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# Evaluation of rapid enzyme inhibition test for pesticides detection and validation by spectrophotometer and GC/MS

**M Bhavadharani, P Janaki, R Sheeba Joyce Roseleen, K Arulmozhiselvan and J Ejilane**

**Abstract**
A perceptible increase of pesticides usage in agriculture needs on-site monitoring of their residues to increase the food safety and avoid health issues. Here, the present investigation was carried out to evaluate a rapid enzyme based test kit to detect the presence of cholinesterase-inhibiting pesticides in vegetables. The inhibition of acetylcholinesterase enzyme (AChE) by OP and pesticides using specific substrate, acetylthiocholine and suitable chromogenic reagent was evaluated and observed for the colour development. Initially it was evaluated with technical standards and fortified vegetable samples to find out the lowest limit of colour development for each pesticide and then extended to the fortified and unknown vegetable samples. Increase in pesticide concentration decreased the yellow colour intensity and the developed colour was also analyzed in spectrophotometer for validating the test technique and calculating the AChE inhibition percent. Under optimized condition, the detection limit of 2 mg/L for chloripyriphos, profenofos and dimethoate and 1 mg/L for triazophos, quinalphos, ethion and dicofol was achieved. Pesticide residues from fortified and unknown vegetables were extracted by QuEChERS method and assayed by the developed rapid enzyme test and spectrophotometer. The total amount of time required for detection of pesticide residue using rapid AChE based test is a minute. The main advantage of the developed rapid test kit is minimum reagent requirement, low-cost and easy to handle. Even non-professional can screen pesticide residue using the developed rapid test kit.

**Keywords:** ACHE, pesticide detection, GC/MS, vegetables

## Introduction
Agriculture is the backbone of the Indian economy. Indian ranks 2nd in the population next to China. Ensuring food safety is an arduous task with declining cultivable land resource and increasing the crops productivity by adopting high yielding varieties, balanced fertilization and indiscriminate use of pesticides. Pesticides are largely used in vegetables such as brinjal, bhendi, tomato, cabbage, cauliflower, cluster bean, chilli and green leafy vegetables since they are highly susceptible to pest and disease attack. Sometimes pesticides are also applied over the vegetables even after the harvest to control in-borne pests and to fetch good market value. Usually, Organophosphorus (OP) pesticides are widely used to control pest and diseases because of their expeditious breakdown and low persistence in the environment. Eventhough, usage of OP pesticides causes many adverse health effects like headache, fatigue, breathing problems, abdominal cramps and tingling in extremities along with depression in the activity of cholinesterase. The OP pesticides controls the pest infestation by inhibiting acetylcholinesterase enzyme activity, which inhibits the activity of neurotransmitter acetylthiocholine (Quinn, 1987) [1]. The same mechanism was followed in all living organism, so it causes severe health impact on other living organisms also.

In order to monitor the pesticide residues, several analytical methods were developed such as Gas chromatography equipped with Mass Spectrometer, High-performance Liquid Chromatography, Liquid Chromatography-Mass Spectrometer and bio-sensors. However, these methods require long time and heavy initial investment (Skerritt, 1998) [2] which will be reflected in the cost of per sample analysis.

An enzyme inhibition method is considered as an effective tool for the measurement of pesticide residues. An enzyme inhibition procedure involves the measurement of the uninhibited activity of an enzyme which is the simple and fast method and doesn't require expensive apparatus. The effect of pesticides on the inhibition of an enzyme in a reaction

system is dependent on their concentration and own chemical property. Also, this method can only measure the total content of the pesticides residue in a sample, and it is unable to analyze individual pesticides.

