot.

ELISA and 6 also negative by indirect plate ELISA at 75µg/ml (Table 4). In case of cabbage, it was found that out of 18 samples, 9 found to be positive by Dot ELISA and 12 were positive by indirect plate ELISA while 8 negative by Dot ELISA and 6 by indirect plate ELISA at 125µg/ml. So it was observed that out of 20 samples of orange 10 were found to be positive by Dot ELISA and 15 were positive by indirect plate ELISA, while 10 negative by Dot ELISA and 5 by indirect plate ELISA at 75µg/ml (Table 4, plate 2).

During the study four other parameters viz. sensitivity, specificity, accuracy, and K value were also determined as shown in tables 4.These results showed K value greater than 0.81 in all the field samples indicating perfect agreement between two techniques followed i.e. plate ELISA and Dot ELISA. This showed that both of the techniques were comparable for detection of pesticide residue detection at 75-125µg/ml dilution of antigen for Dot ELISA and 75-125ng/ml dilution of antigen among the field sprayed samples. Literature reveals that most of the work in the field Dot ELISA has been carried out for the qualitative detection of various diseases and reports on such study regarding the pesticide residue analysis in the field samples is meager. Therefore the present results have been justified on the basis of work done in clinical aspects. Shome *et al.* [19] observed that the overall sensitivity of the Dot ELISA test was found to be 78.9% with tissue infected with A. hydrophila in fish, while it showed 92.8% sensitivity with direct plate ELISA in field conditions. Development of a Dot-ELISA assay for diagnosis of southern rice black-streaked dwarf disease (SRBSDV) in the field of suspected rice was found to be positive for SRBSDV by the Dot-ELISA and confirmed by the One Step RT-PCR method Wang *et al.* [20]. A multidot immunoblot assay was performed for diagnosis of pulmonary tuberculosis by using locally available nitrocellulose membrane (NCM) and it was found that 42 sera were bacteriologically infected with pulmonary tuberculosis with sensitivity of 95% and the specificity of 92 %, thus indicating a good correlation of Dot ELISA with micropipette plate ELISA Rattan and Shriniwas [21]. Various viral, bacterial and parasitic diseases have also been detected by Dot ELISA assay and the finding of these studies has shown effective applicability of this technique [5, 6, 4]. The results obtained in the present study can also be supported by the facts observed by Young *et al.* [22], as these workers has also reported ELISA as sensitive, specific, effective and

suitable immunoassay for detection of chlorpyrifos residue in field samples as compared to other conventionally used techniques.

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CONCLUSION

In the present study comparison of Dot ELISA over indirect ELISA as an detection tool of pesticide has been demostrated.it is clearly indicated that quantitative detection of chlorpyrifos coluld be fulfilled by using Dot ELISA. This simple, rapid and sensitive assay was proven to be a useful and practical detection technique for chlorpyrifos pesticide and could be applied for detecting suspected cases in field level. According to the results of comparision between three techniques GC,indirect ELISA and Dot ELISA. Were equally sufficient and capable for pesticide residue detection with different level of sensitivity and detection limit. Thereupon Dot-ELISA assay provided a new rapid Quantitative detection system with LOD of 75-125µg/ml as this method can be utilized by technicians or even farmers themselves could test their own crops.

List of Non Standard Abbreviations:

LOD-Limit of detection
ACAb-Antichlorpyrifos antibodies
GC-Gas chromatography
CPF-chlorpyrifos
CV-cofficient of variance
LOD-Limit of detection
PBS-Phosphate buffered saline
LC 50- Lethal concentration of 50% of total population
HRPO-Horse radish peroxidase
NCM-Nitrocellulose membrane

