

*Full Length Research Paper*

## Development of ELISA kit for the assay of dichlorodiphenyltrichloroethane in milk and milk products

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Immunoassay of pesticide residues in foods is gaining importance in the past two decades. The objective of the present work is to develop an enzyme – linked immunosorbent assay (ELISA) kit for the assay of dichlorodiphenyltrichloroethane (DDT) in milk and milk products. Here, an ELISA method was developed using rabbit polyclonal antibodies against a hapten, DDT-OH for the detection of organochloride pesticide DDT in milk and milk products. The immunogen for raising the antiserum was prepared from BIS (4-chlorophenyl) ethanol and succinic anhydride coupled with bovine serum albumin (BSA) One ml of immunogen was inoculated subcutaneously in multiple sites of thigh region in rabbits following three boosters at weekly intervals and the antiserum was harvested after the second booster. The ELISA response of the anti DDT antiserum, diluted and undiluted was studied against the antigen (DDT) coated onto the immuno module. The homogenous response of the anti DDT anti serum was adequate to identify DDT. Fifteen samples each of milk, buttermilk, cheese, kalakhand and khoa were quantitatively analyzed for DDT using ELISA as well as GC methods. DDT was present only below detectable limit (BDL) (5 ng g<sup>-1</sup>) in milk and milk products obtained from organized dairies in and around Chennai city, India. The percentage recovery was above 70% ensuring that the ELISA method developed for DDT is dependable. The developed ELISA method can be helpful in detecting the residual amount of DDT present in milk and milk products. The analysis of pesticides in samples by ELISA and GC was comparable.

**Key words:** DDT-BSA conjugate, ELISA-Kit, Hapten synthesis, milk, milk products.

### INTRODUCTION

Food safety is one of the major concerns in every country regardless of the economic and social development. The

deleterious effects of organochlorine pesticides have been witnessed phenomenally both on human and animal

subjects (Dawood et al., 2004; Grilo et al., 2013; Adami et al., 1995). Some of them are of serious in nature causing irreversible damage at a rather low level (WHO, 2003) which necessitates the use of innovative methods to detect the low level pesticides (Bhadekar et al., 2011) present in soil, water, air, food stuffs, breast milk, blood and animal tissues. Residues of DDT have been detected in soil, water and also in air. While Thin Layer Chromatography (TLC), High Performance Liquid Chromatography (HPLC) and Gas Chromatography (GC) are commonly used methods for the detection of pesticides; they are highly laborious and require highly skilled technical expertise (Ramesh et al., 2007); however, ELISA method developed for the detection of pesticides is accurate with high sensitivity, specificity, as well as cost effectiveness for large number of samples and adaptability to field use and rapid test despite gaining wider predominance (Zhang et al., 2013).

ELISAs are widely applied for the quantification of various pesticides and insecticides such as DDT/DDE, HCH isomers, toxaphene and cyclodiene OCPs present in environment (Behnisch et al., 2001; Sherry, 1997). Earlier Amitarani et al. (2002) have reported that DDT residues measured in water samples by means of ELISA were comparable to GC method. In fact, over the years, ELISA methods are developed for the quantification of various pesticide residues in food products including milk and milk products (Hongsibsong et al., 2012; El-Gendy et al., 2011; Botchkareva et al., 2003; Brandon et al., 2002).

