The Pharma Innovation Journal 2018; 7(10): 253-258

www.ThePharmaJournal.com

The Pharma Innovation

ISSN (E): 2277- 7695
ISSN (P): 2349-8242
NAAS Rating: 5.03
TPI 2018; 7(10): 253-258
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www.thepharmajournal.com
Received: 31-08-2018
Accepted: 30-09-2018

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Development of amperometric biosensor against caffeine in solution over a concentration range

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Abstract
Amperometric biosensor was prepared by using of alkaline phosphatase (ALP) against caffeine by a chemical covalent immobilization of alkaline phosphatase (ALP) with a cross-linking agent, glutaraldehyde on a ceramic based gold screen printed electrode that was modified with cysteamine by forming a self-assembled monolayer. Caffeine (CAF) competitively inhibits ALP enzyme and the determination method of caffeine by the biosensor was based on this inhibition effect of caffeine. The principle of the measurement was based on the determination of the differentiation of biosensor responses in the enzymatic reaction catalyzed by ALP in the absence and the presence of caffeine. Differences between the biosensor responses were related to caffeine concentration which was added in to the reaction medium. Caffeine concentration can be determined accurately between 0.2 and 10 µM using the biosensor. Detection limit (LOD) of the biosensor is 0.1 µM. In the optimization studies of the biosensor, glycine buffer (pH 10.5; 50 mM) and 30 ºC were obtained as the optimum working conditions. The optimum pH value was obtained as 10.5 by using of glycine buffer more comparable with another buffer systems such as Tris/NaOH and borate etc. Below and above this pH value decreases in the biosensor response were observed.

Keywords: ALP, biosensor responses, LOD & concentration

1. Introduction
Caffeine (1, 3, 7-trimethylxanthine) is a natural alkaloid occurring in coffee, cocoa beans, cola nuts and tea leaves. It is mildly stimulating and is used as a therapeutic agent (Ritchie *et al.*, 1975) [22] and plays an important role in food and drug chemistry. It is also present in many painkillers and antimigraine pharmaceuticals (Abourashed *et al.*, 2004 & Armenta *et al.*, 2005) [2, 6]. The beverages such as coffee, tea, cola or drug formulations belong to the significant economic products in which the highest quality in international business is demanded.
A biosensor based on inhibition of 3, 5-cyclic phosphodiesterase (CPDE) from bovine heart in combination with a pH electrode for the detection of caffeine in coffee was reported by (Pizzariello *et al.*, 1999) [21]. In respect to an ascending number of samples, the novel and perspective analytical methods for determination of caffeine providing accurate and reliable results are necessary. Electrochemical methods have been commonly exploited as cheap, rapid and simple alternatives to modern separation and spectral methods. To-date however, a comprehensive review on the electrochemical determination of caffeine has not been reported. Herein the present paper gives a summary on the current state in this field. The major part deals with the use of bare and modified carbon-based electrodes as voltammetric sensors for determination of caffeine (Svorc *et al.*, 2013) [35].
The main advantages of these devices are their specificity, sensitivity and ease of sample preparation and the fact that no other reagents besides a buffer and a standard are usually required. Caffeine (1, 3, 7-trimethylxanthine) is a naturally occurring substance found in the leaves, seeds or fruits of some plant species and is a member of a group of compounds known as methylxanthines (Hall *et al.*, 1986 & Joachim *et al.*, 1986) [12, 13].
It is also present in many painkillers and antimigraine pharmaceuticals. The most commonly known sources of caffeine are coffee, cocoa beans, cola nuts and tea leaves. It does not accumulate in the body over the course of time and is normally excreted within several hours of consumption.
Traditional methods for the immobilization of microorganisms include adsorption, encapsulation, entrapment, covalent binding, and cross-linking. Besides these methods, many novel immobilization strategies have been explored in recent years in order to improve the analytical performance and storage stability of the microbial biosensor

(Lowe *et al.*, 1984 & North *et al.*, 1985) [15, 17].
There has been no systematic improvement in reported detection limits during the past 15 years of label-free affinity biosensor research. On-going fundamental studies on mediated and direct electron-transfer electrochemistry, on new sensing principles, and on enzyme stabilization, coupled to extensive commercial efforts, should have a tremendous impact on point-of-care clinical testing, and upon biomedicine, in general.

