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Electrochemical acetylcholinesterase biosensor based on ZnO nanocuboids modified platinum electrode for the detection of carbosulfan in rice

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ABSTRACT

The consumption of carbosulfan-contaminated rice affects the immune and lymphocyte response, germinal centers in the spleen, plasma cells in popliteal lymphoid nodes, bone marrow cells and granulocyte-macrophage progenitor cells. Towards this, a highly sensitive acetylcholinesterase (AChE) cyclic voltammetric biosensor based on zinc oxide (ZnO) nanocuboids modified platinum (Pt) electrode has been successfully developed. The Pt/ZnO/AChE/Chitosan bio-electrode was employed for the electrochemical detection of carbosulfan in rice sample. Under optimum conditions, the Pt/ZnO/AChE/Chitosan bio-electrode detected carbosulfan ranging from 5 to 30 nM with a detection limit (LOD) of 0.24 nM. The developed Pt/ZnO/AChE/Chitosan bio-electrode showed good recovery (99.06–100.96%), thus providing a promising tool for analysis of carbosulfan in rice sample.

1. Introduction

Carbosulfan is extensively utilized for the control of insects on rice (Plese et al., 2005). Carbosulfan pesticide has been utilized widely in the Guntur region of Andra Pradesh, India (Nagaraju and Rathnamma, 2014). The wide use of carbosulfan pesticide at different stages of rice cultivation for amending rice productivity has enhanced the concern of the users regarding the potential contamination of food (Sahoo et al., 1988). According to World Health Organization (WHO) and Food and Agriculture Organization (FAO), carbosulfan is listed together with malathion and dimethoate for toxicological assessment of pesticide residues (Nagaraju and Rathnamma, 2014). Acceptable daily intake of carbosulfan assessed by FAO and WHO is 0–0.01 mg kg^{-1} bw (Soler et al., 2006a). Abnormally, high carbosulfan levels can affect the immune and lymphocyte response, germinal centers in the spleen, plasma cells in popliteal lymphoid nodes, bone marrow cells and granulocyte-macrophage progenitor cells (Chandrasekara and Pathiratne, 2007). Since high concentrations of carbosulfan exhibit health risks, quantification of carbosulfan in rice is therefore crucial (Chandrasekara and Pathiratne, 2007). The dosage, lethality of carbosulfan and duration of exposure determine the toxicity level of carbosulfan (Capkin and Altinok, 2013; Nwani et al., 2010; Giri et al., 2002). For this reason, toxicity of carbosulfan pesticide in rice is one of the most popular issues within the field of food safety (Soler et al., 2006b).

Many techniques employed in environmental monitoring are based on enzyme inhibition, which are primarily concentrated on the detection of environmental toxins such as heavy metal ions (Ghica and Brett, 2008; Oehme and Wolfbeis, 1997) and pesticides (Liu et al., 2011; Arduini et al., 2010). High performance liquid chromatography, spectrophotometry and gas chromatography are the analytical methods used for determination of carbosulfan (Soler et al., 2006a, 2006b; Kabir et al., 2008). These methods have various limitations such as high cost, time-consuming and low accuracy (Singh et al., 2014; Nesakumar et al., 2014; Gumpu et al., 2014; Nesakumar et al., 2013). Owing to specific advantages such as high sensitivity, selectivity, simplicity and low cost, electrochemical acetylcholinesterase biosensor is employed for carbosulfan detection (Singh et al., 2014; Nesakumar et al., 2014, 2013; Gumpu et al., 2014). Many investigations report the fabrication of biosensor for detecting carbosulfan based on acetylcholinesterase (AChE) enzymatic inhibition (Chandrasekara and Pathiratne, 2007;

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Guessan et al., 2003; Capkin et al., 2014). Liu et al. (2007) developed a biosensor based on the inhibition of acetylcholinesterase (AChE) enzyme for the detection of carbosulfan with the IC_{50} value (concentration of carbosulfan that inhibits 50% AChE enzyme activity) reaching 16.20 μM (for female fish) and 26.80 μM (for male fish). Chandrasekara and Pathiratne (2007) used absorbance spectrophotometer for the detection of carbosulfan in which they used acetylthiocholine (ATCh) as the substrate and AChE as the enzyme. The calculated IC_{50} values of carbosulfan pesticide residue for sub-adult, fry and fingerlings stages of fish were 8.72, 3.37 and 7.02 μg L^{-1} respectively. The ultimate aim of the present work is the estimation of carbosulfan pesticide residue in rice by using cyclic voltammetry.

Among various nanoparticles, zinc oxide (ZnO) nanoparticles are extensively utilized in biosensors fabrication (Singh et al., 2014; Nesakumar et al., 2014, 2013; Gumpu et al., 2014). ZnO nanoparticles have isoelectric point (pI) of 9.5, which can immobilize an enzyme of low isoelectric point such as AChE (pI=5.35) and chitosan membrane (pI=6.8) by electrostatic interaction (Kabir et al., 2008; Singh et al., 2014; Liu et al., 2007; Sen et al., 2004). ZnO nanoparticles can act as a three dimensional matrix for enzyme immobilization (Singh et al., 2014; Nesakumar et al., 2014, 2013; Gumpu et al., 2014). In addition, charge transfer and enzyme mimicking ability of ZnO nanoparticles assist as excellent matrices for AChE enzyme. Even though ZnO nanoparticles have been widely utilized as the interface, it is seldom to find report on its usage for the application of carbosulfan detection in rice samples. In this context, the electrochemical properties of ZnO modified Pt electrode were investigated. In addition, the effect of key parameters such as pH, choice of AChE enzyme and acetylthiocholine substrate concentrations and scan rate was carefully optimized. More specifically, the design, development and performance of carbosulfan pesticide residue electrochemical biosensor based on the inhibition of AChE enzyme immobilized on ZnO modified Pt electrode have been reported.