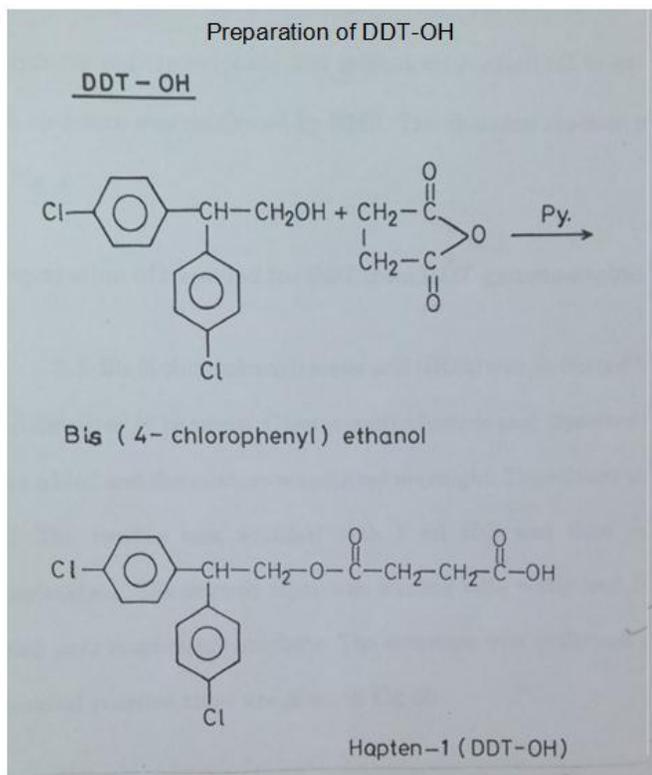
In developed and developing countries, the use of certain pesticides like DDT and HCH was banned. However, the residual effects are still causing problems (Shaker and Elsharkawy, 2015; Zhou et al., 2014). The residues of DDT persist in the environment for long periods and enter the food chain. Hence, reports are coming from Asian, Gulf and European countries. Milk is considered as a universal food consumed by all categories of people. The chances are likely that the trace levels of pesticide residues present in fodder can be transferred to the milk which may affect the consumers. In Iraq, Al-Omar et al. (1985) detected DDT concentrations ranging from 0.01 to 0.05  $\mu\text{g g}^{-1}$  in milk around Baghdad, Iraq. According to Martínez et al. (1997), one percent of milk samples in Spain were contaminated with DDT with mean level of 0.007  $\mu\text{g g}^{-1}$ . Dawood et al. (2004) reported the incidence of DDT in 30% of milk samples with a mean concentration of  $0.1003 \pm 0.19 \mu\text{g g}^{-1}$ . ELISAs have been described that are performed in tubes, plastic-baked nitrocellulose membranes, magnetic particles, etc., but most often 96-well microtiter plates are preferred since these allow the

simultaneous analysis of a large number of samples.

Depending on the immune reagent immobilized on the plate, two main formats can be distinguished when small molecules are analyzed: the direct and indirect. In the direct format, the antibody Ab is usually coated on the active surface and equilibrium is established between the analyte and the enzyme tracer for binding to the Ab. The unbound reagents are washed away and the amount of tracer is measured. The enzyme activity is inversely proportional to the amount of analyte present. In the indirect format, Antigen Ag is immobilized and the amount of analyte is indirectly measured by the quantification of the bound Ab with a second labeled Ab, the bound Ab with a second labeled Ab. There are examples of ELISA for a large number of pollutants, such as carbonates, organochlorine and organophosphorous compounds, triazines, PAHs, PCBs, etc. (Puchades and Maquieira, 1996). Organochlorine pesticides are partly banned in India; however, they are still used in many parts of India for various purposes including agricultural operations. Several reports indicate the incidence of organochlorine pesticides in milk and milk products (Devanathan et al., 2009; Kumar et al., 2005; Pandit et al., 2002).

Kalra et al. (1978) studied DDT contamination of milk collected from different locations in Punjab where the DDT ranged from 0.006 to 0.13  $\mu\text{g g}^{-1}$ . Kaphalia et al. (1990) found DDT in 60% of milk samples with a mean level of 0.028  $\mu\text{g g}^{-1}$ . Dhanalakshmi (1995) found that among the organochlorine pesticides, DDT and DDE were present in traces in all milk samples collected from organized dairies in Chennai, India. Dichlorodiphenyltrichloroethane (DDT) contain the antigen and free DDT. The free DDT then competes with the antigen for the binding site of AuNPs. The resultant strip will show an intense red color of AuNPs in the absence of free DDT. The intensity of the red color decreases with increasing concentration of free DDT. This strip has a LOD at 27  $\text{ng ml}^{-1}$ . Despite the "naked eye" visible test strip, rapid detection of DDT can be done on an immuno-chemiluminescence dip strip with immobilized anti-DDT immunoglobulin Y (IgY) antibodies (Baker et al., 2012). Similarly, in the presence of DDT, DDT competes with the lumino for the antibodies' is hazardous pesticide that was used widely during World War II. It is highly stable in the environment and causes neurological changes and reproductive problems to wildlife. AuNPs based on a competitive immunoassay dip strip has been developed for the rapid testing of DDT (Lisa et al., 2009). The red AuNPs are conjugated to anti-DDT antibodies. The immunocomplex solution is then dipped on a nitro cellulose membrane strip binding site.

