Development of paper based amperometric biosensor for glucose content measurement in Malaysian Stingless Bee Honey

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Abstract. Amperometric biosensor for glucose content measurement in Malaysian stingless bee honey was developed using screen printed carbon electrode (SPCE) integrated with paper disc immobilized with enzyme Glucose Oxidase (GOx) using simple physical adsorption method. The paper-based biosensor required only 8 µL of sample solution for glucose analysis. The calibration of glucose biosensor is linear between 0.5 mM to 4.5 mM ($R^{2=}$ 0.9925) and has a detection limit of 0.15 mM. Interference study on several compound affecting the biosensor response and storage stability was investigated. In addition, its performance was demonstrated in the analysis of six honey samples. The results obtained using glucose biosensor was validated by high performance liquid chromatographic (HPLC) method. The addition of glucose in pure honey at various concentration were also tested by this paper-based biosensor where the current obtained shows increasing trend with the addition of glucose. From this research, it can be concluded that, the prototype sensor to determine glucose adulteration in stingless bee honey was successfully developed.

1. Introduction

Stingless bee honey is a golden sugary liquid with distinct taste and aroma that is valued for their medicinal benefits due to its various components [1]. Main components inside pure honey are carbohydrates which constitute 82.4% (including fructose, glucose and other sugars), 17.1% water and 0.5% minor components (including protein, organic acids, amino acids and other biological compounds) [2]. Due to the grown popularity in stingless bee honey business, there are many commercial honeys available in the market. However, the question has been raised whether the honey is pure or adulterated either with cheap chemicals or commercial sugar. Glucose syrup is one of common adulterants used to confuse consumers due to its colorless, odourless and sweetness characteristics [3]. Methods such as high-performance liquid chromatography, gas chromatography, chemical and optical methods, UV-spectrophotometry are commonly used for glucose determination [4]. However, not all of the tests are suitable as the cost to test samples in the laboratory are expensive, complicated and time consuming. Therefore, the portable on-site testing device or known as Point of

Care (POC) devices are those technologies that are able to provide the alternative methods for detection of honey adulteration.

The POC technologies has sparks the interest in both clinically and industrial as it can provide rapid data output, easy to use, portable and inexpensive [5]. POC devices can be developed using various platforms such as glass, ceramic, plastic and finally paper which has become the most attractive and promising platforms because of its advantages [5]. By using paper as the platform for POC devices, the devices are now known as paper based analytical devices (PAD) or also called paper-based sensor. The idea of PADs is to carry out test on small piece of paper where the most common paper used are filter papers, graphite paper and chromatograpy paper [6,7] With the advanced development of PADs, variety of detection techniques are widely applied to rapidly quantified the fabricated PADs, however, among of these methods, the electrochemical detection (Potentiometry, coulometry, polarography and amperometry) is more relevant with the paper-based analytical device due to the potential for miniaturization and portability, low fabrication cost and higher sensitivity [5,8,9].

Interestingly, in this research, the screen-printed carbon electrode (SPCE) was integrated together with the paper substrate as immobilization matrix for the enzymes. The enzyme, Glucose Oxidase (GOx) were dropped onto paper disc, allowing the enzyme to be physically adsorbed within the porous paper matrix before placing the paper disc on top of SPCE. The SPCE is well known for electrochemical detection in biosensor and often chosen due to their simplicity, ability for mass production, modest cost, portability and ease of chemical alteration [10–12]. However, here, instead of using bare SPCE, the screen printed carbon electrode (SPCE) was first altered by using Prussian Blue (PB) in order to prevent disruption from interference species [13].

This paper-based biosensor was developed using simple fabrication technique and does not required any pre-processing steps. Therefore, the sensor is convenient for POC test application as the analytes were able to be measured not only in the laboratory but also for outdoors application. In addition, this paper based biosensor also were cost effective especially in reagent consumption as only 8 µL of analytes (glucose) was used to detect the glucose adulteration in stingless bee honey [14].