2. Materials and methods

2.1. Materials

Carbosulfan, zinc acetate, 2-pyridine aldoxime methiodide, acetylthiocholine chloride (ATChCl) (≥ 99% purity) with molecular weight of 19,773 g M^{-1} and AChE from electricus eel with specific activity (E.C. 3.1.1.7, activity: 200–1000 U mg^{-1}) were obtained from Sigma-Aldrich, USA. Chemicals namely dibasic sodium phosphate dehydrate, monobasic sodium phosphate monohydrate, ascorbic acid, 0.5 wt% chitosan in 1% acetic acid (degree of deacetylation of 82.5%, molecular weight: 140,000 g mol^{-1}), lactic acid, glucose, potassium hydroxide and sodium hydroxide were purchased from Merck India Ltd., India. Cadmium acetate dihydrate was procured from Loba Chemie Pvt. Ltd., India. Cupric acetate, nickel chloride and urea were purchased from Thermo Fisher Scientific Pvt. Ltd., India. KCl saturated Ag/AgCl reference electrode (CHI111, 0.5 mm diameter), Platinum wire counter electrode (CHI115, 0.5 mm diameter) and platinum (Pt) electrode (CHI102, 2 mm diameter) were purchased from CH Instruments, Inc., USA. All solutions and reagents were prepared using deionized water (Millipore, USA).

2.2. Synthesis of ZnO nanoparticles

Zinc acetate (0.2 M) was added into a 150 mL deionized water and subsequently 0.5 M NaOH solution was slowly added. Then the solution mixture was stirred and heated at 393 K in hot air oven for 12 h. Later the solution was allowed to cool at room temperature and the obtained white colored precipitate was separated by centrifugation. The white colored precipitate was washed several times with deionized water and annealed in muffle furnace at 1023 K for 4 h.

2.3. Fabrication of Pt/ZnO/AChE/Chitosan bio-electrode

Prior to the fabrication of electrode, the Pt working electrode was polished with alumina (0.05 μm) powder. One mg of ZnO powder was suspended in 100 μL of PBS and sonicated for 20 min to get a finely dispersed white colored solution of ZnO. AChE (10 μL) and chitosan (10 μL) were further added to the finely dispersed white colored ZnO solution. Later, 3 μL of the product was taken and immobilized onto the Pt working electrode surface. The resulting Pt/ZnO/AChE/Chitosan bio-electrode was washed with deionized water and left to dry at room temperature for 1 h.

2.4. Characterization of ZnO nanoparticles

Field emission scanning electron microscope (FE-SEM, Model JSM 6701F, JEOL, Japan) was employed to obtain the morphology of ZnO nanoparticles. Image J 1.48 q software was utilized to estimate the grain size of ZnO nanoparticles. The structural property of ZnO nanoparticles was examined by X-ray diffractometer (XRD, D8 Focus, Bruker, Germany) with Cu Kα radiation of wavelength 1.5408 Å.

2.5. Electrochemical analysis

Electrochemical measurements were carried out using an electrochemical work station (CHI600C, CH Instruments, USA). The three electrode system comprised of a platinum wire as auxiliary electrode, a Ag/AgCl saturated with 0.1 M KCl as reference electrode and bare or modified Pt electrode as working electrode. Cyclic voltammetry was carried out in a 20 mL electrochemical cell with 5 mL PBS solution. All cyclic voltammetric studies were performed at room temperature in the potential range of −0.1 to 0.5 V.

2.6. AChE enzyme inhibition and reactivation

The proposed Pt/ZnO/AChE/Chitosan bio-electrode was applied for the determination of carbosulfan using cyclic voltammetry. For inhibition results, the cyclic voltammogram was measured in 0.1 M PBS (7.4 pH) containing 0.08 mM ATChCl and the Pt/ZnO/AChE/Chitosan bio-electrode. This was further incubated in a given concentration of carbosulfan for 20 min. After inhibition, the residual signal was recorded and the degree of inhibition was calculated using the relation,

$$I\% = \frac{J_0 - J_i}{J_0} \times 100$$

where, J_i represents the peak current density of ATChCl on Pt/ZnO/AChE/Chitosan bio-electrode with carbosulfan inhibition and J_0 denotes the peak current density of ATChCl on Pt/ZnO/AChE/Chitosan bio-electrode without carbosulfan inhibition.

Reactivation of Pt/ZnO/AChE/Chitosan bio-electrode was studied with 2-pyridine aldoxime methiodide [2-PAM]. The Pt/ZnO/AChE/Chitosan bio-electrode was firstly immersed in 0.1 M PBS (pH 7.4) comprising 0.08 mM ATChCl and 20 nM carbosulfan for 20 min. After that, AChE reactivation was performed with 2.0 mM 2-PAM for 20 min and the cyclic voltammetric response to 0.08 mM ATChCl before and after reactivation was measured.

$$R(\%) = \frac{J_r - J_{p,exp}}{J_{p,control} - J_{p,exp}} \times 100$$

where, $R(\%)$ is the reactivation efficiency, $J_{p,exp}$ is the peak current

density of ATChCl on Pt/ZnO/AChE/Chitosan bio-electrode with carbosulfan inhibition, $J_{p,control}$ is the peak current density of ATChCl on Pt/ZnO/AChE/Chitosan bio-electrode and J_r is the peak current density of ATChCl on Pt/ZnO/AChE/Chitosan bio-electrode with 2-PAM reactivation.

2.7. Preparation of real samples

The procedure for carbosulfan determination in rice samples is as follows: (1) At first, the rice samples was crushed into powder with the help of mortar and pestle. (2) 30 mg of rice powder was mixed with 100 mL PBS solution and centrifuged at 8000 rpm for 30 min. (3) After centrifugation, 0.2 mL of supernatant was diluted with 20 mL of deionized water. To examine the potential application of the developed Pt/ZnO/AChE/Chitosan bio-electrode in rice sample analysis, the Pt/ZnO/AChE/Chitosan bio-electrode was subjected to recovery tests by adding different concentrations of carbosulfan in rice samples. Finally, the measured degree of inhibition was compared with the calibration curve to quantify the carbosulfan concentration in rice sample.

3. Results and discussion

3.1. Structural and morphological characterizations of nanostructured ZnO samples

As observed from the FE-SEM micrograph (Fig. 1(a)), the ZnO particles were nearly cuboid in shape. The inset Fig. 1(b) depicts that the length of these ZnO cuboids ranges from 341.16 nm to 455.31 nm with mean length of 398.24 ± 57.0 nm ($n=28$, RSD = 14.33%). The inset Fig. 1(c) illustrated that the height of these ZnO cuboids ranges from 134.41 nm to 192.14 nm with mean height of 163.28 ± 28.86 nm ($n=29$, RSD = 17.67%).

