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Research Article

# Acetylcholinesterase-Based Paper Bio-Sensor Assay for the Quick, Cheap and On-Site Detection of Organophosphorus and Carbamate Pesticides in Agricultural Products for the First Time in Iran

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## Abstract

Dramatic increase in pesticide use in agriculture necessitates continuous monitoring and tracking of their residues on the products on the spot to assure the safety of the consumers. At present measurement of pesticide residues on agricultural products is time consuming and costly. Therefore, quick, on-site, simple and cheap methods of measurement of the pesticide residue levels is necessary. In this research by making a paper-based bio-sensor a fast, easy and cheap method to detect organophosphorus and carbamate pesticides by inhibiting the activity of the enzyme acetylcholinesterase was established. The sensor is a device composed of a foldable polyethylene sheet with two separate detection sheets one containing acetylcholinesterase and the other Indoxyl acetate as the substrate prepared and fixed on the strip. Hydrolysis of Indoxyl acetate by the enzyme causes formation of blue color in the test zone and the absence or reduction of the blue color formed indicates the presence of organophosphorus and carbamate pesticides in the sample. By incubating the sample, enzyme and the substrate on the test zone for 10 minutes there will be color formation on the zone. The absence or decrease in the color formed makes it possible to visually determine whether there is any pesticide residue in the sample. The results obtained by using six concentrations (0.1, 0.5, 1, 5, 10 and 25 ppm) of the pesticides Diazinon, Trichlorfon, Carbofuran and Pirimicarb spiked into organic lettuce and rice samples indicate good performance of this bio-sensor for the detection of the residue of the four pesticides under test. The limit of detection (LOD) estimated to be 0.5, 0.1, 0.1 and 0.1 ppm in lettuce and 0.1, 0.1, 0.1 and 0.1 ppm in rice for Diazinon, Trichlorfon, Carbofuran and Pirimicarb respectively. In experiments performed on lettuce and rice samples with unknown levels of pesticide contamination, a comparison was made with the measurement of pesticides residue by the bio-sensor and the liquid chromatography equipment (LC-MS/MS) simultaneously. The results obtained visually by the paper sensor showed high sensitivity and good reproducibility compared with the results obtained by the LC-MS/MS, and it can be acknowledged that the paper-based bio-sensor impregnated with acetylcholinesterase can be a quick, cheap, easy to use, on-site and outside the laboratory alternate method for monitoring and measuring organophosphorus and carbamate pesticide residues in agricultural products.

**Keywords:** Pesticide Residue; Bio-Sensor; Acetylcholinesterase; Quick and On-Site Method; Organophosphates; Carbamates

## Introduction

The indiscriminate use of pesticides and chemical fertilizers in order to increase the yield of agricultural products, has put the world at a great risk of contamination of food products and the environment. Without pest control, 56 to 73% of agricultural products are destroyed, while using different pest control methods, this damage can be reduced to 40 to 60%. Among pest control methods, the use of pesticides, whether as a unique method or in the form of integrated pest management programs, is of particular importance. More than 50% of the pesticides used in the ecosystem do not reach the target plants, and enter the environment in various ways. Some of the pesticides applied remain on the sprayed plants, which is considered a dangerous residue if this amount is higher than the known permitted limits after a certain period of time. This is especially true for products that are consumed fresh (such as cucumbers, tomatoes, and other vegetables). According to the official statistics, during the years 1986 and 1991, about 35,000 to 60,000 tons of different types of pesticides were used annually in the agricultural sector in Iran [1]. The amount of pesticide application in 2020 has reached about 26 thousand tons. Research has been done on the determination of pesticides residue levels in various products and the environment in different parts of the country. There