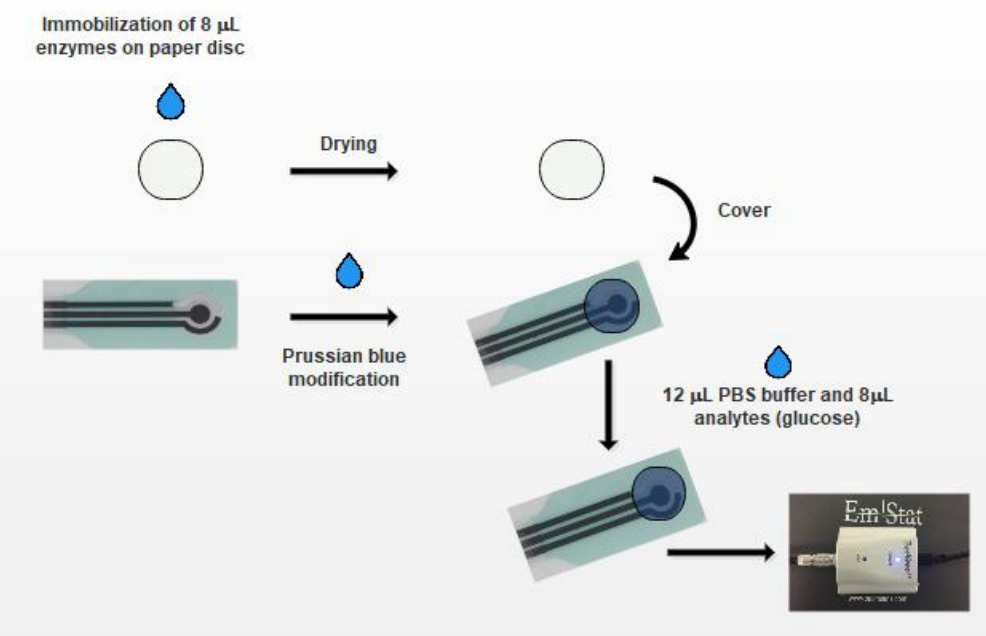

Figure 1. Schematic illustration of paper-based biosensor fabrication process

2. Materials and Methods

2.1 Honey Collection and Preparation

In this study, pure stingless bee honey from *Heterotrigona Itama* (H1) and *Geniotrigona Thoroacica* (H2) species were harvested from Universiti Malaysia Pahang stingless bee farm while four samples of commercial stingless bee honey (H3-H6) were randomly obtained from local market around Malaysia. All the honey samples were stored at 4°C and left at room temperature 25°C for 4–5 h before the analyses.

2.2 Chemicals and Reagents

All of the chemicals and reagents used for this research are analytical grade. Sugar standard (Glucose), Enzyme Glucose Oxidase (GOx) from *Aspergillus niger*, Type VII, lyophilized powder, ≥100,000 units/g solid (without added oxygen), 10KU and hydrochloric acid (HCl), were purchased from Sigma-Aldrich (USA). Potassium dihydrogen phosphate (KH_2PO_4) and dipotassium hydrogen phosphate (K_2HPO_4) were mixed in order to obtain phosphate buffer where both chemicals were purchased also from Sigma-Aldrich (USA). Iron(III) Chloride ($FeCl_3$) and Potassium Ferrocyanide ($K_3Fe(CN)_6$) were obtained from R&M (Malaysia). Whatman Filter paper Grade 1 (90mm Ø, Pore size: 11 µm) were obtained from Tay Scientific (Malaysia), Instrument 3-Channel Screen Printed Electrode (O-ring) 7.20 100 720: Working Electrode (Carbon), Reference Electrode (Ag/AgCl) and Counter Electrode (Carbon) and the connector to the potentiostat were bought from Rapid Genesis Sdn Bhd (Malaysia). Lastly, the water used in this experiment was from a Mililipore Milli-Q purification system.

2.3 Electrode modification

Prussian blue modification of SPEs was done according to method by [15,16] by dropping 30 µL of mixed solution containing 10 µL of 0.1 M $K_3Fe(CN)_6$ (1.65 g were diluted in 10mM 50 mL HCL) with and 10 µL of 0.1 M FeCl3 (0.811 g were diluted in 10 mM 50 ml HCL onto electrode surface. The electrode was dried off for about 10 minutes and rinsed with 10 mM HCL before being left for 90 minutes in the oven at 60 ° C to ensure a safe and active PB layer. At room temperature (25-27 ° C) in the dark, the PB- modified electrodes were then stored intact.

2.4 Construction of Glucose paper-based biosensor

The method of enzymes immobilization on paper disc were done by following method from past literature with some modification [14,17,18]. Filter paper, Whatman Grade 1, was cut into round disks with ca. 10 mm. Then, 8 µL of enzyme GOX solution (50 U/ml in of 0.1 M of phosphate buffer, pH 7.0) was carefully dropped to paper disc surface and allowed to dry at room temperature. If paper discs are to be used in the following days or weeks, it must be kept in dark and at 4 ºC.

2.5 Application to stingless bee honey samples

Stingless bee honey samples and the market samples were prepared by dissolving 1 g of honey in 50mL of Millipore milli-Q water and diluted to 100 times in 0.1M phosphate buffer of pH 7.0 [19].

