JOURNAL OF NATURAL RESOURCES AND DEVELOPMENT

Research article

Enzymatic Inhibition Constant of Acetylcholinesterase for the Electrochemical Detection and Sensing of Chlorpyrifos

Karla Ramírez-Sánchez [a]*, Alvarado-Hidalgo, Fernando [b], Ardao, Inés [c], Starbird-Pérez, Ricardo [d]

[a] School of Chemistry, Costa Rica Institute of Technology, 159-7050 Cartago, Costa Rica

[b] School of Materials Science and Engineering, Costa Rica Institute of Technology, 159-7050 Cartago, Costa Rica

[c] BioFarma group, Center for Research in Molecular Medicine and Chronic Diseases-CiMUS, University of Santiago de Compostela, 15782 Santiago de Compostela, Spain

[d] School of Chemistry, Costa Rica Institute of Technology, 159-7050 Cartago, Costa Rica

Corresponding author: karla22rs@gmail.com

Abstract

Infiltration into soils of pesticides used during agricultural production has led to the contamination of aquatic ecosystems due to their long persistence in the environment. Some pesticides (e.g. Chlorpyrifos) are inhibitors of cholinesterase enzyme activity and their presence in water samples can be indirectly detected by a decrease in enzymatic activity. Biosensors based on cholinesterase enzymes are an alternative for the sensitive detection of important contaminants in the environmental sector. Acetylcholinesterase enzyme (AChE) catalyzes the hydrolysis of acetylthiocholine (ATCh) to produce thiocholine (TCh). This feature can be employed to measure the decrease in AChE activity. The inhibitory characteristics of the AChE-Chlorpyrifos system have been studied through cyclic voltammetry, by evaluation of the oxidation of the thiol group, which corresponds to TCh production on platinum electrodes in the presence of an inhibitor. In the present study, enzymatic curves were obtained at different concentrations of substrate and inhibitor, which were then used to determine the enzymatic kinetics corresponding to a mixed inhibition type, with an inhibition constant (Ki) of (18.26 ± 0.01) μM. TCh electrochemical detection appears to be a promising option for the development of biosensors to identify and quantify pesticides present in the ecosystem.

1. Introduction

The increasing use of pesticides in agricultural production, has led to the contamination of aquatic ecosystems due to their long persistence in soil environments and harmful impacts upon organisms [1], [2]. Organophosphorus and carbamate pesticides are highly toxic compounds. Chlorpyrifos [O, O-diethyl-O-(3, 5, 6-trichloro-2-pyridyl) phosphorothioate] is the most widely used organophosphate insecticide [3], its presence can be harmful to human health as it inhibits some important enzymes such as acetylcholinesterase (AChE), which is essential for the functioning of the central nervous system [4], [5]. AChE catalyzes hydrolysis of the neurotransmitter acetylcholine. As a result of AChE inactivity, acetylcholine is accumulated, causing the nerve order to be reiterated, leading to exhaustion, respiratory paralysis and death [5], [6].

The enzyme inhibition mechanism comprises the formation of a complex through a reversible/irreversible reaction of organophosphorus pesticides with AChE active sites [7]. An enzymatic inhibitor lowers the substrate binding capacity due to direct interaction between the enzyme and the inhibitor. In mixed inhibition, the inhibitor most often binds to a remote site and induces a conformational change that affects the active site, reducing the catalytic turnover and altering the substrate binding capacity [8], [9]. One approach to understanding the mechanism of action of enzymatic inhibitors has been to study the effect of inhibitor concentration on the enzyme reaction rate. The dissociation constant of the enzyme-inhibitor complex (Ki) has been used to describe the extent of inhibition [10].

Ki is proportional to the affinity and the inhibitory capability of the inhibitor from the enzyme. Therefore, it is a fundamental parameter when comparing the inhibition potency of pesticides and assessing the sensitivity of enzymes. The Ki value is different depending on the pesticide, the enzyme conditions and the type of entrapment in the case of immobilized enzymes [11].

