CHAPTER III

EXPERIMENTAL

3.1 Chemicals and reagents

3.1.1 Tetracycline hydrochloride(Sigma)

3.1.2 Chlortetracycline hydrochloride (Sigma)

3.1.3 Oxytetracycline hydrochloride (Sigma)

3.1.4 Doxycycline hydrochloride (Sigma)

3.1.5 Doxycycline hyclate (Sigma)

3.1.6 Hydrogen peroxide (30% w/v, BDH)

3.1.7 Chromium nitrate (Fluka)

3.1.8 Potassium hexacyanoferrate

3.1.9 Potassium chloride (Merck)

3.1.10 Potassium dihydrogen phosphate (BDH)

3.1.11 Disodium hydrogen phosphate (BDH)

3.1.12 Phosphoric acid (85% Carlo Erba)

3.1.13 Sodium hydroxide (Merck)

3.1.14 Potassium hydroxide (Merck)

3.1.15 Sulfuric acid (Merck)

3.1.16 Succinic acid (UNIVAR)

3.1.17 Sodium succinate (UNIVAR)

3.1.18 Citric acid (Carlo Erba)

3.1.19 Boric acid (Merck)

3.1.20 Acetic acid (Merck)

3.1.21 Sodium acetate (Merck)

3.1.22 Ammonium chloride (UNIVAR)

3.1.23 Hydrochloric acid (Merck)

3.1.24 Perchloric acid (Merck)

3.1.25 Ethylene diamine tetraacetic acid (EDTA, Riedel de Haën)

3.1.26 Methanol (Merck)

3.1.27 Acetonitrile (Merck)

3.1.28 Ethyl acetate (Merck)

3.1.29 Ethanol (Merck)

3.1.30 2-Propanol (Merck)

3.1.31 Sodium citrate (Riedel de Haën)

3.2 Apparatus

3.2.1 Boron doped diamond thin film (BDD) (obtain from Prof. A. Fujishima Labarotory, The University of Tokyo) were grown in a chemical vapor deposition system (CVD). The BDD film contained 10^{21} ppm boron. The electrode was rinsed with an ultrapure water prior to use.

3.2.2 A glassy carbon electrode (0.07 cm^2, Bioanalytical Inc.) was pretreated by polishing with alumina power (1 and 0.05 micron, respectively) slurries in ultrapure water on felt pads and rinsed thoroughly with an ultrapure water prior to use.