2.6 Electrochemical characterization

Cyclic voltammetry was monitored from potential of -0.2 V to potential of +0.3 V where the scan rate was at 40 mV s^{-1}. The capabilities of PB layer on SPCE was tested by cyclic voltammetry. The detection of glucose was recorded chronoamperometrically at fixed potential of +0.8 V versus Ag/AgCl for 100 s. The electrochemical response was recorded as current (µA). The cyclic voltammetry (CV) measurements and amperometry evaluation were carried out using a Palmsens Emstat3 (Palmsens, Netherlands) potentiostat controlled by PSTrace 5.5 software. The paper discs were placed on the top of PB-SPCE fully covered the working, counter and reference electrodes before each measurement. For characterization of the biosensor, 12 µL of 0.1 M of phosphate buffer, pH 7.0

and 8 µL of 1 mM glucose was dropped on the paper disc. While for sample analysis, 8 µL of the samples was dropped on paper disc. The obtained values from biosensor were compared with HPLC method [20].

3. Result and Discussion

3.1 Drop Volumes

Prior to glucose detection, various parameters were examined including drop volume. The drop volume is important as it determine the amount of reagent required to cover the entire paper disc [21]. Figure 2 shows various drop volume using pink solution. The drop volume was varied from 5 µL to 1.2 µL. The best drop volume was 8 µL as it nicely spread the entire paper disc compared to other volumes. Volumes greater than 8 µL wet the paper disc with excess solution. Thus, 8 µL volume was chosen to introduce both GOx and reagents onto paper disc.

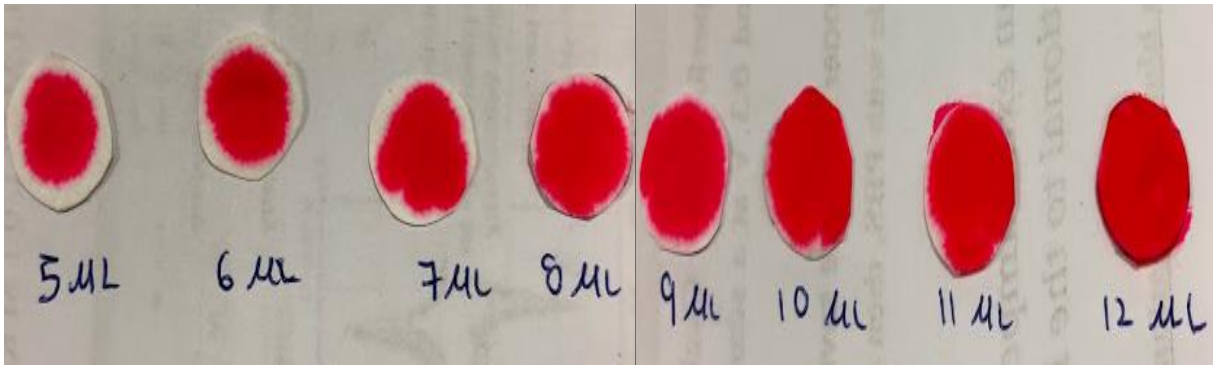

Figure 2. Photographs of various drop volumes investigation of paper disc with10 mm diameter test regions.

3.2 Cyclic Voltammetry (CV) Characterization

The electrochemical behaviour of the bare SPCE, PB modified SPCE, PB-SPCE with immobilized enzymes and analytes additions on paper disc was investigated by cyclic voltammetry and is intepreted in Figure 3. As shown, the bare SPCE does not display any redox peaks. However after the modification of SPCE by PB, there are some increament in reduction current whereas only slight increase in oxidation current was observed. The same trend has been reported in literature where it proves that PB was a great electrocatalyst for hydrogen peroxide (H_2O_2) reduction [14]. After the addition of glucose on the enzymes paper disc, the reduction current were increased significantly however, the oxidation peaks only increased slightly. This also an indicator that only a small volume of sample (8 µL) is required to yield excellent signals.

