A simple paper-based biosensor for on-site visual detection of organophosphate residues

Phromchaloem C.[1,3*] and Muensritharam L.[2,3]
1. Department of Chemistry, Faculty of Science and Technology, Muban Chombueng Rajabhat University, Ratchaburi, THAILAND
2. Department of Food Processing, Faculty of Science and Technology, Muban Chombueng Rajabhat University, Ratchaburi, THAILAND
3. Agricultural Products Inspection Centre, Ratchaburi Province, Muban Chombueng Rajabhat University, Ratchaburi, THAILAND
*chanya.phromchaloem@gmail.com

Abstract
In general, the laboratory method of analyzing pesticides in vegetables is complicated due to the high cost of equipment and chemicals. The process of analyzing pesticide residues generally requires expertise as well as a significant period of time. In this study, a paper-based biosensor was developed for the detection of acetylcholinesterase (AChE) inhibitors, particularly organophosphate pesticides. The paper-based biosensor was constructed based on the Ellman colorimetric assay by immobilizing AChE on cellulose paper with 2% alginate gel, 0.25% glutaraldehyde and the colorimetric reagent 5,5-dithio-bis-(2-nitrobenzoic acid) (DTNB) in phosphate buffer (pH 8.0). As a substrate, acetylthiocholine chloride (ATChCl) was used. The results showed that the developed paper-based biosensor has been stable for 2 weeks with a detection limit of 0.03 mM of chlorpyrifos.

The paper-based biosensor was applied to detect organophosphate pesticides in vegetables from the farmers' market, Ratchaburi Province. It was found that the test results of the paper-based biosensor were similar to the commercial GT-test kit. The paper-based biosensor was 10 times faster than the GT-test kit in terms of testing time and the results were easy to identify due to the color-based indicator. As a result, a paper-based biosensor is rapid, portable and easy to use by the general population.

Keywords: Acetylcholinesterase, Organophosphate, Paper-based biosensor, Pesticide residues, Acetylcholine inhibition.

Introduction

Many pesticides in agriculture cause environmental pollution, food chain contamination and high risks to health. Organophosphate compounds (OPs) are synthetic organic insecticides. These compounds are highly toxic to humans and residues can be present in vegetables and in the environment[7]. There are several compounds in the group of organophosphates that are well-known such as malathion, dimethoate, fenitrothion, pirimiphos methyl and dichlorvos. These compounds are widely used in agriculture. Due to the use of pesticides in agriculture, the detection of pesticide residues is strongly required. The detection methods need to be based on an economical, rapid and reliable method. The detection tools involved should be cost-effective and should be able to target specific pesticides in food and their environmental applications[10].

Gas and liquid chromatography have been introduced for the detection of organophosphate residues. However, the limitation is that they are not suitable for on-site detection. Due to this, a portable sensor has been developed for pesticide residue detection. Recently, biosensors have focused on the development of mobile detection devices[5]. There are several types of biosensors. A paper-based biosensor is suitable for developing low-cost analytical devices[6]. Since paper is a low-cost material and disposable and has a large volume of surface, it is commonly used as a supporter of biosensors. The paper-based biosensors have been developed for on-site applications of diagnostic tools[6]. The biosensor paper tools are typically produced by modification of the paper surface with a biomolecule coating and by adding functional groups of sensors[2].

A number of colorimetric techniques have been developed for paper-based biosensors including paper strip and dipstick techniques. For pesticide detection, the inhibition of specific enzymes on biosensors has been attractive to many researchers[11]. By immobilizing enzyme and colorimetric reagents on the paper, the color will be released upon the reaction of the reagent with the target compounds. For these techniques, colorimetric formats are suitable for a single-step detection process. It also uses specific enzymes for developing the color[12].

Acetylcholinesterase (AChE) is an enzyme that hydrolyzes acetylcholine (ACh) into acetic acid and choline which are specifically inhibited by organophosphate pesticides[8]. Inhibiting AChE can determine the amount of these pesticides in the environment. This means that the enzyme can be used as a common bioindicator for organophosphate detection. It can also enable the monitoring of organophosphate pesticides in samples. The AChE-based paper biosensor is designed for convenience and for combining detection devices with the colorimetric technique. The AChE-based detection is reported in sensor devices for agricultural products such as fruits and vegetables[2,9].