has been a lot of research in the world regarding the measurement of pesticide residues in the environment or agricultural products. Over a five-year period in Ontario, Canada, pesticide residues were measured in 1,536 vegetable and 802 fruit samples. These studies showed that 31.5% of the samples did not contain pesticide residues but in 68.5% of the samples there was one or more pesticide residues. In 48% of the samples, pesticide residue level was less than 0.1 mg/kg and in 86% of them it was less than 1 mg/kg [2]. In a study Viana., *et al*. measured pesticide residues in 48 samples of vegetable, and the highest pesticide residues were observed in ten samples with values ranging from 7 ng/g to 40 ng/g [3]. Kazar Soydan studied 3044 fruits and vegetables in Turkey and found 354 samples had higher than the MRLs and 473 samples had residue levels below the MRLs [4]. Evaluation of pesticide residues in fruits and vegetables from Algeria showed that out of 160 samples of 13 types of fresh fruits and vegetables analyzed 42.5% had no residues and 12.5% of the samples contained pesticide residues above maximum residue limits [5]. In a surveillance of multi-pesticide residues of fruits and vegetables in markets of Botswana a total of 83 fruit and vegetable were examined and the levels of pesticides ranged between 0.0032 ± 0.0009 mg/kg and 70.4 ± 19.4 mg/kg. Only 13% of the samples violated the EU/Codex MRLs [6]. A total of six vegetable samples, 3 fruit samples, 7 soil samples and 6 water samples was examined for pesticides residues in Pakistan and the pesticides having the highest health risk in vegetables were determined to be Bifenthrin and Difenoconazole and in fruits it was Amamectin, bifenthrin and difenoconazole and the same pesticides were detected in soil and water too [7]. In Iran, Imani studied the residue levels of eight types of pesticides from different groups in tomato and cucumber by multi-residue analysis and showed that the residue levels of diazinon, chlorpyrifos, phosalone, dichlorvos, carbaryl, permethrin and fenpropathrin in cucumber reached the permissible levels after 9, 12, 12, 9, 7, 5 and 4 days after spraying, respectively, and in tomato samples the residue levels of diazinon, chlorpyrifos, and phosalone reduced to the permissible levels after 8, 10, and 11 days, respectively [8]. Hadian and Azizi in research on 30 samples of cucumber, tomato, cabbage and lettuce detected 117 pesticides of different groups using GC/ITMS. In these samples 53.33% of them contained insecticide residues [9]. The pesticide residue levels of these pesticides were much lower than those recommended by FAO/Codex and the European Union standards. In a study on 16 cucumber samples from 4 greenhouses in Varamin region, Morowati., *et al.* observed contamination of the samples with imidacloprid residue in 14 samples (87.5%). The mean residue level in 3 greenhouses was higher than the MRLs [10]. In another study, the residues of four insecticides dichlorvos, deltamethrin, imidacloprid and pymetrozine were studied in greenhouse cucumber samples in four cities of Isfahan province. The results showed that overall, 35 to 45% of the samples in each city contained residue levels higher than the MRLs [11]. Nikan and Morowati examined the residue levels of 9 pesticides including chlorpyrifos, imidacloprid, dichlorvos, malathion, diazinon, pirimicarb, tetradifon, bromopropylate and metalaxyl in 39 greenhouse cucumber samples collected from Hamedan province. The results showed that 37.5% of the samples were contaminated above the maximum residue limits [12]. In another study the residue of diazinon was investigated on forty cherry samples collected from the central fruit and vegetable market of Tehran, which were produced in five different cities of Lavasan, Shahriar, Qazvin, Mashhad and Urmia, the results showed that 10% of the tested samples had higher residue levels than the national MRL [13]. Pesticides can cause numerous disorders such as neurological diseases like Parkinson's [14,15], cancers in children and adults [16], miscarriage, birth defects and pathological lesions in various tissues and organs of the body [17,18]. Experiments have shown that there is a direct relationship between 2-4D and NeemAzal-T/S levels and reduction in sperm count and an increase in deformed sperms [19-21]. Therefore, determining the residue levels of pesticides in agricultural products and comparing them with the maximum residue limits is very important in terms of human health and safety. Currently, the highest consumption of pesticides includes two major groups of pesticides namely organophosphates and carbamates and their toxic effects are caused with inhibition of acetylcholinesterase (AChE) in nervous system, which leads to the accumulation of acetylcholine neurotransmitter at the synapses [22,23]. This inhibition causes acute toxicity with symptoms like headache, increased salivation, seizures, shortness of breath and eventually death. At present, sampling and measurement of pesticide residue levels in agricultural products can take about 2 to 3 days and is expensive and requires advanced and sophisticated devices such as GC/MS and LC/MS [24-26]. Although these methods are very accurate and sensitive, but the high cost, skilled technicians and time-consuming preparation methods. In recent decades, many rapid pesticide determination methods based on electrochemical and optical biosensors have been developed and studied as alternative methods for detection of pesticide residues [27-35], however, these biosensors also require experienced technicians and various devices to be used and are not very suitable for on-site testing in places such as farms, markets and other places outside the laboratory. Other methods have been used based on immune-chromatography in which specific antibodies are made for some pesticides, which unfortunately due to the limited ability to identify pesticides and the lack of detection of unknown pesticides and the high cost of antibodies have proved to be not very practical [28,36].

Therefore, the need for a rapid, on-site, simple and low-cost pesticide residue determination method is essential. The current device designed in the present research creates a fast, easy and low-cost method for tracing organophosphorus and carbamate pesticides by inhibiting the activity of AChE on paper strip and formation of color by the hydrolysis reaction catalyzed by this enzyme with different chromogenic chemicals [37-40] for the first time in Iran.

## Materials and Methods

filter paper was used as a base for the enzyme AChE and its substrate indoxyl acetate, according to figure 2. In this method, hydrolysis of indoxyl acetate to indigo by acetylcholinesterase produces blue /green color during the process as shown in figure 1.

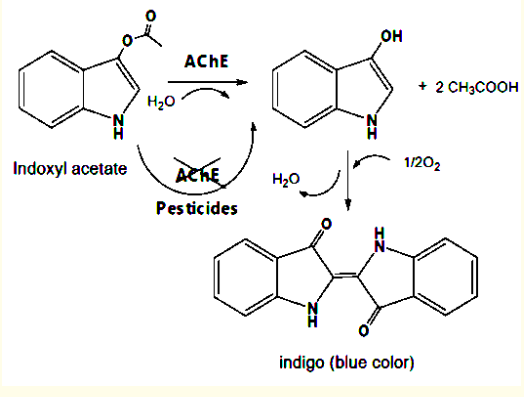



**Figure 1:** Mechanism of hydrolysis of indoxyl acetate catalyzed by the enzyme acetylcholinesterase.

For this purpose, a folded polypropylene plastic strip is used as a small folder. To prepare polypropylene strips, 2 × 10 cm strips are cut and folded in the middle and the two ends are put on top of each other, then two 6 mm diameter punches are made at both the ends of this strip. The punch is used to add buffer during incubation to avoid dryness of the test zone. At one end of the strip a circular Whatman No. 1 filter paper (detector) with a diameter of 6 mm containing the immobilized enzyme, and at the other end a square filter paper measuring 10 × 10 mm containing the substrate was placed. The filter papers were fixed in place by double-sided adhesive tape, which were previously placed on both sides of the strip, and by folding it the tips of the strip would stick together (Figure 2). To prepare the filter papers, five microliter of the enzyme acetylcholinesterase was placed on the circular filter paper and were kept at room temperature for one hour to dry. The papers were then placed in a blocking solution at room temperature for 20 minutes to prevent non-specific binding of other materials and then placed in a washing solution for 30 minutes and dried at room temperature for 2 hours. These enzyme-containing papers were stored in air tight plastic bags at -20°C until use. For the substrate, 20 µl of indoxyl acetate was placed on the square filter papers and after drying, it was placed in air tight plastic bags at -20°C until use. To measure the pesticides residue by the paper-based bio-sensor strips, square and circular filter papers, were mounted on the tips of strip which were punched. To evaluate the efficiency of enzyme and substrate activities, after installing enzyme-impregnated filter papers and indoxyl acetate, to activate the enzyme, 10 µl of phosphate/saline buffer was dropped on it and allowed to incubate for 10 minutes at room temperature. Then the two edges of the tape were glued together and two circular and square filter papers containing enzyme and indoxyl acetate were put into contact with each other and then to avoid the test zone to get dried 10 µl of the phosphate/saline buffer was dropped twice at an interval of 5 minutes until the completion of the reaction. The color produced by the reaction was then examined. This color appears as blue/green and the intensity of the color created is as the result of the activity of AChE, which means that the more the intensity of the color produced, the more enzyme activity and the paler the color, lower is the activity of the enzyme. In the next step, the effect of the pesticides under test in preventing the activity of the enzyme, was investigated in the following three steps

- Adding a drop of the extracted agricultural sample or pesticides standard solution on to the base containing the enzyme (test zone) and incubating for 10 minutes at 30-40 ° C.
- Folding the strip and placing the substrate containing side in contact with the lower part containing the enzyme, and adding buffer from the hole installed on top of it twice at an interval of 5 minutes.
- Opening the folder after 10 minutes of incubation and observing the color intensity formed (Figure 2).