3.2.3 A Ag/AgCl electrode (TCI) with a salt bridge

3.2.2 A home-made platinum wire

3.2.4 A home-made cap cell (plastic bottle cap)

3.2.5 A home-made glass cell

3.2.6 A home-made brass holder

3.2.7 An O-ring viton (0.07 cm^2)

3.2.8 A polishing set of 0.05 and 1 micron alumina powder slurry (Bioanalytical Inc.)

3.2.9 An Autolab Potentiostat (PG-100, Methrom)

3.2.10 A peristaltic pump (Ismatic)

3.2.11 A HPLC pump (Water 5100)

3.2.12 A Rheodyne injection valve, Model 7725 (Altech), with a 20 μL stainless steel injection loop (0.5 mm. i.d.)

3.2.13 A thin layer flow cell (Bioanalytical Inc.)

3.2.14 A silicone gasket (Bioanalytical Inc.)

3.2.15 Milli-Q system, model Millipore ZMQS5V00Y, (Millipore, USA)

3.2.16 Inertsil-ODS3 C_{18} (5 μm, 4.6 mm x 25 cm, GL Science)

3.2.17 C18-E cartridges 500 mg, 6 mL, (Phenomenex, USA).

3.2.18 Teflon tubing (1/16 inch. o.d., Upchurch)

3.2.19 PEEK tubing (0.25 mm. i.d.) and connecting (Upchurch)

3.2.20 A cutting set (Altech)

3.2.21 A 0.2 μM Nylon membrane filter (Altech)

3.2.22 A 0.45 μM Nylon membrane syringe filter with polypropylene (PP) housing (Orange Scientific filter).

3.2.23 A pH meter (Metrohm)

3.2.24 A sonicator (USA, A006651)

3.2.25 An analytical balance (Metler, AT200)

3.2.26 A homogenized (National MK-H1000N Mixer 3 speed)

3.2.27 A Centrifuge (CENTAURA 2, Sanyo)

3.2.28 A Microcentrifuge (MINI CENTRIFUGE, Cole Parmer)

3.2.29 A Scanning Electron Microscope (SEM, JOEL JSM-5410 LV Scanning Electron Microscope)

3.3 Preparation of buffer solution

3.3.1 Preparation of 0.1 M phosphate buffer solution

13.6 g and 17.6 g of potassium dihydrogen phosphate and disodium hydrogen phosphate dehydrate were dissolved in 1 L of deionized water to make the solution of 0.1 M potassium dihydrogen phosphate buffer and 0.1 M disodium hydrogen phosphate. The recipes of each pH solution were shown in Table 3.1

Table 3.1 Recipes of phosphate buffer preparation.

pH	0.1 M Potassium dihydrogen phosphate (mL)	0.1 M Disodium hydrogen phosphate (mL)
5	99.2	0.8
6	88.9	11.1
7	41.3	58.7
8	3.7	96.3

The 0.1 M of potassium dihydrogen phosphate was adjusted by phosphoric acid to make the range of pH from 2 to 4.

3.3.2 Preparation of 0.01 M phosphoric acid solution (pH 2.5)

0.67 mL of phosphoric acid was diluted to the total volume of 1 L to make the solution of 0.01 M phosphoric acid. The pH was adjusted by 0.1 M sodium hydroxide.

3.3.3 Preparation of 0.1 M potassium chloride solution (for hydrogen peroxide determination)

7.4 g of potassium chloride was dissolved in 1 L of deionized water. The pH was adjusted by hydrochloric acid or sodium hydroxide.

3.3.4 Preparation of 0.1 M McIlvaine buffer solution (pH4)

15 g of disodium hydrogen phosphate dihydrate, 13 g of citric acid monohydrate and 3.72 g of EDTA were dissolved in 1 L of deionized water.

3.3.5 Preparation of 1 M citrate buffer solution (pH 5)

2 M solution of citric acid (21.01 g of citric acid in 50 mL of deionized water) and 0.1 M solution of sodium citrate (29.05 g of sodium citrate in 50 mL of deionized water) were prepared. 20.5 mL of 2 M citric acid was mixed with 29.5 mL of 2 M sodium citrate. The mixture solution was further diluted to a total volume of 100 mL with deionized water.

3.3.6 Preparation of 0.1 M acetate buffer (pH 5)

0.2 M solution of acetic acid (5.6 mL of acetic acid (glacial) in 500 ml of deionized water) and 0.2 M solution of sodium acetate (13.61 g sodium acetate trihydrate in 500 mL of deionized water) were prepared. 245 mL of 0.2 M acetic acid

was mixed with 255 mL of 0.2 M sodium acetate. The mixture solution was further diluted to a total volume of 1 L with deionized water.

3.3.7 Preparation of 0.1 M Britton-Robinson buffer (pH 5)

The solution containing 0.04 M of acetic acid, 0.04 M of citric acid and 0.04 M boric acid was prepared. The solution was adjusted with the appropriate volume of 0.2 M sodium hydroxide to make the final pH (pH 5).

3.4 Preparation of anodized BDD electrode

The anodized BDD electrode was prepared by treating an as-deposited BDD electrode in 0.1 M potassium hydroxide solution. The potential was applied between 0 to 2.4 V vs. Ag/AgCl using cyclic voltammetry for 60 min. The anodized BDD electrode was sonicated with propanol for 5 min. The anodized BDD electrode was also rinsed with ultrapure water prior to use.

3.5 Cyclic voltammetry

3.5.1 Apparatus

Electrochemical experiment was conducted in a single compartment three-electrode glass cell. The BDD electrode was pressed against a smooth ground joint at the bottom of the cell, isolated by an O-ring viton (area 0.07 cm^2). Ohmic contact was made by placing the backside of the Si substrate. The GC electrode was used as working electrode for comparison. The Ag/AgCl with salt bridge and Pt wire were used as reference and counter electrodes, respectively. The voltammetric measurement was performed with the three types of electrodes using Autolab Potentiostat 100.

