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Analytical Methods

# Acetylcholinesterase based biosensor for monitoring of Malathion and Acephate in food samples: A voltammetric study

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ABSTRACT

Acetylcholinesterase (AChE) biosensor was developed through silica sol–gel (SiSG) immobilisation of AChE on the carbon paste electrode (CPE) and used as working electrode. AChE catalyses the cleavage of acetylthiocholine chloride (ASChCl or substrate) to thiocholine, which was oxidised to give a disulphide compound by dimerisation at 0.60 V versus saturated calomel electrode. All the experiments were carried out in 0.1 M phosphate buffer solution (PBS) at pH 7.0 and 0.1 M KCl solution at room temperature. The limit of detection and limit of quantification values were found to be 0.058 ppm, 0.044 ppm and 0.194 ppm, 0.147 ppm for Malathion and Acephate, respectively. The response of the biosensor showed a good linearity range with an incubation time of 4 min for Malathion and Acephate, respectively. This biosensor was used for the direct determination of pesticides without any pretreatment and it requires less time for analysis.



## 1. Introduction

Organophosphorous insecticides are very toxic compounds, intensively used in agriculture. Their existence in the environment is very harmful to human health as they irreversibly inhibit the catalytic active sites of AChE, an essential enzyme that permits the transmission of electric signals in the nervous system of most animal beings. However, there is a fear towards the poisoning of drinking water sources and food samples by neurotoxic agents, which can produce acute poisoning even at very low concentrations (Hildebrandt et al., 2008a). In recent years, growing attention has been to the development of reliable, fast and inexpensive analytical systems to monitor pesticides from environmental samples.

Malathion (ML) and Acephate (AP) are organophosphorous insecticides. These pesticides are widely used in agriculture, residential landscaping, public relation areas and in public health pest control programs such as mosquito eradication. The misuse or overdose of these pesticides results in contamination of fields, crops, water and air. Both the compounds are relatively insoluble in water, poorly soluble in petroleum ether and mineral oils, and readily soluble in most organic solvents. The physical, chemical and biological factors are influencing the distribution of above pesticides in air, water, soil and organisms in the environment. These pesticides are also readily taken into the body through skin, inhalation, even though the amount absorbed will depend on where the exposure occurs on the body. Organophosphates such as ML and AP are inhibitors of cholinesterases.

Many methods have been developed in the last few years for the determination of pesticides. Most of these are based on a separation by gas (Maria Dolores, Ana, Amadeo, Luis, & Mariano, 2001) or liquid chromatography (Wang, Wang, & Yuan, 1999), spectrophotometry (Rodrignez, Monferrer-Pons, Esteve Romero, Garcia Alvarez, & Ramis Rames, 1997; Tuxelli, Bag, & Turker, 2001), infrared spectroscopy (Capitan-Valley, Deheidel, & Avivad, 1998; Daghbouche, Garrigues, & Guardia, 1995; Mc Garvey, 1993; Murillo Pulgarin, Molina, & Fernandez Lopez, 2006), fluorescence spectrometry (De kok & Hiemstra, 1992), mass spectrometry (Newsome, Lau, Ducharme, & Lewis, 1995), high performance liquid chromatography (HPLC), UV or electrochemical detectors. Many of the above mentioned methods are accurate and selective, but they require relatively expensive instrumentation and high consumption of toxic organic reagents, becoming expensive and time consuming. The development of alternative methods, which minimises the consumption of reagents and decreases in the time of analysis with the quality of measurements, is of great interest. A numerous biosensing methods have been developed for the detection of pesticides by using enzyme based and affinity – based sensors as well as several types of transducers (Suwansa-ard et al., 2005). Among the electrochemical sensors, the enzyme modified electrode plays a vital role in the development of highly sensitive, selective, low cost and chemical analysis with short time biosensor.

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Enzyme sensors based on the inhibition of AChE with potentiometric or amperometric detection (Kindervater, Kunnecke, & Schmid, 1990; Leon-Gonzales & Townshend, 1991; Martorell, Cespedes, Martinez-Fabregas, & Alegret, 1994; Rouillon, Mionelto, & Marty, 1992; Skaladal, 1992) have been developed for the determination of organophosphorous and carbamate pesticides. The immobilisation of AChE in biosensor technology involves different types of methods. Such as sol–gel entrapment, covalent binding, adsorption and encapsulation of sensing agents with in a polymers. Among these sol–gel immobilisation can be preferred since the other immobilisation methods are tedious, result in poor stability, and require expensive reagents, enzyme leaking and loss of enzyme activity. So many sol–gel derived enzyme biosensors have been developed to monitor glucose, lactate, cholesterol, dopamine, $H_2O_2$, phenols and urea (Pandey, Upadhyay, Tiwari, Singh, & Tripathi, 2001; Park, Lwuoha, Smyth, Freoney, & Mc Shane, 1997; Sampath & Lev, 1996; Yao & Takashima, 1998; Park, 1999; Li, Chia, Goh, & Tan, 1998; Pandey & Singh, 2001).

In this work, we demonstrated that acetylthiocholine chloride (ASChCl) used as a substrate for the enzyme catalysed reaction. We developed a biosensor by using TEOS sol–gel system doped with AChE towards the determination of ML and AP pesticides in spiked food samples. The parameters such as operational, linear range, pH, response and response time have been investigated.