Chromatographic techniques, such as gas chromatography (GC) and high-performance liquid chromatography (HPLC) offer high accuracy and sensitivity for compound monitoring; however, these techniques require highly trained personnel, are time consuming and limited to laboratory analysis [7], [12]. Thus, modern technologies have been developed to perform on-site analysis and obtain reliable and quick results for measuring pesticides.

In recent years, the use of AChE and acetylthiocholine (ATCh) in electrochemical biosensors has shown satisfactory results for pesticide analysis [13], [14], while the use of nanomaterials such as gold-platinum bimetallic nanoparticles [15], gold nanoparticles [16], and multiwalled Carbon nanotubes [17], coupled with AChE biosensors, has resulted in improved electrochemical performance. AChE inhibition-based biosensors are also portable, less expensive and they do not require complicated sample pretreatment [7], [12].

In order to study the electrochemical behavior of the AChE/ATCh system, cyclic voltammetry (CV) allows analysis of the responses from the oxidation of the thiol group of thiocholine (TCh), hydrolysis product of ATCh; where the current peak generated is proportional to the concentration of TCh and is reduced in the presence of an inhibitor, such as Chlorpyrifos [13], [18]. The aim of the present study is to determine the enzymatic inhibition constant for the AChE-Chlorpyrifos complex to use it in the electrochemical determination of these pesticides.

2. Materials and Methods

2.1. Chemicals and reagents
Acetylcholinesterase enzyme (AChE) extracted from electric eel, acetylthiocholine chloride (ATCh), Tris HCl and potassium chloride (KCl) were acquired from Sigma–Aldrich, (San Jose, Costa Rica). Deionized water was used in all experiments.

2.2. Electrochemical Cell
Electrochemical measurements were performed using an Autolab PGSTAT 302N (AUTOLAB, Netherlands) with a three-electrode system: Ag/AgCl (KCl, 3M) was used as the reference electrode and a Platinum sheet as the counter electrode, while Metrohm Screen Printed Platinum electrodes (AUTOLAB, Netherlands) were used as the working electrode. Cyclic voltammetry measurements were carried out by scanning from -0.2 to 1.0 V with a 0.2 V/s scan rate with a ±0.1 μA resolution.

2.3. Enzymatic Reactions
An electrochemical cell was prepared containing KCl (0.2M) and (10.80 ± 0.02) μl of AChE enzyme (139 U/ml), where one enzyme unit is defined as 1 mol of thiol group/min [19]. ATCh and Chlorpyrifos were added to the electrochemical cell according to a full factorial 3x3 design of experiments (DOE). Using Minitab 17 Software (Pennsylvania, USA) and based on preliminary experiments where a standard deviation was estimated, the minimum sample size was found to be of 2, this was obtained by using a two-sided analysis with a 95 % confidence interval with and error of 1. Measurements were recorded at all possible combinations, choosing two experimental factors: ATCh and Chlorpyrifos concentration in the electrochemical cell. To complete the DOE three different levels were chosen for each experimental factor.

Pesticides were added by determining the concentration required to inhibit approximately 50 %, 10 % and 0 % of the enzyme activity under the assay conditions. **Table 1** shows the values for each factor at each level of the experiment design. Enzymatic reactions were followed in accordance with all possible combinations in the design of experiment for 10 minutes each, recording 13 measurements during that period.

Table 1: Design of a full factorial 3x3 design of experiments.

Experimental Factor	Level 1	Level 2	Level 3
ATCh Concentration	(375.00 ± 1.84) μM	(750.00 ± 3.25) μM	(1500.00 ± 6.26) μM
Chlorpyrifos Concentration	0.0 μM	(14.90 ± 0.05) μM	(44.80 ± 0.12) μM

