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An optical fiber biosensor for chlorpyrifos using a single sol–gel film containing acetylcholinesterase and bromothymol blue

Bambang Kuswandi *, Chulaifah Indah Fikriyah, Agus Abdul Gani

Chemo and Biosensors Group, School of Pharmacy, University of Jember, Jember 68121, Indonesia

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Abstract

An optical fiber biosensor consisting of acetylcholinesterase (AChE) and bromothymol blue (BTB) doped sol–gel film was employed to detect organophosphate pesticide chlorpyrifos. The main advantage of this optical biosensor is the use of a single sol–gel film with immobilized AChE and BTB. The compatibility of this mixture (AChE and BTB) with the sol–gel matrix has prevented leaching of the film. The immobilization of the enzyme and indicator was simple without chemical modification. The biosensing element on single sol–gel film has been placed inside the flow-cell for flow system. In the presence of a constant AChE, a color change of the BTB and the measured reflected signal at wavelength 622 nm could be related to the pesticide concentration in the sample solutions. The performance of optical biosensor in the flow system has been optimized, including chemical and physical parameters. The response time of the biosensor is 8 min. A linear calibration curve of chlorpyrifos against the percentage inhibition of AChE was obtained from 0.05 to 2.0 mg/L of chlorpyrifos (18–80% inhibition, $R^2 = 0.9869$, $n = 6$). The detection limit for chlorpyrifos was 0.04 mg/L. The results of the analysis of 0.5–1.5 mg/L of chlorpyrifos using this optical biosensor agreed well with chromatographic method.

Keywords: Optical fiber biosensor; Acethylcholinesterase; Sol–gel; Chlorpyrifos

1. Introduction

A wide variety of pollutants from industrial, agricultural and other human activities can contaminate aquatic environments. Among environmental pollutants, pesticides are of relevant concern, due to their toxicity and the prevalence of their use. In particular, organophosphate pesticides are widely used for agricultural and other domestic purposes. Even, these pesticides have relatively low persistence in the environment; however they have high acute toxicity to human health and ecosystems [1]. Chlorpyrifos is one of the most frequently used organophosphate pesticides in agriculture as commonly insecticide used to control insect and arthropod pests on agricultural and vegetable crops (e.g. grains, nuts, cottons and fruits). Due to agricultural and point source discharges, chlorpyrifos is most responsible for aquatic life toxicity [2]. Therefore, detection of chlorpyrifos is importance, which has enforced different regulatory agencies for chlorpyrifos detection. For example, under joint decision by the Ministers of Health and Agriculture Republic of Indonesia, No. 881/Menkes/SKB VIII/1996 and No. 771/Kpts/Tp. 270/8/1996, the maximum limit permitted for chlorpyrifos as pesticide residue is 0.05 mg/L [3]. Thus, it has strictly limited the discharges, emissions and losses of chlorpyrifos.

A wide variety of analytical methods have been reported in the literature for pesticide determination. Standard analytical methods, such as liquid chromatography (LC) [4], high performance liquid chromatography (HPLC) [5], and gas chromatography (GC) [6], have been commonly used for an efficient determination of chlorpyrifos and its metabolites [7]. However, these methods are time-consuming; require labor-intensive preparation of samples and skilled personnel.

An alternative to chromatographic methods, the enzyme-based biosensors have been described in literature [8–15]. For instance, different kinds of cholinesterases as biosensing elements have been employed to measure the loss of enzyme activity after inhibited reaction by analyte. The detection of organophosphate compounds with acetylcholinesterase-based biosensors have been proposed using potentiometric [13], amperometric [14] and optical transducers (spectrometric or fluorometric) [9–15].

* Corresponding author.
E-mail address: b_kuswandi@farmasi.unej.ac.id (B. Kuswandi).

