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Application of Gold Screen Printed Electrode (GSPE) based Caffeine Biosensor for real sample analysis

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Abstract
Caffeine (1, 3, 7-trimethylxanthine) increases calcium excretion in the urine and so heavy caffeine usage may increase the risk of osteoporosis. Biosensor adoption is increasing every year and the number of biosensor applications is continuously growing. 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 interference effects of the other compounds were negligible. Developed biosensor gold screen printed electrode (GSPE) was also used in real sample analysis. Our study showed minimum response detection ranges with lowest detection limit (LOD) than previous literatures for the same purpose of study and response time was very short just only 40 s by using of isolated screen printed gold electrode for the development of amperometric biosensor against caffeine over concentration range. Caffeine in the soft drinks can be determined sensitively using the biosensor. In this study, it was found that present amperometric biosensor having of 95-100% recovery which was average result by comparing with another methods such as Voltammetry, Flow injection, SFC-FTIR, MIP–PMAA/PVC sensor & UV-VIS (AOAC 12.028).

Keywords: GSPE, UV-VIS, Response time & Detection ranges

1. Introduction
Biosensors are powerful tools aimed at providing selective identification to toxic chemical compounds at ultra trace levels in industrial products, chemical substance, environmental sample (e.g., air, soil & water) or biological system (e.g., bacteria, virus or tissue components) for biomedical diagnosis (Haggett *et al.*, 1986, Bergmeyer *et al*., 1974 & Guilbault *et al*., 1985) [8, 2, 6].

The development and application of new caffeine detection methods remains an active area of investigation, particularly in food and clinical chemistry. Significant research and development activity has been devoted to preparing compact analytical devices comprising a bioactive sensing element integrated with a suitable transducing system, known as biosensors, for determination of various inorganic, organic and biological substances. 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 (Thakur *et al.*, 2003) [26].

Furthermore, recent development in molecular biology offers a novel method to construct genetically engineered microorganisms (GEMs), thus providing a new direction to manipulate the selectivity and sensitivity of microbial biosensors at the DNA level. DNA can be used to identify organisms ranging from humans to bacteria and viruses. Immobilizing microorganisms on transducers plays an important role in the fabrication of microbial biosensors (Kernevez *et al.*, 1983 & Kricka *et al.*, 1986) [12, 13].

Developed gold-chitosan nanocomposite sensor for selective electrochemical determination of caffeine. There was little change on nanocomposites synthesis affect selectivity. The electrochemical behaviour of caffeine at both gold bare and gold electrode modified with AuNPs with different morphology was carried out in acidic medium by cyclic voltammetry (CV), differential pulse voltammetry (DPV) and electrochemical impedance spectroscopy (EIS). Electrochemical parameters were optimized in order to improve the electrochemical response to caffeine (Trani *et al.*, 2017) [28].

Recovery of proposed biosensor with various methods for the determination of caffeine. In this study, it was 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).

2. Materials and Methods

2.1. Enzyme and chemicals

Alkaline phosphatase (EC 3.1.3.1) from bovine intestinal mucosa, glutaraldehyde (25%) Enzyme solution was prepared in Tris–HCl buffer (pH 7.0), Caffeine, 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).

2.2. Instruments

Corundum Ceramic Based Screen Printed Gold Electrode (SPGE) (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 method

Pnitrophenyl phosphate (PNP) is converts to p-nitrophenol and phosphate. p-Nitrophenol loses H^+ ion and turns into the negatively charged compound p-nitrophenolate at medium pH under alkaline conditions (pH >10.0) ALP. This compound can irreversibly oxidize to p-nitro phenoxy cation at anode resulting in a peak at +0.95V (Zhu *et al.*, 2007) [29]. As a result, this product formed is measured chronoamperometric ally at an aplication potential of +0.95V (Bolado *et al.*, 2006 & Patil *et al.*, 2008) [3, 18].

When the caffeine was injected in to the reaction medium with p-nitrophenyl phosphate, both the activity of the enzyme and amperometric response of the biosensor were decreased. Decreases were related to the caffeine concentration added into the reaction medium. The principle of the measurement of the biosensor is based on the determination of these changes in the current that are related to caffeine concentration. As a result, the differences in the current in the absence and in the presence of caffeine were detected by the biosensor to obtain a standard curve for the determination of caffeine. All the measurements were made at 30 ºC using a thermostatic reaction cells and glycine buffer (pH 10.5).