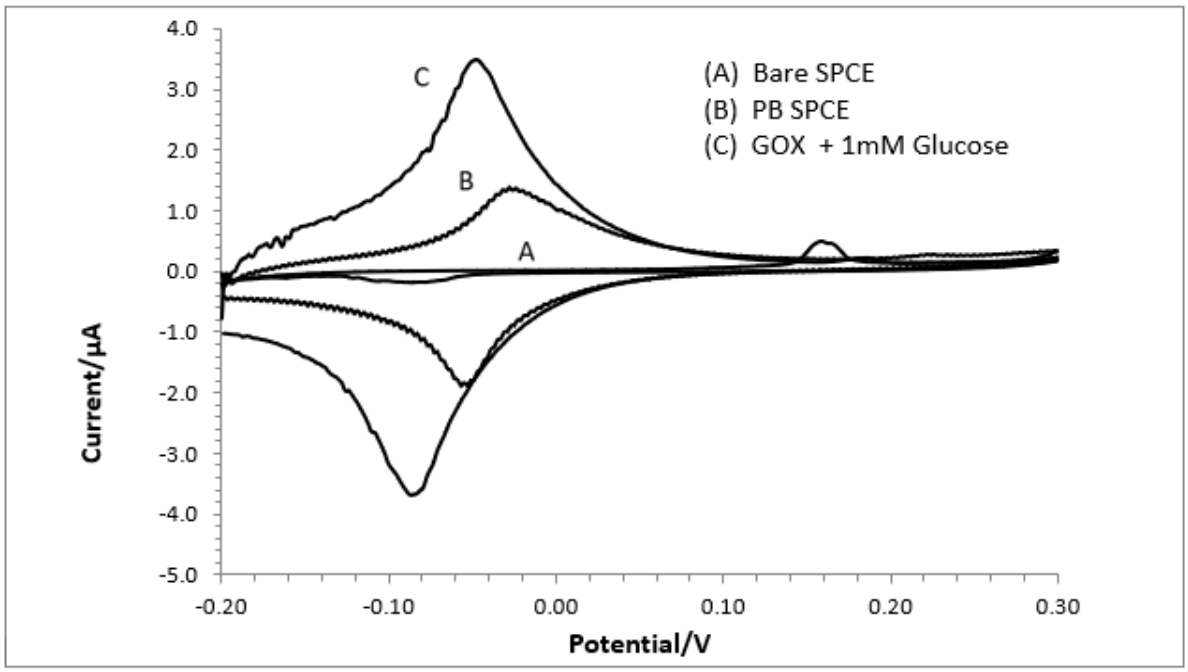

Figure 3. Cyclic voltammograms of the (A) Bare SPCE (B) PB-SPCE (C) GOx immobilized on paper disk + addition of 1 mM glucose in 0.1 M PBS (pH = 7.0) at a scan rate of 40 mV s−

3.3 Effect of applied potential

The effect of applied potential for glucose paper-based biosensor was illustrated in Figure 4. The signals were measured in the potential range of + 0.2 V to + 1.0 V. The biosensor started increased gradually from +0.2 V until reached maximum current at +0.8 V before the response drop beyond +0.8 V to +1.0. The sensitivity were increased with the increasing of applied potential due to the reduction of H_2O_2 upsurge force. [14]. Thus, in order to attained the greater sensitivity, +0.8 V potential was chosen for the following experiments.

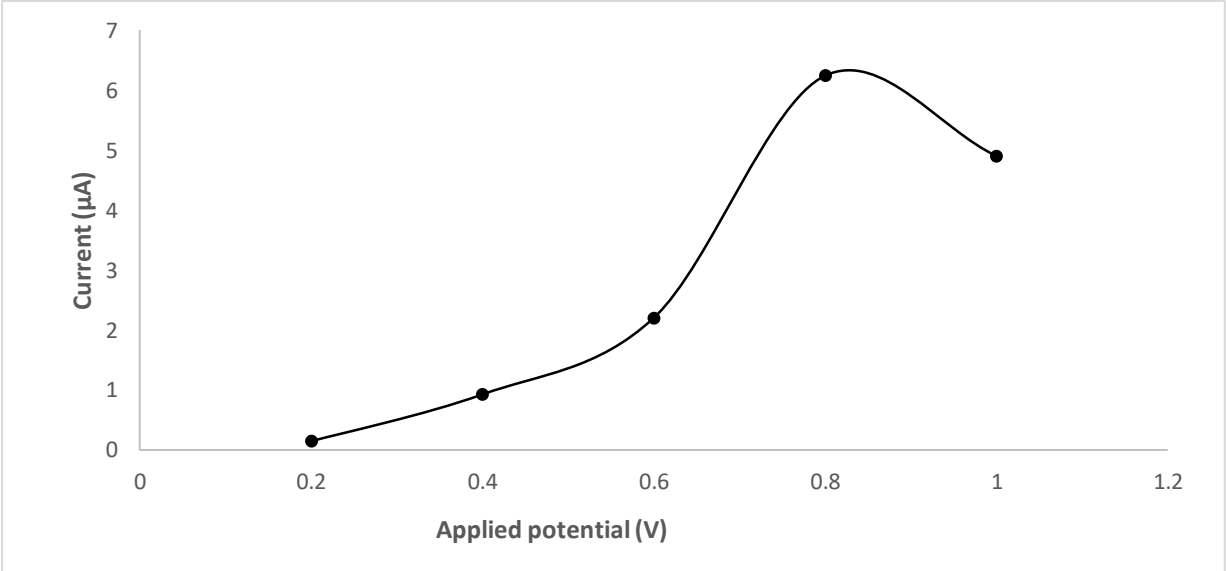

Figure 4. The effect of applied potential on the glucose paper based biosensor response with 1mM glucose in 0.1 M PBS (pH = 7.0)