High stability of biomaterial or biosensor development was the purpose of the bioactive compound immobilization process. Many studies used the concept of enzyme immobilization[1,2]. In this study, AChE was immobilized by crosslinking natural polymer alginate and glutaraldehyde

including colorimetric reagent on cellulose paper. Enzyme substrates need to be used to elicit a response from the color production investigated in the assay technique. The effect of different concentrations of pesticides on the inhibition of AChE was correlated with color intensity.

This method was inexpensive and suitable for detection of organophosphate residues. Therefore, the purpose of this study was to develop paper-based biosensors for the detection of organophosphate pesticide residues in vegetables from the farmers' market.

Material and Methods

Chemicals and reagents: Acetylcholinesterase (AChE) from *Electrophorus electricus* (electric eel), Acetylthiocholine chloride 99% (ATChCl), Glutaraldehyde solution (Grade I, 70% in H_2O), 5,5-dithio-bis-(2-nitrobenzoic acid) (DTNB), Dipotassium hydrogen phosphate (K_2HPO_4), Potassium dihydrogen phosphate (KH_2PO_4), Sodium alginate and Tris(hydroxymethyl) methylamine were all purchased from Sigma-Aldrich Co. (USA). Organophosphate pesticides and chlorpyrifos (99.4%) were obtained from Dr.Ehrenstorfer. All chemicals and reagents were commercially available and of analytical grade. They were used without further purification.

Solution preparation: The AChE enzyme solutions were prepared freshly in 20 mM Tris-HCl buffer (pH 7.5). The enzyme activity was calculated as unit/millilitre (U/mL). The concentration of 10 mM DTNB was prepared in phosphate buffer (pH 8.0). ATChCl and alginate stock solutions were prepared and diluted in distilled water. The solution of 0.25% glutaraldehyde was made by diluting from a commercial solution using distilled water. Pesticide stock solutions were prepared freshly in distilled water on the day of the experiments and used within three hours. Organophosphate pesticides are toxic. These materials should be handled with gloves and masks and used in a fume hood.

Paper-based sensor preparation: Alginate was chosen as the matrix for enzyme immobilization. The alginate gel mixture composition was prepared first following an optimization process for AChE enzyme efficacy, glutaraldehyde (crosslinking agent) and DTNB (chromophore) in phosphate buffer (pH 8.0). The paper-based biosensor was prepared by using 1 x 30 cm cellulose filter paper as an enzyme-gel supporter. The filter papers were incubated at 120^{o}C for 30 minutes prior to coating before being attached with 10 x 30 cm of 100 GSM bond paper for strength. A mixture of alginate gel that was crosslinked with AChE enzyme by glutaraldehyde and DTNB, was applied to the cellulose filter papers by pipetting and painting brushes.

This occurred in filter paper which was referred to as reactive paper in this study. The paper-based biosensor was dried at 35^{o}C for 15 minutes and then cut into 1 x 10 cm. Each paper-based biosensor contained 1 x 1 cm piece of reactive paper. For control experiments, a reactive paper that did not contain AChE was made in the same manner.

Optimization of paper-based sensors: In order to optimize the paper-based sensor, the effects of various parameters including alginate and DTNB concentration, the efficacy of AChE on reactive paper, biosensor stability and color intensity were examined as follows:

(1) Optimization of alginate concentration for reactive paper: The concentrations of alginate were tested for gel formation. The concentrations of 0.5, 1, 2 and 4% were investigated in phosphate buffer pH 8.0. The crosslinking process was optimized by adding 200 μL of 0.25% glutaraldehyde from the stock solution, 20 μL of 10 mM DTNB and 1 μL of AChE in 1 mL of alginate gel solution. The suitable viscosity of the gel for enzyme immobilization was selected.