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**Figure 1.** Chemical reaction steps.

The luminescence intensity is inversely proportional to the concentration of free DDT. The development of immunoassay on pesticide has become mature enough to give reliable screening within 10 min. Hence, the aim of current study was to develop an immunoassay kit for measuring DDT by raising polyclonal antibodies in rabbits.

## MATERIALS AND METHODS

### Milk and milk product sample

Milk, cheese, khoa, kalakhand and buttermilk samples (15 in each) were collected aseptically under refrigerated condition from organized dairies in and around Chennai, India. The representative samples were drawn as per the standard methods for examination of dairy products (Marshall, 1992).

### Chemicals and reagents

#### Solvents

The solvents used in the study were n-hexane (95%), acetonitrile (99.7%) and methanol (99.8%) obtained from Fisher Scientific, USA. All other chemicals and reagents were analytical grade.

### Working buffer (50 mM)

Stock phosphate buffer (PBS) was diluted five times with distilled water to get 50 mM working buffer. For every 100 ml of PBS (50 mM), 1.1 ml of fish gelatin was added. This was kept at 4°C and used for all dilutions.

### Wash buffer

Stock PBS (250 mM) was diluted 25 times to get 10 mM wash buffer. To one liter of wash buffer, 5 ml of 10% Tween- 20 was added. This was used for washing plates.

### DDT standard

DDT standard was prepared in n-hexane (Clarke, 1986).

### Preparation of hapten

BIS (4-chlorophenyl) ethanol (DDT-OH) was dissolved in pyridine and succinic anhydride (10 M) was added to the mixture, stirred overnight at 4°C and ethyl acetate was added. It was washed with water, 1 N HCl and brine. The organic solvent was dried over anhydrous sodium sulphate and solvent evaporated off to get the hapten. NMR confirmed the structure. The chemical reaction steps are shown in Figure 1 (Karanth et al., 1998).

### Chromogen solution

10 mg of tetra methyl benzidine (TMB) was mixed with 1 ml of dimethyl sulphoxide (DMSO) and stored at room temperature.

### Antibody production

One milligram equivalent of the hapten - protein conjugate in Freund's complete adjuvant (1:1 ratio) was injected epidurally at multiple sites to six rabbits of 3-4 months of age as a primary dose. After 30 days, the primary bleed was checked for the antibodies by mean antibody capture assay (Karanth et al., 1998). When the antibody titre was found satisfactory, booster dose was given (500 µg equivalent of protein in incomplete Freund's adjuvant) by intra muscular injection. After 8 days, 3 - 5 ml of blood was drawn from marginal ear vein and checked for antibody titre. When the titre had increased, a second booster was given in incomplete Freund's adjuvant by intra muscular injection. After 8 days, the antibody production was again checked. Since the peak antibody production was noticed at this time, 15-20 ml blood was collected and the serum was separated. Serum IgG was purified using gamma bound sepharose column (Harlow and Lane, 1988).

### Analysis of milk and milk product samples by ELISA

Milk and milk products were analyzed using ELISA reader (Anthos HT II, Austria) as per the protocol given in the operational manual. 30 ml of milk was extracted with 150 ml of methanol and used directly for loading in the ELISA plate and followed similar procedure and detected the contamination level of DDT in milk and milk products by comparing with the standard curve.