2. Materials and Methods

2.1. Enzyme and chemicals

The enzyme used in this study was alkaline phosphatase (EC 3.1.3.1) from bovine intestinal mucosa, glutaraldehyde (25%) was purchased from Sigma Chemical Co. (USA). Enzyme solution was prepared in Tris–HCl buffer (pH 7.0). Caffeine substrate was also purchased from Sigma Chemical Co. (USA).
The main chemicals that we used in this research were cysteamine, p-nitrophenyl phosphate disodium salt hexahydrate, potassium chloride, magnesium chloride, KH_2PO_4, K_2HPO_4, glycine and all the other chemicals were purchased from Sigma Chemical Co. (USA). Electrochemical experiments were carried out in glycine buffer (50 mM; pH 10.5 containing 0.1MKCl + 1m $MMgCl_2$).

2.2. Instruments

Corundum Ceramic Based Screen Printed Gold Electrode (1.0 Mm) from BVT Technologies (CZ), Gibson P100 and P1000 Automatic Pipets from France, Yellow Line Magnetic Stirrer from Germany, Nuve Model Thermostat from TR, Double Beam Spectrophotometer from E.I., H.P. (India), Ultra-pure water from Mili-Q and Milipore RIOS-DI 3 UV (USA), Indikrom Paper (pH meter) from Galaxosmithkiine Pharma. Ltd., Mumbai (India).

2.3. Amperometric detection method for determination of caffeine on reparation of the biosensor on screen printed gold electrode (SPGE)

The principle of the measurement is found on the evaluation of the differentiation of biosensor responses in the enzymatic reaction catalyzed by ALPE in the absence and the presence of CAF. Selectivity study of detection of CAF and theobromine on molecularly imprinted polypyrrole (MPPY) electrode surface. Comparison of the techniques such as pulse amperometric detection (PAD) and electrochemical impedance spectroscopy (EIS) with results shows no statistical difference.
Isolated Screen printed gold electrode immersed cysteamine solution (100mM, in phosphate buffer; 50mM, pH 7.0) to form the structure of self-assembled monolayer (SAM) during 6 h period. After 6 h the electrode was washed with ultra-pure water and cross-linked with glutaraldehyde (5%) solution (in phosphate buffer; 50mM, pH 7.0) for 30 min. After that the electrode was washed again and immersed in alkaline phosphatase (13 U/ml) (in Tris–HCl buffer pH 7.0, 50mM, contained MgCl2) for 22 h. Thus, the immobilization of the enzyme was carried out.
In our study, amperometric detection of caffeine biosensor was observed by using of corundum ceramic based screen printed gold electrode (1.0 mm). Under alkaline conditions (pH >10.0) ALP converts pnitrophenyl phosphate (PNP) to p-nitrophenol and phosphate. p-Nitrophenol loses H^+ ion and turns into the negatively charged compound p-nitrophenolate at medium pH. This compound can irreversibly oxidize to p-nitro phenoxy cation at anode resulting in a peak at +0.95V (Zhu *et al.*, 2007) [44]. As a result, this product formed is measured chronoamperometric ally at an aplication potential of +0.95V (Das *et al.*, 2007, Bolado *et al.*, 2006 & Patil *et al.*, 2008) [8, 7, 20]. The anodic current values resulting for the oxidation of the enzymatically generated p-nitrophenol or p-nitrophenolate were recorded and plotted each substrate concentration. So, caffeine acts to be a competitive inhibitor and decreases these anodic currents by interaction with ALP enzyme.

2.3.1. Sensitivity

Sensitivity (S) is the parameter expressing the change in electrode potential according to the logarithm of the analyte ion activity and is expressed in mV / pIon. The formula of S is given below:

$$S= E_1-E_2/ \log (a_1/a_2)$$

Where E1 and E2 are the values of the potential between which the evaluation is made, both taken in the field of linearity, and a1 and a2 are the ionic potential activities corresponding to E1 and E2, respectively.

2.3.2. Detection Limit

The detection limit (LD) is the ionic activity found from intersection of the extrapolations of the linear portions of the calibration curve.

2.3.3. Linearity Domain

The linearity domain (DL) is the range of analyte activities between which the electrode potential depends linearly on the logarithm of the analyte. DL was calculated as the interval between the LD and the upper limit of the range of activities, otherwise, the upper activity which would meet this criteria was taken into account, for which the square of the correlation coefficient linear range was at least 0.99.

2.3.4. Statistical Analysis

The data was subjected to statistical analysis for the determination of significance by using ANOVA.