3.4 Effect of buffer pH

The effect of buffer pH is important to the paper -based biosensors sensitivity as it controlled both the bioactivity of the GOx and electrochemical performance of PB [14]. Figure 5 showed the response of buffer pH which are ranging from pH 5.0 to pH 9.0. The signal increased until pH value achieved pH 7. At pH values higher than 7.0, the catalytic activity might decrease due to irreversible denaturation of the enzyme. These results are as predicted because they correlate to the optimal pH range of the enzymes as well as pH of biological substances in food [19,22]. Thus, for the following studies, a pH of 7 was chosen which corresponds to the pH commonly used in biological samples.

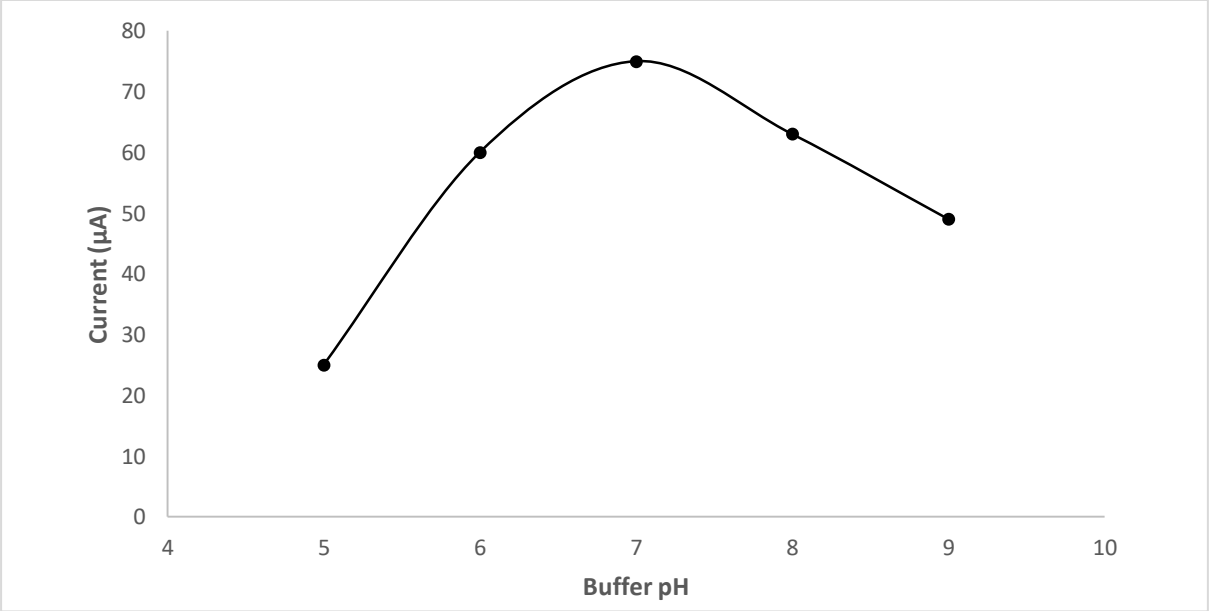

Figure 5. The effect of 0.1 M pH buffer on the glucose paper based amperometric biosensor response with 1mM glucose

3.5 Effect of enzyme concentration

Enzyme activity influenced the effeciency of biosensor thus the effect of enzymes concentration was investigated. Different amounts of GOx (5, 15, 35 and 50 U/ mL) were studied in working buffer were shown in Figure 6. At 35 U/ mL, the graph shows the highest current response and afterwards (>35 U/ml) the PB-SPCE GOx response were dropped. The lower current response may due to support overloading or oxygen and substrates transfer to the bioactive surface diffusion problems [23,24].Thus, for further experiments in glucose paper- based biosensor, concentration of 35 U/ mL was chosen.

