CHAPTER 3

Results and Discussion

3.1 Working electrode investigation

In order to investigate the suitable working electrode for determination of hydroquinone, electrochemistry of quinone (the product from the catalytic oxidation of hydroquinone by laccase enzyme) on different working electrodes was studied by cyclic voltammetry. The voltammetric studies were carried out with SPCE modified with various nanomaterials as working electrode, Ag/AgCl as reference electrode and Pt wire as auxiliary electrode. Potential was scanned from -1.0 V to +1.0 V versus Ag/AgCl at scanning rate of 100 mV s^{-1}. The characteristic of cyclic voltammograms of blank solution (0.1 M citrate buffer pH 5.0) and various concentrations of quinone are shown in Figure 3.1. As the results, the cathodic signal produced from reduction of quinone at about 0 V was employed for the hydroquinone determination in the FI-Amp system. The current signal of the cathodic peak increased when concentration of quinone increased. The second cathodic peak which is smaller was characterized to occur from reduction of dissolved oxygen.

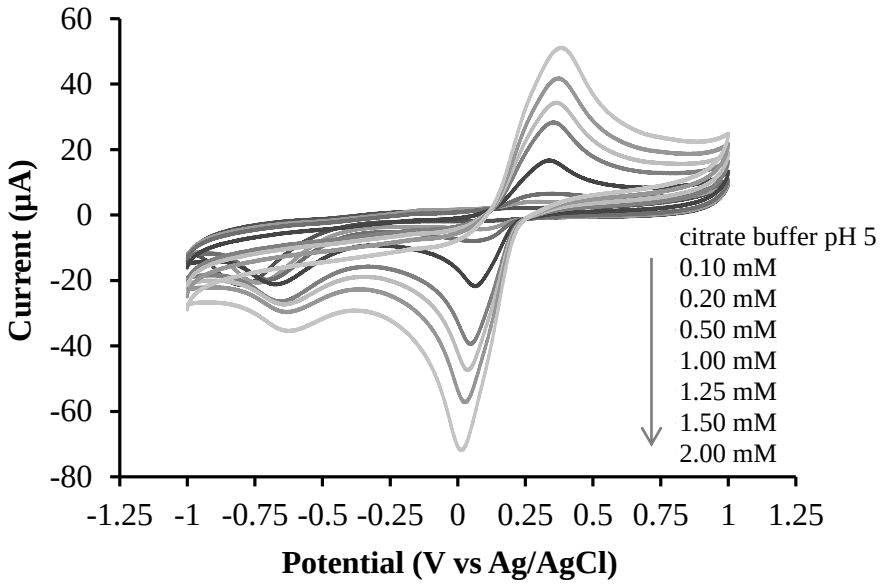

Figure 3.1 Cyclic voltammograms on CNTs modified SPCE of 0.1 to 2 mM quinone in 0.1 M citrate buffer pH 5.

The peak current of the first cathodic peak at 0 V was examined and used to plot a calibration graph. The slopes obtained indicated sensitivity of different modified electrodes as shown in Figure 3.2. Various nanomaterials were modified on SPCE as described in section 2.5. The result shows that the SPCE modified with CNTs and CNTs-AuNPs provided higher sensitivity comparing to the other electrodes. The SPCE modified with CNTs (SPCE-CNTs) was chosen as a working electrode for further experiments because it is simpler, lower cost and already gives high sensitivity. In addition, CNTs offered a faster electron transfer may be mainly due to the increase in the surface area and their good electrical conductivity.

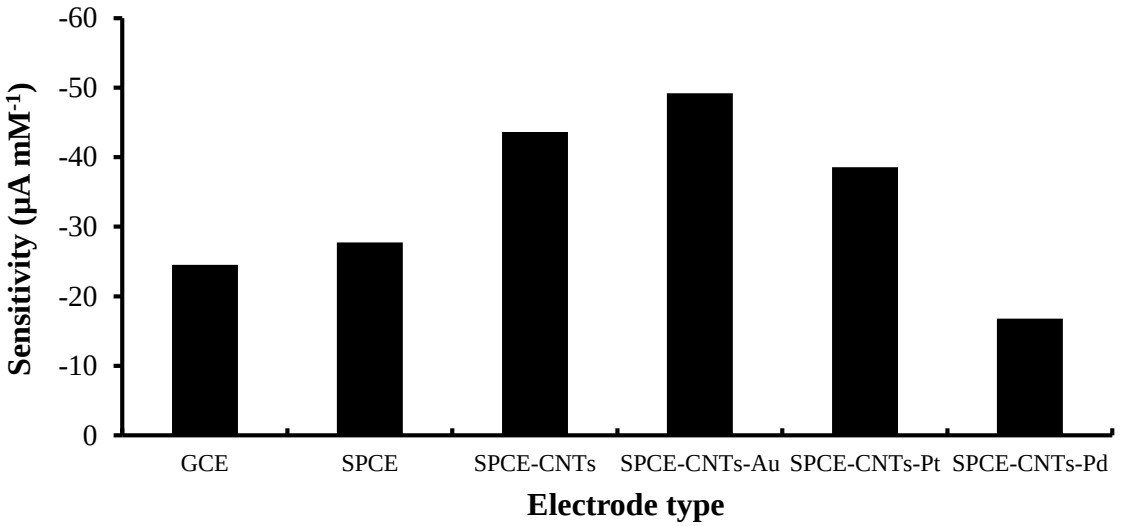

Figure 3.2 The sensitivity of the various electrodes with 0.1 to 2 mM quinone in 0.1 M citrate buffer pH 5.