Fig. 1(d) shows the typical XRD patterns of ZnO nanoparticles. For the as-prepared ZnO nanocuboids, the detected peaks observed at 31.91°, 34.19°, 36.05°, 47.46°, 56.37°, 62.59°, 66.32°, 67.78°, 69.23°, 72.54° and 76.90° can be attributed to the (100), (002), (101), (102), (110), (103), (200), (112), (201), (004) and (202) planes of ZnO according to Joint Committee on Powder Diffraction Standards (JCPDS) card 36-1651. These peaks have confirmed the polycrystalline nature of ZnO nanoparticles with hexagonal wurtzite structure.

3.2. Optimization of experimental variables for carbosulfan detection

3.2.1. Effect of chemically modified Pt electrode

Cyclic voltammetric response of (i) bare Pt, (ii) Pt/AChE/Chitosan, (iii) Pt/ZnO/Chitosan, (iv) Pt/ZnO/AChE/Chitosan electrodes in pH 7.4 PBS containing 2.0 mM ATChCl performed at a scan rate of 0.5 V s^{-1} is shown in Fig. 2(a). The cathodic and anodic potentials of Pt/AChE/Chitosan, Pt/ZnO/Chitosan and Pt/ZnO/AChE/Chitosan bio-electrodes were located at 0.159 V and 0.232 V, 0.130 V and 0.256 V and 0.096 V and 0.292 V with ΔE_p of 0.073 V, 0.126 V and 0.196 V respectively, which was the characteristic potential of thiocholine(ox)/thiocholine(red) couple.

$$\text{Acetylthiocholinechloride} + H_2O \xrightarrow{\text{Modified Pt electrode}} \text{thiocholine} + \text{aceticacid} + Cl^-$$

$$2\text{Thiocholine} \xrightarrow{\text{Anodic potential}} \text{Dithio} - \text{bis} - \text{choline} + 2H^+ + 2e^-$$

$$\text{Dithio} - \text{bis} - \text{choline} + 2H^+ + 2e^- \xrightarrow{\text{Cathodic potential}} 2\text{Thiocholine}$$

The observed anodic and cathodic potentials of various modified Pt electrodes were in good agreement with the previously reported redox potential of AChE modified Nano-Au–$CaCO_3$/SiO_2/Au electrode ($E_{pa}=0.275$ V vs. Ag/AgCl and $E_{pc}=0.192$ V vs. Ag/AgCl) (Chauhan et al., 2011). There was no redox peak noticed at bare Pt electrode, suggesting that there was no redox activity in the applied potential range. When compared to the redox current densities of Pt/ZnO/AChE/Chitosan bio-electrodes, the redox current density of Pt/ZnO/Chitosan bio-electrode was reduced by 25%. This might be due to the absence of AChE enzyme. When compared with the Pt/AChE/Chitosan and Pt/ZnO/Chitosan bio-electrodes, only the Pt/ZnO/AChE/Chitosan bio-electrode showed increased peak current densities both in the cathode and anode. This can be attributed to the high sensitive behavior of Pt/ZnO/AChE/Chitosan bio-electrode towards ATChCl. It also showed that the AChE enzyme was effectively immobilized on the Pt/ZnO working electrode and provided the essential conduction pathways. Based on the electrochemical analysis, Pt/ZnO/AChE/Chitosan bio-electrode was chosen for carbosulfan detection in all further electrochemical studies.

3.2.2. Effect of pH

The pH effect on the performance of Pt/ZnO bio-electrode immobilized with 5 μL of AChE (0.3 U mL^{-1}) was examined at the scan rate of 0.1 V s^{-1} by measuring the current density response to 0.2 mM ATChCl in various pH solutions (Fig. 2(b)). The current density increased with the increasing pH in the range from 5 to 7.4. Later, the current density decreased with the increasing pH in

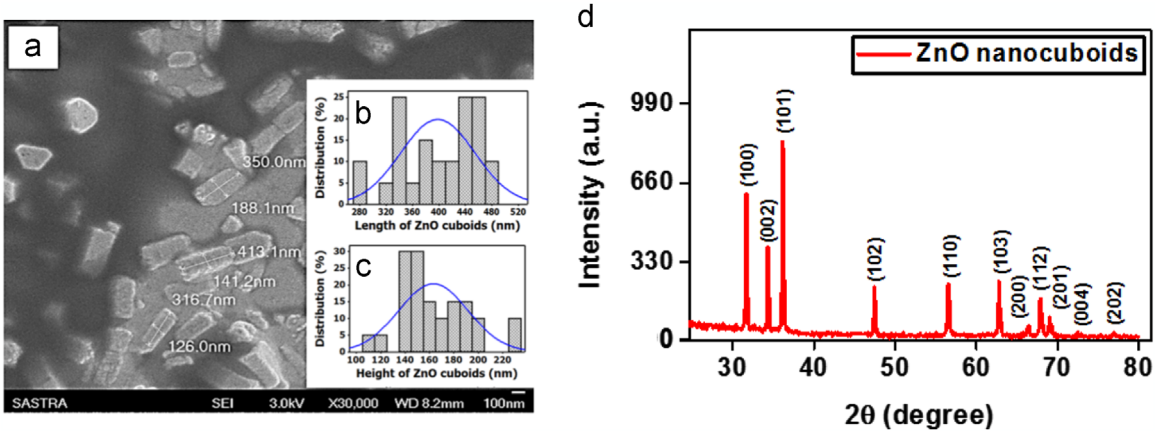

Fig. 1. (a) FE-SEM image of the as-prepared ZnO nanocuboids, distribution of ZnO cuboid's length (b) and (c) height (d) X-ray diffraction patterns of the as-prepared ZnO nanoparticles.