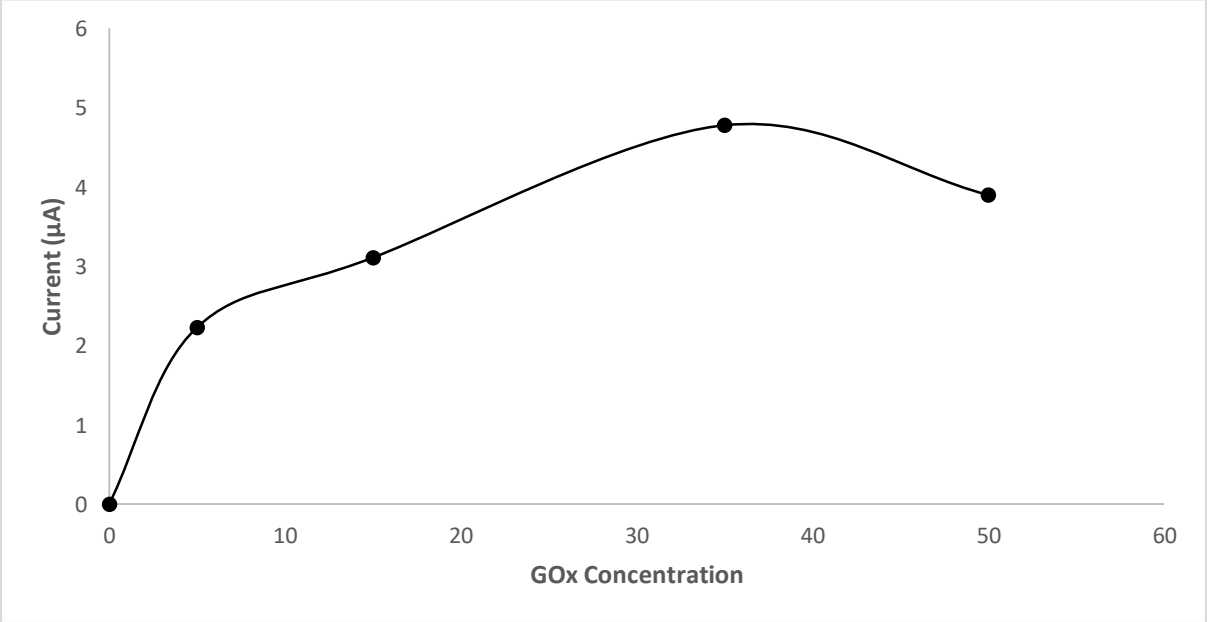

Figure 6. The effect GOx concentration on the glucose paper based biosensor response with 1mM glucose in 0.1 M PBS (pH = 7.0)

3.6 Paper-Based Biosensor response characteristic

The evaluation of glucose paper- based biosensor was determined by using several parameters such as development of glucose calibration curve, selectivity against potential interference, reproducibility of the biosensor and storage ability. These parameters were studied using the best condition that has been done in previous section. The conditions are summarized in Table 1.

Table 1. Operating condition for glucose paper-based biosensor

Conditions	Glucose paper-based biosensor
Applied potential	+ 0.8 V
Buffer pH	pH 7.0
Enzyme concentration	35 U/ mL GOx

3.6.1 Calibration curve and reproducibility. By employing the best conditions from previous section, the sensor calibration graph was shown in Figure 7. The glucose biosensors are linear between 0.5 mM to 4.5 mM where the regression equation obtained is $y = 0.2993x + 2.2843$ and the R^2 value was found to be 0.9925 . The symbol y indicates the current in µA while x represent the glucose concentration in mM. The reproducibility of this paper based biosensor was carried out at n = 3 where the relative standard deviation (RSD) obtained is 3.74% . On the other hand, the limit of detection (LOD) is 0.15 mM.