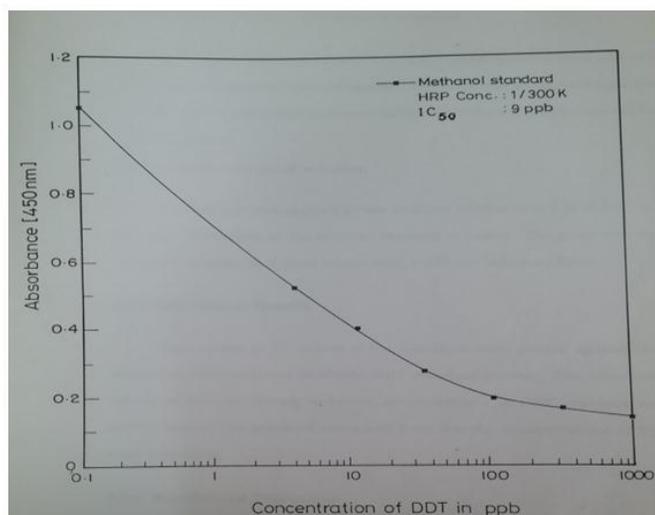


Figure 2. DDT standard curve which was found to be linear.

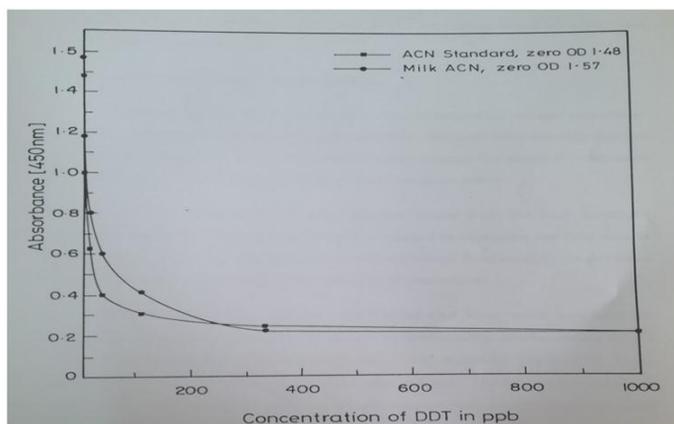


Figure 3. Acetonitrile extract of milk and buttermilk.

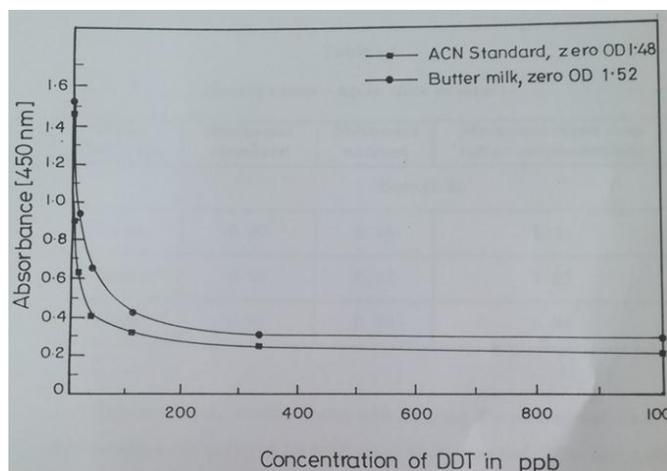


Figure 4. Effect of acetonitrile extract of milk and buttermilk.

### Analysis of milk and milk product samples by GC

Milk and milk product samples have been extracted in n-hexane and analysed using Gas Chromatography (Tracor 540, Tracor Instruments, Austin, Texas, USA) according to International Dairy Federation (IDF) standard methods (2008).

### Statistical analysis

The data were statistically analysed as per the procedure adopted by Snedecor and Cochran (1994).

## RESULTS AND DISCUSSION

The absorbance values obtained from the standards were plotted against their respective concentrations (1, 10, 100 and 1000 ppb) on semi logarithmic graph to obtain the DDT standard curve which was found to be linear (Figure 2). From the standard curve, it was observed that absorbance readings obtained from ELISA were inversely proportional to the concentration of analyte in standard that is, an increase in concentration will be accompanied by a decrease in absorbance values.