### Chemicals

The enzyme Acetylcholinesterase (EC 3.1.1.7, *Electrophorus electricus*), indoxyl acetate, sodium lauryl sulfate and casein, four pesticide standards including two organophosphate insecticides (diazinon and trichlorfon) and two carbamate insecticides (carbofuran and pirimicarb) were purchased from Sigma-Aldrich Company. Other chemicals such as methanol, sodium hydrogen orthophosphate, sodium dihydrogen phosphate was purchased from Merck. Boric acid and sodium dodecyl sulfate were purchased from Bioscience company. In this experiment, Whatman filter paper No. 1 and polypropylene sheets were used to make the bio-sensor strip.

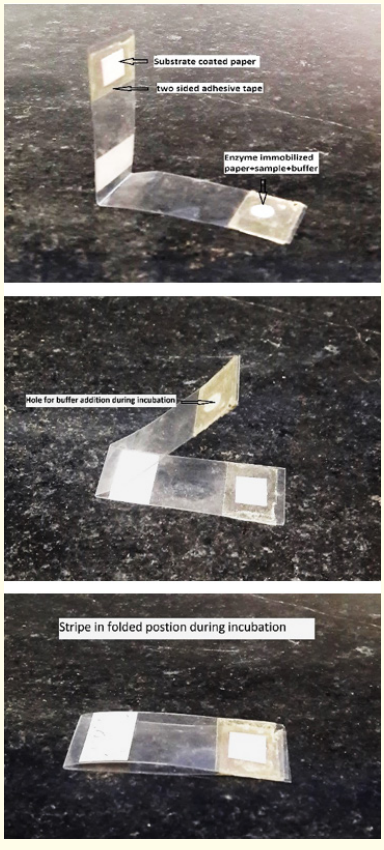



**Figure 2:** Paper-based bio-sensor strip and the location of enzyme and substrate impregnated papers.

## Preparation of reagents

The enzyme acetylcholinesterase was prepared at a concentration of 800 units/ml in phosphate/saline buffer (20 mM, pH 7.4). The substrate indoxyl acetate was prepared with a concentration of 5 mg/ml in methanol and the blocking solution was prepared by boric acid buffer (50 mM, pH 8.3) with 0.5% (w/v) of casein, and the washing solution was made with phosphate buffer (50 mM, pH 7.5) and 0.01% (w/v) sodium dodecyl sulfate. The pesticides stock solutions (1000 mg/L) were prepared in methanol. 20 mM phosphate/saline buffer was used to dilute and make different concentrations of these standards. Eight concentrations of pesticide standard solutions (0.01, 0.05, 0.1, 0.5, 1, 5, 10 and 25 mg/l) were prepared and used and six concentrations (0.1, 0.5, 1, 5, 10 and 25 mg/l) were used for spiking rice and lettuce samples.

## Measurement of pesticides residue levels in rice and lettuce samples

The ability of the paper-based bio-sensor in detecting and measuring the pesticides residue levels was assessed in lettuce and rice samples. Different concentrations of pesticides under test (0.1, 0.5, 1, 5, 10 and 25 mg/kg) were spiked to the pesticide-free samples of ground and mixed lettuce and rice at a rate of 1 ml/g. The spiked samples were then refrigerated for 24 hours and the next day 1 g of each sample was extracted by placing them in phosphate buffer (20 mM PBS) and phosphate buffer (20 mM PBS) plus 20% methanol separately, for 10 minutes. The extract was then filtered and was tested by the paper-based bio-sensor to detect and measure the pesticides residue. Finally, 5 and 7 unknown samples of lettuce and rice were purchased from the market and tested after extraction by PBS buffer plus methanol by bio-sensor strips, respectively. The results were compared with the samples extracted by QuECheRS method and analyzed by LC-MS/MS.

## Processing and interpretation of the data

For qualitative detection of the pesticide residues, the color formed by the reaction of AChE and indoxyl acetate can be observed by naked eye. For semi-quantitative studies, the intensity of color produced was evaluated colorimetrically by ImageJ software (ImageJ 1.45s - National Institute of Health, U.S.A.) by taking a digital image of the bio-sensor strips after testing. The color intensity created in the RGB channel of this software was used as the level of enzyme activity. Thus, the average color intensity created in the reaction zone (Test Zone) was subtracted from the background color and the amount obtained was considered as the amount of enzyme activity and by this data the calibration curves were plotted.

## Results

The sensitivity of the hydrolysis of indoxyl acetate by AChE is affected by various factors, including concentration of the enzyme AChE and its substrate indoxyl acetate, the moisture content of the test zone on the paper and the incubation time of the reaction. Therefore, studies were performed to select the optimal conditions. The hydrolysis is represented as background-subtracted intensity determined by digital-image analysis using ImageJ. Insets represent results at testing zone. Data are the means (± SD) of 3 independent measurements (Figure 3-7).

## Optimization of the concentration and amount of AChE

Different concentrations of AChE from 5 to 1000 units/ml were selected and 5 µl of the enzyme was placed on the round filter paper and, 20 µl of indoxyl acetate (5 mg/ml) on the square paper and after folding of the strip, 10 µl of buffer (20 mM PBS) was added and incubated for 10 minutes so that the color of the hydrolyzed indoxyl acetate by the enzyme was formed. The color intensity increased with increasing the enzyme concentration. By increasing

this amount beyond 800 units/ml, the color intensity remained almost constant and unchanged. Therefore, 800 units/ml of AChE was selected for the experiments (Figure 3).