## 2. Experimental

### 2.1. Apparatus

The electrochemical measurements were conducted in a three electrodes cell at the room temperature 25 ± 2 °C. The working electrode was the enzyme immobilised carbon paste electrode (AChE–SiSG–CPE). The reference electrode was the saturated calomel electrode system and platinum wire was used as the auxiliary electrode. Measurements were carried out using CH – Electrochemical Analyzer (Model CHI – 660D, CH Instruments, USA).

### 2.2. Materials

All chemicals were obtained from commercial sources and used without further purification. Acetyl cholinesterase (E.C. 3.1.1.7 type-VI-S/1.5 mg, electric eel source, 500 U/1.5 mg) and acetylthiocholine chloride were purchased from Sigma–Aldrich chemicals Co. Missouri, USA. Malathion and Acephate were obtained from Riedel–deHaen, Fluka, Missouri, USA. The pesticide stock solution was prepared in acetone (GR grade solution, obtained from Merck specialities Pvt. Ltd., Mumbai (INDIA). Tetraethyl orthosilicate (TEOS), cetyltrimethyl ammonium bromide (CTAB), Triton-X-100 were obtained from Sigma–Aldrich chemicals Co. Missouri, USA. The graphite fine powder was procured from Loba Chemie Pvt. Ltd., Mumbai (INDIA) and silicon oil from HiMedia Laboratories Pvt. Ltd., Mumbai (INDIA). Phosphate buffer solution (PBS) was prepared by mixing 0.1 M sodium dihydrogen phosphate monohydrate and 0.1 M disodium hydrogen phosphate. All the aqueous solutions were prepared with double distilled water. The enzyme stock solution and working solutions of chemicals were stored in cool place.

### 2.3. Preparation of bare carbon paste electrode

The bare carbon paste electrode was prepared by hand mixing of 70% graphite powder and 30% silicon oil in an agate mortar to produce a homogenous carbon paste. The paste was packed into the cavity of homemade PVC (3 mm in diameter) and then smoothed on a weighing paper. The electrical contact was provided by copper wire connected to the paste at the end of the tube (Chandra, Kumara Swamy, Gilbert, & Sherigara, 2010; Chithravathi, Kumara Swamy, Cahndra, Mamatha, & Sherigara, 2010; Gilbert, Kumara Swamy, Chandra, & Sherigara, 2009).

### 2.4. Biosensor/AChE–SiSG–CPE preparation

A homogenous TEOS silica sol was prepared by mixing 2 ml of TEOS, 1 ml of $H_2O$, 50 μL of 0.1 M HCl, and 25 μL of 10% Triton-X-100. The mixture was stirred for 1 h and homogenised before each usage and stored in cool place.

The 5 μL of stock sol–gel solution was mixed with 45 μL of phosphate buffer containing 0.5 U of enzyme stock solution and from this 5 μL of enzyme sol of concentration 0.05 U was spread on the electrode surface. This electrode was allowed to keep for about 3–5 min at room temperature. Finally the electrode was cleaned with PBS (pH 7.0) and was used for further experimental procedures (Anitha, Venkata Mohan, & Jayarama Reddy, 2004).

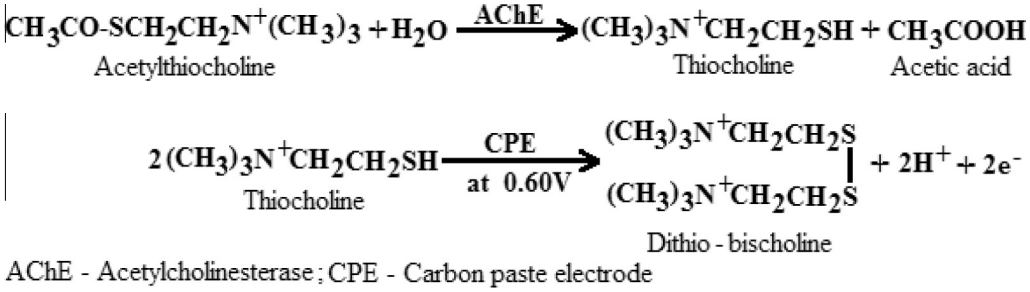
### 2.5. Sample collection

The food samples listed in Table 1B were chosen for the determination of pesticide residues. The food samples were taken from the fields near Swarnamuki River (Chittoor (District), Andhra Pradesh, India). The samples were cleaned with distilled water, chopped and removed outer membrane. From this five grams of each chopped or grained samples were added to 10 ml PBS (pH 7.0) and stirred for 1 h at room temperature. This sample solution was filtered and the filtrate was dissolved in acetone. These samples were spiked with a known concentration of ML and AP derived from stock solution and used immediately for analysis.

## 3. Results and discussion

### 3.1. Cyclic voltammetric behaviour of biosensor

The fabricated AChE based biosensor entrapped through sol–gel immobilisation method onto the carbon paste electrode (CPE) was shown through the mechanism in Fig. 1. The electrochemical behaviour of biosensor was evaluated by using acetylthiocholine chloride (ASChCl) as a substrate. The enzyme catalysed reaction and anionic oxidation of thiocholine was shown below.