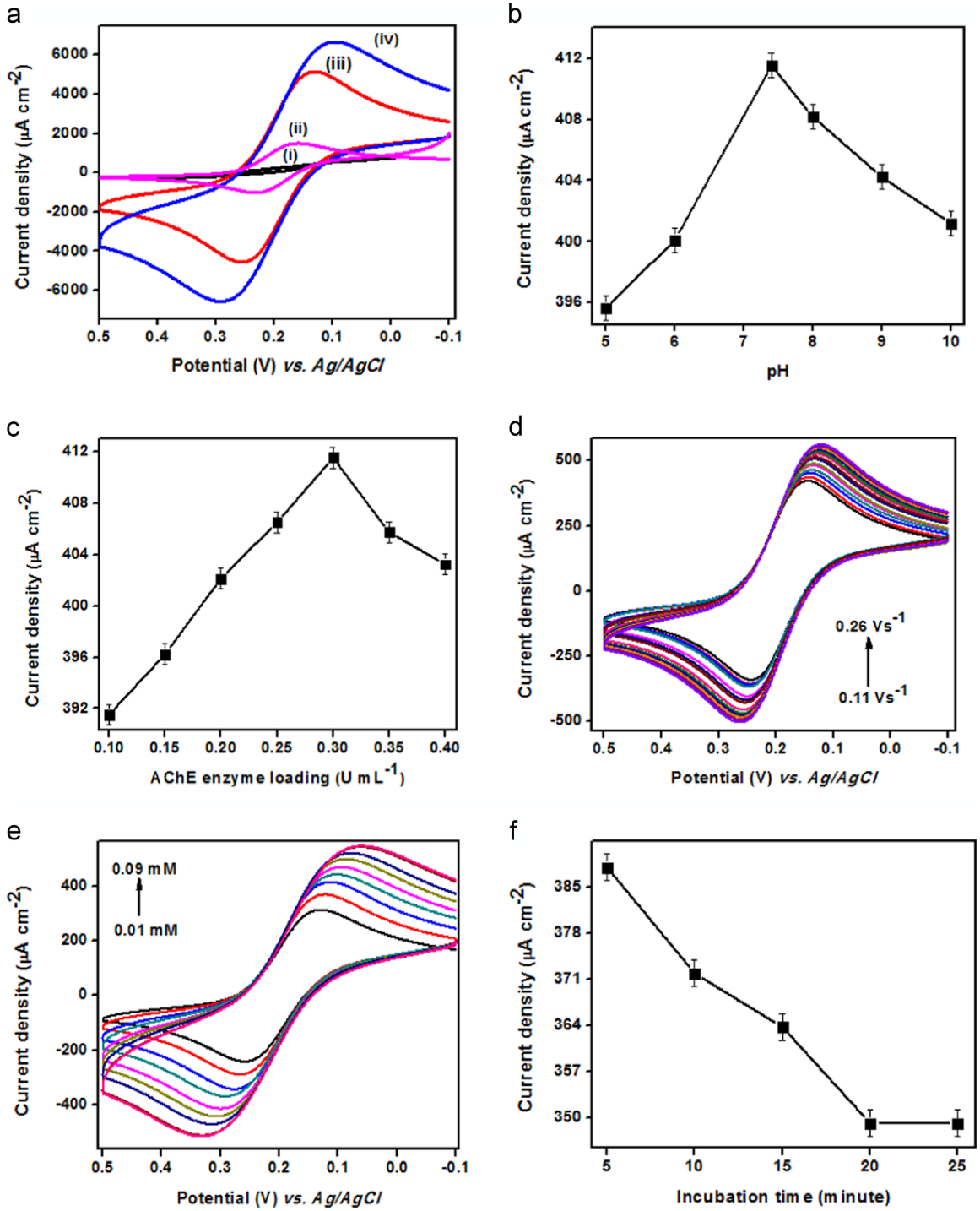

Fig. 2. (a) Effect of different modified Pt electrode, (b) pH, (c) AChE enzyme loading, (d) scan rate, (e) varying ATChCl concentrations and (f) incubation time for the optimization of experimental variables.

the range from 7.4 to 10. In the range of pH values from 5 to 10, a maximal current density response was found at pH 7.4. Therefore, PBS was maintained at pH 7.4 for the electrochemical detection of carbosulfan.

3.2.3. *Effect of amount of immobilized AChE enzyme*

The influence of immobilized AChE enzyme on the cyclic voltammetric response of Pt/ZnO/AChE/Chitosan bio-electrode was studied in the presence of 0.2 mM ATChCl in PBS (pH 7.4) at a scan rate of 0.1 V s^{-1} (Fig. 2(c)). With increasing the amount of immobilized AChE enzyme from 0.1 U mL^{-1} to 0.3 U mL^{-1}, the current density increased obviously and reached the maximum at 0.3 U mL^{-1}. However, the current density response decreased when the amount of immobilized AChE enzyme increased further, which may be attributed to the over-thickness of immobilized AChE layer preventing the transmission of electrons to the ZnO modified Pt working electrode surface. Therefore, 0.3 U mL^{-1} was chosen as the optimal AChE enzyme amount for the electrochemical detection of carbosulfan.

3.2.4. *Effect of scan rate*

The effect of scan rates on the electrochemical response of the

Pt/ZnO/AChE/Chitosan bio-electrode is shown in Fig. 2(d). Both cathodic (J_{pc} ($\mu A\ cm^{-2}$)=766.440 [ν] ($\mu A\ cm^{-2}/V\ s^{-1}$)+347.925, R^2=0.97) and anodic peak current density (J_{pa} ($\mu A\ cm^{-2}$)=−961.516 [ν] ($\mu A\ cm^{-2}/V\ s^{-1}$)+320.538, R^2=0.98) increased linearly with the scan rate from 0.11 to 0.26 V s^{-1} and it was evident that electroactive thiocholine adsorbed onto the Pt/ZnO/AChE/Chitosan bio-electrode surface undergone a surface confined electron transfer.

3.2.5. Effect of ATChCl

Cyclic voltammetric curves of Pt/ZnO/AChE/Chitosan bio-electrode for increasing ATChCl in 0.1 M PBS (pH) 7.4 at 0.11 V s^{-1} is shown in Fig. 2(e). It was found that the J_{pc} increased with the concentration of ATChCl up to 0.08 mM. There was no considerable current density improvement after 0.08 mM of ATChCl concentration. The Pt/ZnO/AChE/Chitosan bio-electrode had attained its saturation level at 0.09 mM. Based on these results, 0.08 mM of ATChCl was chosen as optimum concentration for further electrochemical studies.

3.2.6. Effect of incubation time

Effect of incubation time (5, 10, 15, 20 and 25 min) on AChE inhibition using Pt/ZnO/AChE/Chitosan bio-electrode in PBS (0.1 M, pH 7.4) containing 0.08 mM ATChCl and 5 nM carbosulfan performed at a scan rate of 0.11 V s^{-1} is shown in Fig. 2(f). The current density decreased rapidly with increasing incubation time from 5 to 20 min. No significant decrease in current density was observed for incubation time higher than 20 min. Thus 20 min was selected as the working incubation time.