3. Results and discussion

In this study, an amperometric biosensor based on alkaline phosphatase was developed in order to investigate the effect of caffeine on the activity of alkaline phosphatase enzyme. From the experimental studies we detected an inhibition effect of caffeine on the enzyme activity and this effect increased at higher caffeine concentrations. Using the biosensor we detected a linear concentration range for caffeine in the presence of a constant concentration of p-nitrophenyl phosphate. The developed biosensor was also used in real sample analysis. If we compare the results obtained by the biosensor with results obtained by the derivative spectrophotometric method it can be said that the biosensor is much more sensitive, accurate and easy to use than the derivative spectrophotometric method.
In addition reproducibility of the biosensor is very good and the biosensor can be used as an alternative method for routine analysis of caffeine. The biosensor comprises the biological sensing element, the transducer, amplification and detector systems as shown in Fig. 1 and Fig. 2 showing printed electrode for the amperometric detection of caffeine (CAF) at below.

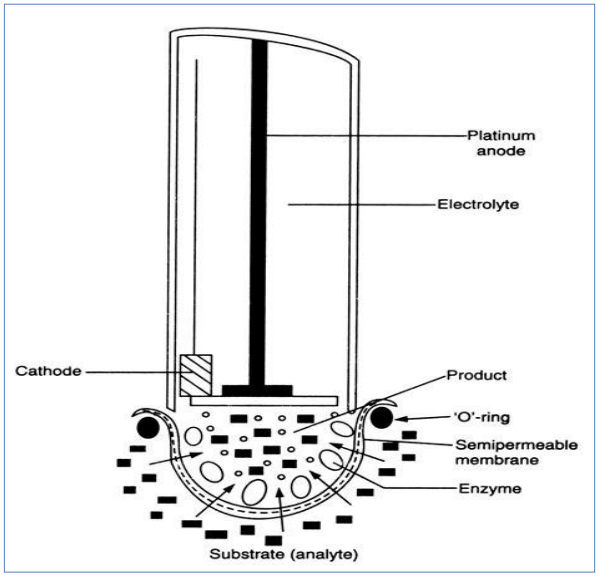

Fig 1: Schematic diagram of the for the biosensor.

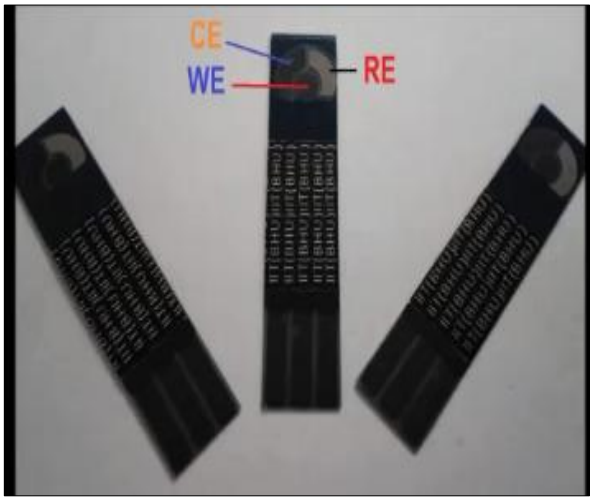

Fig 2: Printed electrode for the electrode used amperometric detection of caffeine (CAF).

3.1. Competitive inhibition effect of Caffeine (CAF) on ALP enzyme

We discussed about biosensor responses in the presence and absence of caffeine with another sensor such as gold-chitosan nanocomposite based sensor for selective electrochemical determination of caffeine. Electrochemical parameters were optimized in order to improve the electrochemical response to caffeine. The most satisfactory result, that means the higher electrochemical improvement, was obtained using a gold electrode modified with AuNPs synthetized in a chitosan matrix in the presence of oxalic acid, in aqueous solution containing $HClO_4$ 0.4 mol L^{-1} as supporting electrolyte. The performance of the sensor was then evaluated in terms of linearity range (2.0×10^{-6}-5.0×10^{-2} mol L^{-1}, R = 0.999), operational and storage stability, reproducibility (RSD = 3.7%), limit of detection (LOD = 1.0×10^{-6} mol L^{-1}) and response to a series of interfering compounds as ascorbic acid, citric acid, gallic acid, caffeic acid, ferulic acid, chlorogenic acid, glucose, catechin and epicatechin (Trani *et al.*, 2017) [36].