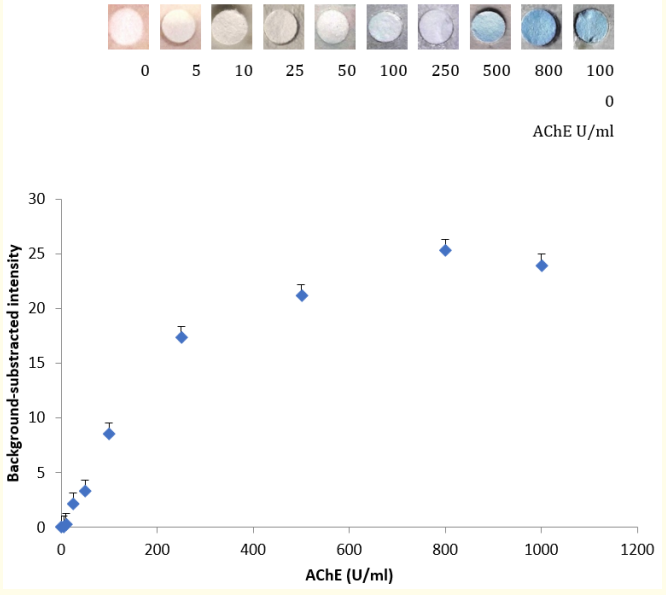



**Figure 3:** Effect of different concentrations of AChE (U/ml) on hydrolysis of 20 µl of indoxyl acetate (5 mg/ml).

In order to optimize the quantity of the enzyme AChE for the experiments, 5 and 10 microliters were used for the experiments. The results showed good performance of both the quantities and the amount of color obtained in both the volumes were clear and sufficient. Although the amount of color intensity produced in the paper-based bio-sensor containing 10 µl was higher (Figure 4. a and b), but due to its higher volume, some of it was spread around the test zone, hence, in order to save the amount of enzyme and to avoid spreading around, in all subsequent experiments, 5 µl of enzyme was used for each strip.

## Optimization of the concentration of indoxyl acetate

The optimal concentration of indoxyl acetate as a substrate was investigated using concentrations of 1 to 30 mg/ml at a rate of 20 µl. The color intensity increased with increase in the concentration of indoxyl acetate up to 5 mg/ml. At concentrations more than 5 mg/ml, there was no significant increase in the color intensity. Therefore, this concentration of the substrate was used as the optimal concentration in subsequent tests (Figure 5).


**Figure 4:** Effect of 5 µl (a) and 10 µl (b) of AChE on hydrolysis of indoxyl acetate.

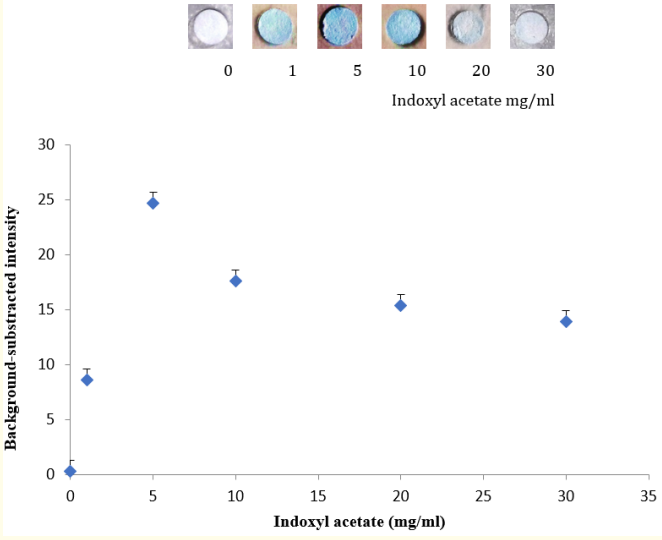



**Figure 5:** Effect of different concentrations of indoxyl acetate (mg/ml) hydrolyzed by 5 µl of AChE (800 units/ml).

## Optimal number of times of buffer addition

One of the weaknesses of this method is the drying of the reaction zone of the enzyme and the substrate during the reaction. As a result, due to this dryness, the reaction is limited. Therefore, the wet system was developed in this method to facilitate the enzyme and substrate reaction. To create this moisture, buffer droplets were added at the reaction zone through a hole made at the top layer of strip to facilitate the reaction. The results showed more color intensity by addition of buffer droplets at a 5-minute interval between them (Figure 6).

Adding the buffer twice at the reaction site produced the most appropriate color intensity, indicating the reaction of the enzyme and the substrate, and further addition did not increase the color. Therefore, the method created by adding buffer during the reaction