3.3. Effect of carbosulfan exposure on AChE activity

In order to examine the carbosulfan inhibition of AChE and ZnO catalyses, Pt/AChE/Chitosan and Pt/ZnO/Chitosan electrodes were used. All electrochemical measurements were performed in 0.8 mM ATChCl solution in the absence and presence of 5 nM carbosulfan. From Fig. 3, it was observed that the ZnO nanoparticles showed better catalytic activity towards thiocholine (red)/thiocholine (ox) redox couple than that of the immobilized AChE enzyme. However, in the presence of 5 nM carbosulfan, the catalytic electrochemical reaction of thiocholine (red)/thiocholine (ox) redox couple at the surface of ZnO and AchE enzyme modified Pt electrodes got affected, which in-turn resulted in lower electrochemical response. These results clearly distinguished the inhibition to electrochemical response and the one to the catalytic response.

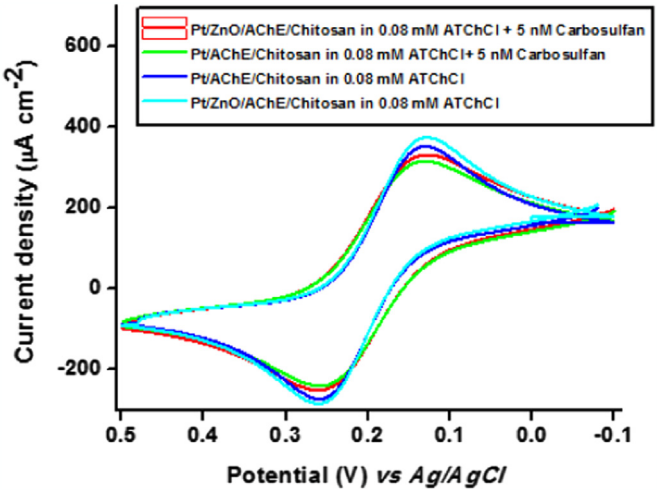

Fig. 3. Comparison of the cyclic voltammograms in 0.08 mM ATChCl solution between before and after the exposure of the various modified Pt electrode to 5 nM carbosulfan.

Cyclic voltammograms of Pt/ZnO/AChE/Chitosan bio-electrode in pH 7.4 PBS solution containing 0.08 mM ATChCl after inhibition with carbosulfan (5–50 nM) for 20 min is shown in Fig. 4(a). Inhibition response curves of Pt/ZnO/AChE/Chitosan bio-electrode calculated from the cathodic and anodic responses are shown in inset Fig. 4(b). It was noticed that the peak current density decreased with increasing amount of carbosulfan and attained the minimum at 50 nM. The decrease in the Pt/ZnO/AChE/Chitosan bio-electrode response was due to the blocking of serine hydroxyl group (present in the active site of AChE) by carbosulfan. It also depicted the predictive ability of Pt/ZnO/AChE/Chitosan bio-electrode to detect carbosulfan at nanomolar concentrations. When the added carbosulfan was 50 nM, the inhibition curves from the cathodic and anodic response was almost leveled off and the degree of inhibition reached maximum suggesting that the blocking of serine hydroxyl group in the active site of AChE by carbosulfan attained saturation at 50 nM. Moreover, at 50 nM carbosulfan, the hydrolysis rate was affected to a greater extent which in-turn resulted in poor release of thiocholine per second. This led to lower Pt/ZnO/AChE/Chitosan bio-electrode response and thus high degree of inhibition was achieved. The inhibition curves from the cathodic and anodic responses follow a typical Michaelis–Menten process. The p, K_M^{app} and I_{max} % estimated from the cathodic and anodic responses were 1.730, 15.702 and 39.721 and 2.209, 17.385 and 31.054, respectively according to Hill equation indicating positive cooperativity. It also depicted that the binding of carbosulfan to serine hydroxyl group in the active sites of AChE increased the affinity of further incoming carbosulfan molecules. The I_{max} % value calculated from the cathodic response was much higher than that of I_{max} % value obtained from the anodic response. Therefore, inhibition curve from the cathodic response was chosen for further calibration study.

As shown in Fig. 4(c), upon Pt/ZnO/AChE/Chitosan bio-electrode was immersed in carbosulfan solution, notable changes in residual AChE enzyme activity ($\text{Residual enzyme activity}(\%) = \frac{J_i}{J_0} \times 100$, where J_0 and J_i are the observed current densities before and after incubation) were observed. It indicated that residual activity of immobilized AChE enzyme depended on the amount of carbosulfan added in the measuring cell. With increasing carbosulfan concentration, the percentage of residual immobilized AChE enzyme activity decreased. This was because carbosulfan showed high acute toxicity and engaged in inhibition action to the immobilized AChE enzyme, thus reduced the activity of AChE to ATChCl. Logarithm of %Inhibition was plotted against logarithm of carbosulfan and linear regression analysis was performed (Fig. 4(d)). A sensitivity of 0.643% nM^{-1} over a linear range of 5–30 nM carbosulfan was observed.

3.4. Determination of limit of detection, limit of quantification and IC_{50}

The limit of detection (LOD) and limit of quantification (LOQ) were calculated as follows:

$$\text{LOD} = \frac{3.3 \times s}{S} \quad \text{and} \quad \text{LOQ} = \frac{10 \times s}{S}$$

where, S is the slope of the calibrated AChE inhibition curve and s is the standard deviation of the lower level of carbosulfan concentration. The calibrated AChE inhibition curve has provided LOD and LOQ at 0.24 nM and 0.72 nM respectively, manifesting that the developed bio-electrode can detect nanomolar concentrations of carbosulfan. The improved analytical performance can be ascribed to ZnO nanocuboids. The Pt/ZnO/AChE/Chitosan bio-electrode was found to be more sensitive to carbosulfan