3.2 Effect of CNTs amount

After that the effect of amount of CNTs being modified on the electrode was studied to obtain higher sensitivity. Because of the limitation of electrode surface, volume of CNTs suspension was varied from 2 to 20 μL. A result in Figure 3.3 shows that the sensitivity slightly increased. At higher CNTs suspension volume, the sensitivity was not significantly higher than the low volume of CNTs suspension due to the thick layer of CNTs that affected the efficiency of electron transfer. Therefore, 5 μL of CNTs suspension was selected to modify on SPCE surface as it gave high sensitivity and the convenience in preparation of the working electrode.

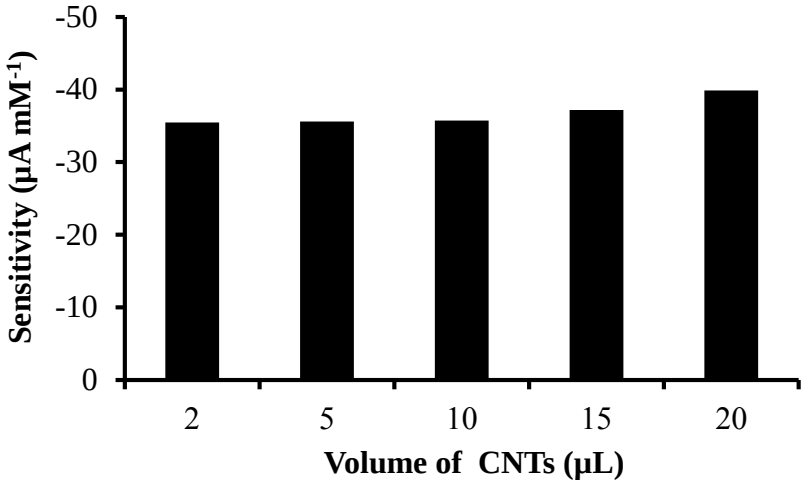

Figure 3.3 The effect of volume of CNTs on sensitivity of 0.1 to 2 mM quinone.

3.3 Characterization of the electrode surface

Scanning electron microscopy (SEM) was used to observe directly the morphology of SPCE-CNTs surface. The SEM images of bared SPCE and SPCE modified with CNTs are shown in Figure 3.4(A) and (B), respectively. The image of SPCE modified with CNTs showed that CNTs mostly covered and well dispersed on the SPCE surface. CNTs diameter was smaller than 10 nm and the result also indicated the successful modification of CNTs on the SPCE surface.

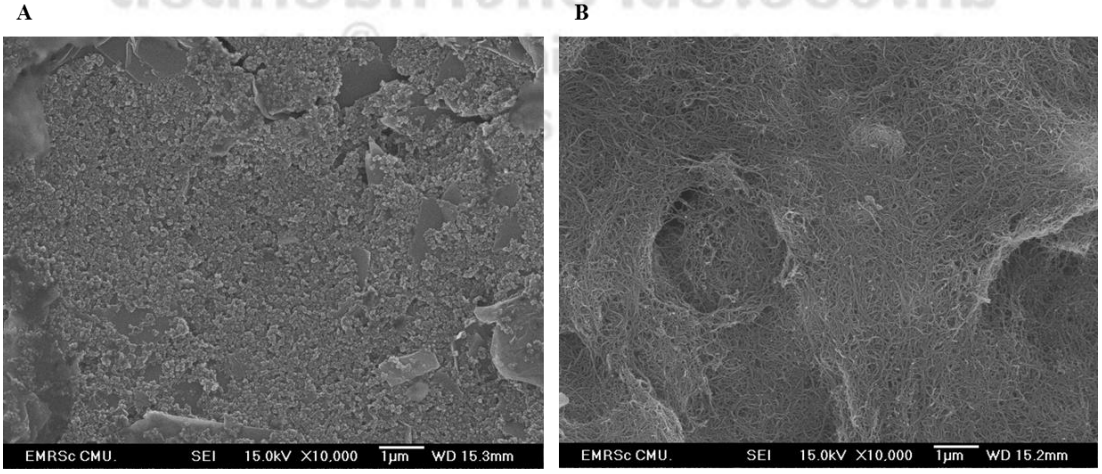

Figure 3.4 SEM images of (A) bare SPCE and (B) SPCE modified with CNTs.

3.4 Optimization of flow injection amperometric system

Optimization of parameters can be increased the sensitivity for hydroquinone determination. FI-Amp system (Figure 2.1) but without the laccase column was optimized for determination of quinone which is the product from catalytic oxidation of hydroquinone. Amperometric measurements were carried out for quinone concentration between 5 to 20 µM. Normally amperometric technique is based on the measurement of the current between WE and AE at a constant applied potential which induced a redox reaction at WE. However in this experiment, the signal was reported in term of voltage because a homemade amperometer converted the current to voltage.

3.4.1 Effect of applied potential

In order to obtain a high sensitivity in the electrochemical detection of the enzymatic product, the applied potential for reduction of quinone was investigated from +100 to −200 mV and the variation of the sensitivity with the applied potential is presented in Figure 3.5. As the result, the more negative applied potential (+100 to −50 mV), the higher sensitivity was achieved until stable at −50 mV hereafter. The applied potential of −50 mV has been selected for further amperometric experiments in order to avoid interference from substances that can be electrochemically reduced. This applied potential was lower than a hydroquinone biosensor using modified core–shell magnetic nanoparticles supported on carbon paste electrode (i.e. −187 mV) [Zhang, 2007].