3.5.2 Voltammetric study of TCs

The 0.5 mM of TCs in 0.1 M potassium dihydrogen phosphate (pH 2) were investigated using BDD electrode by cyclic voltammetry at scan rate 50 mV s^{-1}. Comparison the results using as-deposited BDD and GC electrodes were carried out.

3.5.3 The scan rate dependence study

The 1 mM solution of TCs, experiments were performed at various scan rates including 10, 20, 50, 100, 200 and 300 mV s^{-1} to investigate the adsorption of the analytes on the surface of electrode.

3.5.4 pH dependence study

The 1 mM of the analyte at each pH was measured using anodized BDD electrode by cyclic voltammetry to obtain the optimum pH of the measurement. A scan rate of 50 mV s^{-1} was used. The surface area was 0.28 cm^2. The experiment was carried out in plastic cap cell.

3.6 Flow injection with amperometric detection

3.6.1 Apparatus

The flow injection analysis (FIA) consisted of a thin layer flow cell, a 20 µL stainless steel loop of injection port, peristaltic pump, and electrochemical detection. The carrier solution (0.1 M potassium dihydrogen phosphate pH 2) was regulated at the flow rate of 1 mL min^{-1} by measuring the volume of carrier solution in 1 min. The thin layer flow cell consisted of the Ag/AgCl reference electrode and stainless steel tube as the counter electrode, which also served as the outlet of the system. A 1-mm thick silicone rubber gasket with a geometric area of 0.6 cm^2, was used as spacer in the flow cell. The potential of electrochemical detector was set and controlled using a Autolab Potentiostat 100. The experiments were performed in

copper faradaic cage to reduce the electronic noise. All the experiments were done at room temperature.

3.6.2 Hydrodynamic voltammetry

Hydrodynamic voltammetry was carried out to find the optimum potential to set the Autolab Potentiostat-100 before the amperometric determination to. The data were obtained by recording the background current at each potential and then injecting a series of three replicates of 20 μL of 100 μM each analyte solution. The peak height after each injection was recorded, together with the corresponding background current. These data were plotted as a function of applied potential to obtain hydrodynamic voltammogram.

3.6.3 Calibration and linear range

5 mM stock solutions of each TC were freshly prepared and then diluted to a concentration range from 0.1 to 1000 μM. The experiments were carried out by injecting 3 replicates of each concentration. The peak heights of each concentration were plotted versus the concentration to make the calibration curve and to find the linear range. The detection limit was carried out by injection of low concentrations of analyte solutions under the optimum potential. The detection limit was defined as the concentration that provided a current response more than three time higher than noise signal ($S/N > 3$).

3.6.4 Sample preparation of pharmaceutical formulations.

The details and contents within the pharmaceutical capsules (as described on the label) were shown in the Table 3.2.

Table 3.2 List of TCs samples used in this study.

Analytes	Brand name	Supplier	Amount of analyte in sample
OTC	Oxycline™	General Drug House Co. Ltd.	250 mg
TC	Tetracycline Hydrochloride™	General Drug House Co. Ltd.	250 mg
CTC	Aureomycin™	Summit	250 mg
DC	Medomycin™	Medline Co. Ltd.	100 mg

The powder of twenty capsules of each drug was accurately weighted and homogenized to obtain the mean capsule weight. A portion of the homogenized powder corresponding to the mean capsule weight was transferred into volumetric flask and dissolved in 0.1 M phosphate buffer (pH 2), and then mixed thoroughly. A portion of the sample solution was filtrated through 0.45 μm nylon membrane. The filtrated solutions were further diluted using 0.1 M phosphate buffer (pH 2) to make the final concentration in the range of 1.92-2.98 μg mL^{-1}. The final solution were protected from light by covering with aluminum foil and stored at 4 °C.