Anticholinesterase is nothing but acetylcholinesterase, a member of carboxyl esterase family is a catalyst in the process of breaking down of acetylcholine into acetic acid and choline and also breakdown other cholinesterase, which act as neurotransmitter. It will degrade 25000 molecules/second, since it has high catalytic action (Fukuto, 1990) [3]. It will be more active in sensory neurons while comparing with motor neurons. Organophosphorus insecticides considered as group of irreversible inhibitor for AChE, by the process of hydrolysis of acetylcholine into choline and acetic acid at cholinergic synapses(Sultatos, 2006; Colovic *et al.*, 2013) [4, 5]. This property is used in the detection of OP and carbamate compounds and acetyl cholinesterase inhibitors have more advantages even though it has disadvantage on human health and environmental issues. Therefore, more specific detection techniques were developed for these compounds. Though the cholinesterase inhibition was attempted for insecticide residue detection for over 50 years, still improvements are going on using different sources for acetyl cholinesterase enzyme to have tower sensitivity and precision so as to cover the registered new pesticides.

Action of cholinesterase on acetylthiocholine (a thio-analogue for the natural substrate for the enzyme, acetylcholine) produces acetic acid plus thiocholine. This is the reaction which is inhibited by carbamate and organophosphates (thiophosphates in the sample require conversion before analysis). The extent of conversion to thiocholine is measured spectrophotometrically by use of dithiobisnitro benzoic acid (DTNB); a yellow colour forms with a maximum absorbance at 412 nm.

Winterlin *et al.* (1968) [6] reported that the thin layer chromatography was used to identify pesticides inhibiting cholinesterase activity using benzeneacetate, which results in the development of white colour on the spot against blue background. Drevenkar *et al.* (1981) [7] reported that residues of organophosphorus and carbamate group of pesticides were identified using Ellman's reagent with AChE in surface water sample. Intensity of developed colour was measured in spectrophotometer at 412 nm. Ni *et al.* (2007) [8] explored the application of a spectrophotometric method for the simultaneous kinetic determination of binary mixtures of carbaryl and phoxim. This method was based on the inhibitory effect of the pesticide analytes on acetyl cholinesterase and offered a possible new direction of analysis in this field.

Nagatani *et al.* (2007) [9] developed a disposable pesticide residue detection chip for periodic monitoring of other neurotoxic pesticides of OP and CM family by non-professional end-users in agricultural products by inhibiting the activity of acetyl cholinesterase. It can able to differentiate between the concentrations of 0.1 ppm and 0.2 ppm diazinon – oxon in real samples. Other concentration can be measured by changing the volume of AChE.

Hossain *et al.* (2009) [10] conducted an experiment on reagent less bioactive paper- based solid phase biosensor todetectthe inhibitors of anticholinesterase. In reagent less lateral flow paper sensor, the pesticide residue can be predicted by the intensity of developed colour on paper. The signal intensity can be enhanced using polyvinylamineas a cationic capture agent. From this experiment, they concluded that bioactive paper-based assay platform is good enough for the detection of organophosphate and carbamate pesticide content in food products and habitat.

In this paper, the inhibitory effect of OP pesticides and on AChE of commercially purchased and DTNB as a chromogenic reagent was evaluated for residue detection. The AChE extracted from honey bees was also evaluated for rapid residue detection. It was also validated using fortified samples extracted by QuEChERS and spectrophotometric determination. The evaluated protocol was tested for its applicability to detect the pesticide residues in real vegetable samples.

## Materials and Methods

The experiments were conducted in Centre of Excellence in Sustaining Soil Health, Department of Soil Science and Agricultural Chemistry, Anbil Dharmalingam Agricultural College and Research Institute, Tiruchchirappalli, Tamil Nadu, India.

All reagents and chemicals used were of analytical grade belongs to Hi-media and SD Fine chemicals, Mumbai. The commercial acetylcholinesterase (E.C. 3.1.1.7 from electrophorus electricus, 500U), acetylthiocholine-iodide (ATCI) and DTNB were purchased from Sigma Aldrich. The disodium hydrogen orthophosphate, sodium dihydrogen phosphate, sodium chloride, potassium chloride were purchased from HiMedia laboratories Pvt Ltd. Analytical grade reagents and MilliQ-water were used throughout the experiment.

Borosil glassware's were cleaned with the soap solution and washed with chromic acid followed by Milli Q-water and then rinsed with acetone, dried at room temperature and sterilized in a hot air oven at 80°C for three hours before use. Fresh vegetable samples were collected from local market (Uzhavar Sandhai) and used for pesticides residue extraction and detection.