3. Results and Discussion

Figure 1 shows the cyclic voltammograms corresponding to three different measurement times, in the system containing 1500 µM ATCh and no inhibitor. Curves b and c have an irreversible oxidation peak near 0.7 V which is associated with the oxidation process of thiocholine, a hydrolysis product of ATCh by AChE activity **[18]**. One important aspect when analyzing **Figure 1** is the fact that the oxidation peak is not visible in curve a, this is because that curve was measured at the very beginning of the reaction time, when the enzymatic hydrolysis was barely starting and no appreciable thiocholine was produced. However, as the reaction time increased, the peak became visible and showed an increasing associated current, leading to the curve with the longest enzymatic reaction time, curve c, with the highest associated current. This behavior is related to the increasing thiocholine concentration as a result of AChE hydrolysis.
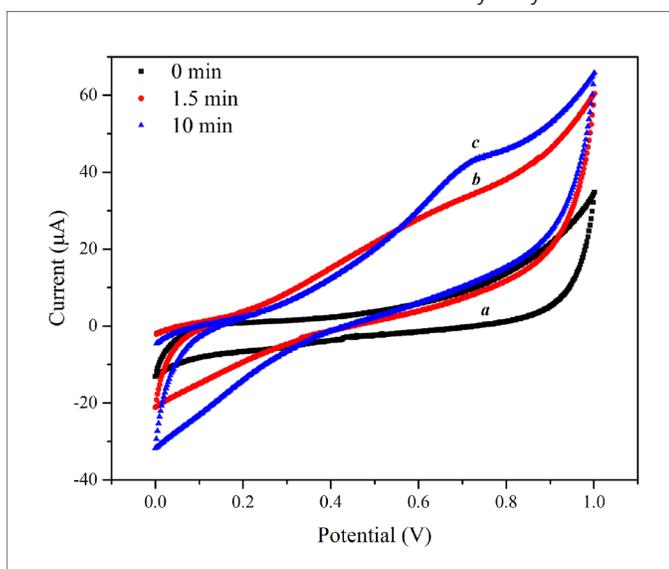

Figure 1: Cyclic Voltammograms of measurements in (1500.00 ± 6.26) µM ATCh without inhibitor at a) 0 min of incubation, b) 1.5 min of incubation and c) 10 min of incubation.

The effect of the substrate and inhibitor concentration on the reaction kinetics catalyzed by free AChE was studied using ATCh chloride and Chlorpyrifos as substrate and inhibitor, respectively. Comparison of the measured current in an enzymatic system provides information about the interaction between the enzyme and substrate. These measurements may vary depending on the substrate concentration as shown in **Figure 2**, which shows the typical enzymatic behavior of the substrate concentration over time.

AChE activity measurements were used to evaluate the inhibition process resulting from the presence of Chlorpyrifos. To obtain the inhibition constant for the system, an incubation time of 10 min was selected for the measurements. **Figure 3** shows enzymatic curves for 14.9 µM and 44.8 µM of inhibitor, respectively. In each figure, ATCh concentration is also varied.
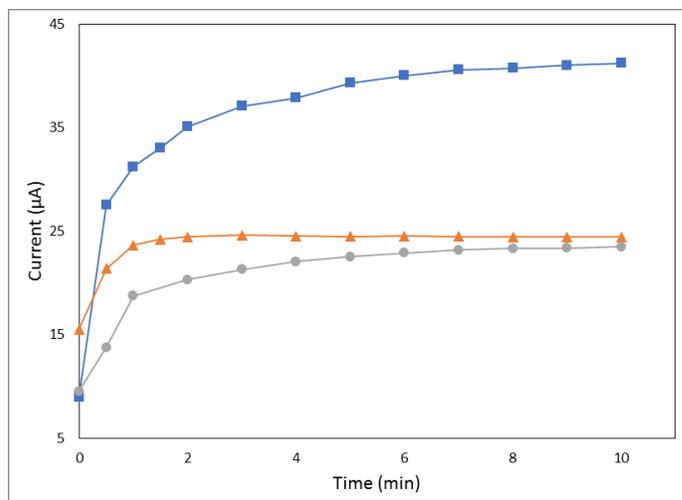

Figure 2: AChE activity behavior curve of current measurements at different ATCh concentrations: (■) (1500.00 ± 6.26) µM (▲) (750.00 ± 3.25) µM and (●) (375.00 ± 1.84) µM.