A stock of standard solution in 0.1 M phosphate buffer (pH 2) and a set of five 10 mL volumetric flasks were prepared. 5 mL of sample solution was pipetted in each volumetric flask, then 0, 0.25, 0.5, 0.75, and 1 mL of stock solution of analyte solution were added to make the final concentration of standard in the solution (Table 3.3).

Table 3.3 The details of the stock standard concentrations and final standard concentrations in samples.

Analytes	Stock standard solution ($\mu g\ mL^{-1}$)	Final standard concentration ($\mu g\ mL^{-1}$)
OTC	49.7	0,1.24,2.48,3.73,4.97
TC	48.1	0.1.20,2.40,3.60,4.81
CTC	51.5	0,1.29,2.58,3.86,5.15
DC	38.5	0.0.96,1.92,2.85,3.85

3.7 HPLC with amperometric detection

3.7.1 Apparatus

The HPLC system consisted of HPLC pump, a syringe-load manual injection equipped with 20 μL injection loop, 5 μm particle size reverse-phase C18 column, a thin layer flow-cell (BAS) and an amperometric detector. The thin layer flow cell consisted of the Ag/AgCl reference electrode and stainless steel tube as the counter electrode, which also served as the outlet of the system. A 1-mm thick silicone rubber gasket with the geometric area of 0.6 cm^2, was used as spacer in the flow cell. The potential of electrochemical detector was set and controlled using a Autolab Potentiostat 100. The carrier solution (81:19 of 0.01 M phosphoric acid (pH 2.5) : acetonitrile) was regulated at the flow rate of 1 mL min^{-1} by measuring the volume of carrier solution in 1 min. The stock mobile phase was degassed by sonicating for 1 hr. prior to use.

3.7.2 Hydrodynamic voltammetry

Hydrodynamic voltammetry were carried out to find the optimum potential to set the Autolab Potentiostat 100 before the amperometric determination. The data were obtained by recording the background current at each potential and

then injecting a series of two replicates of 20 μL of 100 μM mixed TCs standard solution. The peak area after each injection was recorded, together with the corresponding background current. These data were plotted as a function of applied potential to obtain hydrodynamic voltammogram.

3.7.3 Calibration and linear range

5 mM of mixed TCs standard solution was freshly prepared and then diluted to a concentration range from 0.1 to 1000 μM. The experiments were carried out by injection of two replicates of each concentration. Data acquisition in peak area mode was used to obtain the calibration curve. The detection limit was carried out by injection of low concentrations of analyte solutions under the optimum potential. The detection limit was defined as the concentration that provided a current response more than three time higher than the noise (S/N > 3).

3.7.4 Sample preparations of egg samples

Egg samples were purchased from the local market. Various extraction methods were used for the extraction of TCs in the egg samples. Each method was described as the following:

3.7.4.1 Mcvaillane buffer [39] (Method I)

Extraction and cleanup procedures used in this study were described. A blended egg sample of 2.5 g was added with 12.5 mL of 0.1 M Na_2EDTA-McIlvaine buffer (pH 4) and then centrifuged at 3800 ppm for 30 min. The supernatants were filtrated. The filtrated was applied to a Bond Eluted SPE C18 (500 mL, 6 mL) that has been pretreated with 10 mL of methanol followed by 10 mL of water. Then, the samples were loaded through the cartridge. The TCs were eluted from the column with methanol. The eluent was evaporated by nitrogen gas and the residues were filtrated with 0.45 μm nylon filter membrane. The filtrate was diluted with methanol prior to injection to the column.

3.7.4.2 Perchorlic acid [109] (Method II)

An accurately weighed 0.2 g of blend egg sample was taken into a 1.5 mL micro-centrifuge tube and homogenized in 0.4 mL of 10% (v/v) perchloride acid (PCA) solution (in water) with ultrasonic bath for 1 min. Then, the centrifuge tube was placed in micro-centrifuge and centrifuged at 6000 rpm for 10 min. A 0.2 mL portion of supernatant liquid was put into an Ultrafree-MC/PL (0.2 μm cellulose acetate) and centrifuged at 6000 rpm for 5 min. The 20 μL of ultra-filtrated was injected to HPLC system.