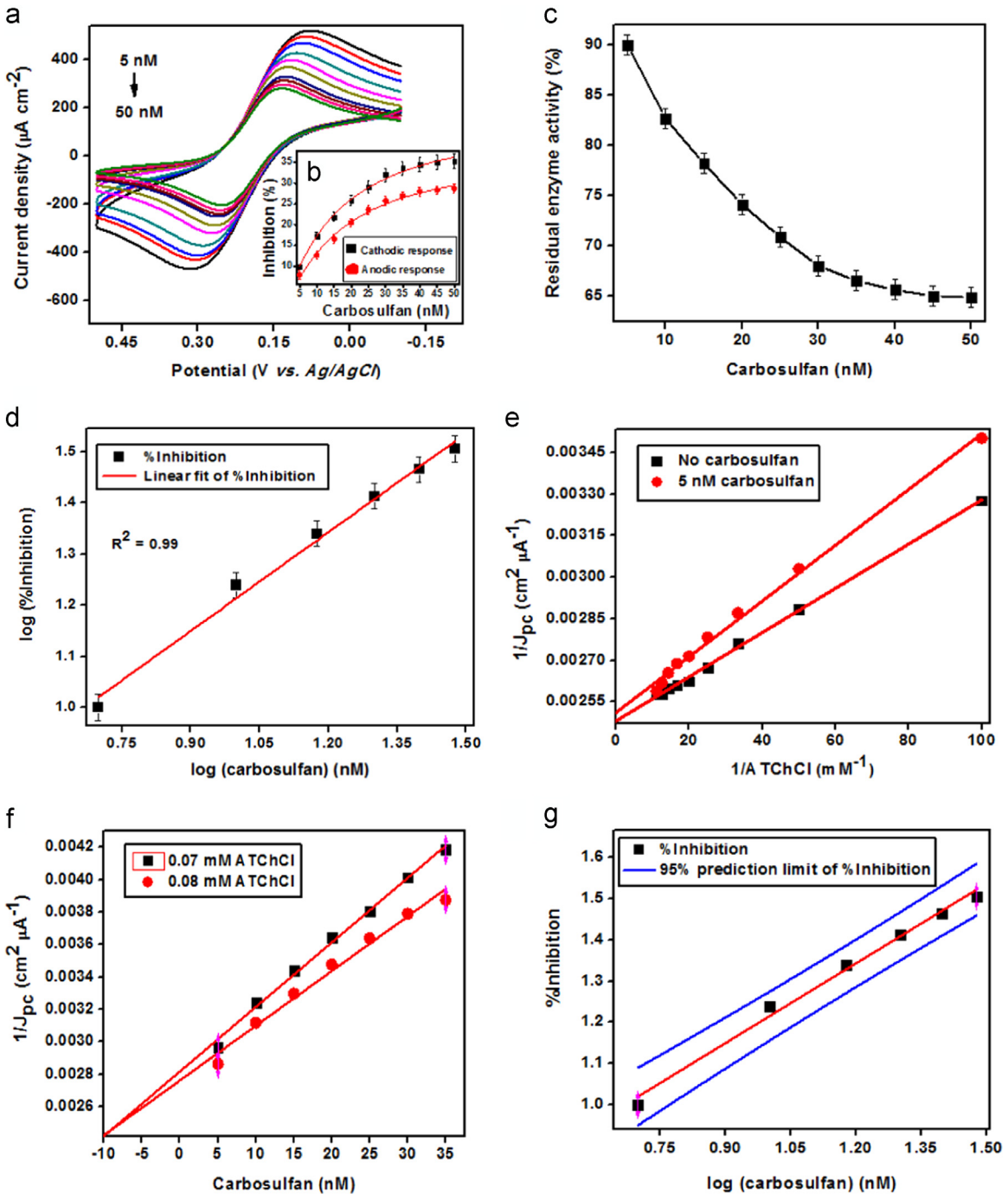

Fig. 4. (a) Cyclic voltammograms and (b) inhibition curves of Pt/ZnO/AChE/Chitosan bio-electrode in pH 7.4 PBS solution containing 0.08 mM ATChCl after inhibition with carbosulfan (5–50 nM) for 20 min, (c) residual enzyme activity for different carbosulfan concentrations, (d) calibration curve of log (%Inhibition) vs. log (carbosulfan), (e) Lineweaver–Burk and (f) Dixon plot of competitive inhibition of AChE immobilized in Pt/ZnO/AChE/Chitosan bio-electrode by carbosulfan and (g) prediction band for the estimated percentage inhibition.

because the LOD and LOQ values were very low. The 50% inhibition value (IC_{50}) for carbosulfan was calculated from the dose response analysis. The required carbosulfan concentration for the 50% inhibition of immobilized AChE enzyme activity was calculated to be 15.702 nM.

3.5. Determination of the type of inhibition and interference study

The inhibitory effect of carbosulfan on AChE enzyme activity was investigated by means of Lineweaver–Burk plot (Fig. 4(e)), which in the presence of carbosulfan depicted a constant maximum degree of inhibition I_{max} % and an increase in apparent Michaelis–Menten constant K_M^{app} indicating a competitive inhibition. The value of inhibition constant K_i was calculated according to Dixon method. This presentation affirmed the competitive character of immobilized AChE inhibition by carbosulfan. From Fig. 4(f), the value of K_i was calculated to be 10 nM. The K_i to K_M^{app} ratio ($K_i : K_M^{app} = 1{:}3.93 \times 10^2$) manifested that immobilized AChE enzyme had a greater affinity towards carbosulfan than ATChCl.

In order to assess the interference of this carbosulfan biosensor, interfering molecules namely glucose, urea, ascorbic acid, lactic acid, Ni^{2+}, Cd^{2+}, Zn^{2+} and Ca^{2+} at a relatively high concentration

Table 1
Intra- and inter-day recovery, accuracy and precision of carbosulfan in rice samples ($n=3$) using Pt/ZnO/AChE/Chitosan bio-electrode.

Intra-day assay						
Sample (30 mg of rice)	**Carbosulfan added**	**Mean carbosulfan detected**	**Recovery**	**RSD ($n=3$)**	**RMSECV**	**RPE**
	(nM)	**(μM ± SD)[a]**	**(%)**	**(%)**		**(%)**
1	7.5	7.47 ± 0.002	99.60	0.28	0.03	0.40
2	12.5	12.53 ± 0.002	100.24	0.17	0.03	0.24
3	17.5	17.47 ± 0.003	99.82	0.12	0.03	0.17
4	22.5	22.51 ± 0.002	100.04	0.03	0.01	0.04
5	27.5	27.49 ± 0.002	99.96	0.02	0.01	0.03
Inter-day assay						
1	7.5	7.43 ± 0.003	99.06	0.66	0.07	0.94
2	12.5	12.62 ± 0.002	100.96	0.67	0.12	0.95
3	17.5	17.42 ± 0.004	99.54	0.32	0.08	0.45
4	22.5	22.55 ± 0.003	100.22	0.15	0.05	0.22
5	27.5	27.56 ± 0.003	100.21	0.15	0.06	0.21

[a] Average of 3 determinations.

