CHAPTER 2

Experimental

2.1 Chemicals

All chemicals used in this research are analytical reagent grade. All solutions were prepared by dissolving in deionized (DI) water obtained from a Millipore water purification system (Millipore, Sweden).

1. Acetone: C_3H_6O (distilled commercial grade, BDH, England)
2. 3−aminopropyltriethoxysilane, 3−APTES: $C_9H_{23}NO_3Si$ (Sigma, USA)
3. Ammonium chloride: NH_4Cl (Carlo Erba, Italy)
4. Calcium chloride dihydrate: $CaCl_2.2H_2O$ (Ajax, Australia)
5. Carbon nanotube with 6-10 nm diameter (Nanomaterial Research Unit, Chiang Mai University, Thailand)
6. Citric acid monohydrate: $C_6H_8O_7.H_2O$ (Merck, Germany)
7. Ethanol 95%v/v: C_2H_5OH (Merck, Germany)
8. Glutaraldehyde: $C_5H_8O_2$ (Fluka, Switzerland)
9. Hydrogen peroxide: H_2O_2 (Carlo Erba, Italy)
10. Hydroquinone: $C_6H_6O_2$ (Sigma, China)
11. Laccase from *Trametes versicolor* (Sigma, Germany) (for more information see Appendix B)

12. Magnesium sulfate heptahydrate: $MgSO_4.7H_2O$ (Merck, Germany)

13. Phenol: C_6H_6O (Rankem, India)

14. Potassium chloride: KCl (Ajax, Australia)

15. Quinone: $C_6H_4O_2$ (Merck, Germany)

16. Silica gel, 45 μm average particle size (Merck, Germany)

17. Sodium chloride: NaCl (Carlo Erba, Italy)

18. Sodium hydroxide: NaOH (Merck, Germany)

19. Uric acid: $C_5H_4N_4O_3$ (Sigma, USA)

2.2 Materials and instruments

1. Peristaltic pump (Ismatec, Switzerland)
2. PTFE tubing (1.59 mm O.D., 0.5 mm I.D.) (Upchurch Scientific, USA)
3. Tygon pump tubing (1.14 mm I.D.) (Ismatec, Switzerland)
4. Six-port injection valve (Upchurch Scientific, USA)
5. Flow through electrochemical cell (by Miss Preeyaporn Reanpang) composes of a 3 mm diameter screen-printed carbon as working electrode (SPCE), a Ag/AgCl as reference electrode (RE) and a stainless steel as auxiliary electrode (AE)
6. Digital multimeter (ProsKit, USA)
7. Homemade amperometric detector (by Assoc. Prof. Dr. Jaroon Jakmunee)
8. E-corder 210 (eDAQ Pty Ltd., Australia)
9. Micropipette 20, 1000 μL (Eppendorf, Germany)
10. pH meter model 744 (Metrohm, Switzerland)

11. Plasma cleaner (PDC−32G, Harrick, USA)

12. Magnetic stirrers with heating (IKA, Germany)

13. Autolab Potentiostat (Metrohm, Netherland)

14. Oven (G-therm, Italy)

2.3 Software

1. Microsoft Excel 2003 (Microsoft, USA)
2. eDAQ Chart (eDAQ, Australia)
3. Autolab (Metrohm, Switzerland)

2.4 Preparation of reagents

2.4.1 Citrate buffer, 0.1 M pH 5

$C_6H_8O_7.H_2O$ 21.01 g was dissolved in 800 mL DI water and the pH was adjusted to 4.5 with 10% NaOH solution. Then the volume was made up to 1000 mL with DI water.

2.4.2 Standard solution of hydroquinone

Hydroquinone 0.0110 g was dissolved in solution of ethanol: 0.1 M citrate buffer pH 5 (1:20) and the volume was made up to 100 mL to obtain 1 mM of hydroquinone solution.

2.4.3 Standard solution of quinone

Quinone 0.0108 g was dissolved in solution of ethanol: 0.1 M citrate buffer pH 5 (1:20) and the volume was made up to 100 mL to obtain 1 mM of quinone solution.