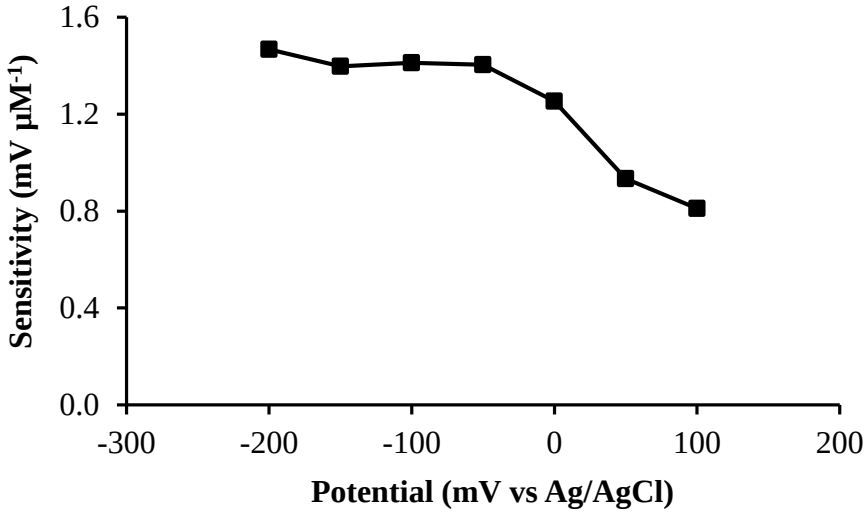

Figure 3.5 Effect of the applied working potential for reduction of quinone on sensitivity of 5 to 20 µM hydroquinone.

3.4.2 Effect of sample volume

Increasing of the injected sample volume is one of the effective ways to achieve higher sensitivity but large amount of sample results in peak broadening and consuming large amount of sample and reagent solutions. Therefore, sample volume was optimized in the range of 50 to 300 µL using different lengths of sample loop. The sensitivities obtained are shown in Figure 3.6. The result showed that the sensitivity increased with increasing of sample volume and the injected sample volume of 200 µL was chosen because it offered sufficient sensitivity and without peak broadening.

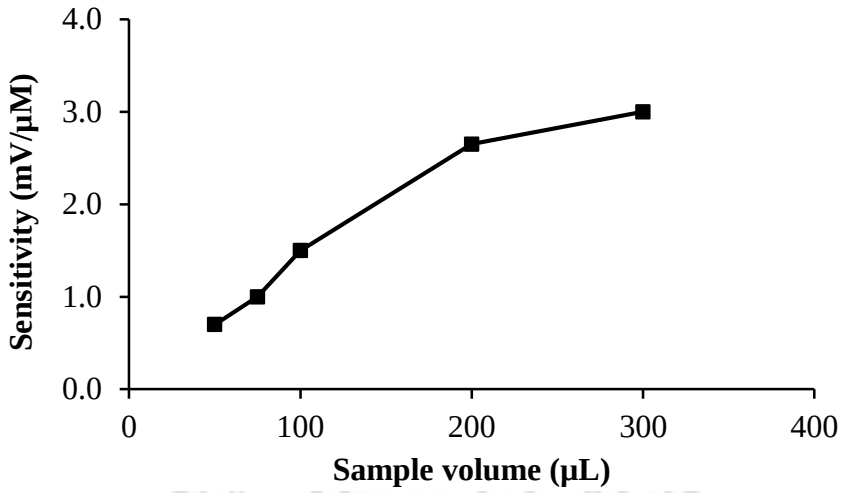

Figure 3.6 Effect of sample volume on sensitivity of 5 to 20 µM hydroquinone.

3.4.3 Effect of mixing coil length

Afterwards, the mixing coil length was studied from 0 to 200 cm. The result was presented in Figure 3.7. It was found that the sensitivity decreased with increasing of the mixing coil length due to larger dispersion of the injected solution at the longer coil length which lead to dilution of the injected hydroquinone concentration and then the signal will be decreased. The longer coil length was very necessary for the system that used to mix the reagent and standard solution for longer time to obtain high quantity of the product. However, in this work, the mixing coil was only used to disperse zone of sample and to obtain the reproducible peak profile. So, the mixing coil length of 30 cm was selected for further experiments because it gave the highest sensitivity, fast analysis time and used small amounts of citrate buffer solution.

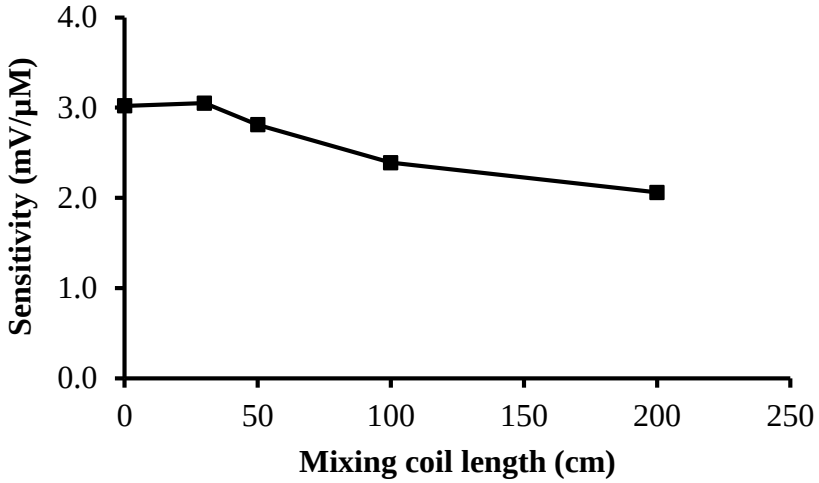

Figure 3.7 Effect of mixing coil length on sensitivity of 5 to 20 µM hydroquinone.

