CHAPTER III

EXPERIMENTAL

3.1 Chemical Reagents

3.1.1 Tetracycline hydrochloride (Sigma)

3.1.2 Oxytetracycline hydrochloride (Sigma)

3.1.3 Chlortetracycline hydrochloride (Sigma)

3.1.4 Doxycycline hydrochloride (Sigma)

3.1.5 Potassium dihydrogen orthophosphate (BDH)

3.1.6 Disodium hydrogen orthophosphate (BDH)

3.1.7 Phosphoric acid (Merck)

3.1.8 Diaminetetraacetic acid disodium salt (Fluka)

3.1.9 Citric acid (J.T.Baker)

3.1.10 Acetonitrile (HPLC Grade, Merck)

3.1.11 Methanol (HPLC Grade, Merck))

3.1.12 2-Propanol (Analytical Grade, Merck)

3.1.13 Oxalic acid dihydrate (Merck)

3.2 Instruments and Equipments

3.2.1 An Autolab Potentiostat (PGSTAT 30, Metrohm)

3.2.2 A Water No. 510 solvent delivery system (Waters Associates Inc, Milford, MA, USA)

3.2.3 A Rheodyne injection valve, Model 7225 (Altech), with a 20 μl stainless steel injection loop (0.5 mm. i.d.)

3.2.4 Automated LC system (HP 1100 series from Agilent Technologies, USA.) consisted of auto-sampler, binary pump, on-line degasser, UV-Visible and fluorescence detector

3.2.5 Milli-Q water system, model Millipore ZMQS5V00Y, (Millipore, USA)

3.2.6 Centrifuge (CENTAURA 2, Sanyo)

3.2.7 Mobile phase filter set included 300 mL glass reservoir, glass membrane holder, 1000 mL flask and metal clip (Millipore, USA)

3.2.8 Autopipette and tips (Eppendorf, Germany)

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

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

3.2.11 A gold rotating disk electrode (Au RDE 0.07 cm^2, Metrohm)

3.2.12 A gold disk electrode (1.0 mm, Bioanalytical System Inc.)

3.2.13 A Ag/AgCl electrode (Bioanalytical System Inc.)

3.2.14 A platinum rod electrode (Metrohm, 6.1204.010)

3.2.15 A platinum wire (Bioanalytical System Inc.)

3.2.16 The as-deposited boron doped-diamond electrode (WD769/1, Purchased from Centre Suisse d' Electronicat de Microtechnique SA (CSEM) and obtained from Associate Prof. Kensuke Honda.

3.2.17 Electrolyte vessel (Metrohm, 6.1415.150)

3.2.18 Control/Power unit for rotating disk electrode (Metrohm, 628-10)

3.2.19 Drive unit for rotating disk electrode (Metrohm, 1.628.0020)

3.2.20 A polish set of 0.05 and alumina powder slurry (Metrohm)

3.2.21 A thin layer flow cell (Bioanalytical System Inc.)

3.2.22 A Teflon cell gasket (Bioanalytical System Inc.)

3.2.23 Peek tubing (0.25 mm i.d.) and connecting (Upchurch)

3.2.24 Teflon tubing (1/10 inch i.d., Upchurch)

3.2.25 A cutting set (Altech)

3.2.26 A 0.45 μm Nylon membrane filter (Altech)

3.2.27 A 0.45 μm Nylon membrane syringe filter with polypropylene (Orange Scientific filter)

3.2.28 A pH meter (Metrohm)

3.2.29 A sonicator (USA, A006651)

3.2.30 An analytical balance (Metler, AT 200)

3.2.31 HPLC vials 2.0 mL with PTFE screw caps (Agilent Technologies, USA)

3.2.32 Glass containers with Teflon screw cap 25 mL

3.2.33 Erlenmeyer flasks 10, 100, and 250 mL

3.2.34 Separatory funnels 500 mL

3.2.35 Volumetric flasks 10, 25, 50 and 100 mL

3.2.36 Beakers 10, 25, 50, 100, 500 and 1,000 mL

3.3 Preparation of chemical solutions

3.3.1 Stock Standard Solutions

Each stock standard solution (1000 μg/mL) of TC, OTC, CTC and DC was prepared daily by weighing 50 mg of each standard into a 50 mL volumetric flask and the volume was adjusted to 50 mL with the mobile phase.