2.4.4 CNTs suspension

CNTs 0.0020 g was dispersed in 1 mL ethanol and the suspension was sonicated for 1 h at room temperature.

2.4.5 Laccase solution

Laccase powder 0.0147 g was diluted in 2 mL of 0.1 M acetate buffer pH 4.5 to obtain 100 U/mL of laccase solution. Then the solution was stored at 4 ^{o}C.

2.5 Preparation of electrode

Before being modified with nanomaterial, SPCE was treated in a chamber of plasma cleaner for 1 min. To modify electrode, 5 µL of the nanomaterial suspension was dropped on surface of the electrode and the solvent was evaporated at room temperature.

2.6 Working electrode investigation

Different working electrodes were studied by cyclic voltammetry in citrate buffer with quinone concentration of 0.1−2.0 mM. The applied potential was scanned from −1.0 V to +1.0 V (vs Ag/AgCl) at the scan rate of 100 mV/s. The peak current (peak height) was examined and used to plot a calibration graph and a slope indicated

the sensitivity of different electrode. The optimal working electrode was selected by considering for the better sensitivity.

2.7 Study on effect of CNTs amount

The effect of amount of CNTs being modified on the electrode was studied by varying volume of CNTs suspension from 2 to 20 µL. This parameter was also studied by cyclic voltammetry with the same conditions. The optimal CNTs amount was selected by considering the slope (sensitivity) of calibration graph in the range of 0.1 to 2.0 mM quinone.

2.8 Characterization of the electrode surface

Surface of bare SPCE and SPCE modified with CNTs were characterized by scanning electron microscope (SEM). The dried electrode surface samples were mounted over SEM stubs and then examined using these conditions: 10,000X magnification, ~15 mm working distance and 15 kV accelerating voltage.

2.9 Manifold of flow injection amperometric system

All the FI−Amp experiments were carried out using the FI−Amp system as shown in Figure 2.1. It consisted of carrier solution which is citrate buffer, a peristaltic pump, an injection valve, a laccase column, a mixing coil, an electrochemical cell which has SPCE electrode as a working electrode (WE), a stainless steel as an auxiliary electrode (AE) and a Ag/AgCl electrode (3M KCl) as a reference electrode (RE), a home−made amperometer as a detector, an analog to digital converter unit for data recording with relevant software and a computer.

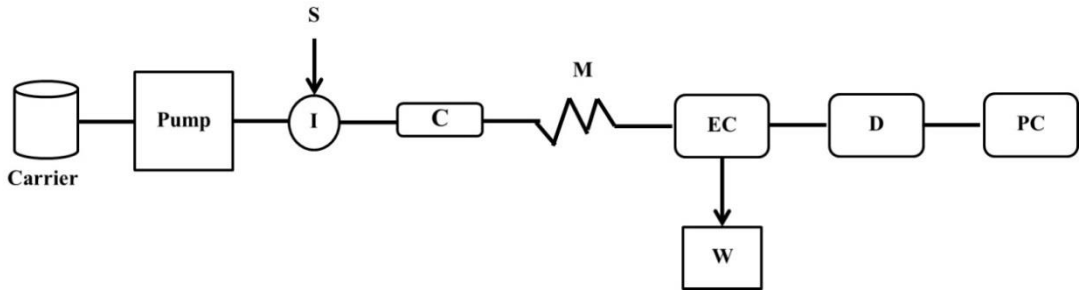

Figure 2.1 FI manifold of flow injection amperometric system for determination of hydroquinone; carrier = 0.1 M citrate buffer, I = injection valves, S = standard/ sample, C = laccase column, M = mixing coil, EC = electrochemical cell (WE, RE and AE), W = waste, D = amperometer and PC = personal computer.

In the experiments without laccase, quinone was used as standard because it was the product from laccase catalytic oxidation of hydroquinone and quinone was reduced at a SPCE working electrode at a constant applied potential of −50 mV vs. Ag/AgCl. Therefore, the current can be measured which is directly proportional to quinone/hydroquinone concentration and the calibration graph was plotted between peak height and quinone/hydroquinone concentration.