A Perkin-Elmer UV-VIS spectrophotometer Lambda 365 controlled by a computer and equipped with a 1 cm path length quartz cell was used for spectral acquisition. The data was acquired using UV express V4.1.0 software. The refrigerated centrifuge was used for centrifugation of plant extracts.

### Extraction of acetyl cholinesterase from honey bees

Honey bees were collected from Bee Garden, Department of Plant Protection, Anbil Dharmalingam Agricultural College and Research Institute, Trichy. After collection, the insects were stored in a deep freezer maintained at -22ºC. One hundred frozen honey bees were selected and the head was decapitated from the body for extraction of AChE. The decapitated honey bee heads were homogenized in 0.25 M sucrose solution (100 mg/mL) using pestle and mortar for 15 minutes. The homogenized solution was centrifuged in cooling centrifuge at 5000 rpm for 1 hour. The centrifuged solution was added with 1 ml of phosphate buffer, KCl, NaCl, glucose and PSA and volume to 100 ml made with 0.25 M sucrose solution and preserved in freezer for pesticide assay.

### Preparation of reagents and inhibiting assay for detecting pesticides

AChE was prepared at 500U/100 mL in a 20mM phosphate buffered solution (PBS, pH 7.4: 1.44 g $Na_2HPO_4$, 0.2 g KCl, 0.24 g $KH_2PO_4$, and 8 g NaCl dissolved in 400 mL distilled water). The 0.4 g of dithiobisnitrobenzoic acid was prepared

by dissolving it in a 100 mL of phosphate buffer.0.2 M acetyl thiocholine was prepared by dissolving 5.7 g in 100 mL of phosphate buffer. All the prepared solutions were refrigerated below -10ºC for further use.

**Standard solutions preparation**

Pesticide standard stock solutions were prepared by dissolving the appropriate amount of the analytes in acetone to obtain a concentration of 100 mg/L for the organophosphate pesticides chlorpyriphos, ethion, profenofos, quinalphos, triazophos, dimethoate and organochlorine compound dicofol. Working solutions of each standard were prepared by diluting the respective stock solution with acetone.

**Evaluation of enzyme based rapid test method**

The reaction was carried out in a 10 mL graduated test tube. 0.5 mL of serially diluted different concentration of pesticide standards were pipetted out into a clean test tube containing 0.5 mL of prepared acetylcholinesterase enzyme and 1mL ATCI. Later, 1 mL of 0.4% of dithio nitrobenzoic acid (Ellman's reagent) was added and the mixure was observed for the colour development.

**Validation of enzyme based colour development method**

Studied pesticides were fortified in pesticide free vegetable samples at different concentrations *viz.,* 0.5, 1.0, 2.0, 5.0 and 10.0 mg/L and allowed to stand for 1 hour. Then the residue was extracted by QuEChERS method and the final residue was re-dissolved in acetone. One mL of extract was added to the test tube containing 0.5 mL of AChE and 1 mL of each ATCI and 0.4% Ellman's reagent. Then colour development was observed in a test tube in comparison with the blank without sample extract and observed for the colour development. Colour intensity was also measured in UV-Visible Spectrophotometer at 412 nm in terms of absorbance to work out the inhibition percentage in comparison with the blank (no insecticide and AChE acted on substrate).

**Analysis of pesticide residue in vegetable samples**

Samples of commercial vegetables were collected and homogenized using a blender. Then 5 g of homogenized samples were transferred to a 50 mL Eppendorf centrifuge tube, 10 mL of acetonitrile (1:2) was added. Then 2 g of anhydrous sodium sulphate and 0.5 g of sodium chloride was also added in order to absorb any water present in the vegetable samples. The sample was hand shaken for 1 minute and centrifuged at 3000 rpm for 2 minutes. The supernatant was collected in another centrifuge tube which contains 0.3 g of primary secondary amine and 1.8 g of anhydrous sodium sulphate. Again, hand shaken for 1 minute and centrifuged at 3000 rpm for 2 minutes. After centrifugation, the supernatant was collected and allowed to dry at room temperature overnight. Diluted to 2 mL using acetone. Diluted sample extracts were taken in a test tube and analyzed as described above.