AChE - Acetylcholinesterase ; CPE - Carbon paste electrode

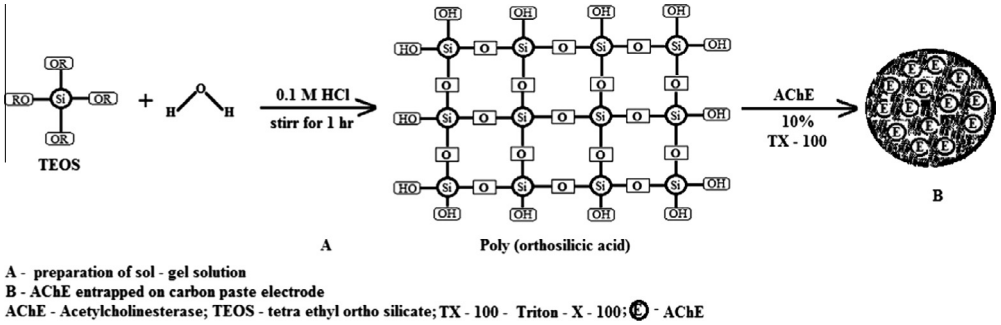



**Fig. 1.** Immobilization of AChE through sol–gel process on the CPE.

All sensor experiments were carried out in 0.1 M PBS/pH (7.0), 0.1 M KCl at room temperature by employing cyclic voltammetry (CV). Fig. 2A shows the cyclic voltammograms of the biosensor in the absence and presence of the substrate (1–6 mM) in PBS (pH 7.0) at the scan rate of 5 $mVS^{-1}$. In absence of substrate no peak can be observed even up to 1.0 V with biosensor. When the substrate is present a larger anodic peak was observed at 0.60 V this was due to the oxidation of thiocholine, which inturn produces dimmer structure (Andreescu, Barthelmebs, & Marty, 2002) and was shown in (R2). The biosensor response increases with the substrate concentration until a saturation point is reached and was seen through the plateau in inset of Fig. 2A. The kinetics of the immobilised enzyme showing Michaelis–Menten plot characteristics, was diphasic in nature. We observed linear dependency of rate for smaller substrate concentration (first order) by a correlation coefficient very close to unity ($[S] << K_m^{app}$, where $[S]$ is the substrate concentration & $K_m^{app}$ is the apparent Michaelis–Menten constant). For higher substrate concentration, the rate was independent of concentration (zero order, $[S] >> K_m^{app}$). The apparent Michaelis–Menten constant ($K_m^{app}$) was 0.57 mM which was obtained from the linear portion of the calibration plot using Lineweaver–Burk equation.

In order to support the enzyme catalysed hydrolysis reaction, the variation of the peak potentials as a function of the scan rate was analysed. The anodic peak potentials depend linearly on the logarithm of the scan rate as predicted by Eqs. (1) & (2) proposed by (Aoki, Akimoto, Tokuda, Matsuda, & Osteryoung, 1984).

$$Ep_a = E^o + m[0.78 + \ln(D^{1/2}/K^o) - 0.5\ln m] + 0.5m\ln\upsilon \quad (1)$$

$$m = RT/[(1-\alpha)nF] \quad (2)$$

Where '$E^o$' is the formal potential of acetylthiocholine, '$D$' is the diffusion coefficient, '$K^o$' is the heterogeneous standard rate constant, '$\alpha$' is the energy transfer coefficient and '$n$' is the number of electrons transferred during the heterogeneous reaction. $R$, $T$ and $F$ are the universal gas constant, absolute temperature and Faraday constant respectively. The formal potential of ASChCl was deduced from the intercept of $Ep$ Vs $\upsilon$ plot. By using the value of acetylthiochline diffusion coefficient $0.12 \times 10^{-6}$ $cm^2$ $S^{-1}$ and from the intercept of $Ep_a$ Vs ln $\upsilon$ plot, the value of $K^o$ was calculated as 0.00026 cm $S^{-1}$. From Eq. (3) (Bard & Faulkner, 2000), it is possible to calculate the value of the energy transfer coefficient ($\alpha$) as 0.35. The '$\alpha$' value and the slope obtained from the plot of $Ep_a$ Vs ln $\upsilon$ was substituted in Eq. (2). From the calculations, the number of electrons involved during ASChCl oxidation process was found to be 1.94 (approx. 2). From this result, we conclude that ASChCl oxidation occurs through, two electron transfer process as indicated in (R2).