### Matrix effect and clean-up procedure

The uptake kinetics of an analyte during analysis is influenced by proteins and this problem is called as matrix effect (Liu et al., 2008). It is suggested that the elimination of matrix effect is necessary and important for the determination of analytes in complex samples (Liu et al., 2008; Hall et al., 2012). Clean-up is an analytical procedure involving series of steps in which the bulk of the potentially interfering compounds are removed by physical or chemical methods. Various clean-up approaches are involved to eliminate the matrix effect in milk samples (Tölgyesi et al., 2012; Xia et al., 2010; Martins et al., 2013). Especially, certain clean-up procedures were successfully employed for eliminating the matrix effect during the analysis of pesticide residues in milk samples (Spinks et al., 2001; Di Muccio et al., 1997; Di Muccio et al., 1996). Trace amounts of DDT in milk has to be dislodged by solvent extraction. In this procedure, a number of constituents from the milk (the matrix) also get partitioned along with the pesticide which may change the assay performance either negatively or positively leading to matrix effect. Milk and milk products are classified under high fat-high moisture content food stuffs. Clean-up procedure is necessary to overcome the food matrix effect (Schenck and Lehotay, 2000; Xia et al., 2010). The approach made for clean-up was derived from sample preparation methods for GC analysis of pesticides. Milk and buttermilk were extracted in acetonitrile. Acetonitrile extract of milk and buttermilk had no matrix effect (Figures 3 and 4). In an earlier work,

**Table 1.** Matrix clean-up in milk products (Mean  $\pm$  S.E).

Milk products	Methanol standard <sup>NS</sup>	Methanol extract	Methanol clean-up (after sulphonation)
Cheese	0.96 $\pm$ 0.01	0.26 <sup>a</sup> $\pm$ 0.01	1.15 <sup>b</sup> $\pm$ 0.01
Kalakhand	0.97 $\pm$ 0.01	0.82 <sup>c</sup> $\pm$ 0.02	1.01 <sup>a</sup> $\pm$ 0.01
Khoa	0.96 $\pm$ 0.01	0.74 <sup>b</sup> $\pm$ 0.04	0.98 <sup>a</sup> $\pm$ 0.01

#Mean of four observations; <sup>NS</sup> Not significant; means bearing different superscripts in the same column differ significantly ( $p < 0.01$ ).

**Table 2.** Percentage recovery of DDT in milk and milk products by ELISA and gas chromatography techniques (Mean  $\pm$  S.E)

Particular	GC (g g <sup>-1</sup> )		ELISA (ng g <sup>-1</sup> )		Percentage Recovery	
	Quantity		Quantity		GC	ELISA
Dairy product	Spiked		Spiked			
Milk	0.98 $\pm$ 0.05 <sup>a</sup>	0.71 $\pm$ 0.02 <sup>b</sup>	0.55 $\pm$ 0.07	0.40 $\pm$ 0.01	73.63 $\pm$ 0.55 <sup>a</sup>	81.25 $\pm$ 0.43 <sup>b</sup>
Significance	$p < 0.01$		NS		$p < 0.01$	
Buttermilk	0.51 $\pm$ 0.03 <sup>a</sup>	0.35 $\pm$ 0.01 <sup>b</sup>	0.51 $\pm$ 0.03 <sup>a</sup>	0.40 $\pm$ 0.01 <sup>b</sup>	69.75 $\pm$ 0.85 <sup>a</sup>	80.25 $\pm$ 0.85 <sup>b</sup>
Significance	$p < 0.05$		$p < 0.05$		$p < 0.01$	
Cheese	0.50 $\pm$ 0.05	0.38 $\pm$ 0.01	5.05 $\pm$ 0.06 <sup>a</sup>	3.73 $\pm$ 0.01 <sup>b</sup>	74.25 $\pm$ 0.85 <sup>a</sup>	77.50 $\pm$ 0.65 <sup>b</sup>
Significance	NS		$p < 0.01$		NS	
Khoa	0.97 $\pm$ 0.05 <sup>a</sup>	0.82 $\pm$ 0.01 <sup>b</sup>	4.95 $\pm$ 0.07 <sup>a</sup>	4.25 $\pm$ 0.07 <sup>b</sup>	81.00 $\pm$ 0.41 <sup>a</sup>	85.50 $\pm$ 0.65 <sup>b</sup>
Significance	$p < 0.05$		$p < 0.01$		$p < 0.05$	
Kalakhand	1.00 $\pm$ 0.04 <sup>a</sup>	0.81 $\pm$ 0.01 <sup>b</sup>	4.95 $\pm$ 0.12 <sup>a</sup>	4.05 $\pm$ 0.06 <sup>b</sup>	81.00 $\pm$ 1.58	80.50 $\pm$ 0.65
Significance	$p < 0.05$		$p < 0.01$		NS	