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Optical transducers in particular, have many interests in recent years [9]. Since, many new materials can be used in optical biosensors such as zeolite, conducting polymers, sol–gel, etc. [8,9]. Sol–gel for instance, there are many advantages, such as its optical clarity, the ability to entrap biomolecules (e.g. enzymes, DNA, antibody), thermal and chemical stability, simplicity of preparation and flexibility in controlling pore size and geometry [10]. Many works have reported the use of sol–gel and enzymes as optical biosensors for the determination of pesticides [10,11,16–18]. For optical biosensor based on sol–gel using pH indicator as transducer, the immobilization of indicator is an essential part of the biosensor fabrication process. A number of pH indicators have been incorporated into sol–gel film and in most cases, covalent binding is required to prevent leaching and loss of the pH indicator from the film [11,16]. In addition, a simple approach has been proposed by Ahmad and co-workers [18] using a chromoionophore (ETH5294) doped sol–gel film interfaced with another sol–gel film immobilized with acetylcholinesterase (AChE). However, this optical biosensor has been used double sol–gel layers for biosensing element, which in turn can lengthening sensor response time (12 min), reducing detection limit (0.5 mg/L) and reproducibility (<16%) for organophosphate pesticides (dichlorvos), since the measurements were also performed in batch mode.

In this work, we have entrapped a bromothymol blue (BTB) and AChE in a single sol–gel film with simple procedure without further chemical modification. The optical fiber biosensor proposed has faster response time (8 min), lower detection limit (0.04 mg/L) and better reproducibility (<1.5%) for organophosphate pesticide (chlorpyrifos) compared to previous work [18]. The suitability of this biosensor in the flow system for detection of chlorpyrifos has also described, which allow its utilization as alarm warning system in the pesticides monitoring. We also describe the validation of the biosensor results by comparison with a chromatographic method.

2. Experimental

2.1. Reagents

Acetylcholinesterase (E.C. 3.1.1.7) from electric eel (Type V I-S, 480 U/mg solid, AChE) and acetylcholine chloride (ACh) were purchased from Sigma Chemicals, chlorpyrifos was purchased from Riedel-de Haen, tetraethoxysilane (TEOS), a precusor of sol–gel, 2-pyrimidine aldoxime methiodide (2-PAM) and bromothymol blue were purchased from Merck. All other chemicals were of analytical grade and used as received without further purification. All solutions were prepared with double distilled or deionized water.

2.2. Enzyme and pH indicator immobilization

The sol–gel was prepared as described before with minor modification [10,18]. The acid-catalyzed sol solution was prepared by mixing 4.5 mL of tetraethoxysilane (TEOS), 1.4 mL of double distilled and deionized water and 0.10 mL of 0.1 M HCl in a glass vial. The mixture was then stirred (5 h) until a clear solution was formed and left overnight at room temperature before used. A solution of 1.5 mg/mL BTB was prepared by dissolving the BTB in absolute ethanol as a solvent. For the preparation in sol–gel film, 3 μL of BTB solution, 3 μL of sol solution and 10 μL of Tris–HCl buffer 0.1 M (pH 8) were mixed together. Then 30 μL 100 U/mL enzyme solutions in 0.1 M Tris–HCl buffer (pH 8) was added to the mixture of sol solution before spin coated with speed of 300 rpm for 10 s. The mixture was then put in designed circular mold (1 cm in i.d. and 1 mm depth) left for 5 days at a refrigerator (4 °C) where the film formed. The final biosensor film was taken from the mold and stored in a sealed container and kept in a refrigerator (4 °C) until used. Here, the addition of Tris–HCl buffer in sol–gel process is needed in order to reserve enzyme activity in the sol–gel matrix.

2.3. Optical fiber biosensors construction

The construction of the optical fiber biosensor has been carried out by carefully placed a single sol–gel film doped with AChE and BTB into the specially designed flow-cell [12,19]. In this biosensor design, it allows reducing the effect of other incident light levels on the flow-cell and optical system. Furthermore, the single film used was possible in this biosensor construction, due to the fact that AChE did not inhibited by BTB, either in sol–gel or in solution [20]. Thus, this single film makes the biosensor really simple and eases to be constructed in such optical fiber biosensor design.