3.5 Testing efficiency of the laccase column

After immobilization of laccase enzyme on activated silica gel, the enzyme suspension was filled into the column as described in section 2.11 and the laccase column was incorporated to the FI-Amp system as shown in Figure 2.1. The activity of laccase that contained in column was preliminary evaluated to confirm the achievement of laccase immobilization. The signal of 10 µM hydroquinone was compared between the system with and without the laccase column. As a result in Figure 3.8, the system with the laccase column showed clear signal response that indicating catalytic oxidation of hydroquinone, while the system without the column presented lower peak signal. In addition, the efficiency of the laccase column was carried out by constructing a calibration graph of hydroquinone in the range of 1–100 µM. The FIAgram obtained from the both systems are shown in Figure 3.9 and 3.10, respectively.

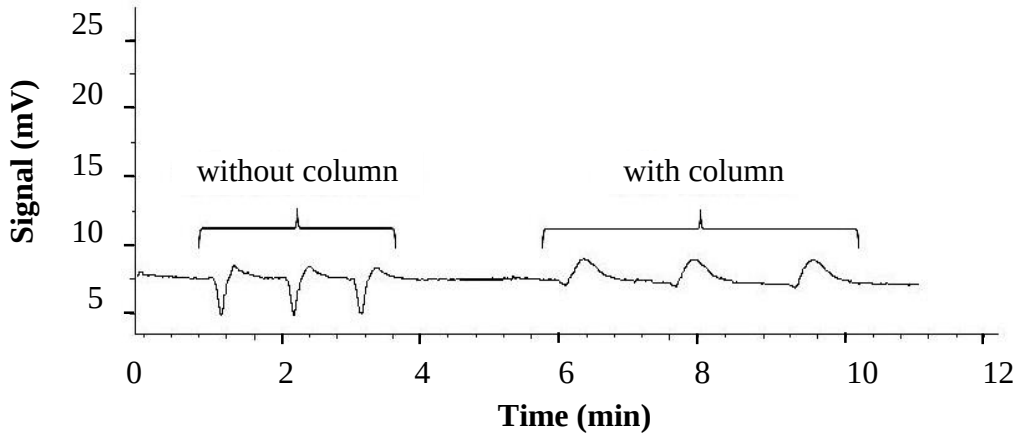

Figure 3.8 FIAgram of the signal of 10 µM hydroquinone obtained from the FI-Amp system with and without the laccase column.

The signal of hydroquinone in the range of 1–100 µM obtained from the system without the laccase column cannot constructed the calibration curve for determination of hydroquinone as presented in Figure 3.9 because the signal was not proportional to concentration of hydroquinone. This result was set as a blank to evaluate the success of laccase immobilization.

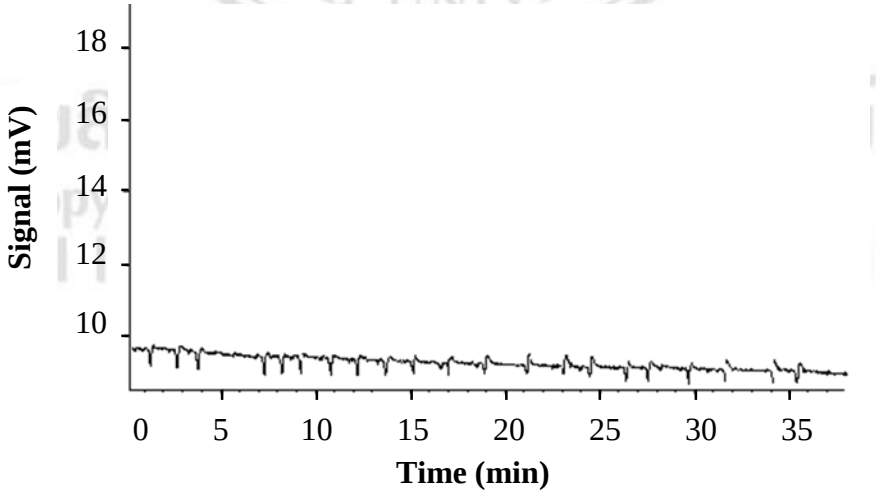

Figure 3.9 The signal of hydroquinone in the range of 1–100 µM obtained from the system without the laccase column.

On the other hand, the system with the column showed the good correlation between the signal and concentration of hydroquinone in the range of 1–100 µM with R^2 = 0.9958 as illustrated in Figure 3.10 and Figure 3.11, respectively. The result can confirmed that laccase was successfully immobilized on the activated silica gel and the laccase column offered good efficiency for catalytic oxidation of hydroquinone.

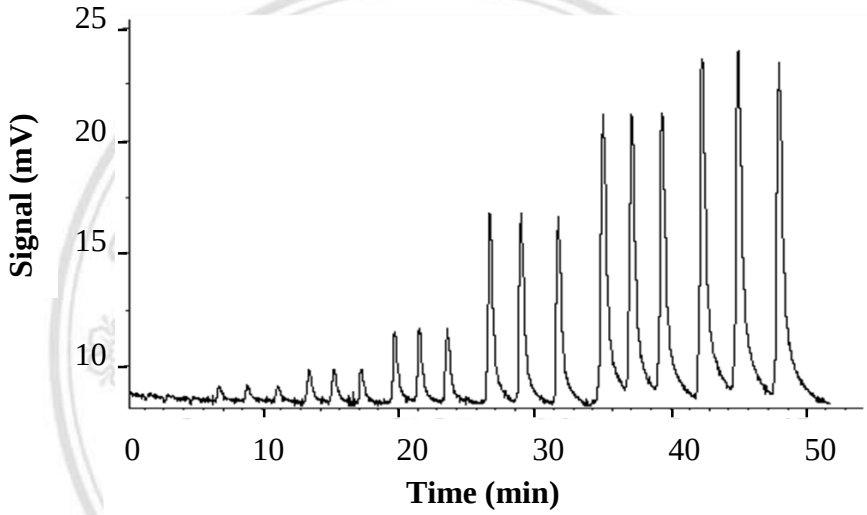

Figure 3.10 The signal of hydroquinone at 1, 5, 10, 20, 50, 75 and 100 µM obtained from the system with the laccase column.