$$i_p = 0.227\,FAC_o^* K^o \exp[-\alpha f(E_p - E^o)] \quad (3)$$

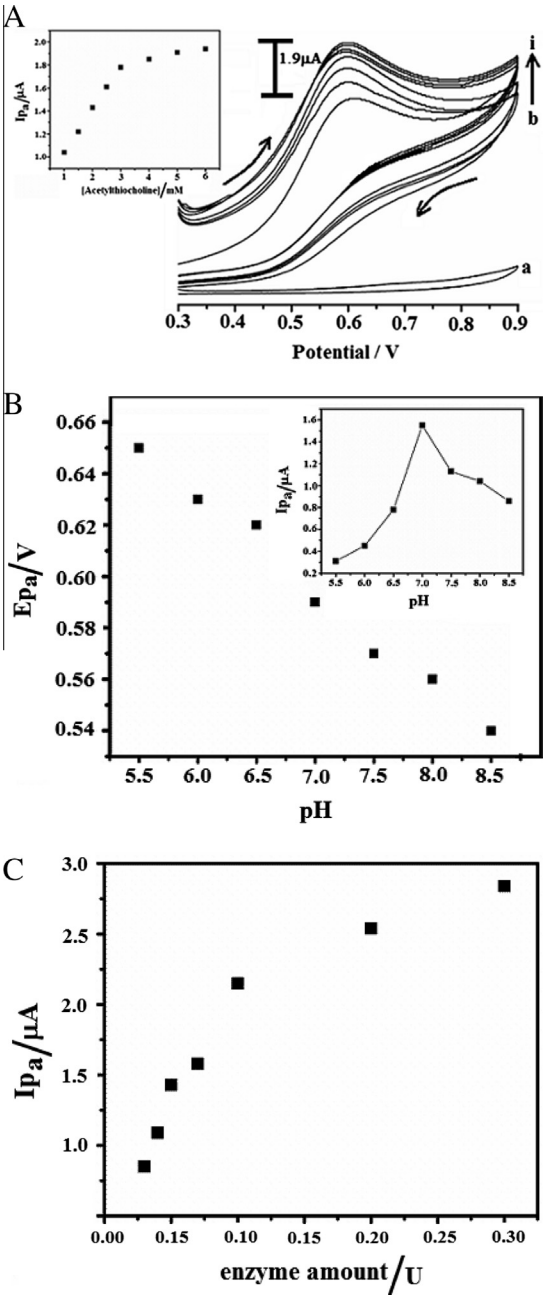



**Fig. 2.** (A) Cyclic voltammograms of AChE–SiSG–CPE in 0.1 M PBS (pH 7.0)/0.1 M KCl and at the scan rate of 5 mV $s^{-1}$ (a) without substrate (b) 1.0 (c) 1.5 (d) 2.0 (e) 2.5 (f) 3.0 (g) 4.0 (h) 5.0 and (i) 6.0 mM substrate and the calibration plot has been shown in the inset. (B) Effect of pH on the anodic peak potential ($Ep_a$) and inset shows plot of $Ip_a$ Vs pH to 1 mM substrate. (C) The variation of biosensor response against the concentration of enzyme on AChE–SiSG–CPE.