(2) Optimization of DTNB concentration for reactive paper: The different volumes of DTNB were tested by adding 20 and 50 μL of 10 mM DTNB from the stock solution to the gel matrix (1 mL) containing 2% alginate in phosphate buffer pH 8.0, 0.25% glutaraldehyde (200 μL) and 1 μL of AChE (activity = 27.91 units/mL) and applied to the reactive paper. The optimum volume of DTNB was selected by observing the color intensity produced by the enzymatic hydrolysis of ATChCl.

(3) Efficacy of AChE on reactive paper: ATChCl as a specific substrate of AChE was tested at 75 mM as the optimum concentration for the development of the yellow color according to the method of Ellman et al.[4] The papers that were coated with the gel mixture contained 0.5, 1, 2 and 4% alginate, 200 μL of 0.25% glutaraldehyde and DTNB tested for their efficacy. The performance of the paper sensor under optimized conditions was assessed directly by immersion of the paper strip into the ATChCl solution (75 mM). The performance can be assessed by direct addition of substrate solution to the sensing area or by immersion of the paper sensor into the substrate solution and incubation at 35^{o}C for 5 minutes to allow the yellow color to develop.

(4) Stability of the reactive paper: The developed paper sensors were separated into five replicates in sealed plastic bags and stored at 4^{o}C. The stability of the paper sensors was tested at 1, 2, 3 and 4 weeks. The process was to dip the paper sensors into ATChCl for 30 seconds and then they were incubated at 37^{o}C for 5 minutes. The appearance of yellow color was observed.

For the optimization of alginate concentration, DTNB amount and stability of the reactive, the intensity of the yellow color on each paper sensor was measured by eluting a mixture from paper with phosphate buffer (pH 8.0) and quantified in a spectrophotometer by measuring the absorbance at 412 nm.

Inhibition of AChE activity by pesticides: As shown in figure 1, AChE hydrolyzes ATCh to produce thiocholine and acetic acid. Thiocholine's free sulfhydryl group (-SH) can react with DTNB, cleaving the disulfide bond to create 5-thio-2-nitrobenzoic acid (TNB) that has a yellow color. This TNB has maximal absorbance at 412 nm. The inhibition of AChE activity by pesticides can be quantified by this method. In order to measure the concentration of inhibitory molecules, the difference of absorbance at 412 nm between initial AChE activity and after incubation with inhibitors is evaluated.

Chlorpyrifos was represented as an organophosphate pesticide in this study. It was used as an AChE inhibitor. The variations of chlorpyrifos concentration of 7.5 x 10^{-4}, 7.5 x 10^{-3}, 0.015, 0.030, 0.06, 0.12 and 0.24 mM were added into an enzyme color reagent (it contained AChE and DTNB in phosphate buffer pH 8.0) and then incubated in darkness for 15 minutes. The mixture solution was added to 10 μL of 75 mM acetylthiocholine chloride and the absorbance was measured at 412 nm. The concentration of chlorpyrifos that inhibited cholinesterase was calculated at 50% (IC_{50}). The calculation used the linear equation between chlorpyrifos concentrations and the percentage of cholinesterase inhibition. The enzyme-inhibitor, chlorpyrifos, was not present in the control. The enzyme activities were considered by inhibition percentages against the control.

Detection of pesticides using a paper-based biosensor: The chlorpyrifos pesticide was used to evaluate the effectiveness of the paper-based biosensor by assessing the colorimetric assays. This assay measured the decrease in yellow color intensity of TNB production. The concentration of chlorpyrifos was determined by the color that developed on the paper-based biosensor. The paper-based biosensor was incubated with 7.5 × 10^{-4} to 0.24 mM of chlorpyrifos solutions for 1 minute and dried for 1 minute followed by dipping in the ATChCl (75 mM) solution and incubated at 37°C for 5 minutes for color development. The same procedure was applied to the other organophosphate pesticide groups such as palathion, terbufos, malathion and cyanofenfos.

Application for vegetables in farmers' market: The paper sensor was applied to testing for organophosphate residues in vegetables from the farmers' market, Ratchaburi Province. Baby corn, Cos lettuce and Chinese kale, which are common vegetables in this market, were selected for testing. The results of the paper-based biosensor were compared to those of a commercial GT-Test kit that followed the manufacturing instructions. Both test kits used the same extraction method as required by a commercial GT-Test kit.