In our study, limit of detection (LOD) was about 0.1 µM with caffeine concentration about 0.2-10 µM. Cyclic voltammetric experiments were carried out. CV measurements were performed between + 0.3 and +1.1V in glycine buffer (pH 10.5, 50 mM). Measurements were carried out for bare electrode, ALP modified electrode in the presence of p-nitrophenyl phosphate without caffeine and ALP modified electrode in the presence of p-nitrophenyl phosphate with caffeine. Voltammograms obtained from the experiments were given in Fig. 3. From the figure, the inhibition effect of caffeine on ALP enzyme can be easily seen. Because of the competitive inhibition effect of caffeine on ALP, the biosensor response markedly decreased.

CV voltammograms obtained for the determination of the inhibition effect of caffeine on alkaline phosphatase at a scan rate of 50 mVs^{-1}. (Glycine buffer; pH 10.5, 50mM; T: 30 ºC.) (—) baseline without PNP, (- -) with 0.2 mM PNP and without caffeine, (+ +) with 0.2 mM PNP and 5.0 µM caffeine) (in Fig. 3). Fig. 4 shows the net effect of caffeine on the activity of alkaline phosphatase and also differences in the biosensor responses in the presence and absence of caffeine. Calibration curve for PNP obtained with the alkaline phosphatase biosensor in the presence and absence of caffeine. (Glycine buffer, pH 10.5; 50 mM, T: 30 ºC.) (▲) Without caffeine and (●) with 2.5 µM caffeine (Fig. 4).

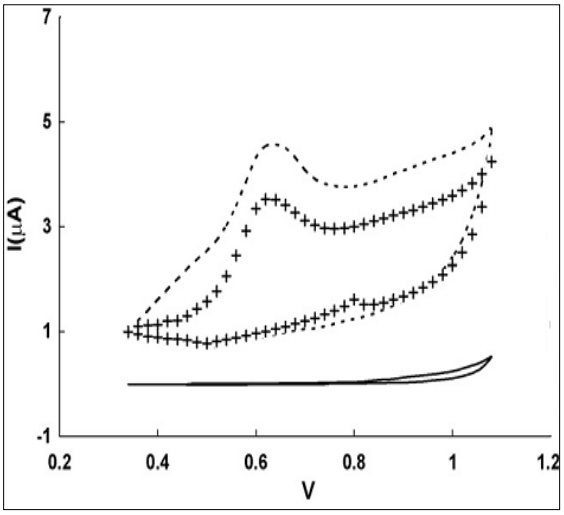

Fig 3

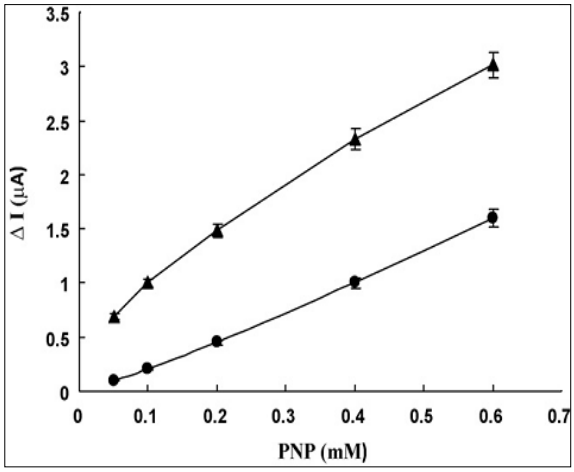

Fig 4

3.2. Optimization of experimental conditions for Caffeine (CAF) biosensor

3.2.1 PH and buffer system

The optimum pH value of the free alkaline phosphatase is

nearly 10.5 and as a result it can be said that the immobilization procedure used in the biosensor preparation, did not change the optimum pH value of the enzyme.

The results obtained from the experiments. The effect of the buffer system on the biosensor response was also studied. In order to investigate the effect of buffer system on the biosensor response, three different buffer systems glycine, Tris/NaOH and borate, which were of pH 10.5 and 50 mM were used. Although the biosensor response obtained with glycine buffer were nearly the same as with the Tris–NaOH buffer, the highest biosensor responses were obtained using glycine buffer. When borate buffer system was used decreases in the biosensor response were detected.

In another study, amperometric principle-based biosensor containing immobilized enzyme tyrosinase was used for detection of polyphenols in tea. The immobilized tyrosinase based biosensor could detect tea polyphenols in the concentration range 10–80 mmol L^{-1}. Immobilization of the enzyme by the cross-linking method gave good stable response to tea polyphenols. The biosensor response reached the steady state within 5 min. The voltage response was found to have a direct linear relationship with the concentration of polyphenols in black tea samples. Enzyme membrane fouling was observed with number of analyses with a single immobilised enzyme membrane. The tyrosinase-based biosensor gave maximum response to tea polyphenols at 30 ºC. The optimum pH was 7.0. This biosensor system can be applied for analysis of tea polyphenols (Abhijith *et al.*, 2007) [1].