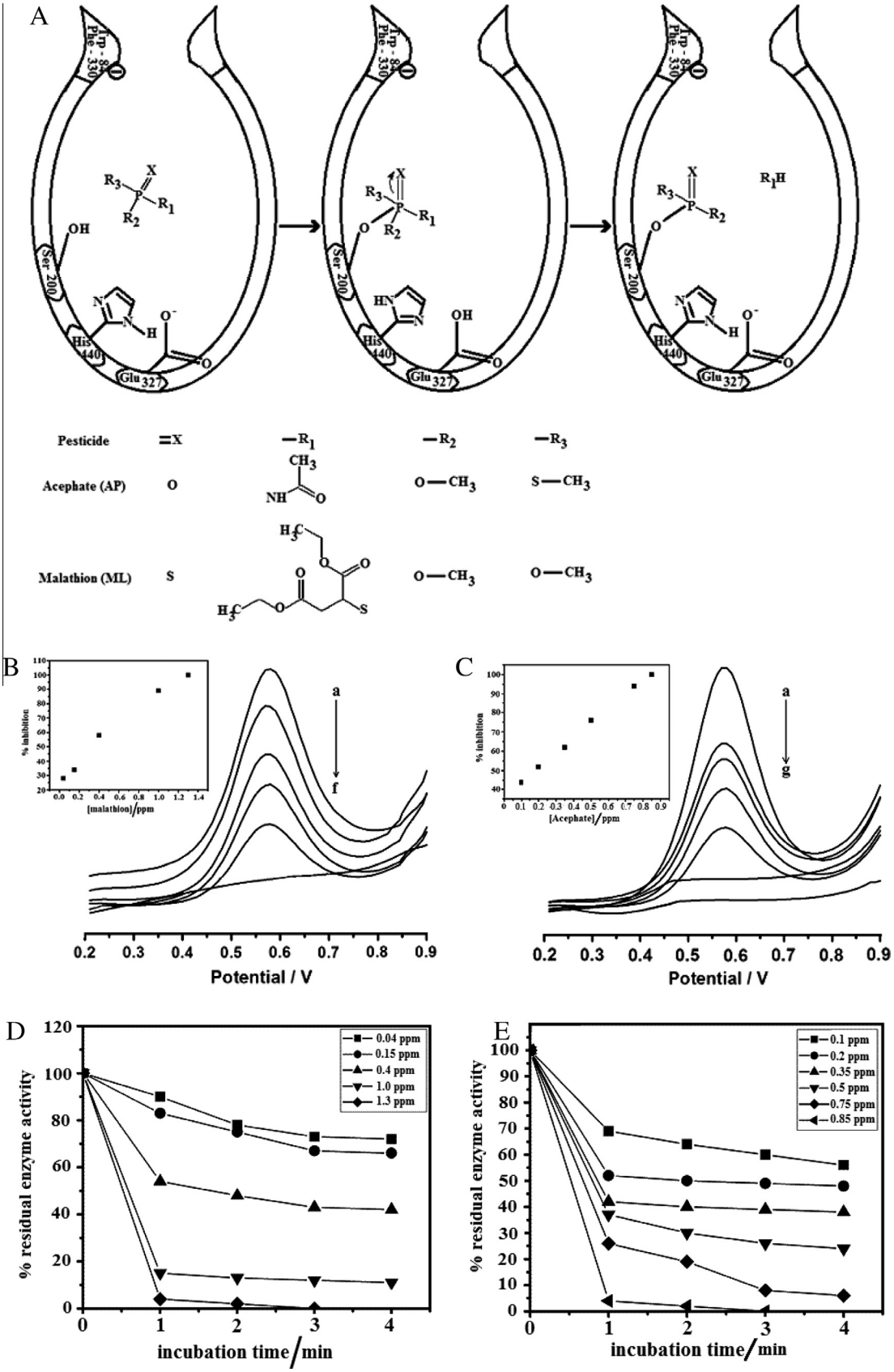



**Fig. 3.** (A) Inhibition of AChE in the presence of pesticides. (B) Differential-pulse-voltammograms recorded with AChE–SiSG–CPE in a 1 mM ASChCl + 0.1 mM PBS (pH 7.0)/0.1 M KCl after addition of ML in different concentrations (a) 0 (b) 0.07 ppm (c) 0.15 ppm (d) 0.4 ppm (e) 1.0 ppm (f) 1.3 ppm. Inset: calibration plot of % inhibition against ML concentration with an incubation time of 4 min. (C) Differential-pulse-voltammograms recorded with AChE–SiSG–CPE in a 1 mM ASChCl + 0.1 mM PBS (pH 7.0)/0.1 M KCl after addition of AP in different concentrations (a) 0 (b) 0.1 ppm (c) 0.2 ppm (d) 0.35 ppm (e) 0.5 ppm (f) 0.75 ppm (g) 0.85 ppm. Inset: analytical curve of % inhibition against AP concentration with an incubation time of 4 min. (D) and (E) Effect of incubation time on various inhibitor concentrations in 0.1 M PBS (pH 7.0)/0.1 M KCl for ML and AP, respectively.

Where $A$ is the electrode surface area, $C_o^*$ is the ASChCl concentration and $f = F/RT$.

### 3.2. Effect of pH

The pH of the solutions containing substrates can affect the enzyme activity. The denaturation of enzyme can occur at extreme pH values due to conformational changes observed in a native tertiary structure of AChE enzyme. The response of the AChE – biosensor as a function of pH has been studied between pH 5.5–8.5 and was shown in inset of Fig. 2B. The enzyme activity decreases approximately 70% at pH 5.5 compared to that of pH 7.0. When the pH is more than 8.0 the response is very small. The maximum currents appeared at pH 7.0. The anodic peak potentials shifted to less positive side with increasing the pH of the buffer solution and were shown in Fig. 2B. The graph has good linearity with slope of 53 mV/pH. This slope value was close to the theoretical slope of (59 mV/pH), which is in accordance with Nernst equation for transfer of equal number of electrons and protons in the reaction (Chandra, Kumara Swamy, Gilbert, & Sherigara, 2010; Gilbert, Kumara Swamy, Chandra, & Sherigara, 2009).

### 3.3. Effect of enzyme concentration

Fig. 2C shows the response of the biosensor and was found to be dependent on the amount of immobilised enzyme on the surface of CPE. All experiments were carried out in 1 mM substrate solution prepared in 0.1 M PBS (pH 7.0). It can be concluded from the obtained data that the enzyme concentration from 0.03 to 0.3 U into the sol–gel layer, produced increased response of the biosensor. The enzyme concentration of 0.05 U was chosen as best compromise between a low enzyme loading and enough substrate signals. In fabrication of biosensor the lowest feasible concentration of enzyme was necessary towards the determination of pesticides with lower detection limits (Mohammadi, Amine, Cosnier, & Mousty, 2005; Shane, Mousty, & Cosnie, 2004).

### 3.4. Determination of pesticides using AChE–SiSG–CPE/biosensor

AChE biosensor could potentially provide important information about the relative inhibitory effect of ML and AP. In order to obtain lower detection limit and for rapid analysis, incubation time of 4 min was selected for inhibition measurements of both pesticides. Two pesticides are investigated under the optimum conditions (0.1 M PBS, 0.1 M KCl, pH 7.0 and 1 mM substrate). The standard solutions of the two pesticides were freshly prepared before the experiments to avoid decomposition.

Measurement of organophosphorus pesticides was carried out in a two step procedure. The first step involves in measuring the response of biosensor, when the substrate was added to the buffer and this value is corresponds to $I_i$ (current before the inhibition of pesticide). In the second step the electrode was washed with the same buffer and was placed in the sample containing a known concentration of pesticide solution and measured the response, this second value corresponds to $I_F$ (current after the inhibition of pesticide). The percentage of enzyme inhibition and residual enzyme activity percentage was determined according to the following formula (Anitha et al., 2004).

$$\text{Inhibition } \%(I\,\%) = [(I_i - I_F)/I_i] \times 100 \quad (4)$$

$$\text{Residual enzyme activity } \%(\text{REA}\,\%) = [I_F/I_i] \times 100 \quad (5)$$