REFERENCES

1. Cho Y, Lee HS, Park EY, Lee YT, Hammock BD, Ahn KC, Lee JK. Development of an ELISA for the organophosphorus insecticide chlorpyrifos. Bulletin of the Korean Chemical Society. 2002;23(3):481-7.
2. Brun A, Castilla J, Ramírez MA, Prager K, Parra B, Salguero FJ, Shiveral D, Sánchez C, Sánchez-Vizcaíno JM, Douglas A, Torres JM. Proteinase K enhanced immunoreactivity of the prion protein-specific monoclonal antibody 2A11. Neuroscience research. 2004 Jan 1;48(1):75-83.
3. Pappas MG, Hajkowski R, Cannon Sr LT, Hockmeyer WT. Dot enzyme-linked immunosorbent assay (dot-ELISA): comparison with standard ELISA and complement fixation assays for the diagnosis of human visceral leishmaniasis. Veterinary Parasitology. 1984 Jun 1;14(3-4):239-49.
4. Kapur J, Sekhon CP, Sodhi SS. Production of anti-aflatoxin B 1 antibodies in rabbits and development of an immunoassay for their detection. Annals of Biology. 1996;12(1):15-8.
5. Romer TR. Screening method for the detection of aflatoxins in mixed feeds and other agricultural commodities with subsequent confirmation and quantitative measurement of aflatoxins in positive samples. Journal-Association of Official Analytical Chemists. 1975 May;58(3):500-6.
6. Coker RD, Jones BD, Nagler MJ. Tropical development and research Institute, mycotoxin training manual. Overseas development administration, London, Sec B. 1984;3:5-6.
7. Xue H C, Quiu L S, Zang Y H and Zhu Z X. Dot ELISA in the diagnosis of schistomiasis.Clin. Med J. 1987; 100:113-15.
8. Parmar P, Kocher D. Comparative study of immunoassay and Gas chromatography for chlorpyrifos detection in laboratory spiked water samples. International journal of scientific research. 2018 Nov 13;6(2).
9. Krotzky AJ, Zeeh BE. Pesticides report 33. Immunoassays for residue analysis of agrochemicals: Proposed guidelines for precision, standardization and quality control (Technical Report). Pure and applied chemistry. 1995 Jan 1;67(12):2065-88.
10. Krotzky AJ, Zeeh BE. Pesticides report 33. Immunoassays for residue analysis of agrochemicals: Proposed guidelines for precision, standardization and quality control (Technical Report). Pure and applied chemistry. 1995 Jan 1;67(12):2065-88.
11. Venkatesh G. *Development of recombinant antigens for diagnosis of avian mycoplasmosis* (Doctoral dissertation, Tamil Nadu Veterinary and Animal Sciences University).
12. Wu J, Xu F, Zhu K, Wang Z, Wang Y, Zhao K, Li X, Jiang H, Ding S. Rapid and sensitive fluoroimmunoassay based on quantum dots for detection of melamine in milk. Analytical Letters. 2013 Jan 15;46(2):275-85.

13. You Q, Liu M, Liu Y, Zheng H, Hu Z, Zhou Y, Wang B. Lanthanide-labeled immunochromatographic strip assay for the on-site identification of ancient silk. ACS sensors. 2017 Mar 23;2(4):569-75.
14. Ramesh A, Thirugnanam PE, Balakrishnamurthy P. Hapten synthesis, generation of polyclonal antibodies and development of ELISA for determination of thiamethoxam residues in food and environmental samples.
15. Halimah M, Osman H, Ainie K, Tan YA, Pauzi MD. Determination of chlorpyrifos in refined palm olein by GC-FPD and GC-ECD. Journal of Oil Palm Research. 1999;11(2):89-97.
16. Brown RL, Farmer CN, Millar RG. Optimization of sweep codistillation apparatus for determination of coumaphos and other organophosphorus pesticide residues in animal fat. Journal-Association of Official Analytical Chemists. 1987;70(3):442-5.
17. Agüera A, Contreras M, Fernandez-Alba AR. Gas chromatographic analysis of organophosphorus pesticides of horticultural concern. Journal of chromatography a. 1993 Dec 3;655(2):293-300.
18. Sullivan JJ, Chen YG, Goh KS. Performance assessment and validation of a paramagnetic particle-based enzyme-linked immunosorbent assay for chlorpyrifos in agricultural runoff waters. Journal of agricultural and food chemistry. 2007 Aug 8;55(16):6407-16.
19. Shome R, Shome BR, Gaur KK. Rapid detection of Aeromonas hydrophila (Chester, 1901) infection in freshwater fish by Dot ELISA. Indian Journal of Fisheries. 2005;52(3):269-73.
20. Wang Z, Yu D, Li X, Zeng M, Chen Z, Bi L, Liu J, Jin L, Hu D, Yang S, Song B. The development and application of a Dot-ELISA assay for diagnosis of southern rice black-streaked dwarf disease in the field. Viruses. 2012 Jan 23;4(1):167-83.
21. Rattan A and Shriniwas B. Standardisation of nitrocellulose membrane Dot ELISA for serodiagnosis of tuberculosis: a technique suitable for India. Ind. J. Tub. 1990; 37: 17-19.
22. Young JG, Eskenazi B, Gladstone EA, Bradman A, Pedersen L, Johnson C, Barr DB, Furlong CE, Holland NT. Association between in utero organophosphate pesticide exposure and abnormal reflexes in neonates. Neurotoxicology. 2005 Mar 1;26(2):199-209.