Results and Discussion

Paper-based sensor preparation: An alginate gel mixture was prepared by crosslinking alginate gel with AChE, glutaraldehyde and DTNB in phosphate buffer and then coating the alginate gel mixture onto cellulose filter paper. This process created a reactive paper. The reactive paper was attached to 100 GSM bond paper as a supporter. This paper is called a paper-based biosensor for further use to detect pesticide residues in samples (Figure 2).

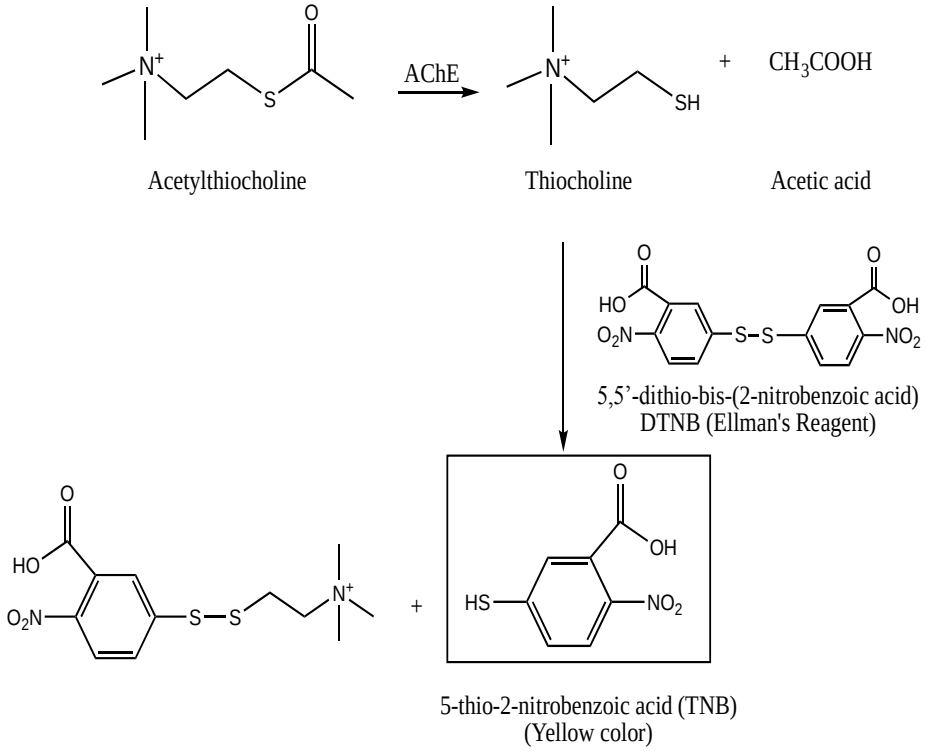

Figure 1: Acetylcholinesterase (AChE) hydrolyzes the acetylthiocholine and forms thiocholine base which then reacts with dithiobisnitrobenzoate (DTNB) to generate 5-thio-2-nitrobenzoate (TNB, an anion) which is yellow

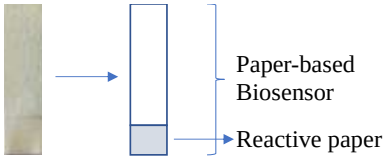

Figure 2: The paper-based biosensor

Optimization of bioactive paper-based sensors

(1) Optimization of alginate concentration for reactive paper: Enzyme immobilization is a technique that changes a liquid biological catalyst into an insoluble or slightly soluble solid biological catalyst[13]. This technique had various methods. This study used a physical method by crosslinking an enzyme with a polymer that allowed the substrates and chemicals to pass. Alginate is soluble in water and becomes a high viscosity polymer. Concentrations of 0.5, 1, 2 and 4% of alginate were chosen for immobilization. It was found that the alginate concentrations slightly affected the color intensity according to figure 4. However, 2% alginate was the most suitable for the experiment because of its viscosity.

(2) Optimization of DTNB concentration for reactive paper: An enzyme immobilized on reactive paper reacted with ATChCl to produce thiocholine. A reaction between 5,5-dithio-bis-(2-nitrobenzoic acid) or DTNB and thiocholine could result in a yellow color with a maximum absorbance at 412 nm. As a result, the reactive paper containing DTNB turned yellow which was investigated for an appropriate reaction.