In order to monitor a color change of the film as a function of the biosensor response, the flow-cell was then directly faced and geometrically adapted to the circular end of a fiber optic bundle probe. The flow-cell device incorporated two stainless-steel needles (1 mm o.d., 20 mm length), positioned at 120° opposite each other, to serve as inlet/outlet for solution flow. To provide a reflective surface backing, the circular bottom end of the flow-cell was also fitted with a with PTFE disk (at 2 mm distance from the probe tip). The internal flow-cell has a measuring volume of approximately 20 μL.

2.4. Flow manifold and measurement procedure

The flow-cell set-up described above was integrated with flow injection analysis (FIA) system, with the flow manifold configuration as shown in Fig. 1. The FIA system comprises three injection valves (Omnifit 1106) and four channels peristaltic pump equipped with Tygon tubing (1.0 mm i.d). Teflon® tubing was used between all components in the FIA system (FIA 5010, Tecator). The determination of chlorpyrifos is based on inhibition of AChE in the presence of the substrate (ACh). In the absence of chlorpyrifos, acetylcholine is bio-catalytically hydrolyzed to yield acetic acid and choline. The release acid originates a pH decrease that is sensed by the BTB, the color of which varies from green to yellow leading to an increase in the reflected signal intensity (at 622 nm) measured by optical fiber. In the presence of increasing amount of pesticide and a constant ACh concentration, the activity of the enzyme AChE decreases so that a smaller pH change occurs and a change in the reflected signal intensity of the sensor is measured using

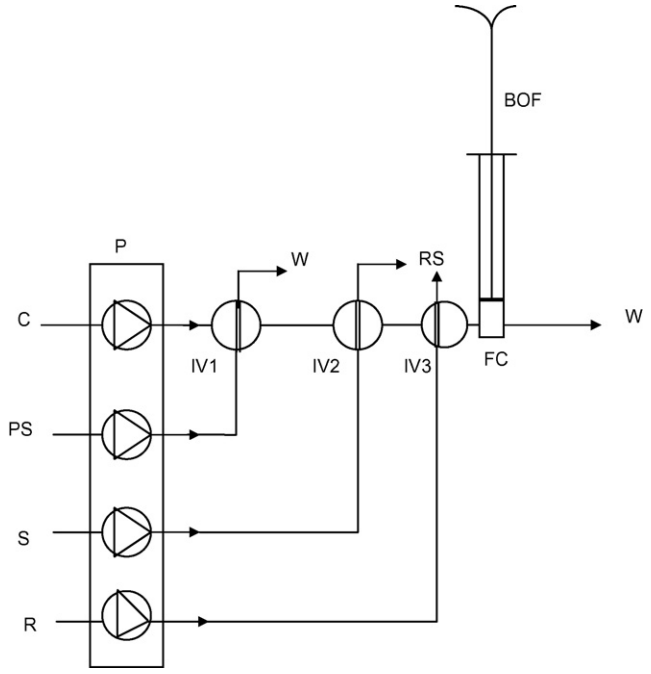


Fig. 1. Flow manifold used for measurement of enzymatic determination of chlorpyrifos. C, carrier (pH 7.5, 7.5 mM Tris buffer); PS, sample (pesticides solution); S, substrate (acetylcholine solution); R, reactivation solution; P, peristaltic pump; IV, injection valve; RS, reservoir; FC, flow-cell; BOF, bifurcated optical fiber; W, waste.

an optical fiber spectroscopy (USB2000 spectrometer) as that described previously [12,19].