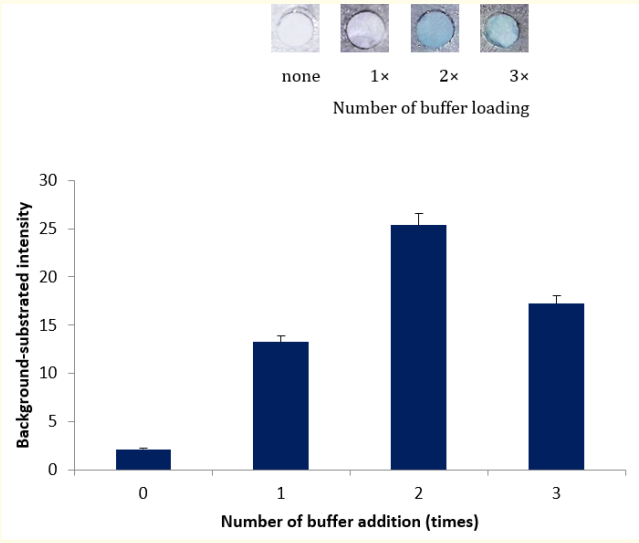



**Figure 6:** Effect of the number of times 10 µl of buffer was added to the reaction zone in the wet system.

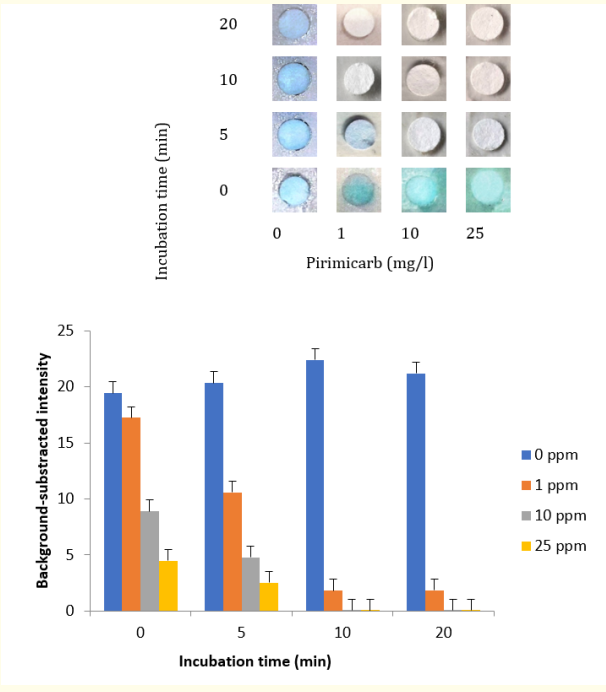



**Figure 7:** Effect of the incubation time of enzyme, pesticide and substrate reaction.

has a significant role in performance of the reaction between the enzyme and the substrate and is a strong point compared to other similar methods.

## Optimization of the incubation time for the reaction

For this purpose, pirimicarb was used to determine the appropriate incubation time to evaluate the inhibitory efficiency of AChE enzyme by the OP and CM pesticides. 10 µl of 0, 1, 10 and 25 mg/l of this insecticide were added to the test zone and were incubated for 5, 10 and 20 minutes. During the incubation period PBS buffer was added to the test zone twice from the hole at the top of the strip to ensure sufficient moisture at the zone. The results showed a decrease in color intensity by increasing the concentration of pirimicarb up to 10 minutes and further no changes were observed at the reaction zone. In the control strip with no pesticide also, increase in incubation time showed an increase in color intensity up to 10 minutes and later than that no significant change was observed. Therefore, 10 minutes was chosen as the optimal time of incubation (Figure 7).

## Performance of paper-based bio-sensor for detection of the pesticides

After optimization of the test conditions, to evaluate the performance of this strip for pesticide detection, different concentrations of OP (diazinon and trichlorfon) and CM pesticide (carbofuran and pirimicarb) standards were used. For this purpose, eight concentrations (0.01, 0.05, 0.1, 0.5, 1, 5, 10 and 25 mg/l) of the pesticide standards were prepared in PBS buffer. Tests were performed and the color intensity formed was examined. The blue color created at the reaction zone decreased dramatically with the increase in the concentration of pesticides and could be observed with the naked eyes within 10 minutes (Table 1).

According to the images obtained, the limit of detection (LOD) of diazinon, trichlorfon, carbofuran and pirimicarb are estimated to be 0.1, 0.1, 0.05 and 0.01 mg/l respectively. The difference between the observed sensitivities among these pesticides is due to the difference in the biomolecular rate constant of AChE inhibition in the pesticides.

Although for qualitative analysis the color generated by the reaction of AChE and indoxyl acetate can be observed by the naked eyes, for a semi-quantitative measurement of the pesticide residues, color intensity at the reaction zone was photographed by digital camera and analyzed for colorimetry by ImageJ software. The color signal at the reaction zone was analyzed by measuring the mean intensity in the RGB channel. The mean intensity value of each test zone was obtained by subtracting the intensity from that of the background. The background subtracted intensity values obtained was plotted against various concentrations of the pesticide standards under test to get the calibration curves. (Figure 8-11). In this method, the limit of detection is calculated and shown by IC50, which means the amount of pesticide that inhibits 50% of the activity of the enzyme AChE. The LODs calculated by the graphs were 0.1, 0.109, 0.044 and 0.057 mg/L for diazinon, trichlorfon,

carbofuran and pirimicarb, respectively, which are approximately consistent with the visual limits of detections except for pirimicarb (Table 1). These results indicate a relatively good performance of visual detection compared to the semi-quantitative residue determination.

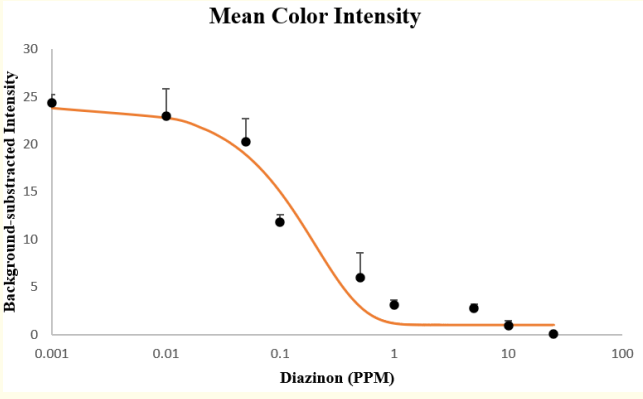



**Figure 8:** Mean color intensity values calculated by the analysis of digital images using ImageJ software against different concentrations of diazinon. The curve is fitted by the Multistage equation. Data are the means (± SD) of three independent measurements.

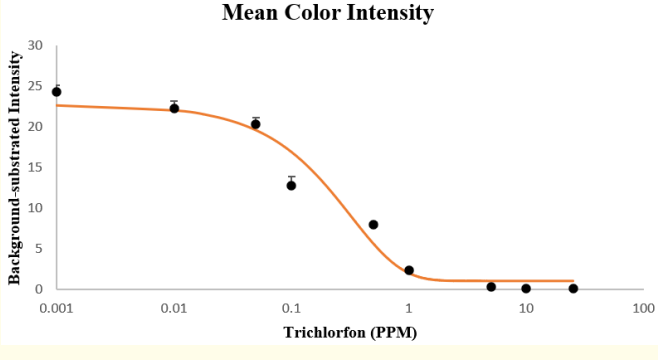



**Figure 9:** Mean color intensity values calculated by the analysis of digital images using ImageJ software against different concentrations of Trichlorfon. The curve is fitted by the Multistage equation. Data are the means (± SD) of three independent measurements.