The results of 20 and 50 μL of 10 mM DTNB were investigated as shown in figure 3. The usage of 50 μL of DTNB revealed a greater absorbance than 20 μL at a wavelength of 412 nm. Therefore, 50 μL of DTNB was effective for yellow color vision in this reaction.

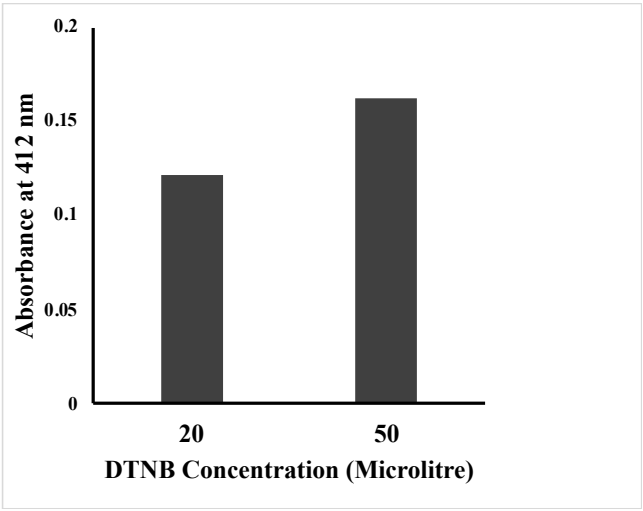

Figure 3: The absorbance at 412 nm of the paper-based biosensor contained 10 mM DTNB in volumes of 20 and 50 μL

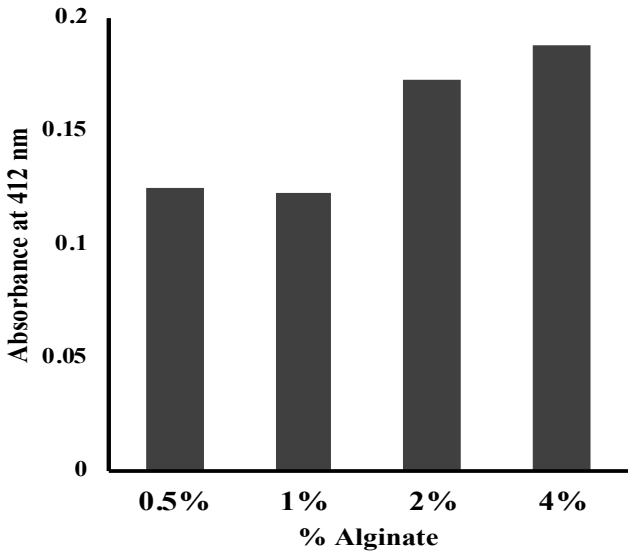

Figure 4: The absorbance at 412 nm of the paper-based biosensor varied with concentrations of alginate (0.5, 1, 2 and 4%)

(3) Efficacy of AChE on reactive paper: The AChE of the electric eel was employed. It was immobilized on cellulose paper with alginate and glutaraldehyde using molecular crosslinking. The AChE was degraded into acetic acid and thiocholine when it interacted with the substrate (ATChCl)[3]. The yellow color resulted from the interaction of thiocholine with DTNB that was presented on the reactive paper. This indicated that AChE was immobilized on the paper. According to figure 4, the color intensity was shown to be higher when there was a higher alginate concentration. This result showed that the concentration of alginate affected AChE immobilization.

(4) Stability of the reactive paper: The paper-based biosensor was stored at 4°C and its stability was tested by determining the AChE activity. The activity was measured by color intensity determination. After the first week of storage, the activity of AChE was reduced from 100% to 95% (Figure 5) which represented only a small decrease. However, as storage time was increased, the activity gradually decreased.

According to figure 6, absorbance at 412 nm decreased from week 1 to week 2, then stabilized between week 2 to week 3 and then decreased again. Additionally, the color intensity decreased the most when the paper-based biosensor was stored for 4 weeks. This phenomenon could be caused by AChE decomposition. In terms of this result, the bioactive paper-based sensor can be stored up to 2 weeks without affecting the results.