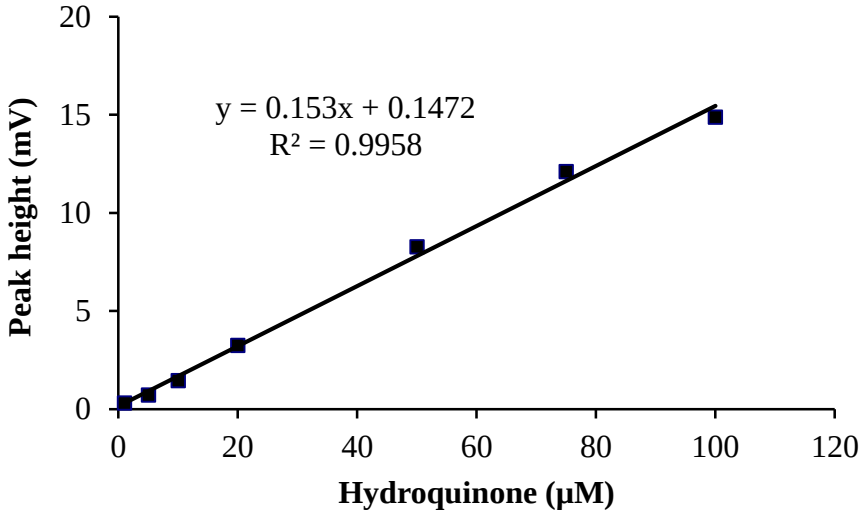

Figure 3.11 Calibration graph of hydroquinone in the range of 1–100 µM.

3.6 Optimization of the parameters of the biosensor

Afterwards, several parameters, i.e., flow rate, pH, ionic strength and temperature affecting the analytical performances of the biosensor were optimized. The principle of biosensor is based on catalytic oxidation of hydroquinone by laccase to produce 1,4-benzoquinone which was detected amperometrically as described above. The effect of some parameters was investigated as presented below.

3.6.1 Effect of flow rate

Flow rate is one important parameter in FI-Amp sensor system because it defines the duration that hydroquinone being in the column. Flow rate was investigated from 0.5 to 1.5 mL min^{-1} and the result is shown in Figure 3.12. It was found that flow rate did not significantly effect to the sensitivity, indicating that laccase column has high efficiency for catalytic oxidation hydroquinone to quinone in short time. Sample throughputs achieved for using flow rate of 0.5, 0.8, 1.0, 1.2 and 1.5 mL min^{-1} were 15, 20, 26, 30 and 36 h^{-1}, respectively. Flow rate of 1.0 mL min^{-1} was chosen because it offered good sensitivity without consuming excessive amounts of buffer comparing with faster flow rate, fast analysis time and high sample throughput.

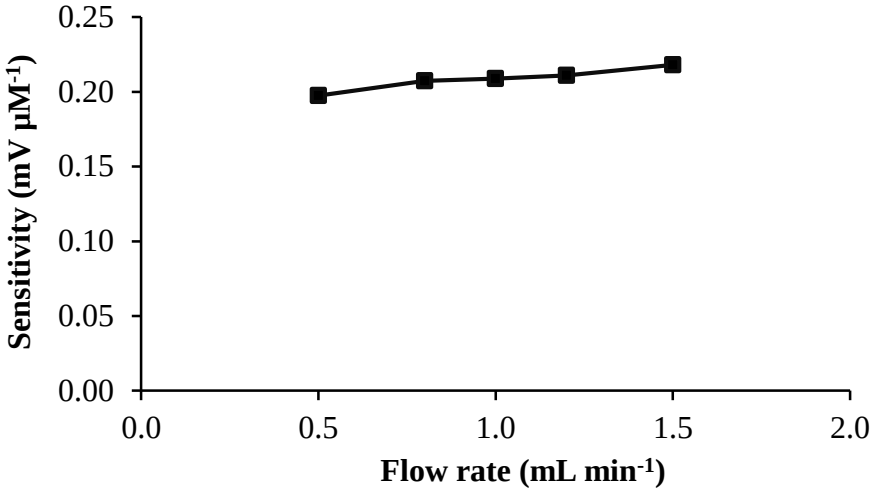

Figure 3.12 Effect of flow rate on sensitivity of 5 to 20 µM hydroquinone.

3.6.2 Effect of pH

The influential of pH on the amperometric biosensor response was studied in the pH range of 3.5–5.5 because the optimum pH of laccase is more acidic and usually found in the range of pH 3 to pH 5 [Madhavi, 2009]. The results are reported in Figure 3.13, showing that the highest sensitivity is obtained at pH 4.5, which is in agreement with those previously obtained for detection of different kinds of phenols [Portaccio, 2013] At lower or higher pH than 4.5, sensitivities decreased probably due to loss or inactivation of the enzyme activity. Therefore, a 0.1 M citrate buffer at pH 4.5 was selected for further experiments.