Initially, a carrier stream of a Tris buffer was pumped through the FIA system in order to obtain a baseline signal. Three injection valves were connected in series; the substrate (ACh) solution was injected manually through injection valve 2 to determine the initial activity of enzyme (i.e., absence of inhibition (E_0)). The sample solution of chlorpyrifos was injected, using valve 1, into the carrier stream. After inhibition, the residual enzyme activity was revealed by substrate (ACh) injection and the percentage inhibition (E_c) was calculated according to the following expression:

$$\text{inhibition}\,(\%I) = \left[\frac{(E_0 - E_c)}{E_0}\right] \times 100 \quad (1)$$

where E_0 and E_c are the peak height reflected signal intensity for substrate and substrate plus inhibitor, respectively. After the inhibition measurement, the reactivation solution (2-PAM) had to be introduced via injection valve 3 in order to remove chlorpyrifos from the immobilised enzyme effectively and recover its activity. The whole cycle measurement was repeated for other concentrations of chlorpyrifos. Furthermore, the sol–gel film had to be carefully washed with water after each set of measurement in order to avoid the precipitation of the salts present in the buffering solution that could also be block the flow system.

2.5. Determination with chromatographic methods

To compare the results of analysis with standard methods for chlorpyrifos determination, gas chromatography using mass spectrometry detection (GC–MS) method was chosen [21]. Water samples for analysis were first spiked with different concentrations of chlorpyrifos from 0.5 to 1.5 mg/L. For GC–MS analysis, the samples were first extracted with solid-phase extraction SPE column (Bakerbond spe^{TM}). Each column contained 6 mL disposable extraction cartridges packed with 200 mg styrene divinylbenzene copolymer. To extract and concentrate all samples, an Autotrace SPE workstation and TurboVap LV evaporator were used. Water samples of 500 mL, in which 5 mL of methanol had been added, were passed over the conditioned sorbent. Afterwards, the sorbent was dried under nitrogen for 20 min. Elution was performed by soaking and eluting the cartridge with 5 mL of ethyl acetate. The eluted was then dried and the final volume was precisely adjusted to 0.5 mL of isooctane.

A GC 8000 series capillary gas chromatograph coupled to a MD800 mass spectrometer (Fisons Instruments) was used for chlorpyrifos analysis. Chromatographic separations were conducted on a DB-5 MS fused-silica capillary column, 30 m × 0.25 mm i.d. × 0.25 μm film thicknesses, with helium as a carrier gas, at a flow rate of 1.5 mL/min. Injections of 1 μL volume of sample were performed on a splitless injector using an AS 800 autosampler (CE Instruments). The mass spectrometer operated in the EI mode. The injector temperature was set at 270 °C, whilst the interface and ion source temperatures were maintained at 270 and 200 °C, respectively. The identification and quantification of chlorpyriphos were carried out by monitoring *m/z* 314 and 316 with the instrument. The linear plot (peak area versus concentration) was constructed.

3. Results and discussion

3.1. Optimization of experimental parameters

In order to determine the optimum operating parameters (chemical and physical) for this method a series of preliminary investigations were performed and Table 1 shows the range over which each of the parameter was investigated and its optimum value. Within the chemical parameters, the most importance ones was found to be those directly involved in the enzymatic process, i.e. the buffer, pH and substrate (ACh) concentration. For the enzymatic reaction, Tris buffer was used as the buffer at a concentration of 7.5 mM, where both the blank and the inhibition signals were optimum. Above this concentration the reaction

Table 1
Optimisation of experimental parameters

Experimental conditions	Value range	Optimal value
[ACh] (mM)	2.5–100	40
pH	6.0–8.0	7.5
Buffer concentration (mM)	1.0–10.0	7.5
Substrate injection volume (mL)	1.0–5.0	2.0
Pesticide injection volume (mL)	1.0–5.0	1.5
Flow rate (mL/min)	0.1–5.0	0.2
Inhibition time (min)	1.0–10.0	7.0
Reactivation solution (mL)	1.0–10.0	1.0

rate decreases along with inhibition degree, and below this concentration the reproducibility of response is slightly poor. A pH 7.5 was found to be optimum for the enzymatic process. Here, both ionic strength (buffer concentration) and pH are importance in determining the biosensor response, due to the fact that the biosensing process is based on the net of pH change before and after inhibition of the enzyme with pesticides. Furthermore, though low concentrations of ACh produced measurable blank signal and inhibition degree, it resulted in a low level of the blank signal. Thus, a concentration of 40 mM of ACh was chosen as a compromise.