Inhibition of AChE activity and detection of pesticides using paper-based biosensors: The developed paper-based biosensor was tested with chlorpyrifos, an organophosphate pesticide. Concentrations of 7.5×10^{-4}, 7.5×10^{-3}, 0.015, 0.030, 0.06, 0.12 and 0.24 mM were used. The yellow color on the paper-based biosensor could be seen with the naked eye and the intensity of the color was inversely proportional to the amount of insecticide present. As a result of the chlorpyrifos inhibiting AChE activity, ATChCl was not hydrolyzed to thiocholine. When DTNB was exposed to ATChCl, it was unable to produce a yellow form[9]. Therefore, the reduction of the yellow color indicated higher level of pesticides.

The paper-based biosensor showed no yellow color when tested with samples containing chlorpyrifos concentrations greater than 0.06 mM or 20 ppm. This meant that the enzyme may have been completely inhibited by chlorpyrifos. For inhibition of AChE activity, the concentration of 0.03 mM chlorpyrifos inhibited cholinesterase at 50% (IC_{50}). Meanwhile, the IC_{50} values for other organophosphate pesticide groups such as palathion, terbufos, malathion and cyanofenfos were 0.03, 0.03, 0.02 and 0.03 mM respectively. This demonstrated the efficacy of the paper-based biosensor in determining the level of organophosphate pesticide residues.

Application for vegetables in the farmers' market: The paper-based biosensor was used to investigate organophosphate residue in three kinds of vegetables (Baby corn, Cos lettuce and Chinese kale) from the farmers' market.

Figure 5: The color of the paper-based biosensor when stored at 4°C for (A) 1 day, (B) 1 week, (C) 2 weeks, (D) 3 weeks and (E) 4 weeks (after testing with substrate)

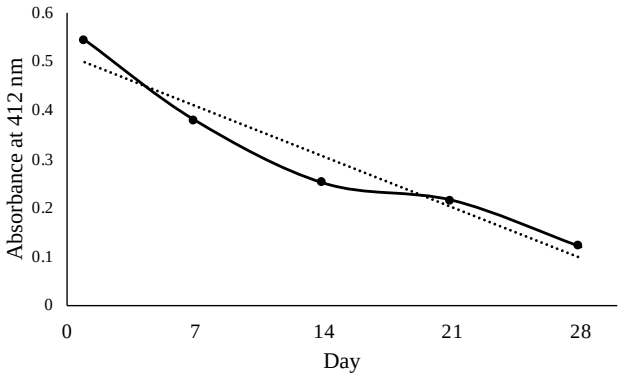

Figure 6: The absorbance at 412 nm of the paper-based biosensor when stored at 4°C for 1-4 weeks (after testing with substrate)

The results showed that all samples had a yellow color on the paper-based biosensor but the color intensity of Chinese kale was lower than the color intensity of the control sample. This implied that pesticide residues were present in Chinese kale. According to the results of the spectrophotometry, the absorbance at 412 nm was also the highest with a blank while baby corn and cos lettuce showed the same level. Chinese kale had the lowest absorbance. It is reasonable to conclude that Chinese kale contains more pesticide residues than baby corn or cos lettuce. Correlating to the results of the commercial GT-test kit, organophosphate residue was also found in Chinese kale. This demonstrated that a paper-based biosensor could be a reliable tool for investigating organophosphate residue in samples and for on-site detection.

Conclusion

The paper-based biosensor was developed based on using an AChE inhibitor sensor for determining the concentrations of organophosphate residue in samples. The visual colorimetric and enzyme immobilization method had been applied as the detection method. The developed paper-based biosensor was tested with a variety of organophosphate pesticides. In terms of results, the developed biosensors performed admirably. It detected chlorpyrifos successfully with a detection limit of 0.03 mM and was also capable of detecting other organophosphate pesticides such as palathion, terbufos, malathion and cyanofenfos. The paper-based biosensor was also performed in the field at farmers' market and it was found to be effective in detecting organophosphate residue in vegetables. Farmers and local customers, for example, acknowledged that the novel paper-based biosensor was simple to use.