The electrochemical determination of organophosphorus pesticides was performed through the inhibition reaction of AChE. After inhibition, the thiocholine produced was in less concentration from the enzymatic reaction and decrease of oxidation peak current was observed and this was due to the blocking active sites of the AChE. The AChE has active site gorge that penetrates basically halfway through the enzyme and it consists of two sub sites, namely an anionic site and an esteratic site. The anionic site is located at the opening of the gorge with Tryptophan-84 (Trp-84) and Phenylalanine-330 (Phe-330). The esteratic site is formed by Serine-220 (Ser-200), Histidine-440 (His-440) and Glutamate-327 (Glu-327) (i.e. catalytic triad) and was blocked by the pesticides through the proposed mechanism. In the first step the proton of the serine is accepted by imidazolic nitrogen of the hystidine residue (His 440). The proton of the imidazol ring is exchanged with anion of glutamate (Glu 327). The activated serine is a strong nucleophile, which can attack the carbonyl/thiocarbonyl of the pesticide. The negative charge formed on carbonyl oxygen/sulphur of pesticides is rearranged to produce blocked serine residue and $-R_1H$. The mechanism of inhibition of pesticide was illustrated in the Fig. 3A.

**Table 1A**
The % inhibition of AChE for unspiked and spiked food samples.

| Sample matrix | $n$[a] | Unspiked mean inhibition% | Pesticide | Spiked (ppm) | Spiked mean inhibition % | (Spiked–unspiked value) % | Expected (ppm) | Found (ppm) | Recovery % |
|---|---|---|---|---|---|---|---|---|---|
| Grape | 3 | 23 | AP | 0.2 | 54 | 31 | 0.25 | 0.22 | 88 ± 1.4 |
| | 3 | 27 | ML | 0.6 | 62 | 35 | 0.65 | 0.61 | 94 ± 1 |
| Apple | 3 | 19 | AP | 0.2 | 52 | 33 | 0.24 | 0.21 | 87 ± 1.7<br>89 ± 3[b] |
| | 3 | 24 | ML | 0.6 | 61 | 37 | 0.63 | 0.6 | 95 ± 1<br>99 ± 3[b] |
| Mango | 3 | 18 | AP | 0.2 | 50 | 32 | 0.22 | 0.18 | 82 ± 2 |
| | 3 | 22 | ML | 0.6 | 59 | 37 | 0.61 | 0.57 | 93 ± 1 |
| Orange | 3 | 41 | AP | 0.2 | 53.5 | 12.5 | 0.28 | 0.21 | 75 ± 2.5 |
| | 3 | 48 | ML | 0.6 | 67 | 19 | 0.87 | 0.71 | 82 ± 2 |
| Banana | 3 | 17 | AP | 0.2 | 50.5 | 33.5 | 0.24 | 0.18 | 75 ± 3 |
| | 3 | 21.5 | ML | 0.6 | 75 | 53.5 | 0.67 | 0.59 | 88 ± 2 |
| Tomato | 3 | 17.5 | AP | 0.2 | 52 | 34.5 | 0.25 | 0.2 | 80 ± 2 |
| | 3 | 18 | ML | 0.6 | 63.5 | 45.5 | 0.65 | 0.62 | 95 ± 2 |
| Rice | 3 | 19 | AP | 0.2 | 54 | 35 | 0.25 | 0.23 | 92 ± 2 |
| | 3 | 22 | ML | 0.6 | 61 | 39 | 0.68 | 0.60 | 88 ± 4.5 |
| Wheat | 3 | 24 | AP | 0.2 | 51 | 27 | 0.26 | 0.21 | 81 ± 4.5 |
| | 3 | 28 | ML | 0.6 | 63 | 35 | 0.73 | 0.62 | 85 ± 3 |

[a] $n$ = number of assays.
[b] Results obtained with molecularly imprinted matrix solid phase dispersion–gas chromatography (MIMSPD–GC) (Wang et al., 2013) was compared with the present method.