In general the FIA parameters were important in the optimisation of the inhibition process, in particular, flow-rate, reagents and sample injections, and time of inhibition. A low flow-rate of 0.2 mL/min was chosen as it allowed the blank signal to develop to a greater extent with relatively low concentration of ACh. The injection volume of substrate (ACh) was found to be optimum at 2.0 mL, as the smaller volume caused a decrease in the blank signal, which in turn, the degree of inhibition was decreased as well. The optimum time of inhibition was selected at 7 min, as compromise between the sensitivity, sample throughput and lifetime of the enzyme. The sample injection volume was optimised in the range of 1.0–5.0 mL, and 1.5 mL was selected as a trade of between sensitivity and sampling rate. The reactivation (1 mM 2-PAM,) injection volume was also optimised in the range of 1.0–5.0 mL, and 1.0 mL was selected as desired volume for reactivation of the inhibited AChE.

3.2. The response of optical biosensor toward chlorpyrifos

The biosensor resulting from immobilisation of AChE and BTB on sol–gel film has been tested towards chlorpyrifos as organophosphate pesticides. In uninhibited scheme or blank solution, the biosensor measures a pH change generated by the release of acetic acid during the enzymatic reaction according to the Eq. (2). While in inhibited scheme, the biosensor also measures a pH change where enzyme AChE is inhibited by pesticides. As a whole measurement, actually the biosensor measures a net of pH change before and after inhibition of the enzyme AChE. The inhibition of AChE is normally attributed to the acylation of the serine–OH in the active site of the enzyme by this compound [22]. The reaction with AChE is analogous to the enzyme–substrate reaction, whereby a Michaelis enzyme–pesticide complex is first formed, followed by the transfer of the pesticide acyl groups to the serine–OH of enzyme, and the concomitant release of the side product (HB). Therefore, the enzymatic inhibition by chlorpyrifos is carried out according to the Eq. (3):

$$\text{acethylcholine} + H_2O \xrightarrow{\text{AChE}} \text{acetic acid} + \text{choline} \qquad (2)$$

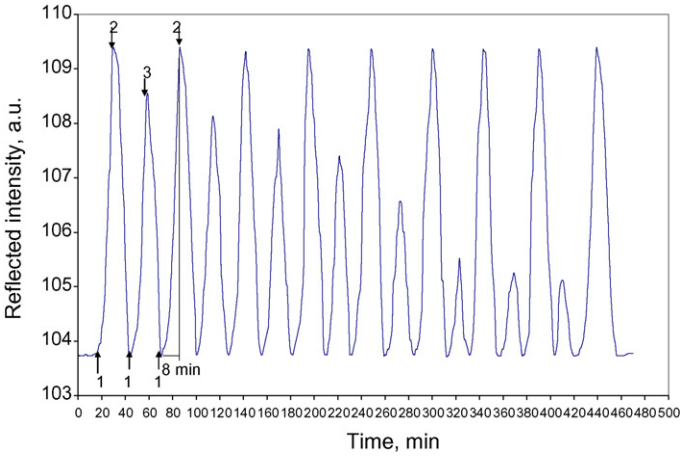
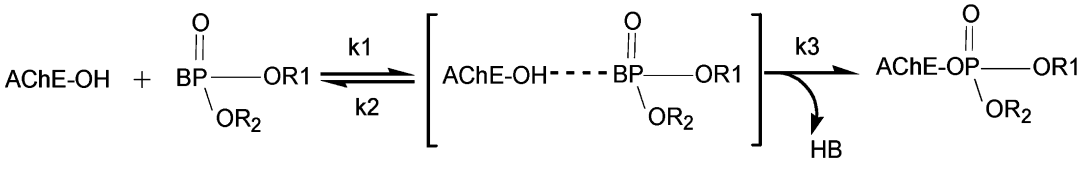