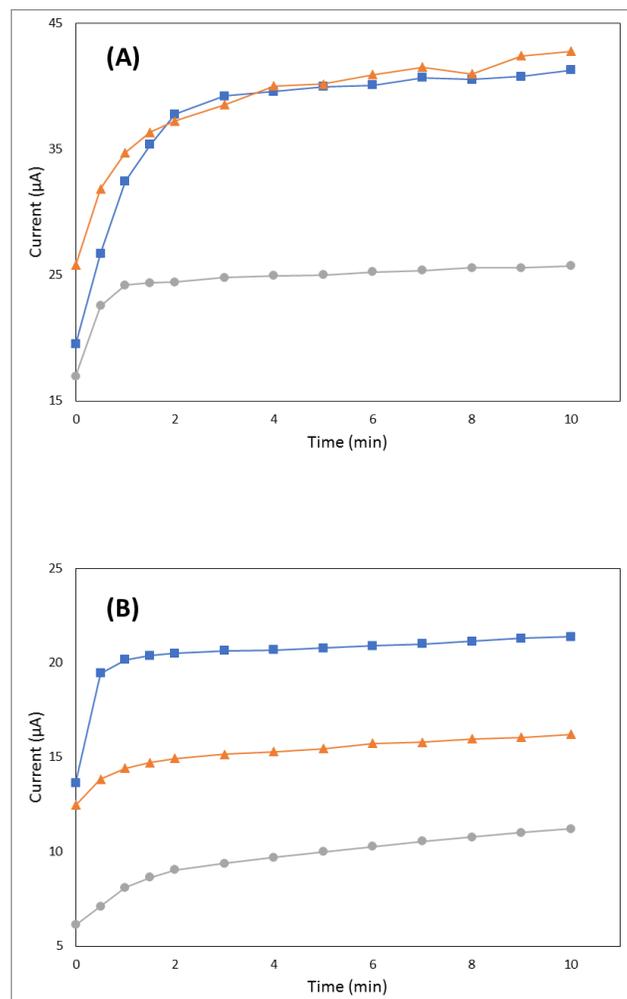**Figure 3:** AChE activity behavior curves of current measurement with A) 14.9 µM and B) 44.8 µM of inhibitor at different substrate concentrations: (■) (1500.00 ± 6.26) µM (▲) (750.00 ± 3.25) µM and (●) (375.00 ± 1.84) µM.

The results show that by increasing the inhibitor concentration in the AChE-ATCh solution, the maximum current measured decreased, indicating that the enzyme has a lower affinity for the substrate, which is caused by the competition between the substrate and inhibitor for the active site, causing the loss of enzyme flexibility [6]. These responses are associated with AChE inhibition and the quantity of pesticide added (**Figure 3**). The decrease in current can be explained as a result of the blocking of the serine hydroxyl group on the enzyme structure by covalently bonding to the phosphate group, thus reducing the charge of the catalytic active site [19]. ATCh is reduced to TCh. The peak of anodic oxidation of TCh should increase according to the concentration of substrate added to the electrochemical measurements [17]. However, in the experiment with 14.9 μM of chlorpyrifos, no current change was observed compared to 750 μM and 1500 μM of ATCh (**Figure 3**A). Unfortunately, this result indicates that the acetylcholinesterase reaction did not occur normally.

Figure 2 and **Figure 3**A show that the concentration of 14.9 μM of Chlorpyrifos does not have a significant inhibitory effect against AChE, and as a result, the measured current did not decrease significantly. In this study, the calculated Ki value is slightly higher than that of 14.9 μM. A lower concentration of pesticide comparing with Ki is hardly detectable. AChE immobilization is a strategy to build biosensors to detect pesticides in aqueous systems [20]-[22]. Therefore, the inhibition process may provide a method to quantify the amount of pesticides in real samples and Ki establishes an approximate value for the lower detection limit for AChE biosensors.