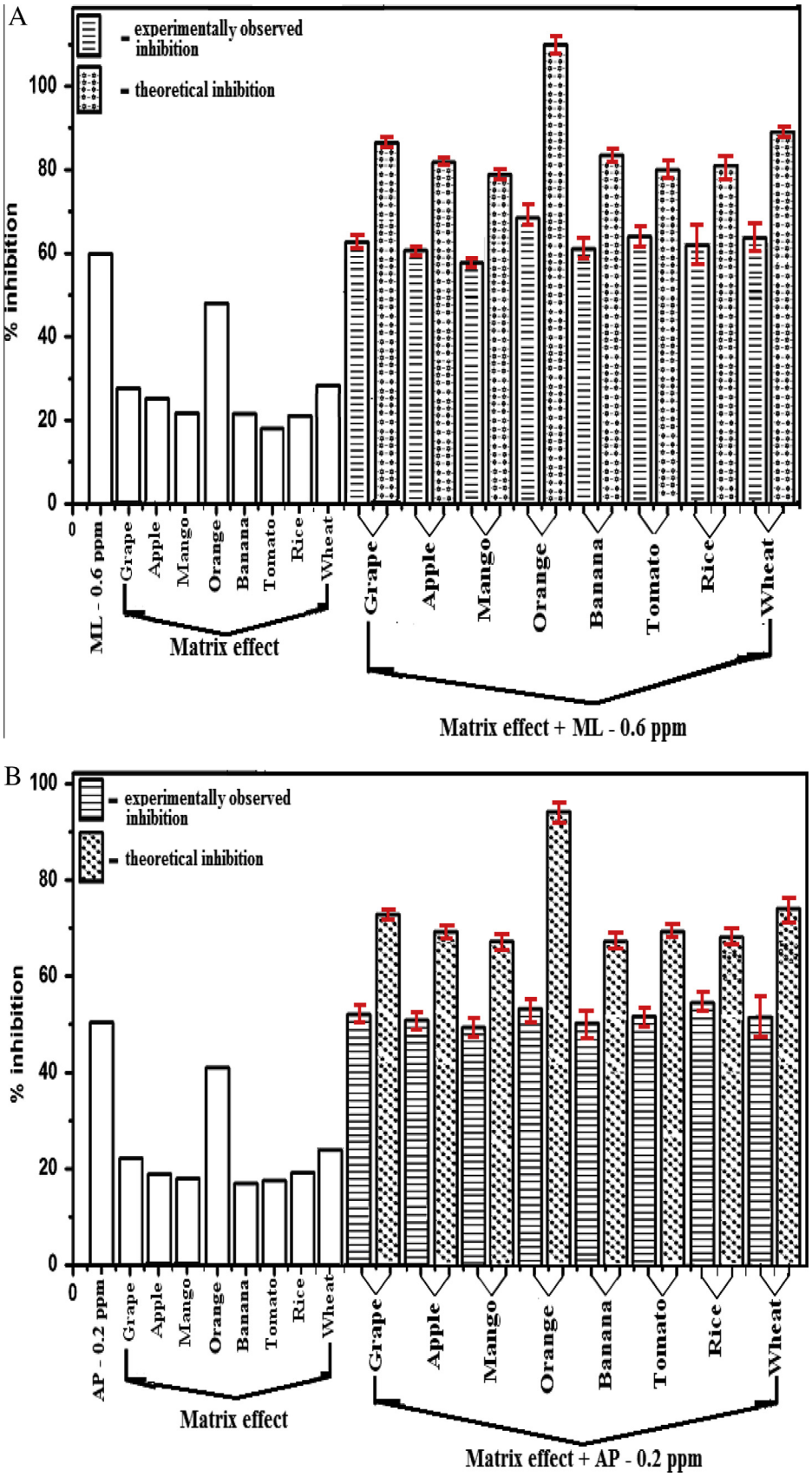



**Fig. 4.** Bar diagram showing theoretical and experimental inhibition values of (A) ML – 0.6 ppm and (B) AP – 0.2 ppm in different food samples.

The detection of individual pesticides was carried out according to the above procedure. Differential-pulse voltammograms (DPV) shows that the peak current decreased with increasing concentration of pesticides and their calibration plots based on the dependence of the % inhibition on concentration were linear and was shown in Fig. 3B and C for ML and AP, respectively. Fig. 3D and E shows the effect of incubation time on residual enzyme activity at different known concentrations of ML and AP, respectively. The percentage of residual enzyme activity decreased with increasing incubation time for both the pesticides. This is because the longer is the incubation time, the interaction between inhibitor and enzyme is more that provides greater inhibition. A complete inhibition was observed at 4 min incubation time with the 1.3 ppm of ML and 0.85 ppm of AP. Limit of detection (LOD) and limit of quantification (LOQ) calculations were carried out by using the following expressions (Madhusudana Reddy & Reddy, 2004; Madhusudana Reddy, Sreedhar, & Reddy, 2003).

$$\mathrm{LOD} = 3\,S_b/S \tag{6}$$

$$\mathrm{LOQ} = 10\,S_b/S \tag{7}$$

Where $S_b$ is the standard deviation of mean values for ten differential-pulse voltammograms of blank solution, $S$ is the slope of the working curve. A good linear relationship was obtained in the range from 0.07–1.3 ppm for ML and 0.1–0.85 ppm for AP (inset of Fig. 3B and C). The regression equations of $I\ \% = 0.0601C$ (%/ppm) + 26.2% ($r = 0.9899$, S.D. = 5.1834) and $I\ \% = 0.0756C$ (%/ppm) + 36.64% ($r = 0.9990$, S.D. = 1.1150) was obtained for ML and AP, respectively. The LOD values were 0.058 ppm, 0.044 ppm and LOQ values were 0.194 ppm, 0.147 ppm for ML and AP, respectively. The lowest LOD value is achieved in the case of AP, which was slightly more toxic than ML.

The robustness of the developed method was evaluated by studying the concept of repeatability (new electrode, same standard solution, same day, same analyst, '*n*' is number of assays) and reproducibility (new electrode, new standard solution, different days, different analyst, '*n*' is number of assays) of the biosensor towards the inhibition of pesticides (Madhusudana Reddy & Reddy 2004; Madhusudana Reddy et al., 2003). To study this experiment the chosen concentration of the stock solutions of ML and AP were 0.5 ppm. The repeatability and reproducibility values were studied in terms of % RSD and were found to be as 2.59, 3.14 for AP and 3.52, 4.19 for ML, respectively. The long term stability of the AChE biosensor was investigated under the storage conditions (in cool place), it was noticed that the activity of immobilised AChE was stable up to one month.