3.7.4.3 Acetonitrile [41] (Method III)

Whole egg was homogenized using a homogenizer. 0.5 g of the homogenate was mixed with 1.2 mL of 1 M citrate buffer (pH 5) using the magnetic stirrer for 5 min. 5 mL of acetonitrile was added and the whole mixture was stirred by magnetic stirrer for 5 min. The sample was then centrifuged for 10 min at 3000 rpm. The supernatant was transferred into the beaker. The residue was mixed with 1.2 mL of Milli-Q water. The further of 10 mL of acetonitrile was added to the sample and the extraction procedure was then repeated. The supernatant was combined and evaporated by nitrogen gas. The residue was reconstituted with methanol and filtrated with 0.45 μm nylon filter membrane.

3.7.2.4 Ethyl acetate [42] (Method IV)

0.5 g of blended egg was mixed with 0.3 mL of 1 M citrate buffer (pH 5) and 3 mL of ethyl acetate using magnetic stirrer for 15 min. Then, the sample was centrifuged for 20 min at 3500 rpm. The supernatant was decanted and the residue re-extracted twice with ethyl acetate (2 x 3 mL). The extract was evaporated by nitrogen gas and the residue was reconstituted by methanol and filtrated through 0.45 μm nylon filter membrane

3.7.3 Recovery test

The recoveries of TCs from blank samples spiked at 0.5, 1, 5, and 10 μg mL^{-1}, respectively, were determined. These fortification levels were prepared by the addition of appropriated volume of mixed TCs standard solution to separate the portion of the samples. Fortified samples were mixed prior to the test.

3.8 Determination of hydrogen peroxide using chromium (III) hexacyanoferrate (II) modified BDD electrode

3.8.1 Preparation of modified working electrode

Electrodeposition experiments were carried out using a bottle cap cell. The BDD electrode was pressed against a smooth ground joint at the bottom of the cell, isolated by a silicon rubber spacer (area 0.07 cm^2). Ohmic contact was made by placing the backside of the Si substrate. The Ag/AgCl with salt bridge and Pt wire were used as reference and counter electrodes, respectively. The voltammetric measurement was performed with the three types of electrodes using Autolab Potentiostat 100.

Chromium (III) hexacyanoferrate (II) modified BDD electrode was prepared in 0.1 M KCl solution (pH 3) containing 10 mM $Cr(NO_3)_3.9H_2O$ and 5 mM $K_3Fe(CN)_6$. The electrode can be prepared by cycling the potential between -0.2 and +0.95 V vs. Ag/AgCl for 1 hr at the scan rate 0.01 mV s^{-1}. Subsequently, the modified electrode was conditioned in 0.1 M KCl electrolyte (pH 3) for an additional 1 hour.

3.8.2 The pretreatment methods of electrode before deposition of chromium(III)hexacyanoferrate(II)

Several electrode pretreatment methods were carried out to find the optimum electrode pretreatment method for the preparation of chromium (III) hexacyanoferrate (II) on BDD electrode. The procedures of each electrode pretreatment methods were described in Table 3.4.

Table 3.4 The description of procedure of electrode pretreatment methods.

Pretreatments	Procedure
As-deposited BDD electrode	Not have any pretreatment
Anodized BDD electrode treated with 0.1 M potassium hydroxide	The electrode was cycling from 0 to 2.4 V vs. Ag/ AgCl for 1 hr using cyclic voltammetry at the scan rate 10 mV s^{-1} in 0.1 M potassium hydroxide.
Anodized BDD electrode treated with 0.1 M sulfuric acid	The electrode was cycling from -2 to 2 V vs. Ag/AgCl for 0.5, 1 and 1.5 hr using cyclic voltammetry at the scan rate 10 mV s^{-1} in 0.1 M sulfuric acid
Anodized BDD electrode treated with 0.1 M sulfuric acid (abrasive electrode)	The electrode was abrasived 1 time with the abrasive paper no Cw-1000. After that, the electrode was cycling from -2 to 2 V vs. Ag/AgCl for 1 hr using cyclic voltammetry at the scan rate 10 mV s^{-1} in 0.1 M sulfuric acid
Anodized BDD electrode treated with 0.1 M sulfuric acid (fixed current)	The electrode was carried out in 0.1 M sulfuric acid using potentiometry with fixed current 10 mA cm^{-2} for 20 min
Anodized BDD electrode treated with 0.1 M potassium hydroxide and 0.1 M sulfuric acid	The electrode was cycling from 0 to 2.4 V vs. Ag/ AgCl for 1 hour using cyclic voltammetry at the scan rate 0.01 V/s in 0.1 M potassium hydroxide. Then, the electrode was cycling from -2 to 2 V vs. Ag/AgCl for 1 hr using cyclic voltammetry at the scan rate 10 mV s^{-1} in 0.1 M sulfuric acid