3.3.2 Working Standard Solutions

The mixture of 4 TCs solutions containing 100 μg/mL was prepared by pipetting 1.0 mL of each standard solution (1000 μg/mL) into a 10 mL volumetric flask and the volume was adjusted to 10 mL with mobile phase. Working standard solutions were prepared by diluting the standard mixture solutions (100 μg/mL) with mobile phase. All of the solutions were protected from exposure to light and stored in the refrigerator.

3.3.3 Preparation of Mobile Phase

The mobile phase for HPLC condition was consisted of acetonitrile and the phosphate buffer solution. The phosphate buffer solution of pH 2.5 was prepared daily by mixing of 0.01 M H_3PO_4 + 0.1 M Na_2HPO_4 (a few drops to adjust the pH). The phosphate buffer was filtered with a 0.45 μm Nylon membrane filter.

3.3.4 Preparation of Na_2EDTA-McIlvaine Buffer

Na_2EDTA -McIlvaine buffer solution (pH 4) was prepared by dissolving 15 g of disodium hydrogenphosphate dihydrate, 13 g of citric acid monohydrate and 3.72 g of EDTA in deionized water in 1 L volumetric flask.

3.3.5 Sample Preparation

The shells, fins and tails of shrimp were removed and ground in a conventional meat grinder. The ground shrimp were stored at below -10°C until analysis.

2.50±0.05 g of ground shrimp was placed in 25 mL centrifuge tube, 12.5 mL of Na_2EDTA-McIlvaine buffer (pH 4) was added, then the mixture was centrifuged at 3500 rpm for 20 min. The supernatant was loaded into SPE cartridge, previously activated with 10 mL of methanol and 10 mL of Milli-Q water. After sample loading, the SPE cartridge was washed with 10 mL of Milli-Q water and finally tetracyclines were eluted by 10 mL of methanol. The residues were diluted in the total volume of 10 mL and the solutions were filtered with a 0.45 μm PTFE filter. Then, 20 μL of aliquot was injected to the HPLC system.

3.4 Preparation of Anodized BDD Electrode

An anodized BDD electrode was prepared by treating an as-deposited BDD electrode in 0.1 M KOH solution. The potential was applied between 0 to 2 V versus Ag/AgCl using cyclic voltammetry for 30 min. The anodized BDD electrode was also rinsed with ultrapure water before use.

3.5 Cyclic Voltammetric Study

Each of 1 mM of OTC, TC, CTC and DC standard solutions was prepared by weighing 0.0495, 0.0481, 0.0515, and 0.0481 g, respectively and transfering into 100 mL volumetric flask. The mobile phase (acetonitrile: phosphate buffer; 20:80, v/v) was used for diluting this aliquot to the mask. These solutions were used for the investigation of the oxidation of TCs by cyclic voltammetry at the Au and the anodized BDD electrode.

3.5.1 Cyclic Voltammetry at Au Electrode

The rotating Au disk electrode (0.07 cm^2) was used as the working electrode. A Ag/AgCl and a platinum rod electrode were used as the reference and auxiliary electrodes, respectively. A commercial rotating disk apparatus was used to rotate the electrode at the rotation speed of 250 rpm. These experiments were carried out at the scan rate of 50 mV s^{-1}.

3.5.2 Cyclic Voltammetry at Anodized BDD Electrode

The anodized BDD electrode (0.07 cm^2) was used as the working electrode. A Ag/AgCl and a platinum wire electrode were used as the reference and auxiliary electrodes, respectively. These experiments were carried out at the scan rate of 50 mV s^{-1}.

3.6 PAD Optimization

The PAD waveform parameters were optimized by injection of 5 ppm mixed standard solution in the HPLC system. The average current responses for each parameter were plotted versus the varied parameters to obtain the optimal values. The optimal PAD waveform parameters were depicted in Table 4.1.

3.7 HPLC Optimization

A HPLC method equipped with a C_{18} column and phosphate buffer (pH 2.5) and acetonitrile as the mobile phase was used to develop the separation of 4 tetracyclines; i.e. oxyetracycline (OTC), tetracycline (TC), chlortetracycline (CTC) and doxycycline (DC). The injection volume was 20 μL and the detector was pulsed amperometric detection (PAD). The optimization was determined by vary the mobile phase strength. The separation of tetracyclines was first tested with standard mixture and the condition was later tested with shrimp samples spiked mixed standard. The optimal HPLC conditions for the separation of tetracyclines were depicted in Table 4.2. The selectivity and resolution on the separation of these compounds were studied as depicted in Table 4.3.