## Tracking and detection of OP and CM residue levels in food samples

Lettuce and rice were used as food samples to evaluate the performance of the paper-based bio-sensor in tracking pesticides in food products. For this purpose, samples of organic pesticide-free lettuce and rice were used and spiked with 0.1, 0.5, 1, 5, 10 and 25 mg/kg of the four pesticides. The samples were tested by paper-based bio-sensor under optimal conditions (Table 2) and

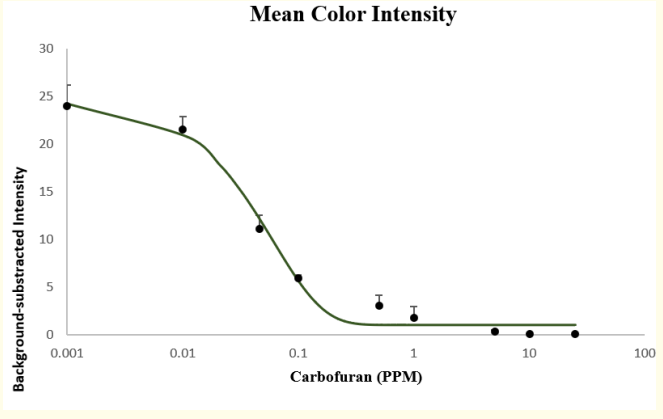



**Figure 10:** Mean color intensity values calculated by the analysis of digital images using ImageJ software against different concentrations of Carbofuran. The curve is fitted by the Multistage equation. Data are the means (± SD) of three independent measurements.

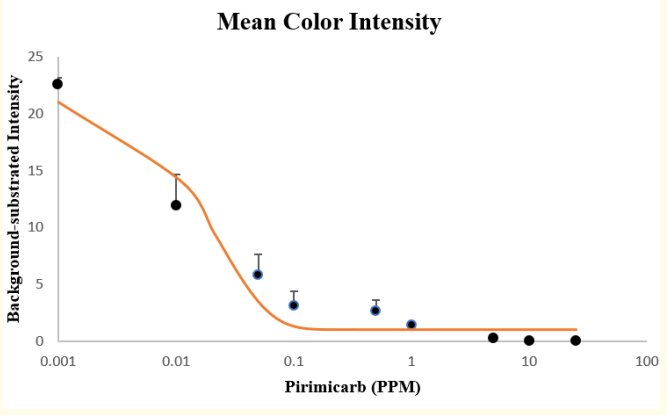



**Figure 11:** Mean color intensity values calculated by the analysis of digital images using ImageJ software against different concentrations of Pirimicarb. The curve is fitted by the Multistage equation. Data are the means (± SD) of three independent measurements.

after quantifying the color intensity by ImageJ software, the calculated values of color intensity in the spiked samples (Ispiked) were subtracted from the color intensity created in organic samples free from pesticides (Iblank) and the result obtained as Delta I (ΔI = Iblank - Ispiked) was plotted against different concentrations of the pesticides (Fig. 12, a and b). The value of ΔI increased with increase in pesticide concentration and the change in color intensity was also clearly visible visually (Tables 2, a and b). According to several studies, to create more sensitivity in the paper-based bio-sensor, extraction and purification by PBS buffer (20 mmol) plus 20% methanol with pH 7.4 was used, which was found to perform better extraction because of the presence of methanol. In order to further

improve the sensitivity of the paper bio-sensor, pre-concentration method was used, too. In this method, addition of 3, 5 and 15 times of the sample to the test zone before starting the activity of the enzyme and substrate was considered. This number of sample additions can help the analyte accumulation at the test zone and ultimately increase the sensitivity of the paper bio-sensor to detect and measure the pesticides residue more accurately. The problem with pre-concentration is that if the addition of the samples is done consecutively, it will cause the sample to spread to the area adjacent to the test zone and the surroundings. Therefore, to prevent this problem, each time the sample was added to the test zone, this area was dried on a hot-plate at 37-40º C and then a new drop of the sample was added to the zone. In these studies, up to 5 times addition of the sample increased the sensitivity of the sensor to detect a concentration of 0.1 mg/kg of the pesticides, which is equivalent to the lowest MRL for these pesticides and the products tested. The pre-concentration operation sometimes increases the test time to 40 minutes. However, to increase the sensitivity of the bio-sensor, sample extraction can be done by QuECheRS method, too. Due to the fact that the cost of QuECheRS extraction method is higher and it is also more time consuming, it is not cost-effective for out-of-laboratory evaluations. However, for samples that are analyzed in the laboratory, QuECheRS method of extraction can be used and therefore, increase the sensitivity. Since the extraction of the samples with buffer along with methanol, makes it possible to measure the pesticides residues up to 0.1 mg/kg (the LODs obtained visually in Table 2), it is good enough to use it for daily tasks outside the laboratory. It is also relatively more cost effective than the QuECheRS method.

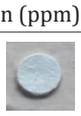
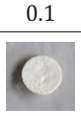
| Colorimetric observations | LOD | National MRLs ppm |
|---|---|---|
| Diazinon (ppm) | 0.1 ppm | |
|  0  0.01  0.05  0.1  25  0.5  1  5  10 |  0.1  0 | 0.3- 2 |
| Trichlorfon (ppm) | 0.1 ppm | |
|  0  0.01  0.05  0.1  25  0.5  1  5  10 |  0.1  0 | 0.1- 1 |
| Carbofuran (ppm) | 0.05 ppm | |
|  0  0.01  0.05  0.1  25  0.5  1  5  10 |  0.05  0 | 0.05 |
| Pirimicarb (ppm) | 0.01 ppm | |
|  0  0.01  0.05  0.1  25  0.5  1  5  10 |  0.01  0 | 0.1- 3 |

**Table 1:** Determination of Limit of Detection of the paper-based bio-sensor for diazinon, trichlorfon, carbofuran and pirimicarb. Visual images of AChE inhibition with various concentrations of test pesticides are shown in the table. the results shown are of three replications in the experiment.