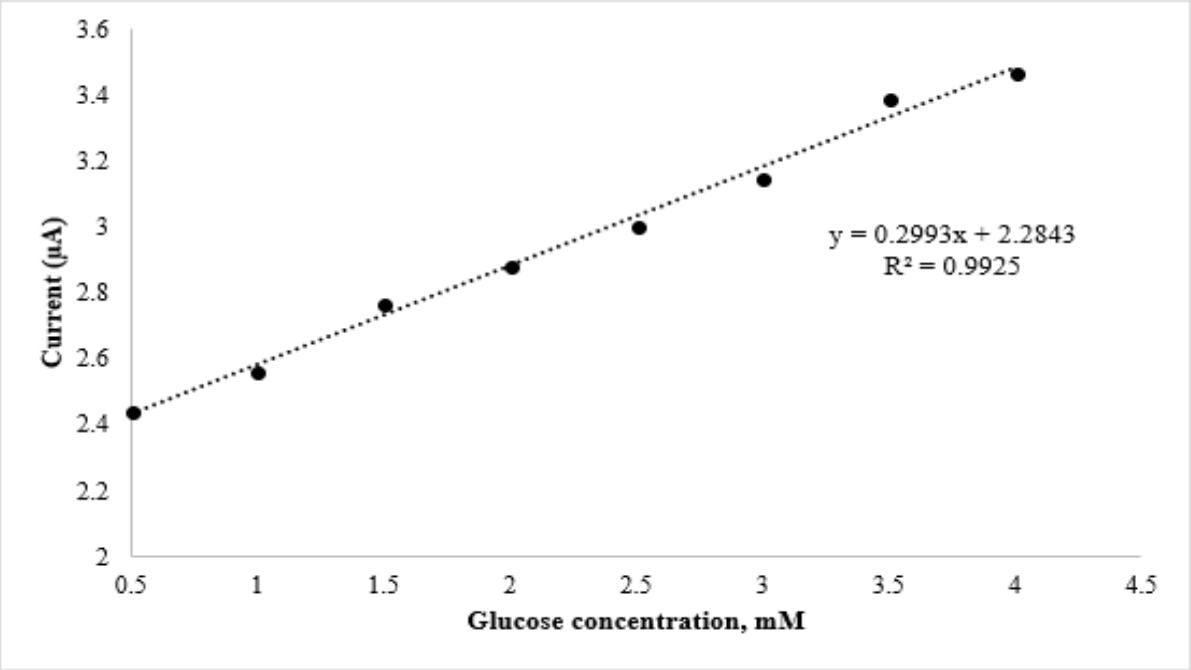

Figure 7. Calibration graph for glucose paper-based biosensor with different glucose concentrations. Applied potential + 0.8 V; 0.1 M PBS at pH = 7.0

3.6.2 Selectivity against interferences. The possible interference on the paper-based biosensor was investigated. The selection of interference was done according to the presence of sugars found in stingless bee honey, thus for glucose paper-based biosensor, the potential sugar interferences are sucrose and fructose. The current for each sugar interference at 1mM concentration were evaluated in the presence of 1 mM glucose (I^{θ}) [14,17]. Then, the current obtained is compared to the current of 1mM glucose alone (I) where the results are exhibited in Table 2. As can be seen, the fructose and sucrose do not cause any significance interference. This may due to the enzyme specificity as only enzyme GOX were presence in glucose paper- based biosensor.

Table 2. The selectivity study of interference substance for glucose paper-based biosensor

Interference substance	Current ratio [a] (I^{θ}/I)
Fructose	0.8
Sucrose	1.0

[a]Current ratios ($I\theta/I$) of 1 mM interfering element with comparison to the presence of 1 mM glucose alone (0.1 M PBS, pH 7.0, [GOX] = 35 U/mL)

3.6.3 *Storage stability.* Several glucose paper disc were prepared forehand and kept at 4˚C as it is the optimum stored temperature for enzymes and in dark until their further use. The stability was observed out in different times 1 ,5, 15, 20 and 30 days duration. As shown in Figure 8, the stability of the sensor based on immobilized GOx was studied. As a result, the response shows a decrease by 24.6% after a week and on the 30th day, the activity had lost about 75.2%. Therefore, it is believed that the biosensor can stay long for 20 days for future use.

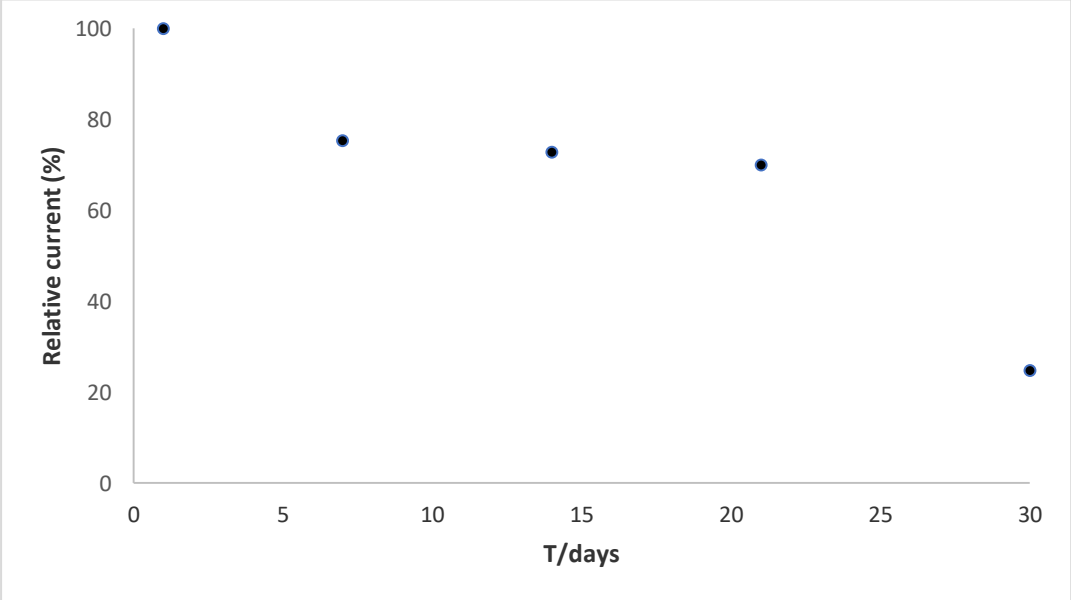

Figure 8. Stability of glucose paper-based biosensor for 30 days duration.