Figure 4 shows the Lineweaver-Burk plot, which is the double reciprocal plot of the enzyme reaction velocity (V) versus substrate concentration (1/V versus 1/[S]). There are four types of reversible inhibition: competitive, noncompetitive, uncompetitive and mixed inhibition. Each may be determined by graphical means using the Lineweaver-Burk plot [23]-[25]. For this study, it can be seen that the data line on the plot intersect in the second quadrant, under these conditions, exhibits a characteristic behavior of fully mixed inhibition. Therefore, the substrate must be competing with the analyte for the active enzymatic sites and for other sites in the enzyme structure. According to this, inhibition at low analyte concentrations could not be detected.

For pesticide analysis one of the most influential parameters is time [7]. Beyond 3 minutes of inhibition time, the curve tends toward a stable value, indicating the binding interaction between the pesticide and the enzyme, which reaches saturation (**Figure 3**). This change in current reflected the alteration of enzymatic activity, which resulted in a change of the interactions with the substrate. However, the enzyme was not totally inhibited, which was likely due to the binding equilibrium between pesticide and binding sites on the enzyme [12]. For AChE inhibition studies, the inhibition constant (Ki) provides a measurement of the dissociation (or binding) of the enzyme-inhibitor complex, and it is dependence on the structural and steric properties of the molecule. It is regarded as a useful parameter for the estimation of the inhibitory potency of a pesticide to AChE [26].
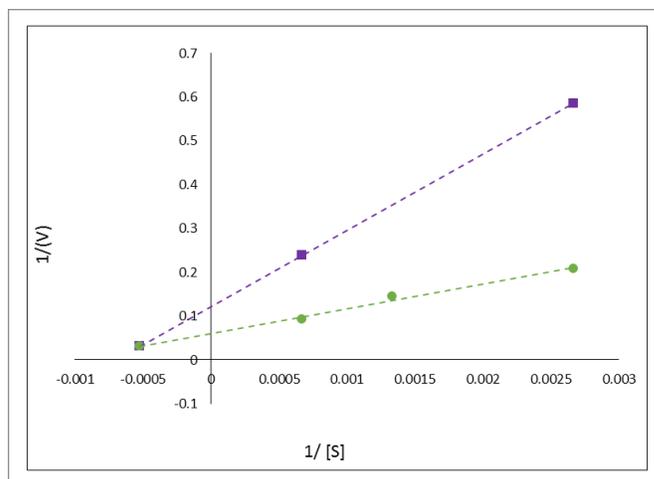

Figure 4: Determination of fully mixed inhibition from Chlorpyrifos to AChE according to substrate concentration and reaction velocity reciprocal for (■) (44.80 ± 0.12) μM (●) (14.90 ± 0.05) μM of inhibitor.

In our study, it was determined that the inhibition constant for the AChE-Chlorpyrifos complex was 18.26 ± 0.01 μM (**Figure 5**). Ki may be regarded as an affinity constant, and therefore, the smaller the value of Ki, the tighter the complex. Pesticides with small values for Ki are strong inhibitors, and those with large Ki values are weak inhibitors [27]. In this regard, when comparing this to other previously reported inhibition constants for AChE (**Table 2**) [28], Chlorpyrifos acts as a strong inhibitor. Previous studies explain that Chlorpyrifos has an adequate fit to the enzyme's active sites, making it a potent pesticide [29], [30].

Table 2: Ki values for inhibition of AChE from different organo-phosphate pesticides.

Compound	Ki value (μM)	Reference
Diazoxon	0.79	[28]
Malaoxon	5.6	[28]
Isomalathion	7.2	[28]
Malathion	130	[28]

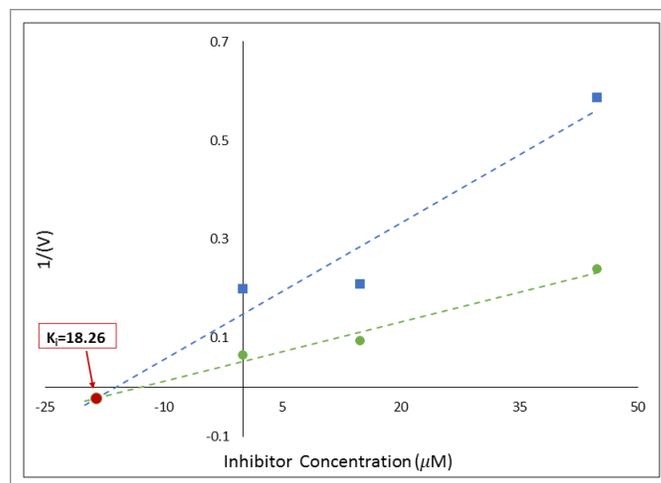**Figure 5:** Inhibition constant (Ki) according to inhibitor concentration and reaction velocity: (■) (1500.00 ± 6.26) μM (●) (375.00 ± 1.84) μM.