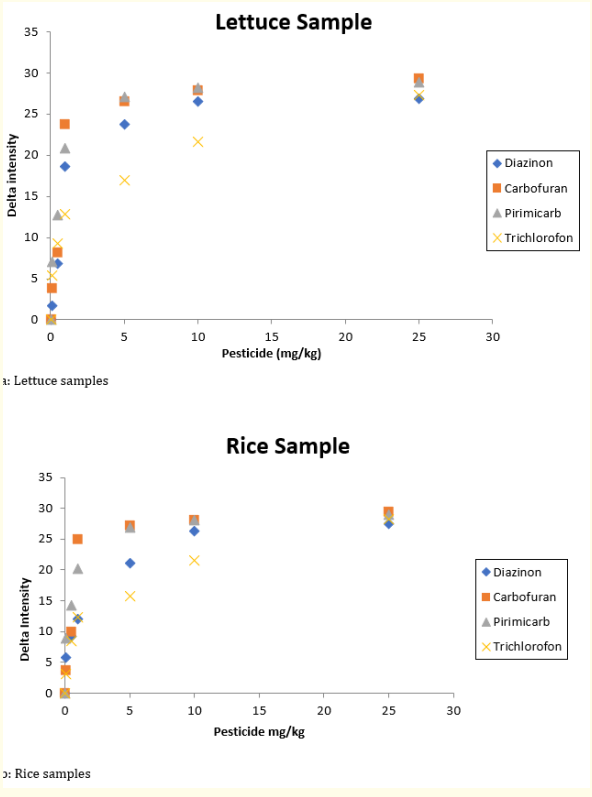



**Figure 12:** Detection of pesticide residues at different concentrations spiked into the organic lettuce (a) and rice (b) by paper-based bio-sensor strip based on inhibition of the AChE activity by measuring the mean intensity (Δ intensity) of the test zone on paper-based bio-sensor versus different pesticide concentrations.

Considering the national MRLs (http: //www.iripp.ir/HomePage), the LODs obtained by the paper-based bio-sensor is acceptable and practical for on-site and rapid monitoring of the pesticide residue levels in the agricultural products.

Table 2 (a and b) shows the activity of the enzyme AChE in the presence of six different concentrations of diazinon, trichlorfon, carbofuran and pirimicarb spiked into the pesticide-free lettuce and rice. Significant reduction in color intensity resulted from the reaction of AChE and substrate indoxyl acetate is observable at the concentration of 0.5 mg/kg diazinon spiked lettuce and 0.1 mg/kg in the samples spiked with the remaining pesticides. There is a decrease in color intensity with increase in pesticide concentrations and at concentrations of 5, 10 and 25 mg/kg almost no color is observed indicating no activity of AChE. However, in spiked samples of rice, a decrease in the color intensity is observable visually at the concentration of 0.1 mg/kg, and in the spiked samples with concentrations of 1, 5, 10 and 25 mg/kg, a drastic decrease in the color intensity is evident. To facilitate detection of pesticide residue levels outside the laboratory where no image can be taken or where the ImageJ software may not be available and it could be time consuming to do so, for semi-quantitative measurement of the pesticide residue levels, the color intensities obtained were categorized into 4 levels which could show the approximate pesticide residue levels visually. By comparing the color intensity created in the paper-based bio-sensor with these four levels, the pesticide residue levels could approximately be determined. Level 1 shows the lowest amount of color formation and the highest amount of pesticide residue, which is more than 5 mg/kg, and level 2 shows the residue level of about 1 to 5 mg/kg, Level 3 indicates the presence of approximately 0.1 to 0.9 mg/kg of pesticides residue concentration and level 4 indicates the lowest residual amount or below the detection limit, i.e. approximately no pesticide residue (0 to 0.09 mg/kg) (Figure 13). The advantage of this method is speed, low cost and the ability to perform the test on-site at the farm or central market.

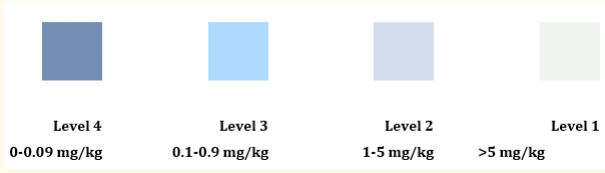



**Figure 13:** Different levels (1 to 4) of color produced in the presence of different concentrations of pesticides in spiked lettuce and rice samples for quick and visual estimation of pesticides residue.

To evaluate the efficiency of the paper-based bio-sensor strip 5 unknown lettuce and 7 unknown rice samples were collected from the market and extracted and purified by the above-mentioned method for the evaluation of the pesticide residue levels and compared the results of the same samples which were extracted and purified by QuEChERS method and analyzed by LC-MS/MS. The residue levels of the pesticides under test in the unknown samples of lettuce and rice purchased from the market analyzed and measured by the LC-MS/MS are compared with the color generated using paper-based bio-sensor in the following table. To estimate the residue levels quantitatively, four levels of the color intensity created in figure 13 was used.

The above results indicate the relatively good accuracy of the paper-based bio-sensor compared to the results obtained by measuring the pesticides residue in the lettuce and rice samples by the QuEChERS method and the LC-MS/MS. According to the proposed color leveling created the extent of contamination of the products with pesticide residues can be visually estimated at an acceptable level (Tables 3 and 4).

| Lettuce samples | Diazinon ppm | Trichlorfon ppm | Carbofuran ppm | Pirimicarb ppm | Level | Bio-sensor |
|---|---|---|---|---|---|---|
| L1 | 1.6 | ND | ND | ND | 2 |  |
| L2 | 1.07 | ND | ND | ND | 2 |  |
| L3 | 3.72 | ND | ND | ND | 2 |  |
| L4 | 0.95 | ND | ND | ND | 3 |  |
| L5 | 0.05 | ND | ND | ND | 4 |  |

**Table 3:** Pesticides residue levels measured by LC-MS/MS in lettuce samples and its comparison with the results of paper-based bio-sensor and levels created for the pesticide residues.

| Rice samples | Diazinon ppm | Trichlorfon ppm | Carbofuran ppm | Pirimicarb ppm | Level | Bio-sensor |
|---|---|---|---|---|---|---|
| R1 | 0.8 | ND | ND | ND | 3 |  |
| R2 | 1.46 | ND | ND | ND | 2 |  |
| R3 | 1.61 | ND | ND | ND | 2 |  |
| R4 | 0.85 | ND | ND | ND | 3 |  |
| R5 | ND | ND | ND | ND | 4 |  |
| R6 | 1.69 | ND | ND | ND | 2 |  |
| R7 | 0.89 | ND | ND | ND | 3 |  |

**Table 4:** Pesticides residue levels measured by LC-MS/MS in rice samples and its comparison with the results of paper-based biosensor and levels created for the pesticide residues.