**Result and discussion**

The higher concentration of the standards (5, 10, 20, 25, 50 and 100 mg/L)of the selected pesticides viz., chlorpyriphos, profenofos, quinalphos, ethion, triazophos, dimethoate belongs to OP group and dicofolof OC group were initially screened for AChE enzyme inhibition test using the commercial source and observed for the development of yellow colour. Then the this rapid colour test was extended to the lower concentrations from 0.01 to 1 mg/L and the results obtained for 10 mg/L pesticides concentration are presented in fig 1. Among the tried insecticides, dimethoate inhibits the AChE and gives bright clear yellow colour, while the profenofos and quinolphos produced lower intensity of yellow colour. Other pesticides viz., chlorpyriphos, triazophos, ethion and dicofol produced turbid yellow colourand the intensity was higher for the chlorpyiphos and lower for ethion. Development of white turbidity by chlorpyriphos and dicofol upon reaction with AChE enzyme and DTNB mixture was also reported by Nandhinidevi (2013) [11] for 500 and 1000 mg/L concentration. Though dicofol is a OC group of pesticides, it also inhibited the AChE enzyme due to the same mode of action as that of OP compounds.

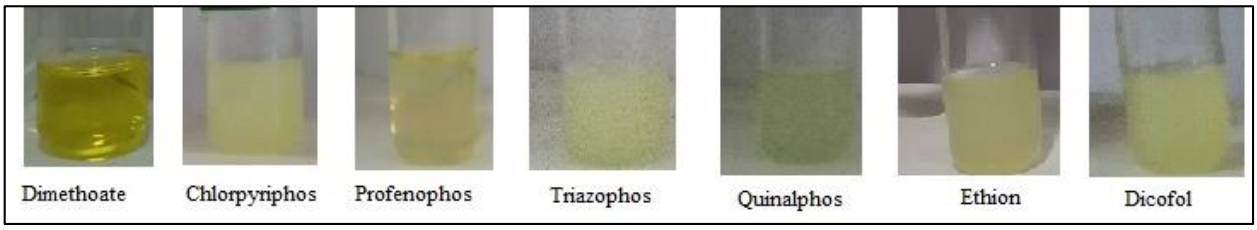


**Fig 1:** Inhibition of acetyl cholinesterase enzyme by OP and OC insecticides (10 mg/L) using Ellman's reagent

The working standard solutions from 0.05 to 10 mg/L of the selected pesticides viz., chlorpyriphos, profenofos, quinalphos, ethion, triazophos, dimethoate and dicofol were tested with rapid AChE enzyme inhibition test using the commercial source and color development was observed. All pesticides were responded well to AChE inhibition upto 1.0 mg/L concentration. The results of yellow colour development and lowest detection level obtained are presented in the table 1. When AChE is mixed with pesticides, it hydrolyses the acetyl thiochloine to yield thiochloine and acetic acid, this thiocholine reacts with DTNB and produces 5-thio-2 nitrobenzoic acid anion which imparts yellow colour (Skerritt, 1998) [2] to the solution. In general, the brilliance of colour decreased with increasing level of concentration. Variation in the colour is attributed to the degree and extent of AChE inhibition by the pesticides.Voss *et al.* (1971) [12] reported that the colour reaction either reduces or stays depending on the incomplete or thorough inhibition of the AChE enzyme by the insecticide and pointed out that the degree to which the reaction proceeds serves as a measure of the amount of the pesticide residue.