# Mean of four observations; <sup>NS</sup> Not significant; \* Means bearing different superscripts in the same row between two data differ significantly ( $p < 0.05$ ), ( $p < 0.01$ )

while studying the matrix effect during the analysis of organophosphates in milk, Erney et al. (1993) reported high percentages of recovery implicating less interference of matrix effect. The zero O.D (no pesticide) and IC<sub>50</sub> were on par with the neat solvent. The IC<sub>50</sub> values for milk and buttermilk extract differed by 2 ng g<sup>-1</sup>, respectively when compared with acetonitrile standard (IC<sub>50</sub> = 14 ng g<sup>-1</sup>). Cheese, kalakhand and khoa showed matrix interference that is attributable to high-fat content in certain milk products affecting the recovery of certain pesticides (Ranganathan et al., 2014)

Therefore, the methanol extract of the samples were treated with sulphuric acid and washed with water three times. There after the extract was partitioned with petroleum ether, the organic layer was collected, evaporated and made up in original solvent. Clean-up was achieved up to 70% using this method. The zero O.D. of the neat solvent was more or less on par with that of the samples (Table 1).

The matrix clean-up effect seen in various milk products was represented as mean  $\pm$  SE in Table 1. It can be noticed that the matrix clean-up effect for methanol extract for the three milk products (cheese, kalakhand and khoa) was statistically significant ( $p < 0.01$ ) between the products. For the methanol clean-up (after sulphonation), the level of significant ( $p < 0.01$ ) were noticeable between cheese and kalakhand and between cheese and khoa. However, no statistically different changes were observed between Kalak hand and khoa.

DDT estimation in milk and milk products were carried out using Gas Chromatography in all the same samples as in ELISA. The amount of DDT recovered (mean  $\pm$  SE) and percentage recovery of DDT from milk and various milk products as measured by means of GC and ELISA methods were represented in Table 2. For milk, the quantity of DDT recovered was statistically significant from the spike value, whereas ELISA data in this case did not show any significant changes between spike and

recovery. In the case of buttermilk, khoa and kalakhand, there was significant difference in the quantity of DDT recovered after spike in both ELISA and GC methods.

For cheese samples, only ELISA method showed significance in the amount of DDT recovery after spike. The percentage recovery of DDT in the case of cheese and kalakhand samples was almost the same by GC and ELISA techniques. On contrary, significant variations were seen between ELISA and GC methods for the percent recovery of DDT from milk, buttermilk and khoa samples. The recovery percentage was 70 and above in both techniques. Hence, it was confirmed that ELISA technique developed for DDT was dependable. The levels of DDT in milk and milk products were found to be well below detectable limits in both methods. According to Pandit et al. (2002), organochlorine pesticide residues analyzed in milk and milk products from different regions of Maharashtra state, India were also well below the detectable limits. Moreover, they reported higher levels of DDT in butter samples when compared with cheese. Earlier, John et al. (2001) reported increased residual levels of organochlorine pesticides during winter season in milk samples collected from Jaipur city, Rajasthan, India. It is a welcoming feature that DDT was present only below detectable limit (BDL) ( $5 \text{ ng g}^{-1}$ ) in milk and milk products obtained from organized dairies in and around Chennai city, India.

## Conclusions

Despite enormous benefits of pesticides in agronomy and food crop protection several risk factors and public health hazards are implicated due to their prolonged accumulation in soils, which is indirectly a source for contamination in milk. Since milk and milk products are essential food for children and infants, it is necessary to monitor the pesticide residues especially, DDT in milk. Immunoassay method developed is more sensitive, rapid than conventional method like TLC. In the present investigation, no alarming DDT levels were found, as the samples were obtained from organized dairies. Owing to the risk factors from unorganized dairies and local vendors, the developed ELISA method can be beneficial in detecting alarming DDT levels present in milk and milk products.

## Conflict of Interests

The authors have not declared any conflict of interests.

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