3. Results and discussion

3.1. Analysis of the various samples

In the sample analysis, cola, coffee and tea were chosen owing to their high caffeine content. Samples were purchased from the local markets. In order to degas cola, the samples were mixed for 15 min by using a magnetic stirrer. Coffee and tea samples were prepared by dissolving 1 g of each in 100 ml boiling water and before the measurements they were filtered through a dry filter paper. Using the biosensor, caffeine contents of the samples were analyzed and the results obtained were compared with those obtained by the UV-spectrophotometric method (Alpdogan *et al.*, 2002) [1]. Five measurements were carried out for each sample and average value (x), standard deviation (SD), and coefficient of variation (CV %) of each sample were calculated.

Table 1: Sample analysis by using biosensor and UV-spectrophotometric method.

Sample	The biosensor method			UV-spectrophotometric method		
	Average (µM)	n=5		Average (µM)	n=5	
		SD (µM)	CV (%)		SD (µM)	CV (%)
Tea	4.878	±0.052	1.06	4.734	±0.041	0.86
Coffee	4.802	±0.164	3.41	4.686	±0.168	3.59
Cola-1	5.058	±0.142	2.81	4.894	±0.151	3.10
Cola-2	5.228	±0.069	1.33	4.825	±0.084	1.75
Cola-3	5.052	±0.136	2.69	4.846	±0.145	2.99

The results obtained from the experiments are given in Table 1. According to the results it can be said that caffeine in the soft drinks can be determined sensitively using the biosensor.

Piezoelectric quartz sensor was developed for caffeine based on molecularly imprinted polymethacrylic acid. In his study, the sensor response was quite reproducible, exhibiting a relative standard deviation of 9% for three replicates. It varied with the concentration of caffeine in the solution. A highly linear relationship was observed between the response and the logarithm of the caffeine concentration, in the range of 1×10^{-9} up to 1 mg/mL, the correlation coefficient being 0.9974. High sensitivity of the sensor was found about (ca. 53 Hz/ln [conc. caffeine, mg/mL]). The detection limit for the sensor was found to be 5.9×10^{-11} mg/mL based on three standard deviations. According to (Ebarvia *et al.*, 2005) [5]. But in our study the detection limit was 0.1 µM with detection range about 0.2- 10 µM. so, our amperometric biosensor is more effective than this piezoelectric quartz sensor.

Table 2: Comparison of some biosensors developed for caffeine determination.

Biosensor	Detection range	Detection limit	Response time	Ref.
Whole cell based	0.1–1.0 mg ml^{-1}	-	3min	Shrivas *et al.*, 2007
pH electrode	0.0–4.0 mg ml^{-1}	0.6 mg ml^{-1}	2–4 min	Pizzariello *et al.*, 1999
Piezoelectric	10^{-9}–10^{-3} mg ml^{-1}	3.75×10^{-11} mg ml^{-1}	10 min	Ebarvia *et al.*, 2004 [4]
Membrane electrode	10^{-6}–10^{-2} M	50 µM	–	Katsu *et al.*, 2008
Present biosensor	0.2–10 µM	0.1 µM	40 s	-

Table 2 shows comparison of detection performance of our biosensor and the others to caffeine. According to the Table 2, it can be said that the most sensitive biosensor is piezoelectric based but its response time is so long. If we consider both of detection limit and response time of the biosensors, it is obvious that our biosensor is much better than the others.

After comparison of the present amperometric biosensor against caffeine with various another biosensor methods, it is clear that our biosensor shows best results in regarding of response detection ranges between 0.2 µM to 10 µM with detection limit about 0.1 µM. Response time was very short just only 40 s. 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.

The electrochemical biosensor functioning was based on the inhibition effect of benzoic acid on the biocatalytic activity of the polyphenol oxidase (PPO) to its substrate (catechol) in 0.1 M phosphate buffer solution (pH 6.5). A potential value of −50 mV versus SCE, and a constant catechol concentration of 20 μM were selective to carry out the amperometric inhibition measurement. The inhibiting action of benzoic acid on the polyphenol oxidase electrode was reversible and of the typical competitive type, with an apparent inhibition constant of 38 μM. This proposed biosensor detected levels of benzoic acid as low as 2×10^{-7} M in solution (Shan *et al.,* 2007) [24]. Our study shows response detection ranges between 0.2 μM to 10 μM with detection limit about 0.1 μM and response time was very short just only 40 s by using of isolated screen printed gold electrode.

A piezoelectric quartz sensor coated with molecularly imprinted polymer (MIP) for caffeine. A steady-state response was achieved in less than 10 min. The performance characteristic of the sensor shows a promising and inexpensive alternative method of detecting caffeine. Surface studies were carried out for the reagent phase of the sensor using SEM, AFM, and XPS analysis in order to elucidate the imprinting of the caffeine molecule. The SEM micrograph, AFM image, and XPS spectra confirmed the removal of caffeine by Soxhlet extraction in the imprinting process and the rebinding of caffeine to the MIP sensing layer during measurement (Ebarvia *et al.,* 2004) [4]. But in our present study, our amperometric biosensor for caffeine shows the response about 40 s only. So, present biosensor is more valuable in respect of time consumption.