4. Conclusions

In this study, the inhibition between Chlorpyrifos and Acetylcholinesterase is found to be a fully mixed inhibition type, with a characteristic inhibition constant with a value of (18.26 ± 0.01) µM. These results represent a useful tool for the study of the extent of inhibition and the mechanism of action of enzymatic inhibitors. It also sets an approximate value for the lower detection limit of biosensors based on AChE immobilization, as their application is important to enhance the sensitivity of enzymatic biosensors in pesticide detection.

References

[1]   M. Arias-Andrés, R. Rämö, F. Mena Torres, R. Ugalde, L. Grandas, C. Ruepert, L. Castillo, P. Van den Brink and J. Gunnarsson, "Lower tier toxicity risk assessment of agriculture pesticides detected on the Río Madre de Dios watershed, Costa Rica," *Environmental Science and Pollution Research*, Oct. 2016. Doi: https://doi.org/10.1007/s11356-016-7875-7

[2]   J. S. Van Dyk and B. Pletschke, "Review on the use of enzymes for the detection of organochlorine, organophosphate and carbamate pesticides in the environment," *Chemosphere*, vol. 82, no. 3, pp. 291–307, Jan. 2011. Doi: https://doi.org/10.1016/j.chemosphere.2010.10.033

[3]   X. Wu, Y. Yu, M. Li, Y. Long, H. Fang and S. Li, "Prediction of bioavailability of chlorpyrifos residues in soil to earthworms," *Journal of Soil Science and Plant Nutrition*, vol. 11, no. 1, pp. 44-57, 2011. Doi: https://doi.org/10.4067/s0718-95162011000100004

[4]   S. Andreescu, V. Magearu, A. Lougarre, D. Fournier and J. Marty, "Inmobilization of enzymes on screen printed sensors via an Histidine Tail application to the detection of pesticides using modified Cholinesterase", *Analytical Letters*, vol. 34, no. 4, pp. 529-540, 2001. Doi: https://doi.org/10.1081/al-100002593

[5]   S. Andreescu, L. Barthelmebs and J. Marty, "Immobilization of acetylcholinesterase on screen-printed electrodes: comparative study between three immobilization methods and applications to the detection of organophosphorus insecticides," *Analytica Chimica Acta,* vol. 464, no. 2, pp. 171-180, Aug. 2002. Doi: https://doi.org/10.1016/s0003-2670(02)00518-4

[6]   F. Şahin, G. Demirel and H. Tümtürk, "A novel matrix for the immobilization of acetylcholinesterase," *International Journal of Biological Macromolecules*, vol. 37, no. 3, pp. 148-153, Nov. 2005. Doi: https://doi.org/10.1016/j.ijbiomac.2005.10.003

[7]   N. Chauhan, J. Narang and C. Pundir, "Immobilization of rat brain acetylcholinesterase on ZnS and poly(indole-5-carboxylic acid) modified Au electrode for detection of organophosphorus insecticides," *Biosensors and Bioelectronics*, vol. 29, no. 1, pp. 82-88, Nov. 2011. Doi: https://doi.org/10.1016/j.bios.2011.07.070

[8]   P. Andrić, A. Meyer, P. Jensen and K. Dam-Johansen, "Reactor design for minimizing product inhibition during enzymatic lignocellulose hydrolysis: I. Significance and mechanism of cellobiose and glucose inhibition on cellulolytic enzymes,"

Biotechnology Advances, vol. 28, no. 3, pp. 308-324, May. 2010. Doi: https://doi.org/10.1016/j.biotechadv.2010.01.003