## Discussion and Conclusion

Paper-based bio-sensor is almost a new technology that includes a complete measurement method performed on paper strips [38-41]. Such strips have the potential for measuring pesticide residues in agricultural products outside the laboratory due to their ease of use, low cost, portability, and rapid testing. At present, a lot of research has been done on measuring the inhibition of AChE enzyme on paper for the analysis of pesticides residue using the AChE hydrolysis quantification method. The amount of color produced is dependent on the concentration of the insecticide that inhibits the activity of AChE. These experiments are performed using various chromogenic chemicals, the best of which is indoxyl acetate [42,43]. In such methods, which are not complicated and time-consuming, the remarkable point is its good efficiency in detecting the residue levels of organophosphate and carbamate pesticides. Although paper bio-sensors have many advantages, they have low detection sensitivity due to the low reactivity of the reagents on paper due to dryness. But in the current method, the wet system on the paper is used to eliminate this defect so that the chemical reaction on the paper can be done completely. To do this, a hole was punched in the polypropylene strip from which the buffer droplets were poured every 5 minutes to keep the reaction zone moist. This is a new method that greatly enhances the reaction of the enzyme, substrate and pesticides [41].

The sensitivity of the paper-based bio-sensor method containing AChE is affected by various factors, including concentration of the enzyme and its substrate indoxyl acetate, the moisture content of the paper, and the incubation time. The optimal concentration of AChE was found out to be 800 units/ml and the amount to be used was 5 microliters on the filter paper. The optimum amount of indoxyl acetate was found to be 5 mg/ml. Eight concentrations of each insecticide, were studied and based on the color created, the Limits of detection of 0.1, 0.1, 0.05 and 0.01 mg/l were detected for diazinon, trichlorfon, carbofuran and pirimicarb, respectively. Tests on pesticide-free lettuce and rice samples when spiked with six concentrations of the pesticides, and examined by the bio-sensor strips showed an acceptable performance of these strips for the detection of the residue levels of these four pesticides and the limits of detection of 0.5, 0.1, 0.1 and 0.1 mg/kg for diazinon, trichlorfon, carbofuran and pirimicarb in lettuce and 0.1, 0.1, 0.1 and 0.1 mg/kg for diazinon, trichlorfon, carbofuran and pirimicarb in rice were determined, respectively (Table 2. a, b and Figure 12. a, b). The difference between the limits of detection obtained in the use of the standards of the pesticides under test is due to the biomolecular rate constant of AChE inhibition in the pesticides, and in the spiked lettuce and rice it could also be due to the matrix effect. In order to create better sensitivity and more accurate performance of the bio-sensor strip, extraction by PBS buffer with a concentration of 20 mmol and pH of 7.4 in combination with 20% methanol was used in the experiments. Another important point to increase the sensitivity is the pre-concentration method. In the pre-concentration method, addition of the sample for 5 times to the test zone increased the sensitivity of the sensor to 0.1 mg/kg, which is equal to the lowest MRL for these pesticides and the products tested. This number of sample additions can help the analytes to accumulate at the test zone and ultimately increase the sensitivity of the biosensor for the measurement of the pesticide residue. The only drawback of the pre-concentration method is that if this number is performed consecutively, the sample will be spread to the area adjacent to the test zone and the surrounding area. Therefore, to prevent this, each time the sample was added to the test site, the strip was dried at a temperature of about 40 °C and a new sample drop was added to the site. To increase the sensitivity of the bio-sensor further QuEChERS extraction method can be used, but since the cost of QuEChERS method is higher and it requires more time to extract than the PBS buffer plus methanol method, it is not cost-effective for out-of-laboratory environments. However, for samples that are in the laboratory, QuEChERS extraction method can be used and the measurement of pesticide residue can be done by a bio-sensor strip to have a higher sensitivity. However, extraction of samples with PBS buffer along with methanol, makes it possible to measure pesticide residues up to 0.1 mg/kg, which makes it suitable and cost effective for daily tests outside the laboratory which have been observed in similar studies [37,41,44,45]. As seen in the results, for colorimetric studies by ImageJ software, the residue of pesticides can be quantified after calibrating the specific color and pesticide concentrations [41,46,47]. As it may be difficult to quantify the color generated in the bio-sensors by ImageJ software outside the laboratory and on-site in the farms or markets, to facilitate the approximate quantification of the pesticide residue levels, the colors produced by the enzyme reaction were categorized into 4 levels and the approximate contamination was classified accordingly. Level 1 indicates the lowest amount of color production and hence the highest amount of pesticides residue present in the sample, which is more than 5 mg/kg, and in level 2 according to the color produced, indicates pesticide residue of about 1 to 5 mg/kg. Level 3 indicates the presence of approximately 0.1 to 0.9 mg/kg of the pesticide residue and Level 4 indicates the lowest pesticide residue level or below the limit of detection i.e., approximately 0 to 0.09 mg/kg. The advantages of the paper-based bio-sensor strip method is the speed of operation, low cost and the ability to use it on-site on the farms or markets [46]. The results obtained by measuring the OP and CM residue levels in lettuce and rice (unknown in terms of pesticide residues) collected

from market by paper-based bio-sensor strip method and analysis of the same samples by QuEChERS and LC-MS/MS residue levels determined were within an acceptable level (Tables 4). According to the results obtained, it could be concluded that the paper-based bio-sensor strip impregnated with AChE can be used as an on-site, fast, cheap, simple method (in terms of application) for the measurement of pesticide residue levels in the agricultural products.

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