As a conclusion, users of the paper-based biosensor have three advantages: 1) Low cost and ease of use. The paper-based biosensor is easy to use and handle as well as inexpensive due to the paper material. 2) Simple method and technology to implement. This paper-based biosensor could detect pesticide residue at low concentrations and could display the results in color. Since the results are only color and not figures, they are easier for a layperson to interpret. 3) Real-time results. This technique was performed in a short period of time in order to present the results and did not require the use of any additional equipment.

Acknowledgement

The authors wish to thank the farmers' market, Ratchaburi Province for their continued support and collaboration. The support from the Department of Chemistry, Muban Chombueng Rajabhat University with their research facilities is highly appreciated. This project was supported by Muban Chombueng Rajabhat University Research Fund.

References

1. Alex A.V. and Mukherjee A., Review of recent developments (2018-2020) on acetylcholinesterase inhibition based biosensors for organophosphorus pesticides detection, *Microchemical Journal*, **161**, 105779 **(2021)**

2. Badawy M.E. and El-Aswad A.F., Bioactive paper sensor based on the acetylcholinesterase for the rapid detection of organophosphate and carbamate pesticides, *International Journal of Analytical Chemistry*, **2014**, 1-8 **(2014)**

3. Chrouda A., Zinoubi K., Soltane R., Alzahrani N., Osman G., Al-Ghamdi Y.O., Qari S., Al Mahri A., Algethami F.K., Majdoub H. and Jaffrezic Renault N., An acetylcholinesterase inhibition-based biosensor for aflatoxin B1 detection using sodium alginate as an immobilization matrix, *Toxins*, **12(3)**, 173 **(2020)**

4. Ellman G.L., Courtney K.D., Andres V. Jr. and Feather-Stone R.M., A new and rapid colorimetric determination of acetylcholinesterase activity, *Biochemical Pharmacology*, **7**, 88-95 **(1961)**

5. Jaffrezic-Renault N., New trends in biosensors for organophosphorus pesticides, *Sensors*, **1(2)**, 60-74 **(2001)**

6. Kavruk M., Özalp V.C. and Öktem H.A., Portable bioactive paper-based sensor for quantification of pesticides, *Journal of Analytical Methods in Chemistry*, **2013**, 1-8 **(2013)**

7. Kazemi M., Tahmasbi A.M., Valizadeh R., Naserian A.A. and Soni A., Organophosphate pesticides: A general review, *Agricultural Science Research Journals*, **2**, 512-522 **(2012)**

8. Kwong T.C., Organophosphate pesticides: biochemistry and clinical toxicology, *Therapeutic Drug Monitoring*, **24(1)**, 144-149 **(2002)**

9. Patel H., Rawtani D. and Agrawal Y.K., A newly emerging trend of chitosan-based sensing platform for the organophosphate pesticide detection using Acetylcholinesterase-a review, *Trends in Food Science and Technology*, **85**, 78-91 **(2019)**

10. Sánchez-Bayo F., Baskaran S. and Kennedy I.R., Ecological relative risk (EcoRR): another approach for risk assessment of pesticides in agriculture, *Agriculture, Ecosystems and Environment*, **91(1-3)**, 37-57 **(2002)**

11. Songa E.A. and Okonkwo J.O., Recent approaches to improving selectivity and sensitivity of enzyme-based biosensors for organophosphorus pesticides: A review, *Talanta*, **155**, 289-304 **(2016)**

12. Sulaiman I.C., Chieng B.W., Osman M.J., Ong K.K., Rashid J.I.A., Yunus W.W., Noor S.A.M., Kasim N.A.M., Halim N.A. and Mohamad A., A review on colorimetric methods for determination of organophosphate pesticides using gold and silver nanoparticles, *Microchimica Acta*, **187(2)**, 1-22 **(2020)**

13. Taqieddin E. and Amiji M., Enzyme immobilization in novel alginate–chitosan core-shell microcapsules, *Biomaterials*, **25(10)**, 1937-1945 **(2004)**.

(Received 22nd March 2021, accepted 27th May 2021)