2.10 Optimization of flow injection amperometric system

This part used FI−Amp system as shown in Figure 2.1, but without the laccase column. Quinone standard solutions were used for optimization. Parameters of flow injection amperometric system were optimized to improve the efficiency of the method by fixing the others parameters. The studied parameters consisted of applied potential, sample volume and mixing coil length as summarized in Table 2.1. All of the results were compared in term of sensitivity, which obtained from the slopes of the calibration

graphs in the range of 5−20 µM quinone. The optimal condition was selected for further experiments.

Table 2.1 The conditions for the study of each parameter

Parameter	Conditions
Applied potential	+100, +50, 0, −50, −100, −150 and −200 mV
Sample volume	50, 75, 100, 200 and 300 µL
Mixing coil length	0, 30, 50, 100 and 200 cm

2.11 Procedure for laccase column preparation

Immobilization method was carried out according to the reference procedure. 1 g of dry silica gel was pretreated with 30% hydrogen peroxide for 30 min at room temperature and washed with deionized water. Then the treated silica gel was mixed with 15% 3−APTES in 20 mL acetone and incubated at 50 °C for 2 h with constant stirring. After that, the treated silica gel was washed with water and dried at 60 °C for 2 h and suspended in 0.1 M citrate buffer (pH 5). Thereafter, glutaraldehyde was added to the suspension and mixed at room temperature for 2 h. The activated silica gel was washed with 0.1 M citrate buffer and resuspended in the same buffer. To immobilize laccase, the suspension was stirred in solution containing 2 unit of laccase at room temperature for 10 h. Next, laccase immobilized silica gel was washed with deionized water to remove weakly adsorbed enzyme and resuspended in 0.1 M citrate buffer. The product was stored at 4 °C.

To prepare the laccase column, column is made of PMMA. Inner diameter and length of column are 0.3 and 2 cm, respectively. The cotton wool was capped at both ends. Then 1 mL of the enzyme suspension was divided to 5 parts and filled layer by layer with cotton wool as shown in Figure 2.2. After preparation, the laccase column was stored at 4 °C.

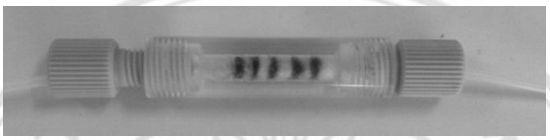

Figure 2.2 The laccase column.

2.12 Testing efficiency of the laccase column

After immobilization of laccase enzyme, the laccase column was evaluated to confirm the success of laccase immobilization by comparing the signal of 10 µM hydroquinone between the system with and without enzyme column. Moreover, the efficiency of the laccase column was carried out by constructing a calibration graph of hydroquinone in the range of 1–100 µM.

2.13 Optimization of parameters of the biosensor

Several parameters (i.e., flow rate, pH, buffer concentration 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 quinone which was detected amperometrically as described above. The laccase column was prepared as described in section 2.11 and was incorporated to the system as shown in Figure 2.1. All of the results were compared in term of sensitivity, which obtained

from the slope of the calibration graphs of hydroquinone concentration in the range of 5–50 µM.

2.13.1 Effect of flow rate

The effect of flow rate was investigated in the range of 0.5 to 1.5 mL min^{-1}. The conditions for the study were summarized in Table 2.2 and the optimal flow rate was selected for further experiments.

Table 2.2 The conditions for the study of effect of flow rate

Parameter	Value
Working electrode	SPCE−CNTs
pH	5.0
Buffer concentration	0.1 M
Temperature	27 ºC

2.13.2 Effect of pH

The influential of pH on the amperometric biosensor response was studied in the pH range of 3.5 to 5.5. The optimal pH was chosen by considering the slope of the calibration graphs of hydroquinone in the range of 5–50 µM. The conditions for the study were summarized in Table 2.3.