### 3.5. Application towards the determination of pesticides in food samples

Organophosphates are some of the most widely used insecticides in fruit cultures. The pesticides were determined in fruits samples without any previous extraction, clean up or preconcentration steps. Different inhibition values were observed depending on the matrix analysed. However, the fruit samples showed an initial matrix effect. We have noticed slight initial matrix effect for fruit samples due to denaturing conditions (pH) or presence of ascorbic acid (Hildebrandt, Bragos, Lacorte, & Marty, 2008b). The matrix effect was minimised by measuring the difference in response between a spiked sample and blank of similar matrix. Table 1A shows differences from 12.5% to 53.5% which were obtained depending upon the matrix. The higher is the difference the lower is the matrix effect, as observed for banana. High matrix effects were observed for orange giving a difference of only 12.5%. The difference between blank values and spiked values for the rest of the samples has given values higher than 30%. The biosensor was able to find the pesticide residues such as ML and AP in the fruit samples. We have observed through the Fig. 4A and B, that the percentage of inhibition of ML and AP were found to be lower in comparison with the sum of the spiked pesticide and matrix effect of individual samples. All measurements were performed in triplicate. The % inhibition of AChE for the spiked apples sample was

**Table 1B**
Comparison of different electrochemical/other techniques for the determination of AP and ML.

| Biosensor | Analyte (linear conc. range) | Technique (incubation time) | Limit of detection (LOD) | Refs. |
|---|---|---|---|---|
| AChE–AuNPs[a]–$CaCO_3$[b]–Au[c]–SiSG[d] | ML (0.1–100 nM) | CV[m] (10 min) | 0.1 nM | Narang, and Pundir (2011a) |
| AChE–Pin5COOH[e]–ZnS[f]/Au[c] | ML (0.1–50 nM) | SWV[n] (10 min) | 0.1 nM | Chauhan, Narang, and Pundir (2011b) |
| AChE-$Fe_3O_4$NP[g]-MWCNTs[h]/Au[c] | ML (0.1–40 nM) | Amperometry (10 min) | 0.1 nM | Chauhan and Pundir (2011c) |
| AChE–PAn[i]–PPy[j]–MWCNTs[h]/GCE[k] | ML (0.01–25 μg/mL) | CV[m] (15 min) | 1 ng/mL | Du, Ye, Cai, Liu, and Zhang (2010) |
| AChE-CHIT[l]–AuNPs[a]–Au[c] | ML (0.1–20 ng/mL) | LSV[o] (15 min) | 0.03 μg/L | Du, Ding, Tao, and Chen (2008) |
| | AP (7.5–500 ng/mL) | μLC-FPD[p] | 2.8 ng/mL | Hooijschuur, Kientz, Dijksman, and Brinkman (2001) |
| | AP (5–140 ng/mL) | ciELISA[q] | 2 ng/mL | Lee, Chang Ahn, Stoutamire, Gee, and Hammock (2003) |
| AChE–SiSG[d]–CPE | ML (0.07–1.3 ppm) | DPV[r] (4 min) | 0.058 ppm | Present work |
| | AP (0.1–0.85 ppm) | DPV[r](4 min) | 0.044 ppm | Present work |

[a] AuNPs – Au nanoparticles.
[b] $CaCO_3$ – calcium carbonate.
[c] Au – gold electrode.
[d] SiSG – silica sol–gel.
[e] Pin5COOH – poly (indole-5-carboxylic acid).
[f] ZnS – zinc sulphide.
[g] $Fe_3O_4$NP – iron oxide nanoparticle.
[h] MWCNTs – multiwalled carbon nanotubes.
[i] PAn – polyaniline.
[j] PPy – polypyrrole.
[k] GCE – glassy carbon electrode.
[l] CHIT – chitosan.
[m] CV – cyclic voltammetry.
[n] SWV – square wave voltammetry.
[o] LSV – linear sweep voltammetry.
[p] pμLC-FPD – microcolumn liquid chromatography-flame photometric detector.
[q] ciELISA – competitive indirect enzyme-linked immunosorbent assay.
[r] DPV – differential pulse voltammetry.

compared with the reference method i.e., Molecularly Imprinted Matrix Solid Phase Dispersion–Gas Chromatography (MIMSPD–GC) (Wang, Qiao, Ma, Zhao, & Xu, 2013) and was found to be in good agreement.

The recovery efficiencies ($R$ %) for the different systems were calculated using the following equation.

$$\%\,R = 100\,([\text{pesticide}]\ \text{found}/[\text{pesticide}]\ \text{expected}) \quad (8)$$

The precision and accuracy of methodologies were tested with different standard solutions of each pesticide and the relative standard deviations (RSD) were calculated using the following equation.

$$\text{RSD} = S_b/X \quad (9)$$

Where $S_b$ is standard deviation of mean inhibition values obtained and $X$ is the mean inhibition (Pedrosa, Caetano, Machado, & Bertotti, 2008). The detection limit of various electroanalytical/other methods proposed for the determination of ML and AP was compared with the present method and shown in Table 1B.

## 4. Conclusion

AChE biosensor has the ability for the direct detection of ML and AP in food samples and it requires no extraction or preconcentration steps. The present study demonstrates the simple sol–gel entrapment procedure for the development of AChE biosensor, which requires less amount of enzyme. This biosensor was highly sensitive to the pesticide determination and it operates at a relatively low cost. In addition, the developed procedure can be employed for the monitoring of different types of organic pollutants and thus enlarging the future applicability of the biosensor.

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