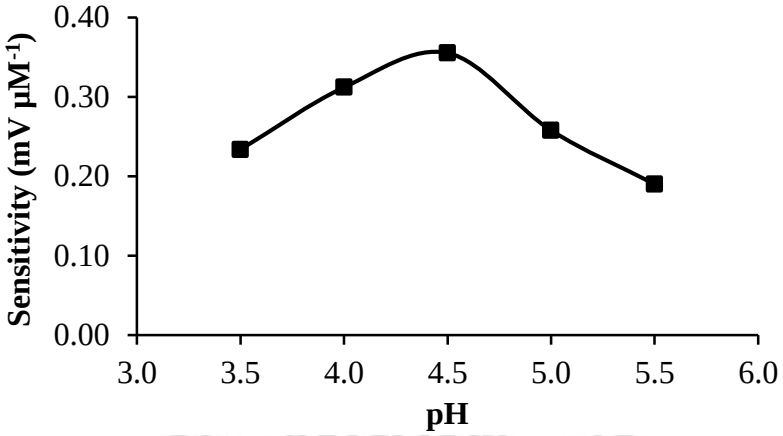

Figure 3.13 Effect of pH on sensitivity of 5 to 20 µM hydroquinone.

3.6.3 Effect of buffer concentration

After that, the effect of citrate buffer concentration from 0.01 to 0.3 M at pH 4.5 was investigated as shown in Figure 3.14. The 0.1 M citrate buffer gave the best sensitivity, which is in good agreement with previous studies [Das, 2014 and Amatatongchai, 2013]. Consequently, this concentration was used for the next experiments.

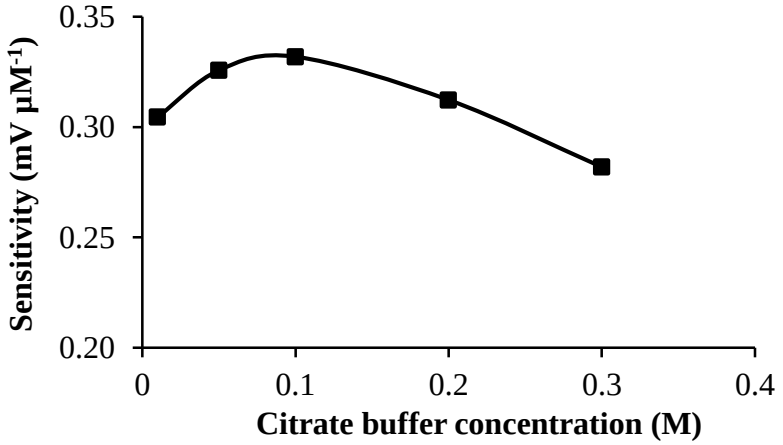

Figure 3.14 Effect of citrate buffer concentration on sensitivity of 5 to 20 µM hydroquinone.

3.6.4 Effect of temperature

The effect of temperature on the laccase column was also studied. From Figure 3.15, the sensitivity increased with the increasing temperature and reached the maximum response at about 27 °C (room temperature). Then the sensitivity began to decrease as the temperature increased above 27 °C because thermal has effect to inactivate the laccase activity. So, 27 °C was selected as the optimum temperature for the amperometric biosensor.

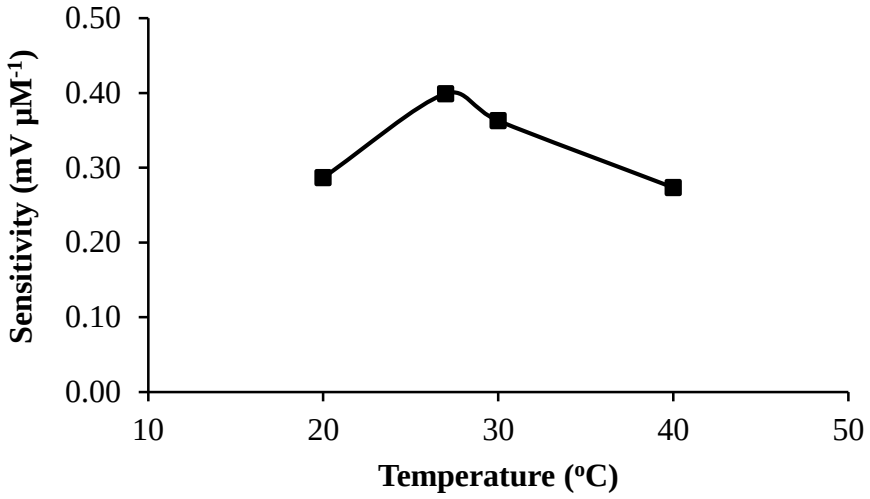

Figure 3.15 Effect of temperature on sensitivity of 5 to 20 µM hydroquinone.

3.7 Analytical characteristics of the procedure

3.7.1 Calibration graph and detection limit

Under the optimum condition, the performance of the proposed system was studied. FIAgram and the calibration graph of the signal response for hydroquinone in the range of 1–100 µM is shown in Figure 3.16 and Figure 3.17. As a result, the peak height slightly increased and began to saturate at hydroquinone concentration higher

than 50 µM due to the limitation of active electrode surface. The linear range for hydroquinone determination was 1–50 µM and the linear regression equation obtained was y = 0.3523x–0.2006, R^2 = 0.9996 (where y is peak height in mV and x is concentration of hydroquinone in µM. The limit of detection (LOD) calculated from LOD = 3SD of intercept of calibration graph/slope of the calibration graph was 0.86 µM which was lower than 2 µM of laccase immobilization onto 1-aminopyrene functionalized reduced graphene oxides (rGOs)-modified glassy carbon electrode by encapsulation with chitosan [Zhou, 2013]. The proposed method consumed 200 µL of sample and 2.2 mL of carrier solution for 1 sample analysis. Sample throughput of 26 h^{-1} was achieved.