In previous study a bare boron-doped diamond based electrode based biosensor was developed 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. Linear response of peak current on the concentration in the range from 4×10^{-7} to 2.5×10^{-5} M, good repeatability (RSD of 2.1%) and detection limit of 1.5×10^{-7} M without any chemical modifications and electrochemical surface pretreatment were evaluated. The effect of possible interferents appeared to be negligible which evidently proved very good selectivity (Svorc *et al.,* 2012) [25]. So, it is clear that our biosensor against caffeine shows best results than this study which was showed by same literature in regarding of response detection ranges between 0.2 μM to 10 μM with detection limit about 0.1 μM and response time was very short just only 40 s by using of isolated screen printed gold electrode.

In previous study, a modified surface of boron-doped diamond (BDD) electrodes by Nafion was developed. The polymer film was applied onto the BDD electrode surface by solvent evaporation. Nafion-BDD electrode was used as a sensor for caffeine detection in e.g. cola beverage samples. In cyclic voltammetric measurements, favorable ionic interaction between the Nafion film and caffeine enhances the current response, and thus the sensitivity, compared to that at the bare electrode. The modified electrode exhibits a stable and sensitive response to caffeine and may represent a new analytical tool, offering a significant improvement over other electroanalytical methods (e.g.: Nafion-modified glassy carbon electrode) and the accepted method for caffeine analysis (HPLC-MS). The analysis of residuals from the linear regression proved that a linear response exists from 2.0×10^{-7} to 1.2×10^{-5} M, obtaining a limit of detection of about 1.0×10^{-7} M (Huitle *et al.,* 2010) [9]. Our study shows response detection ranges between 0.2 μM to 10 μM with detection limit about 0.1 μM and response time was very short just only 40 s by using of isolated screen printed gold electrode for the development of amperometric biosensor against caffeine over concentration range.

In another literature, electrochemical sensor was developed that was based on imprinted sol-gel and nanomaterial for determination of caffeine. This sensor was prepared onto a glassy carbon electrode modified with multiwall carbon nanotubes (MWCNTs)/ viny ltrimethoxysilane (VTMS) recovered by a molecularly imprinted siloxane (MIS) film prepared by sol–gel process. MWCNTs/VTMS was produced by a simple grafting of VTMS on MWCNTs surface by in situ free radical polymerization. The siloxane layer was obtained from the acid-catalyzed hydrolysis/condensation of a solution constituted by tetraethoxysilane (TEOS), methytrimethoxysilane (MTMS), 3-(aminopropyl) trimethoxysilane (APTMS) and caffeine, as a template molecule. The morphology and performance of the imprinted siloxane film was characterized by scanning electron microscopy (SEM) and differential pulse voltammetry (DPV). The MIS/ MWCNTs- VTMS/ GCE sensor was tested in a solution of the caffeine and other similar molecules. After optimization of the experimental conditions, the sensor showed a linear response range from 0.75 to 40 μmol L^{-1}, with a detection limit (LOD) of 0.22 μmol L^{-1}. The imprinted sensor was successfully tested to detect caffeine in real samples (Santos *et al.,* 2012) [22].

Inhibition effect of caffeine increased at higher caffeine concentrations on activity of alkaline phosphatase (ALP) enzyme and linear concentration range for caffeine in the presence of a constant concentration of p-nitrophenylphosphate (pNPP). The differences between the biosensor responses were related to caffeine concentration which was added in to the reaction medium. It is also found that the highest biosensor responses were observed at +0.95 V. Below this potential value decreases in the biosensor response were observed. The interference effects of the other compounds were negligible. In our study, biosensor against caffeine shows best results than this study which was showed by same literature in regarding of response detection ranges between 0.2 μM to 10 μM with detection limit about 0.1 μM and response time was very short just only 40 s by using of isolated screen printed gold electrode. This developed amperometric biosensor against caffeine was also used in real sample analysis.

4. Conclusion

Our study shows minimum response detection ranges with lowest detection limit than previous literatures for the same purpose of study and response time was very short just only 40 s by using of isolated screen printed gold electrode for the development of amperometric biosensor against caffeine over concentration range. It was showed that comparison of detection performance of our biosensor and the others to caffeine. It can be said that the most sensitive biosensor is

piezoelectric based but its response time is so long.

If we consider both of detection limit and response time of the biosensors, it is obvious that our biosensor is much better than the others. It is clear that our biosensor against caffeine showed best results than other literatures in all senses. So, present biosensor is more valuable in respect of time consumption also. 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.

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