[9]   T. Darreh-Shori and H. Soininen, "Effects of cholinesterase inhibitors on the activities and protein levels of cholinesterases in the cerebrospinal fluid of patients with Alzheimer's disease: a review of recent clinical studies," *Curr. Alzheimer Res.*, vol. 7, no. 1, pp. 67–73, 2010. Doi: https://doi.org/10.2174/1567209198607252050

[10] C. Yung-Chi and W. Prusoff, "Relationship between the inhibition constant (KI) and the concentration of inhibitor which causes 50 per cent inhibition (I50) of an enzymatic reaction," *Biochemical Pharmacology*, vol. 22, no. 23, pp. 3099-3108, Dec. 1973. Doi: https://doi.org/10.1016/0006-2952(73)90196-2

[11] N. Ben Oujji, I. Bakas, G. Istamboulié, I. Ait-Ichou, E. Ait-Addi, R. Rouillon, and T. Noguer, "Sol-gel immobilization of acetylcholinesterase for the determination of organophosphate pesticides in olive oil with biosensors," *Food Control,* vol. 30, no. 2, pp. 657–661, Apr. 2013. Doi: https://doi.org/10.1016/j.foodcont.2012.09.005

[12] D. Du, S. Chen, J. Cai and A. Zhang, "Immobilization of acetylcholinesterase on gold nanoparticles embedded in sol–gel film for amperometric detection of organophosphorous insecticide," *Biosensors and Bioelectronics*, vol. 23, no. 1, pp. 130-134, 2007. Doi: https://doi.org/10.1016/j.bios.2007.03.008

[13] J. Gong, L. Wang and L. Zhang, "Electrochemical biosensing of methyl parathion pesticide based on acetylcholinesterase immobilized onto Au–polypyrrole interlaced network-like nanocomposite," *Biosensors and Bioelectronics*, vol. 24, no. 7, pp. 2285-2288, Mar. 2009. Doi: https://doi.org/10.1016/j.bios.2008.11.012

[14] L. Betancor and H. R. Luckarift, "Bioinspired enzyme encapsulation for biocatalysis," *Trends Biotechnol.*, vol. 26, no. 10, pp. 566–572, Oct. 2008. Doi: https://doi.org/10.1016/j.tibtech.2008.06.009

[15] S. Upadhyay, G. R. Rao, M. K. Sharma, B. K. Bhattacharya, V. K. Rao, and R. Vijayaraghavan, "Immobilization of acetylcholineesterase-choline oxidase on a gold-platinum bimetallic nanoparticles modified glassy carbon electrode for the sensitive detection of organophosphate pesticides, carbamates and nerve agents," *Biosensors and Bioelectronics*, vol. 25, no. 4, pp. 832–838, Dec. 2009. Doi: https://doi.org/10.1016/j.bios.2009.08.036

[16] Z. Miao, P. Wang, A. Zhong, M. Yang, Q. Xu, S. Hao, and X. Hu, "Development of a glucose biosensor based on electrodeposited gold nanoparticles-polyvinylpyrrolidone-polyaniline nanocomposites," *Journal of Electroanalytical Chemistry*, vol. 756, pp. 153–160, Nov. 2015. Doi: https://doi.org/10.1016/j.jelechem.2015.08.025

[17] Y. Zhang, M. A. Arugula, M. Wales, J. Wild, and A. L. Simonian, "A novel layer-by-layer assembled multi-enzyme/CNT biosensor for discriminative detection between organophosphorus and non-organophosphrus pesticides," *Biosensors and Bioelectronics*, vol. 67, pp. 287–295, May. 2015. Doi: https://doi.org/10.1016/j.bios.2014.08.036

[18] T. Liu, H. Su, X. Qu, P. Ju, L. Cui and S. Ai, "Acetylcholinesterase biosensor based on 3-carboxyphenylboronic acid/reduced graphene oxide–gold nanocomposites modified electrode for amperometric detection of organophosphorus and carbamate pesticides," *Sensors and Actuators B: Chemical*, vol. 160, no. 1, pp. 1255-1261, Dec. 2011. Doi: https://doi.org/10.1016/j.snb.2011.09.059