Fig. 2. The response of the biosensor towards various concentrations of chlorpyrifos (0–4 mg/L). The response time was 8 min. The arrows indicate the injection point of substrate (ACh) [1], chlorpyrifos [2] and reactivation solution, 2-PAM [3]. From the left to the right, first peak is for uninhibited biosensor response (blank response), second peak for chlorpyrifos 0.05 mg/L, third inhibited peak for 0.1 mg/L, fourth inhibited peak for 0.2 mg/L, fifth inhibited peak for 0.5 mg/L, sixth inhibited peak for 1.0 mg/L; seventh inhibited peak for 2.0 mg/L, eighth inhibited peak for 3.0 mg/L and ninth inhibited peak for 5.0 mg/L of chlorpyrifos concentration respectively.

where $k_{(1–3)}$ are rate constants. The rate-limiting step is the formation of the AChE-organophosphate complex. However, the rate of hydrolysis of phorphorylated AChEs was very slow. Consequently, when the enzymes are reacted again with the natural substrate (ACh), a lower pH change would be detected since the enzyme not fully reactivated (remains inhibited). In order to activate the enzyme again, the reactivation/regeneration solution is needed, where in this case 2-PAM (1 mM) was used [11]. The difference in the pH change before and after the pesticide inhibition provides information on the amount of the pesticide, which in turn can be utilized for chlorpyrifos determination.

The response of the biosensor towards various concentrations of chlorpyrifos is given in Fig. 2, for which the response time of the optical biosensor is 8 min. This lower response time compared to previous work [18] would be due to a single sol–gel film and flow system used, which in turn can increase response time. Here, the time limiting step is controlled by the diffusion of analyte within the film in a flow mode, while in a batch mode, it controlled by the convective mass extraction from the film of the aqueous solution that goes on until the required amount of analyte is reached [23].

Based on Fig. 2 the calibration curves for chlorpyrifos could be constructed. The percentage inhibition increase linearly with increasing chlorpyrifos concentration in the range of 0.05–2.0 mg/L ($\%I = 0.0333$ [chlorpyrifos] + 18.028) equal



(3)

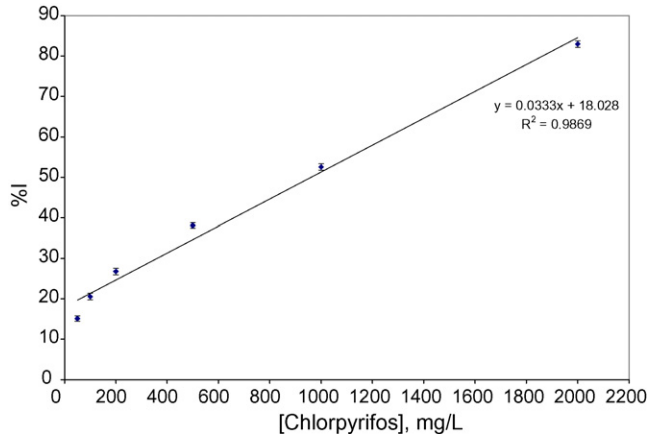


Fig. 3. The percentage inhibition increase linearly with increasing chlorpyrifos concentration in the range 0.05–2.0 mg/L (each data point is an average of three replicates).