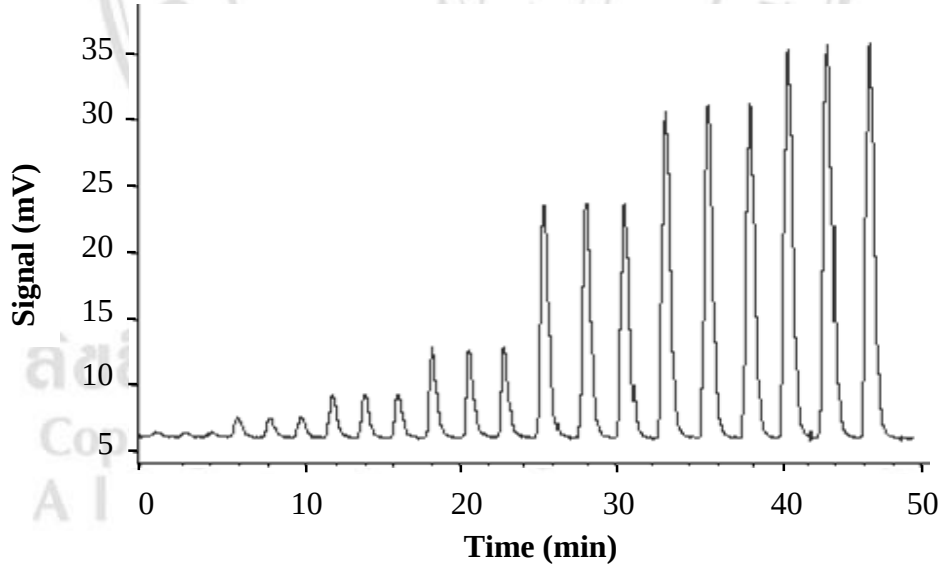

Figure 3.16 FIAgram of signal response to hydroquinone concentration at 1, 5, 10, 20, 50, 75 and 100 µM using the FI-Amp sensor.

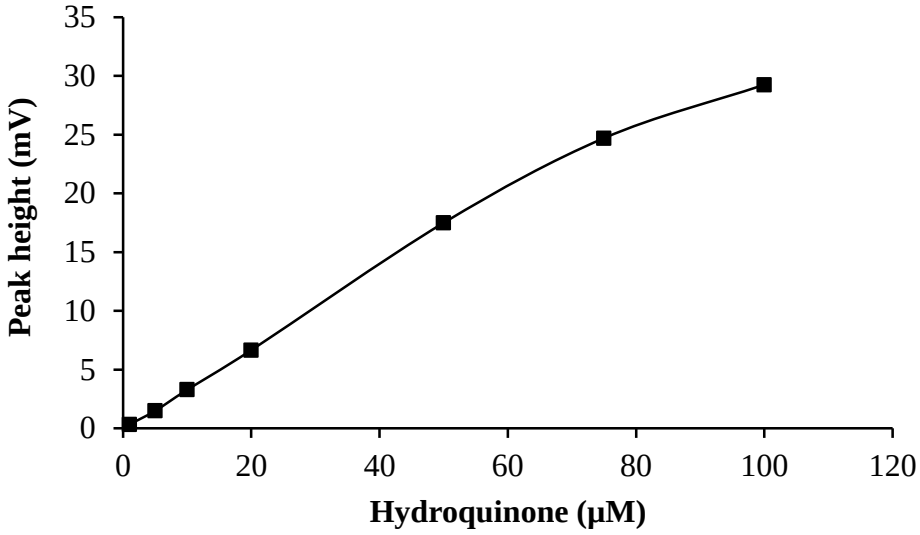

Figure 3.17 Calibration graph obtained in the range of 1–100 µM hydroquinone.

3.7.2 Precision study

3.7.2.1 Precision of working electrode

The percentage of relative standard deviation (%RSD) values was used for evaluating the precision which calculated from the equation 2.1 in section 2.13.2. First, the repeatability of the signal response of SPCE-CNTs to 10 µM quinone was examined for 11 replicate injections and %RSD was 0.89. Then the reproducibility of the modified electrode was studied by measuring the signal of 10 µM quinone in triplicate injections and the result obtained 4.4 %RSD for 9 electrodes as shown in Table 3.1. The stability of the FI-amperometric system was also examined by continuously injecting 10 µM quinone for about 3 h (111 injections), resulting in the RSD of 3.6%, which indicated excellent stability.

Table 3.1 The peak height signal obtained of 10 µM quinone from 9 modified electrodes

Electrode	Peak height (mV)*
1	24.8
2	25.7
3	25.7
4	26.6
5	24.3
6	23.9
7	24.4
8	26.4
9	23.4
Mean	25.0
S.D.	1.13
%RSD	4.52

* triplicate injection

3.7.2.1 Precision of biosensor

Precision of the biosensor was investigated by comparing the signal response of the system to 10 µM hydroquinone for 11 replicate injections and %RSD was 1.34. Precision of the biosensor was investigated by comparing the signal response of the system to 10 µM hydroquinone for 11 replicate injections and %RSD was 1.34. From the results, the proposed system provided good precision for determination of hydroquinone by considering from %RSD obtained.

3.7.3 Stability of biosensor

Moreover, the stability of the biosensor was investigated by measuring 10 µM hydroquinone for 3 h (111 replicate injections) %RSD was 4.2, indicating very good stability of the system for continuous analysis. Then, the same column and the same electrode were tested for the robustness and stability within 30 days. The laccase enzymatic column was stored at 4°C when it was not in use. It was found that the biosensor retained around 80% of initial response after 30 days as shown in Figure 3.18, indicating that there were strong covalent interactions between laccase and the activated silica gel.