**Table 1:** Detection limit obtained for pesticides by AChE inhibition rapid colour test

| Name of the pesticides | Colour of solution | Lowest concentration detected (mg/L) |
|---|---|---|
| Dimethoate | Bright Yellow | 1.00 |
| Chlorpyriphos | Dull yellow | 2.00 |
| Profenofos | Yellow | 1.00 |
| Triazophos | Dull yellow | 2.00 |
| Quinalphos | Dull yellow | 2.00 |
| Ethion | Yellow | 1.00 |
| Dicofol | Yellow | 1.00 |

**Spectrophotometric detection and validation of rapid AChE colour test**

The colour developed by the pesticide residue extracted from fortified samples at different concentrations was measured at 412 nm using the known volume (Fig 2). The reduction in the absorbance in fortified samples were obtained by comparing the fortified samples absorbance with AChE blank and the percent inhibition was worked out using the formula reported by Tran Van An *et al*. (1998) [13]. Results obtained for dimethoate fortification in cauliflower vegetable are presented in Fig 3.

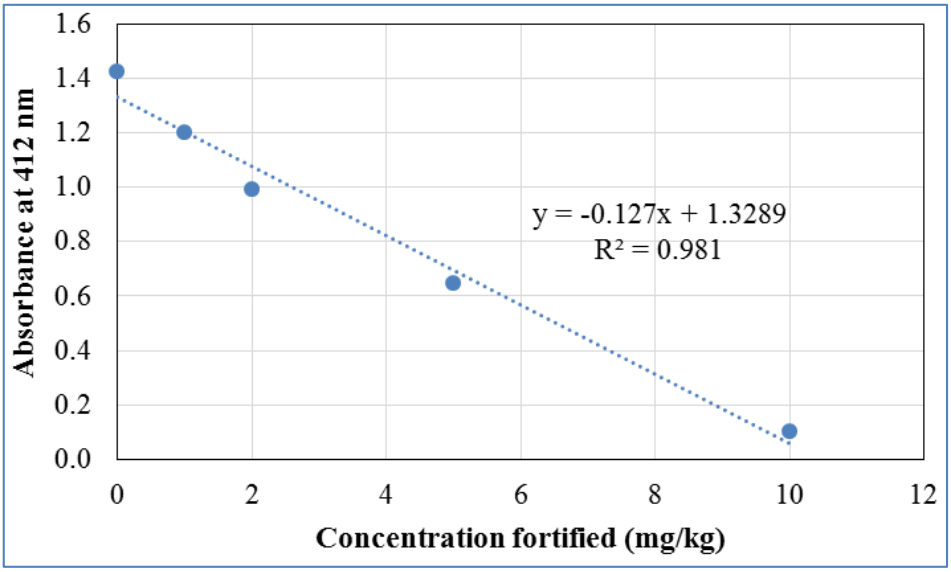


**Fig 2:** Absorbance of AChE inhibition in dimethoate fortified vegetable sample

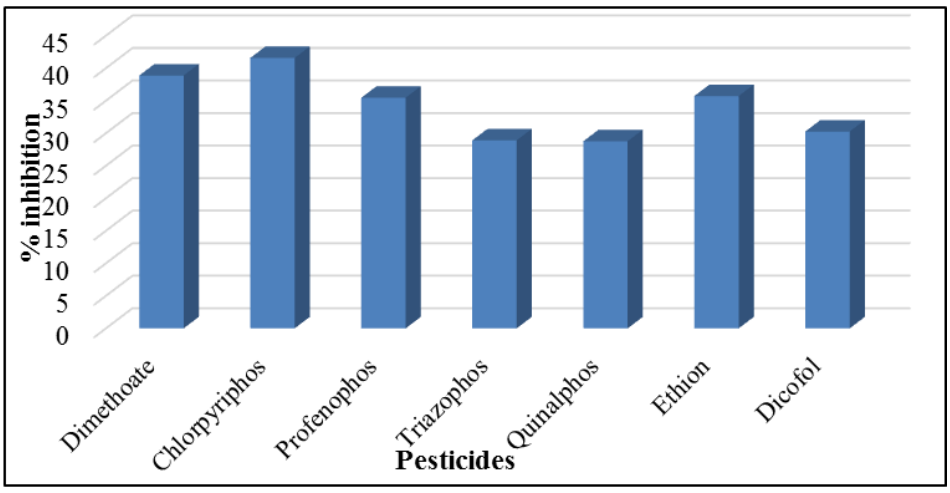


**Fig 3:** Percent inhibition of AChE activity at 2.0 mg/L fortified level in vegetable

It was found that the absorbance decreased with increased concentration of dimethoate in fortified vegetable sample and was owing to the reduced availability of thiocholine to react with DTNB reagent. The gradation of inhibition for 2 mg/L fortified concentration of each pesticide was calculated and found a positive response. This confirms the sensitivity of the evaluated rapid AChE colour test. All the pesticides showed more than 25% inhibition.