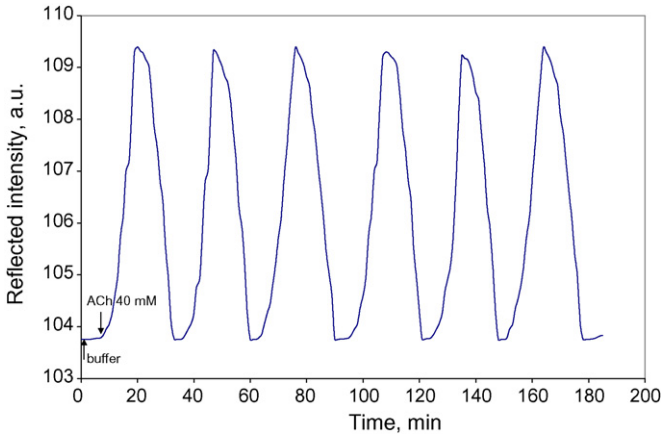


Fig. 4. The responses of the biosensor toward substrate, acethylcholine [40 mM ACh].

to 18–80% inhibition ($r^2 = 0.9869$, $n = 6$) where each point was the average of three injections (Fig. 3). For higher chlorpyrifos concentrations, flat response of the biosensor was reached. The reactivation of the immobilized AChE was achieved by injection with the reactivation solutions (2-PAM). The detection limit (3ε) of the biosensor for chlorpyrifos was 0.04 mg/L. This value was 10-fold lower than previously work reported [18], and could allow the detection of chlorpyrifos within the maximum residue concentration permitted by Indonesian Government (0.05 mg/L) [3].

3.3. Reactivation and reproducibility

It is desirable for inhibited enzyme-based optical biosensor that its enzyme activity be recovered easily and efficiently. Thus, it will allow a more economical use of the sensor and facilities its easier handling [19]. However, like in this case, results have shown that the AChE that has been exposed to chlorpyrifos, a spontaneous recovery of AChE activity was not achieved, even with a repetitive treatment of substrate (ACh) solution. This can be due to the strong ester linkage between the dimethyl phosphate group and the serine moiety of enzyme. Significant reactivation of the enzyme-based biosensor signal was attained after the biosensor has been treated with 1 mM 2-PAM as shown in Fig. 2. The reactivation effect of 2-PAM is due to its strong nucleophilic character. Thus, it makes reactive toward the electrophilic phosphorous atom of the phosphorylated AChE. By doing so, it displaces the AChE from its attachment to the phosphorus, then yielding the free and active form of AChE again [11,12].

The reproducibility of the biosensor has been evaluated toward substrate (ACh) as a blank signal, pesticides (inhibited AChE) as inhibition measurements, and the reactivation/regeneration process. The biosensor was injected with substrate only, for the blank signal reproducibility, while for inhibition, the biosensor was alternately reacted with 1 mg/L chlorpyrifos and then regenerated using 1 mM 2-PAM. The results have been shown that the reproducibility of biosensor towards substrate as a blank signal was good (R.S.D. < 1.5% for $n = 6$) as given in Fig. 4. While for inhibition and reactivation steps were fairly reproducible (R.S.D. inhibition = 1.5% for $n = 3$ and R.S.D. reactivation = 1.2% for $n = 3$) as given in Fig. 5. The stability of the biosensor was good and even after repetitive use for a week, in the presence of chlorpyrifos, the biosensor retained 80% of its original activity. In fact, the biosensor produces reproducible and stable responses up to 2 months, as long as, the sol–gel film of AChE–BTB keep in 4 °C. After this time the biosensor behaviour gradually deteriorates. This is due to the fact that the biosensor response reduced within 25–30% compared to its original signal.