[19] N. Chauhan and C. Pundir, "An amperometric biosensor based on acetylcholinesterase immobilized onto iron oxide nanoparticles/multi-walled carbon nanotubes modified gold electrode for measurement of organophosphorus insecticides," *Analytica Chimica Acta*, vol. 701, no. 1, pp. 66-74, Sep. 2011. Doi: https://doi.org/10.1016/j.aca.2011.06.014

[20] V. Dhull, A. Gahlaut, N. Dilbaghi, and V. Hooda, "Acetylcholinesterase biosensors for electrochemical detection of organophosphorus compounds: A review," *Biochemistry Research International*, vol. 2013, 2013. Doi: https://doi.org/10.1155/2013/731501

[21] C. S. Pundir and N. Chauhan, "Acetylcholinesterase inhibition-based biosensors

for pesticide determination: A review," *Analytical Biochemistry*, vol. 429, no. 1, pp. 19–31, Oct. 2012. Doi: https://doi.org/10.1016/j.ab.2012.06.025

[22]   K. Quesada-Montero, K. Ramírez-Sánchez, and R. Starbird-Pérez, "Fijación de la enzima acetilcolinesterasa (ACHE) en una matriz de poli(alcohol vinílico) (pva) utilizando benzoato de sodio como agente entrecruzante" *Revista Iberoamericana de Polímeros y Materiales*, vol. 18, no. 4, pp. 215–228, 2017.

[23]   J. Yon-Kahn and G. Hervé, *Molecular and cellular enzymology*, Berlin, Heidelberg, Springer, 2010. Doi: https://doi.org/10.1007/978-3-642-01228-0

[24]   M. Yoshino and K. Murakami, "A graphical method for determining inhibition constants," *Journal of Enzyme Inhibition and Medicinal Chemistry*, vol. 24, no. 6, pp. 1288–1290, Nov. 2009. Doi: https://doi.org/10.3109/14756360902829766

[25]   M. A. T. Phan, J. Wang, J. Tang, Y. Z. Lee, and K. Ng, "Evaluation of α-glucosidase inhibition potential of some flavonoids from Epimedium brevicornum," *LWT - Food Science and Technology*., vol. 53, no. 2, pp. 492–498, Oct. 2013. Doi: https://doi.org/10.1016/j.lwt.2013.04.002

[26]   T. Fukuto, "Mechanism of Action of Organophosphorus and Carbamate Insecticides," *Environmental Health Perspectives*, vol. 87, p. 245-254, 1990.

[27]   Y. Sagane, "Molecular characterization of maize acetylcholinesterase. A novel enzyme family in the plant kingdom," *Plant Physiol.*, vol. 138, no. 3, pp. 1359-1371, Jul. 2005. Doi: https://doi.org/10.1104/pp.105.062927

[28]   M. B. Colovic, D. Z. Krstic, T. D. Lazarevic-Pasti, A. M. Bondzic, and V. M. Vasic, "Acetylcholinesterase inhibitors: pharmacology and toxicology," *Curr. Neuropharmacol.*, vol. 11, no. 3, pp. 315–335, 2013. Doi: https://doi.org/10.2174/1570159x11311030006

[29]   K. Kamoshita, I. Ohno, T. Fujita, T. Nishioka and M. Nakajima, "Quantitative structure-activity relationships of phenyl N-methylcarbamates against house fly and its acetylcholinesterase," *Pesticide Biochemistry and Physiology,* vol. 11, no. 1-3, pp. 83-103, Jul. 1979. Doi: https://doi.org/10.1016/0048-3575(79)90050-6

[30]   B. Xuereb, P. Noury, V. Felten, J. Garric, and O. Geffard, "Cholinesterase activity in Gammarus pulex (Crustacea Amphipoda): Characterization and effects of chlorpyrifos," Toxicology, vol. 236, no. 3, pp. 178–189, Jul. 2007. Doi: https://doi.org/10.1016/j.tox.2007.04.010