(0.01 mM) was added to PBS (0.1 M, pH 7.4) containing 5 nM carbosulfan and 0.08 mM ATChCl. Even at the high interferent concentration used in the study, only 0.41–1.52% degree of inhibition (Ni^{2+}: 0.92%, Cd^{2+}: 0.44%, Zn^{2+}: 0.41%, Cu^{2+}: 0.05%, glucose: 1.52%, urea: 0.64%, ascorbic acid: 0.53% and lactic acid: 0.82% of inhibition response) was observed indicating good selectivity. All these results showed that the tested interferents did not significantly interfere with the determination of carbosulfan.

In order to evaluate further the selectivity of Pt/ZnO/AChE/Chitosan bio-electrode towards carbosulfan determination, cross-reactivity of carbosulfan with various interfering molecules was investigated (Table 1).

$$\text{Cross} - \text{reactivity}(\%) = \frac{IC_{50}(carbosulfan)}{IC_{50}(interferents)} \times 100$$

From Table 1, it can be seen that only the glucose biomolecule manifested the highest cross reactivity of 4.153×10^{-3} whereas the other tested potential interferents showed minimal cross-reactivity values. All these results indicated that the detection of carbosulfan would not be influenced by potential interferents.

Prediction band for the calculated percentage inhibition is shown in Supplementary Fig. 4(g). 95% Prediction bands for selective determination of carbosulfan in rice sample were developed by inducing uncertainty in the true position of calibrated log (J_{pc}) vs. log (carbosulfan) curve so that the Pt/ZnO/AChE/Chitosan bio-electrode can selectively detect carbosulfan in rice sample when the same experiment was repeated.

The reproducibility and repeatability of Pt/ZnO/AChE/Chitosan bio-electrode were measured by the relative standard deviation of inter- and intra- assay ($n=10$). The inter- and intra-assay were carried out by measuring rice samples containing 5, 25 and 50 nM carbosulfan. Each rice sample was repeatedly assessed 10 times using 5 parallely prepared Pt/ZnO/AChE/Chitosan bio-electrode. Fig. S1 depicts a control chart in which the central, upper and lower values represent the mean value of current density and mean current density ± 3 times the standard deviation of its mean value, respectively. It can be seen that the current density values were within the control limits. The outcome RSD values for 5, 25 and 50 nM added carbosulfan were 0.47%, 0.46% and 0.60%, respectively. It showed an excellent reliability of Pt/ZnO/AChE/Chitosan bio-electrode. It also manifested that the AChE enzyme was bound tightly on the surface of Pt/ZnO modified electrode. The inter-assays were performed under the same conditions and the measured RSD values were 0.64%, 0.84% and 0.86%, respectively (Supplementary Fig. S2). Moreover, the observed current density values were within the control limits. These results showed that the fabrication procedure was highly reproducible. It also depicted that ZnO nanocuboids exhibited a biocompatible microenvironment to maintain the activity of immobilized AChE enzyme effectively. The carbosulfan concentration ± relative error values for the assay of 6, 12 and 18 nM carbosulfan added into the 0.1 M PBS (pH 7.4) were 6 ± 0.04, 12 ± 0.08 and 18 ± 0.12 nM for six measurements, suggesting good accuracy of the carbosulfan biosensor.

Control chart for Pt/ZnO/AChE/Chitosan bio-electrode for 18 days in the presence of 0.1 mM ATChCl in pH 7.4 PBS at room temperature is shown in Supplementary Fig. S3. It was observed that the current density values were obtained within the control limits for 18 days indicating that the immobilized ZnO/AChE/Chitosan film was rigid over the Pt electrode surface. A clear enhanced stability ($\%\text{Stability} = 100 - [\frac{J_n - J_0}{J_0} \times 100]$, where J_n and J_0 are the measured current density in the 'n' and first day) of 94% even after 18 days was observed. The increase in stability can be attributed to the biocompatible microenvironment provided by ZnO nanocuboids around the immobilized AChE enzyme.

The immobilized AChE enzyme on ZnO matrix inhibited reversibly by carbosulfan can be reactivated with the help of 2-PAM. It was seen that Pt/ZnO/AChE/Chitosan bio-electrode could retain 94.6% original activity after immersing it in 2 mM 2-PAM for 20 min. It showed that the developed bio-electrode could be employed repeatedly with a satisfactory reproducibility.

3.6. Carbosulfan detection in rice sample

Three replicates of rice samples at 5 different carbosulfan concentrations (7.5, 12.5, 17.5, 22.5 and 27.5 nM) were measured on the same day and also on three separate days of analysis to estimate inter- and intra-day precision and accuracy (Table 2). The carbosulfan concentration in the rice sample was estimated using the calibrated first-order polynomial equation. As shown in Table 1, the values of recovery ranged from 99.06% to 100.96% and from 99.60% to 100.24% respectively, which depicted low matrix effect on Pt/ZnO/AChE/Chitosan bio-electrode response. It also indicated that the interferent molecules present in rice sample didn't interfere the detection of carbosulfan. In order to investigate further the accuracy of Pt/ZnO/AChE/Chitosan bio-electrode, correlation between the added and predicted concentrations of carbosulfan was calculated. An acceptable correlation coefficient of 0.99 was obtained with regression equation being

Table 2
Intra- and inter-day recovery, accuracy and precision of carbosulfan in rice samples ($n=3$) using Pt/CeO_2/AChE/Chitosan bio-electrode.

Intra-day assay						
Sample (30 mg of rice)	**Carbosulfan added**	**Mean carbosulfan detected**	**Recovery**	**RSD ($n=3$)**	**RMSECV**	**RPE**
	(nM)	**(μM ± SD)[a]**	**(%)**	**(%)**		**(%)**
1	7.5	7.46 ± 0.003	99.466	0.378	0.040	0.536
2	12.5	12.54 ± 0.003	100.320	0.226	0.040	0.319
3	17.5	17.45 ± 0.004	99.714	0.202	0.050	0.286
4	22.5	22.54 ± 0.003	100.177	0.126	0.040	0.177
5	27.5	27.54 ± 0.002	100.145	0.103	0.040	0.145
Inter-day assay						
1	7.5	7.41 ± 0.002	98.800	0.854	0.090	1.214
2	12.5	12.65 ± 0.003	101.200	0.843	0.150	1.186
3	17.5	17.39 ± 0.005	99.371	0.446	0.110	0.632
4	22.5	22.58 ± 0.003	100.355	0.251	0.080	0.354
5	27.5	27.59 ± 0.005	100.327	0.231	0.090	0.326

[a] Average of 3 determinations.