3.2.2 Temperature dependence of the Caffeine (CAF) biosensor

In our study, we determined the effect of temperature on the biosensor response, experiments were carried out between 20 and 40 ºC. The enzyme activity depends on the temperature and the medium conditions. From the experiments, the highest biosensor responses were observed at 30 ºC. Below and above this temperature, decreases in the biosensor responses were recorded.

In another study, developed an amperometric xanthine biosensor based on zinc oxide nanoparticles (ZnO- NPs) polypyrrole composite film. The biosensor exhibited optimum response within 5 s at pH 7.0, 35 ºC and linearity from 0.8 µM to 40 µM for xanthine with a detection limit 0.8 µM (S/E = 3). Michaelis Menten constant (K_m) for xanthine oxidase was 13.51 µM and I_{max}0.071 µA. The biosensor measured xanthine in fish meat and lost 40% of its initial activity after its 200 uses over 100 days, when stored at 4 ºC (Devi *et al.*, 2011) [9].

3.2.3 Effect of applied potential on the Caffeine (CAF) biosensor response

In this part of the study for the investigation of the effect of applied potential on the biosensor response different potential values were investigated. For this purpose measurements were carried out at +0.75, +0.8, +0.85, +0.9 and +0.95 V. From the experiments, the highest biosensor responses were observed at +0.95 V. Below this potential value decreases in the biosensor response were observed. When the potential increased from +0.75 to +0.95 V, the response sensitivity of the biosensor for caffeine increased significantly. A potential of +0.95V (vs. Ag/AgCl) was selected as the applied potential in the amperometric measurements of caffeine.

3.2.4 Reproducibility of the Caffeine (CAF) biosensor

The reproducibility of the biosensor was also investigated for 5 µM caffeine concentration (n=6) in the presence of 0.2 mM PNP. From the results of six successive measurements, the average value (x), the standard deviation (SD) and coefficient of variation (CV%) were calculated as 5.26±0.0903 µM, and 1.71%, respectively. It can be said that caffeine can be determined more sensitively using the biosensor.

3.2.5 Interference effects of some compounds in Caffeine (CAF) determination

To investigate the interference effects of some compounds in caffeine determination 5.0 µM concentration of d-glucose, d-fructose, l-phenylalanine, l-tyrosine, l-ascorbic acid, catechin and theophylline were used. The biosensor response obtained for caffeine was accepted as 100% and compared with the biosensor responses of other compounds.

Table 1: Interference effects of some compounds on caffeine biosensor response.

Substrate (5.0 µM)	Relative activity (%)*
Caffeine	100
Glucose	100
l-Phenylalanine	110
l-Tyrosine	100
Fructose	100
l-Ascorbic acid	110
Catechin	110
Theophylline	100

***** Average of five (05) measurements.

According to the results obtained by the biosensor only ascorbic acid showed a little interference effects. However, the interference effects of the other compounds were negligible. The results obtained were given in Table 1.

Table 2: Comparison of the recovery of proposed biosensor with various methods for the determination of caffeine.

Method	Recovery (%)	Ref.
Voltammetry	98-104	Zen *et al.*, 1998
Flow injection	98–103	Lima *et al.*, 1998
SFC-FTIR	-	Norton *et al.*, 1996
MIP–PMAA/PVC sensor	52–122	Ebarvia *et al.*, 2004
UV-VIS (AOAC 12.028)	98–101	Ebarvia *et al.*, 2004
Present biosensor	95-100	-

In Table 2 compares of the recovery of proposed biosensor with various methods for the determination of caffeine. In this study, it is found that present amperometric biosensor having of 95-100% recovery which is average result by comparing with another methods such as Voltammetry, Flow injection, SFC-FTIR, MIP–PMAA/PVC sensor & UV-VIS (AOAC 12.028).

In another study, bare boron-doped diamond based electrode was develpoed for voltammetric determination of caffeine in beverage samples. It was found that caffeine (1, 3, 7-Trimethylxantine) provided highly reproducible and well-defined irreversible oxidation peak at very positive potential. The effects of supporting electrolyte, pH and scan rate on the voltammetric response of caffeine oxidation were studied to select the optimum experimental conditions. Present

Biosensor compared with another various methods for the determination of caffeine. Recovery of our caffeine biosensor was about 95-100%.