**Assay of real samples for residues by rapid AChE colour test**

Different vegetable samples collected from local market were extracted for residues using QuEChERS and detected using the evaluated rapid AChE test and also by GC/MS. The results obtained are presented in table 2. Most of the pesticides were not detected in vegetables; however the profenophos, ethion and quinalphos detected. The

profenophos was detected in both chilli and cluster bean bove 0.2 mg/kg whereas ethion and quinalphos was around 0.1 mg/kg. When the same extracted samples were subjected to AChE enzyme based rapid test, the inhibition of 28.5 and 21.7% was obtained for chilli and cluster bean. This showed that this test can be used to detect the total pesticide residue present in vegetables and only disadvantage is that it can't be used to detect the individual pesticide/concentration. Tran Van An *et al*. (1998) [13] suggested that the samples above 43% inhibition contained residues above MRL and hence those samples with less than 20% inhibition can be used for marketing and those with > 50% should be advised to extend the interval between the pesticide applicaion and harvest of the vegetable.

The results showed that there are many factors influencing the pesticide detection level in vegetables by AChE-DTNB colour test though it is rapid when compared to chromatograph methods. Hence following stringent measures from reagent preparation to colour development is essential to have accurate results and lower sensitivity. Selection of suitable source of acetylcholinesterase is also essential. The methods could be used for semi-quantitative determination of total OP, carbamate and other OC pesticides (of similar mode of action to OP group) residue in vegetables.

**Table 2:** Comparing the residues detected from vegetables by rapid AChE-DTNB inhibition with the GC/MS data

| Vegetable crop | Name of the pesticides | Residue level (mg/kg) detected by GC/MS | % inhibition by rapid AChE-DTNB colour test |
|---|---|---|---|
| Chilli | Dimethoate | ND | 28.5 |
| | Chlorpyriphos | ND | |
| | Profenofos | 0.442 | |
| | Triazophos | ND | |
| | Quinalphos | 0.086 | |
| | Ethion | 0.146 | |
| | Dicofol | ND | |
| Cluster bean | Dimethoate | 0.078 | 21.7 |
| | Chlorpyriphos | ND | |
| | Profenofos | 0.284 | |
| | Triazophos | ND | |
| | Quinalphos | 0.099 | |
| | Ethion | 0.189 | |
| | Dicofol | ND | |

**Performance of the AChE prepared from honey bees**

The AChE extracted from honey bee heads were used to detect the different concentrations of each studied pesticides and as well as the residue extracted from fortified vegetables. Though the detection level of 2 mg/L was achieved with this source of enzyme, turbidity was more and obtained higher absorbance for each concentration when compared to the commercial source of AChE. This showed that the type of enzyme source is also important for achieving good sensitivity and accuracy for residue detection using this method. Further refinement of the AChE extraction procedure from honey bee is essential for its applicability to assay the pesticide residues in real samples.

**Conclusion**

The evaluated AChE-DTNB colour test is rapid to detect the pesticides particularly the organophosphorous and OC compounds having similar mode of action. The test is sensitive to detect the residue concentration of 2 mg/L in vegetables which can't be used to meet the MRL's of different agencies like FAO, WHO, FSSAI etc. However, this test can be considered for the rapid detection of total pesticides residue in vegetables for screening and making decision to market and consume the produce or not. The positive samples can be assayed in chromatographic instruments for quantification and used as a complement technique. Though the evaluated method is rapid, transfer of this test technique to simple paper based rapid test could be a cost effective and viable option which needs to be evaluated in future. Application of this test for residue detection in fruits, cereals, packed food products etc., should also be evaluated in future for real time assessment of total residue level in food chain and contamination to the public health.

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