3.7 Application to honey samples

All six stingless bee honey samples were measured by the glucose paper-based biosensor and the results were recorded in Table 3**.** The results were compared with the results obtained from high performance liquid chromatography methodology. For glucose measurement, most of the results obtained are lower compare HPLC result however the results are still comparable compared to the literature [14,18]. The difference of the glucose paper-based biosensor is ranging from 2.6 % to 17.9% which still considered as a good result. It shows that the glucose biosensor was successfully developed.

Table 3. The measurement of glucose for stingless bee honey samples with the paper-based biosensor; Data are given as average ± SD (n=3)

Samples	Reference (g/100g)	Glucose paper-based biosensor (g/100g)	% Difference
H1	16 ± 0.80	13.43 ± 0.17	17.5
H2	12.17 ± 0.47	10.16 ± 1.0	17.9
H3	26.27 ± 0.63	23.09 ± 0.08	12.5
H4	30.09 ± 0.70	27.44 ± 0.05	9.2
H5	31.94 ± 0.07	28.38 ± 0.43	10.3
H6	25.47 ± 0.50	24.62 ± 0.66	2.6

Besides that, to conform the paper-based biosensor were able to identify the difference between pure and adulterate honey, the pure harvested honey (*Heterotrigona Itama)* was prepared along with the adulterate honey. The adulterate honey was prepared by adding 10 -70 % of glucose standards into pure honey (0%). Figure 9 shows the current output for pure honey (0%) and various concentration of adulterate honey. As glucose concentration increase, the current increase linearly. Pure honey represents 0% adulterant concentration where it shows the lowest current value at 3.064 µA. Hence, it shows that the biosensor is able to distinguish the difference between pure and adulterate honey.

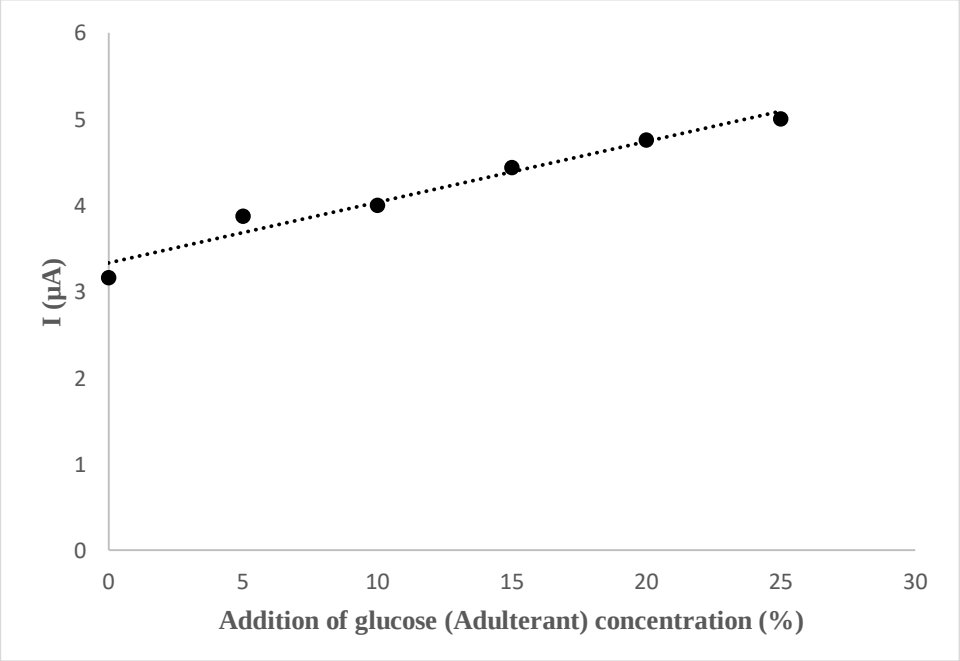

Figure 9. Paper-based biosensor application on adulterated honey

4. Conclusion

A low cost filter paper biosensor was developed to determine the glucose concentration where the enzyme GOx enzyme was immobilized by a simple adsorption process. The developed paper-based biosensor was used to quantify the sugar adulteration in stingless bee honey. The best conditions for this paper based biosensor performance were studied and furthermore, it displayed a good linear range. But sadly, the biosensor had lost more than 70% of its activity on the 30th day. Based on the results of the experiments, it was confirmed that the proposed biosensor was sensitive and capable detecting addition amount of glucose in stingless bee honey. In future, to improve this developed biosensor, more study should be done such as advanced modification of SPCE for example addition of polymers. Nevertheless, this paper-based biosensor has bright future for applications in the detection of sugar adulteration in stingless bee honey.

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