$carbosulfan_{predicted} = 1.000[carbosulfan_{added}] - 0.013$ (for inter-assay) and $carbosulfan_{predicted} = 1.003[carbosulfan_{added}] - 0.050$ (for intra-assay). Moreover, RMSECV and RPE of added carbosulfan in the rice sample were 0.01–0.12% and 0.03–0.95% respectively, depicting the acceptable accuracy of proposed first-order polynomial equation. And also, student's paired *t*-test at 95% confidence interval between the added and predicted carbosulfan. The computed experimental *t* values were lower than the critical tabulated *t*-value. Thus it was concluded that there were no significant differences between the added carbosulfan and the spiked one.

The calculated RSD values for intra- and inter-assay were 0.02–0.28% and 0.15–0.67% respectively, showing the good repeatability and reproducibility of the Pt/ZnO/AChE/Chitosan bio-electrode. In order to validate further the precision of the calibrated method, *F*-test was employed at 95% confidence. No significant differences between the standard deviation of mean predicted and added carbosulfan were observed indicating good reliability of the proposed method.

Comparison of analytical parameters of the developed carbosulfan biosensor with the previously reported sensors for carbosulfan determination is given in Supplementary Table S1. Compared with the previously reported carbosulfan sensor, the proposed Pt/ZnO/AChE/Chitosan bio-electrode was more sensitive for detecting nanomolar concentrations of carbosulfan than micromolar concentrations of carbosulfan. Moreover, it can be seen that the proposed carbosulfan biosensor Pt/ZnO/AChE/Chitosan bioelectrode exhibited high accuracy, lower detection and quantification limit than the previously reported sensor in detecting low concentrations of carbosulfan.

4. Conclusions

The present work gives a simple strategy for acetylcholinesterase immobilization onto the ZnO nano-matrix. The proposed Pt/ZnO/AChE/Chiotsan bioelectrode showed good conductivity and biocompatibility. The Pt/ZnO/AChE/Chiotsan bioelectrode is not only an effective tool for assisting the communication between AChE enzyme and Pt electrode, but also it provided a good microenvironment for AChE-ATChCl interaction. The final outcomes affirmed that the developed Pt/ZnO/AChE/Chiotsan bioelectrode can be employed to detect carbosulfan in rice samples with the limit of detection of 0.24 nM. Such a low limit of detection makes the Pt/ZnO/AChE/Chiotsan bioelectrode to be suitable candidate for the application of food safety testing and environmental monitoring. Further, chemometric investigations are in progress to improve the selectivity of the proposed biosensor.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2015.11.010.

References

Arduini, F., Amine, A., Moscone, D., Palleschi, G., 2010. Microchim. Acta 170, 193–214.
Capkin, E., Altinok, I., 2013. Environ. Toxicol. Pharmacol. 36, 80–87.
Capkin, E., Boran, H., Altinok, I., 2014. Turk. J. Fish. Aquat. Sci. 14, 643–650.
Chandrasekara, L.W.H.U., Pathiratne, A., 2007. Ecotoxicol. Environ. Saf. 67, 109–119.
Chauhan, N., Narang, J., Pundir, C.S., 2011. Int. J. Biol. Macromol. 49, 923–929.
Ghica, M.E., Brett, C.M.A., 2008. Microchim. Acta 163, 185–193.
Giri, S., Giri, A., Sharma, G.D., Prasad, S.B., 2002. Mutation Res. 26, 75–82.
Guessan, R.N., Darriet, F., Guillet, P., Carnevale, P., Lamizana, M.T., Corbel, V., Koffi, A. A., Chandre, F., 2003. Med. Vet. Entomol. 17, 19–25.
Gumpu, M.B., Nesakumar, N., Sethuraman, S., Krishnan, U.M., Rayappan, J.B.B., 2014. Sens. Actuators B 199, 330–338.
Kabir, K.H., Rahman, M.A., Ahmed, M.S., Prodhan, M.D.H., Akon, M.W., 2008. Bangladesh J. Agric. Res. 33, 503–513.
Liu, H., Yi, M., Shi, X., Liang, P., Gao, X., 2007. Fish. Physiol. Biochem. 33, 29–34.
Liu, T., Xu, M., Yin, H., Ai, S., Qu, X., Zong, S., 2011. Microchim. Acta 175, 129–135.
Nagaraju, B., Rathnamma, V.V., 2014. Toxicol. Res. 3, 177–183.
Nesakumar, N., Sethuraman, S., Krishnan, U.M., Rayappan, J.B.B., 2013. J. Colloid Interface Sci. 410, 158–164.
Nesakumar, N., Thandavan, K., Sethuraman, S., Krishnan, U.M., Rayappan, J.B.B., 2014. J. Colloid Interface Sci. 414, 90–96.
Nwani, C.D., Lakra, W.S., Nagpure, N.S., Kumar, R., Kushwaha, B., Srivastava, S.K., 2010. Food Chem. Toxicol. 48, 202–208.
Oehme, I., Wolfbeis, O.S., 1997. Microchim. Acta 126, 177–192.
Plese, L.P.M., Paraiba, L.C., Foloni, L.L., Trevizan, L.R.P., 2005. Chemosphere 60, 149–156.
Sahoo, A., Sethunathan, N., Sahoo, P.K.J., 1988. Environ. Sci. Health. Part B 33, 369–379.
Sen, S., Gulce, A., Gulce, H., 2004. Biosens. Bioelectron. 19, 1261–1268.
Singh, M., Nesakumar, N., Sethuraman, S., Krishnan, U.M., Rayappan, J.B.B., 2014. J. Colloid Interface Sci. 425, 52–58.
Soler, C., Manes, J., Pico, Y.J., 2006a. Chromatography A 1109, 228–241.
Soler, C., Hamilton, B., Furey, A., James, K.J., Manes, J., Pico, Y., 2006b. Anal. Chim. Acta 571, 1–11.