3.8 Calibration and Linearity

Each concentration of the mixed standard TCs solutions at 0.1, 0.5, 1.0, 5.0, 8.0, 10, 25, 50 and 100 mg/L was injected in duplicate. The calibration curves were plotted between the peak areas and the concentrations. The calibration characteristics were summarized in Table 4.4 and 4.5.

3.9 Limit of Detection (LOD) and Limit of Quantitation (LOQ)

The LOD and LOQ were determined by various concentrations under HPLC conditions and optimal PAD parameters. The detection limit (LOD) and quantitation limit (LOQ) are defined as the concentration that provided a current response higher 3 times than the noise ($S/N \geq 3$) and 10 times than the noise ($S/N \geq 10$), respectively.

3.10 Precision and Accuracy

For intra-day precision, the repeat of analysis of spiked sample was studied in one day. For inter-day precision, the repeat of analysis of spiked sample was studied on different days. The spiking at level 0.5, 1.0, 5.0 and 10 mg/kg was used in the study and at each level was repeated in triplicate.

3.11 Applications

The HPLC-PAD using the anodized BDD electrode was applied to detect tetracyclines in shrimps. Two types of shrimp that purchased from the local markets were farming-shrimp and sea-shrimp. The developed method was compared to the AOAC Official method (995.09) and Laboratory Center for Food and Agricultural Products Company Limited (LCFA).

3.12 AOAC Official Method (995.09)

This method could be used to detect tetracycline, chlortetracycline and oxytetracycline. The column used in this method was the same as the one used in HPLC-PAD systems. The HPLC conditions were carried out using the mobile phase of oxalic acid (0.1 M) – methanol – acetonitrile (60:10:30; v/v) on a C_{18} column at a flow rate of 1.0 mL/min at room temperature. The injection volume was 20 μL and the detector was UV-Visible detector at 350 nm.

3.12.1 Preparation of Chemical Solutions

3.12.1.1 Preparation of Mobile Phase

The oxalic acid solution was prepared by weighing 1.26 g of oxalic acid dihydrate and dissolved in 1 L volumetric flask with Milli-Q water. Then, this 600 mL oxalic solution was combined with 300 mL acetonitrile and 100 mL methanol.

3.12.1.2 Preparation of McIlvaine buffer-EDTA solution

28.4 g of anhydrous Na_2HPO_4 was added into 1 L volumetric flask and dissolved with deionized water. Then, 21.0 g citric acid was added into another 1 L volumetric flask and dilute to 1 L with deionized water. The mixed solution was the combination of 1 L citric acid solution and 625 mL Na_2HPO_4 solution in 2 L beaker. The mixed solution was adjusted to pH 4.0 by adding 0.1 M HCl or 0.1 M NaOH. Finally, 60.5 g disodium EDTA dihydrate was dissolved with 1.625 L of mixed solution.

3.12.1.3 Preparation of Methanolic acid

1.26 g oxalic acid dihydrate was dissolved with methanol in 1 L volumetric flask.

3.12.2 Sample Preparation

5.00±0.05 g of sample was placed into 50 mL centrifuge tube, added 20 mL McIlvaine buffer-EDTA solution and blended for 30 sec with homogenizer. Then, this tube was centrifuged for 10 min at 2500 rpm and put the supernatant into another 50 mL centrifuge tube. 20 mL McIlvaine buffer-EDTA solution was added in the first centrifuged tube containing the sample and repeated all steps to obtain the second extraction. Finally, 10 mL McIlvaine buffer-EDTA solution was added in the first centrifuged and repeated all steps. The supernatants from 3 extractions were collected in the second tube, then loaded to the SPE extraction.

SPE cartridges were set, and conditioned with 20 mL methanol and 20 mL Milli-Q water. The supernatant was loaded into SPE cartridge. After sample loading, the SPE cartridge was washed with 10 mL of Milli-Q water and finally tetracyclines were eluted by 6 mL of methanolic oxalic acid. The solution was diluted in the total volume of 10 mL, and filtered with a 0.45 μm PTFE filter. Then, 20 μL of aliquot was injected to the HPLC system.