3.4. Comparison with chromatographic methods

The validation of optical biosensor was performed by using gas chromatography coupled to mass spectrometry (GC–MS) as the reference method. Samples spiked with chlorpyrifos at five different concentrations: 0.5, 0.75, 1.0, 1.25 and 1.5 mg/L were analyzed by both methods. The variability of the method was studied by injecting three replicate of each chlorpyrifos

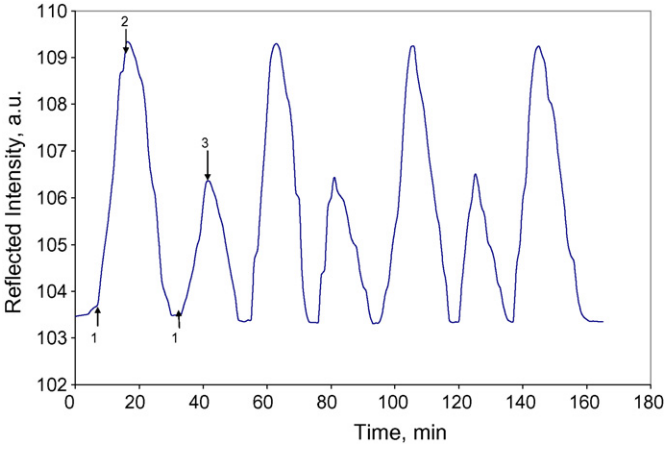


Fig. 5. The responses of the biosensor toward substrate [40 mM ACh] (1), inhibition [chlorpyrifos, 1 mg/L] (2) and reactivation solution [1 mM 2-PAM] (3).

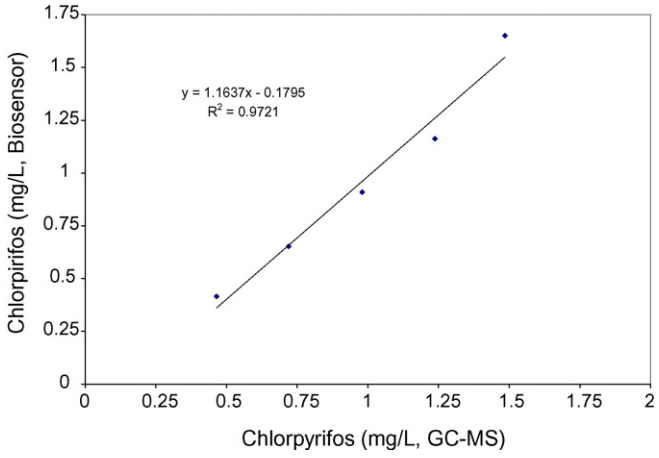


Fig. 6. A correlation of the concentrations of chlorpyrifos determined by the biosensor directly with that determined by GC–MS after solid-phase extraction procedure.

concentration. The linear regression analysis provided the calibration equation ($y = 1.1637x - 0.1795$) as shown in Fig. 6. The correlation data indicates a good agreement between methods ($r^2 = 0.9721$) and no significant discrepancies were found when comparing the biosensors and GC–MS methods, as shown by the slope of the linear regression analysis (1.16). However, the deviation from ideal value (1.0) might be due to the different on the recovery factor as a result of the random errors that could be occurred in both methods.

4. Conclusions

This work shows the utilization of optical fiber biosensor based on single sol–gel film containing of AChE–BTB in flow system for detection of widely used chlorpyrifos based on enzyme inhibition scheme. The main advantage of this optical biosensor is the use of a single sol–gel film with AChE and BTB, which in turn, resulting fast response time, low detection limit and good reproducibility. Furthermore, the immobilization of the biosensing element on sol–gel film was simple without any chemical modification. The biosensor developed can be used for the screening of chlorpyrifos in water samples. The comparison of the biosensor measurements with classical chromatographic method (GC–MS) demonstrated the suitability of biosensor for rapid analysis of pesticides, as compared with the long extraction pre-treatment methods required by the conventional method.

The fully automation of the optical fiber biosensor sensor in the flow system, could be achieved by using microfluidics incorporated in a platform of reduced size. This approach would provide very low reagents and sample used, which in turn, can achieved lower detection limit of pesticides, simple, fast analysis and real-time monitoring, which is currently been developed in our group.

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