In Table 3 shows the various amperometric sensors for determination of CAF. Amperometric microbial biosensor (MIC-B) developed for the analysis of CAF in beverages using immobilized whole cells of *Pseudomonas alcaligenes* (PA) capable of degradation of CAF. Interestingly, this biosensor was highly specific for CAF and the response to interfering compounds such as THO, theobromine and sugars was found to be negligible. Another biosensor based on the alkaline phosphatase enzyme (ALPE) immobilized on gold screen printed electrode (GSPE). The CAF competitively inhibited ALPE and thus determination was based on this inhibition effect. The principle of the measurement was founded on the evaluation of the differentiation of biosensor responses in the enzymatic reaction catalyzed by ALPE in the absence and the presence of CAF. In another literature, it was focused on the selectivity study of detection of CAF and theobromine on molecularly imprinted polypyrrole (MPPY) electrode surface. Many of the techniques were used with comparison such as pulse amperometric detection (PAD) and electrochemical impedance spectroscopy (EIS) with results showing no statistical difference. Table 3 shows the overview of amperometric sensors for the determination of CAF.

Table 3: Amperometric sensors for determination of CAF.

Electrode	Technique	Detection limit	LCR (µM)	DL (µM)	Application	Ref.
GCE	FIA-AD	120	10-80	0.21	Soft drinks	Lima *et al.*, 1998
BDDE	FIA-MPAD	140	5.1-1620	0.87	Drugs	Silva lo *et al.*, 2011
BDDE	BIA-DPAD	> 60	1.8-20	0.72	Drugs	Silva *et al.*, 2011
PA-MIC-B	AD	N/A	510-5130	N/R	Coffee, tea, cola	Sarath Babu *et al.*, 2007
GSPE-ALPE-B	AD	N/A	1-10	0.08	Coffee, tea, cola	Akyilmaz *et al.*, 2010
MPPY/Pt	PAD, EIS	N/A	1000-20000	N/R	Coffee, tea	Vinjamuri *et al.*, 2008
Present Electrode	AD	N/A	0.2-10	0.1	Coffee, tea, cola	-

Electrode: ALPE-B: alkaline phosphatase enzyme biosensor, BDDE: boron-doped diamond electrode, GCE: glassy carbon electrode, GSPE: gold screen printed electrode, MPPY: molecularly imprinted polypyrrole, PA-MIC-B: Pseudomonas alcaligenes microbial biosensor,

Technique: AD: amperometric detection, BIA: batch injection analysis, DPAD: dual pulse amperometric detection, EIS: electrochemical impedance spectroscopy, FIA: flow injection analysis, MPAD: multiple pulse amperometric detection, PAD: pulse amperometric detection,

Others: DL: detection limit, LCR: linear concentration range, N/A: not applicable, N/R: not reported.

In our study, we used isolated screen printed gold electrode immersed cysteamine solution (100mM, in phosphate buffer; 50mM, pH 7.0) to form the structure of self-assembled monolayer (SAM) during 6 h period for developing of amperometric biosensor against caffeine at various concentration ranges with detection limit & detection range about 0.1 µM & 0.2-10 µM respectively that is more suitable compared with another electrode for the various beverages like as tea, coffee & cola etc.

3.2.5 Conclusions

Amperometric biosensor was prepared by using of alkaline phosphatase (ALP) against caffeine by a chemical covalent immobilization of alkaline phosphatase (ALP) with a cross-linking agent, glutaraldehyde on a ceramic based gold screen printed electrode that was modified with cysteamine by forming a self-assembled monolayer. Caffeine (CAF) competitively inhibits ALP enzyme and the determination method of caffeine by the biosensor was based on this inhibition effect of caffeine. Caffeine concentration can be determined accurately between 0.2 and 10 µM using the biosensor. Detection limit (LOD) of the biosensor is 0.1 µM. In the optimization studies of the biosensor, glycine buffer (pH 10.5; 50 mM) and 30 ºC were obtained as the optimum working conditions.

Recovery of our amperometric biosensor against caffeine was about 95-100%. The optimum pH value was obtained as 10.5 by using of glycine buffer more comparable with another buffer systems such as Tris/NaOH and borate etc. Below and above this pH value decreases in the biosensor response were observed.

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