3.8.3 The electrodeposition methods

Several electrodeposition methods were carried to find the optimum electrodeposition method for the preparation of chromium (III) hexacyanoferrate (II) on BDD electrode. The descriptions of each electrodeposition methods were shown in Table 3.5.

Table 3.5 The description of procedure of electrodeposition methods.

Deposition methods	Procedure
Amperometry (fixed potential)	Fix the potential at -1 V vs. Ag/AgCl for 1 hr using chronoamperometric technique
Cyclic voltammetry	Cycling the potential between -0.2 and +0.95 V vs. Ag/AgCl for 1 hr at the scan rate 10 mV s^{-1}.
Pulse amperometry (Potential pulse)	Using the potential pulse with the pulse length of 0.5 sec with minimum potential of -0.2 V vs. Ag/AgCl and maximum potential of 0.95 V vs. Ag/AgCl

3.8.4 pH dependence

The 1 mM of the hydrogen peroxide at each pH were measured using chromium (III) hexacyanoferrate (II) modified BDD electrode by cyclic voltammetry to obtain the optimum pH of the measurement. A scan rate of 50 mV s^{-1} was used.

3.8.5 Effect of electrolyte/buffer systems

The 1 mM of the analyte at each compostion was measured using chromium(III) hexacyanoferrate (II) modified BDD electrode by cyclic voltammetry at the scan rate of 50 mV s^{-1}. In this study, several electrolyte/buffer systems were used as following:

3.7.4.1 Potassium chloride (pH 5)

3.7.4.2 Potassium chloride + Succinic acid (pH 5)

3.7.4.3 Succinic acid (pH 5)

3.7.4.4 Succinic acid + Ammonium chloride (pH 5)

3.7.4.5 Succinic acid + Acetate (pH 5)

3.7.4.6 Acetate buffer (pH 5)

3.7.4.7 Phosphate buffer (pH 5)

3.7.4.8 Britton-Robinson buffer (pH 5)

3.8.6 Voltammetric comparison of hydrogen peroxide using as-deposited BDD, anodized BDD and chromium (III) hexacyanoferrate(II) modified BDD electrodes.

The 1 mM of hydrogen peroxide in the chosen buffer solution from the previous experiments were investigated using diamond electrode by cyclic voltammetry at scan rate 50 mV s^{-1}. Comparison of the results with as-deposited BDD and anodized BDD electrodes were carried out.

3.8.7 Sample preparation of hydrogen peroxide disinfection solution

Hydrogen peroxide sample solution was filtrated through 0.45 μm nylon filter. 1 mL of filtrated sample was transferred to a 100 mL volumetric flask and then diluted with 0.1 M potassium chloride (pH 5). An aliquot of the solution was further diluted again with appropriated volume of 0.1 M potassium chloride (pH 5) to yield a final concentration of 0.60 μg mL^{-1}. The amount of hydrogen peroxide was assumed that the actual content of hydrogen peroxide corresponds to the one reported by the manufacturing laboratories.

A stock solution of 30 μg mL^{-1} in 0.1 M potassium chloride (pH 5) and a set of five 10 mL volumetric flasks were prepared. 5 mL of sample solution was pipetted in each volumetric flask, then added 0, 0.1, 0.2, 0.3, and 0.4 mL of stock solution of hydrogen peroxide solution were added to make the final concentration of standard. 0, 0.30, 0.60, 0.90, and 1.20 μg mL^{-1}, respectively.