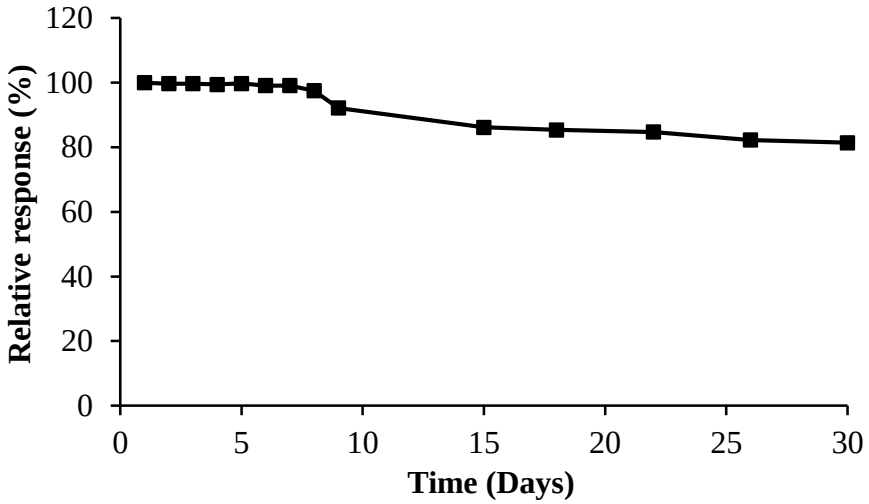

Figure 3.18 The stability of the biosensor for hydroquinone measurement in a period of 30 days.

3.7.4 Interference study

To evaluate the selectivity of the biosensor, the influence of some substances which usually found in water sample and phenolic compounds were investigated. Comparison of peak heights of 10 µM hydroquinone without and with the studied interference species was carried out. The results were summarized in Table 3.2 and suggested that 100-folds NH_4^+, Ca^{2+}, Mg^{2+}, Na^+, K^+, Cl^-, SO_4^{2-} and 10-folds H_2O_2, anthraquinone, 2,5-dihydroxybenzenesulfonate and 2-folds uric acid, phenol, guaiacol, gallic acid and tannic acid did not interfere with the hydroquinone determination (peak height difference below 5.0%), indicating high selectivity of the biosensor.

Table 3.2 Interfering effect of the studied substances on the FI-Amp sensor

Substance	Concentration (µM)	Peak height (mV)	%difference
Hydroquinone	10	2.666	-
Ammonium	1000	2.584	-3.08
Calcium	1000	2.536	-4.88
Magnesium	1000	2.712	+1.70
Sodium	1000	2.597	-2.59
Potassium	1000	2.543	-4.61
Chloride	1000	2.600	-2.48
Sulfate	1000	2.660	-0.23
Hydrogen peroxide	100	2.773	+4.01
Anthraquinone	100	2.644	-0.83
2,5-dihydroxybenzenesulfonate	100	2.784	+4.43

Table 3.2 (continued)

Substance	Concentration (μM)	Peak height (mV)	%difference
Uric acid	20	2.537	-4.84
Phenol	20	2.651	-1.50
Guaiacol	20	2.793	+4.76
Gallic acid	20	2.766	+3.75
Tannic acid	20	2.795	+4.84

3.7.5 Real samples analysis

The analytical application to real sample was investigated to evaluate the accuracy of the proposed method. It was found that concentration of hydroquinone in all 11 water samples were below the detection limit of the method which is 0.85 μM. The maximum allowable amount of hydroquinone in water defined by the European Community is 1.8 μM [IPCS, 1996], therefore all the water samples being analyzed are good quality in term of hydroquinone presented. Since the concentration of hydroquinone in samples were lower than detection limit, the accuracy of the proposed method was studied by spiking hydroquinone into waste water and natural water samples. The percentage recovery was calculated by comparing the expected concentration of hydroquinone in sample to the measured concentration found by the proposed method. The percent recoveries were obtained in the range of 94.5-106.6 as summarized in Table 3.3. Considering from the recoveries obtained, the results

indicated that the proposed method is reliable for quantitative determination of hydroquinone.

Table 3.3 The percentage of recovery at various spiked hydroquinone concentration into water samples

Sample	Spiked (µM)	Found (µM)	%Recovery
Sample 1	0	ND	-
	5.00	4.84	96.8
	20.00	20.46	102.3
	40.00	42.62	106.6
Sample 2	0	ND	-
	5.00	4.87	97.4
	20.00	19.81	99.1
	40.00	41.56	103.9
Sample 3	0	ND	-
	5.00	4.89	97.8
	20.00	20.39	102.0
	40.00	41.84	104.6
Sample 4	0	ND	-
	5.00	5.11	102.2
	20.00	20.55	102.8
	40.00	40.80	102.0

Table 3.3 (continued)

Sample	Spiked (µM)	Found (µM)	%Recovery
Sample 5	0	ND	-
	5.00	5.02	100.4
	20.00	19.61	97.66
	40.00	40.64	101.6
Sample 6	0	ND	-
	5.00	4.79	95.8
	20.00	20.15	100.8
	40.00	40.22	100.6
Sample 7	0	ND	-
	5.00	4.78	95.6
	20.00	18.89	94.5
	40.00	39.83	99.6
Sample 8	0	ND	-
	5.00	4.94	98.8
	20.00	19.31	96.6
	40.00	41.18	103.0
Sample 9	0	ND	-
	5.00	4.77	95.4
	20.00	19.24	96.2
	40.00	39.15	97.9

Table 3.3 (continued)

Sample	Spiked (µM)	Found (µM)	%Recovery
Sample 10	0	ND	-
	5.00	4.86	97.2
	20.00	19.25	96.3
	40.00	41.94	104.9
Sample 11	0	ND	-
	5.00	4.79	95.8
	20.00	18.99	95.0
	40.00	39.70	99.3

ND = Non detected