Table 2.3 The conditions for the study of effect of pH

Parameter	Value
Working electrode	SPCE−CNTs
Flow rate	1.0 mL min^{-1}
Buffer concentration	0.1 M
Temperature	27 ºC

2.13.3 Effect of buffer concentration

The effect of buffer concentration from 0.01 to 0.3 M of citrate buffer was investigated. The optimal condition was chosen by considering the slope of the calibration graphs and the conditions for the study were summarized in Table 2.4.

Table 2.4 The conditions for the study of effect of buffer concentration

Parameter	Value
Working electrode	SPCE−CNTs
Flow rate	1.0 mL min^{-1}
pH	4.5
Temperature	27 ^{o}C

2.13.4 Effect of temperature

The effect of temperature on the laccase column was studied in the range of 20 to 40 ^{o}C and temperature was controlled by immersing the laccase column in the water bath. The optimal condition was chosen by considering the slope of the calibration graphs and the conditions for the study were summarized in Table 2.5.

Table 2.5 The conditions for the study of effect of temperature

Parameter	Value
Working electrode	SPCE−CNTs
Flow rate	1.0 mL min^{-1}
pH	4.5
Buffer concentration	0.1 M

2.14 Analytical characteristics of the procedure

2.14.1 Calibration graph and detection limit

The optimum conditions of FI−Amp system were used to construct the calibration graph of the method by injecting a series of hydroquinone in the range of 1–100 µM. Peak height was plotted against hydroquinone concentration. The limit of detection (LOD) was calculated the criteria, LOD = 3SD of intercept of calibration graph/slope of the calibration graph as described in the appendix A.

2.14.2 Precision study

2.14.2.1 Precision of working electrode

The precision of working electrode was examined including of repeatability and reproducibility. The repeatability of the signal response of SPCE−CNTs was investigated by injection of 10 µM quinone for 11 replicated injections and the reproducibility was also studied by 9-replicate electrode preparation. Stability of the FI-amperometric detection system was examined by repeatedly injecting of quinone standard solution for 3 h.

2.14.2.2 Precision of biosensor

The precision of biosensor was examined by analysis of 10 µM hydroquinone for 11 replicated injections. The percentage of relative standard deviation (%RSD) value was used to evaluate the precision which calculated from the equation 2.1.

$$\%RSD = \frac{S.D.}{\bar{X}} \times 100 \qquad 2.1$$

When; S.D. = Standard deviation

$\bar{X}$ = Mean value

2.14.3 Stability of biosensor

Stability of laccase column was performed by comparing the response signal of 10 µM hydroquinone obtained from the developed system for 3 h (111 replicated injections) and the inter-day stability test was performed for 30 days. The column was stored at 4 ^{o}C when it was not in use.

2.14.4 Interference study

Interference study was investigated by adding interferences including NH_4^+, Ca^{2+}, Mg^{2+}, Na^+, K^+, Cl^-, SO_4^{2-}, H_2O_2, uric acid, guaiacol, gallic acid, tannic acid, anthraquinone, 2,5-dihydroxybenzenesulfonate and phenol into 5 µM hydroquinone solution. The prepared solutions were injected into the FI−Amp system. Ratio of the obtained signals compared to the signal of 5 µM hydroquinone was used to calculate the recovery percentage. The tolerance limit of interfering substances was evaluated based on the percentage different between signal of hydroquinone with and without interference species which should not exceed ± 5%.

2.14.5 Real sample analysis

All of 11 water samples being collected are surface water, including 7 waste water samples from Northern Region Industrial Estate, 3 natural water samples from the Kuang river near by Industrial Estate in Lamphun and 1 natural water sample from the Ping river in Chiang Mai, Thailand. Each sample was taken from riverbank and kept in amber bottle. Before being analyzed, samples were filtered through a filter paper (Whatman No.1). The analytical application to real sample was investigated to evaluate the accuracy of the proposed method by spiking standard of hydroquinone into water samples with 10 times dilution to obtain the final added concentration at 5, 20 and 40 µM. The recovery percentages were calculated from the equation 2.2.

$$\%\text{Recovery} = \frac{C_{found}}{C_{spiked}} \times 100 \qquad 2.2$$

When; C_{found} = The concentration obtained from the spiked sample

C_{spiked} = The concentration of added analyte to the sample