CHAPTER III

EXPERIMENTAL

3.1 Chemicals and Reagents

3.1.1 Tetracycline hydrochloride (Sigma)
3.1.2 Chlortetracycline hydrochloride (Sigma)
3.1.3 Doxycycline hydrochloride (Sigma)
3.1.4 Oxytetracycline hydrochloride (Sigma)
3.1.5 Boron trioxide (Wako)
3.1.6 Acetone (Wako)
3.1.7 Methanol (Wako)
3.1.8 Potassium dihydrogen orthophosphate (BDH)
3.1.9 Di-sodium hydrogen orthophosphate-dihydrate (BDH)
3.1.10 Acetonitrile (Merck)
3.1.11 Sodium hydroxide (Merck)
3.1.12 Hydrochloric acid (Merck)
3.1.13 Phosphoric acid (Merck)
3.1.14 Ethylenediaminetetraacetic acid disodium salt dehydrate (Fluka)
3.1.15 Citric acid monohydrate (Baker analyzed)
3.1.16 A standard buffer solution pH 4 and 7 (Metrohm)
3.1.17 Tetracycline hydrochloride capsule (TC Mycin 250 mg)

3.2 Apparatus

3.2.1 Silicon wafer
3.2.2 Microwave plasma chemical vapor deposition (MPCVD) system (ASTeX Corp., Woburn, MA)
3.2.3 Rutherford backscattering spectroscopic analyzer with particle induced x-ray emission and ion implantation (RAPID)
3.2.4 Raman Spectrometer (Renishaw system 2000)
3.2.5 Digital microscope (Keyence)

3.2.6 Milli-Q water system, model Millipore ZMQS 5 VOOY, Millipore, USA.

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

3.2.8 Ag/AgCl electrode (TCI) with a salt bridge

3.2.9 Home-made platinum wire

3.2.10 Home made glass cell

3.2.11 Home made brass holder

3.2.12 O-ring viton (0.07 cm^2)

3.2.13 Polishing set of 0.05 and 1 micron alumina powder slurry (Bioanalytical System Inc.)

3.2.14 Polishing set of 0.05 micron powder slurry (Element six)

3.2.15 Autolab Potentiostat (PG-30, Methrom)

3.2.16 Peristaltic pump (Ismatic)

3.2.17 A water No.510 solvent divery system (Water Associates Inc, Milford, MA, USA)

3.2.18 Automated LC system (HP 1100 series from Agilent Technologies, USA) consisted of auto-sample, binary pump, on-line degasse, UV-Visible and fluorescence detector

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

3.2.20 HPLC column was ODS-3 Inertsil C18, 5 µM 4.6 x 250 mm i.d. (GL Science Inc.)

3.2.21 Solid phase extraction (Phenomenex)

3.2.22 C18-E cartridges 500 mg, 60 mL (Phenomennex,USA)

3.2.23 Centrifuge, CENTAURA 2, (Sanyo)

3.2.24 Thin layer flow cell (Bioanalytical System Inc.)

3.2.25 Thin layer flow cell (GL Science)

3.2.26 Teflon cell gasket (Bioanalytical System Inc.)

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

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

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

3.2.30 Auto pipette and tips (Eppendrof, Germany)

3.2.31 Cutting set (Altech)

3.2.32 0.2 μm Nylon membrane filter (Altech)

3.2.33 0.45 μm Nylon membrane syringe filter with polypropylene (PP) housing (Orange Scientific filter)

3.2.34 pH meter (Metrohm)

3.2.35 Sonicator (USA)

3.2.36 Analytical balance (Metler)

3.2.37 HPLC vial 2.0 mL with PTFE screw cap.

3.3 The Preparation of supporting electrolyte solution and standard solution

All solutions were prepared using deionized water obtained from a Milli-Q system (Milford, MA, USA). The preparations of buffer solutions are shown below:

3.3.1 0.1 M Phosphate buffer

13.60 g of potassium dihydrogen phosphate was dissolved in 1.0 L of deionized water and then adjusted with 0.1 M sodium hydroxide or 85% phosphoric acid to the required pH.

3.3.2 0.1 M Oxalic acid

The 0.1 M Oxalic acid dihydrate was prepared by weighing 13.60 g Oxalic acid dihydrate into 1.0 L volumetric flask and dissolve in Milli-Q water.

3.3.3 McIlvaine buffer

28.40 g di-sodium hydrogen orthophosphate-dihydrate was weighed into 1.0 L volumetric flask, dissolved and diluted to volume with Milli-Q water. Separately weigh 21.0 g citric acid monohydrate into 1.0 L volumetric flask and dilute to volume with Milli-Q water and mix well. The buffer was made by combining 1.0 L citric acid solution with 625 mL di-sodium hydrogen orthophosphate-dihydrate solution in 2.0 L flask and finally adjusting the pH to 4.0 with either 0.1 M hydrochloric acid or 0.1 M sodium hydroxide.

3.3.4 McIlvaine buffer-EDTA solution

The McIlvaine buffer-EDTA solution was prepared by dissolving 60.5 g disodium EDTA dehydrate in1.625 L McIlvaine buffer (3.3.2).

3.3.5 Methanolic oxalic acid

Oxalic acid dihydrate 1.26 g was dissolved in 1.0 L of methanol in 1.0 L volumetric flask. Dilute to volume with methanol and mix.

3.3.6 Mobile phase for HPLC-ECD

Pipette 0.73 mL of 85% phosphoric acid and transfer into 1.0 L volumetric flask. Adjust the pH to 2.5 by adding drop-wise 0.1 M di-sodium hydrogen orthophosphate-dihydrate. Combine 800 mL of this phosphate buffer solution with 200 mL acetonitrile solution in 1.0 L flask and then mix thoroughly to complete dissolution and degas by ultrasonic bath.

3.3.7 Carbon source

Boron trioxide 1.09 g was dissolved in 8 ml of methanol and 72 mL of acetone and then brought to a complete dissolution by ultrasonic bath.

3.3.8 Tetracycline hydrochloride solutions

The 1 mM tetracycline hydrochloride solution was prepared by weighing 0.0481 g tetracycline hydrochloride powder and transferring into 100 mL volumetric flask. The 0.1 M phosphate buffer (pH 2) was used for diluting this aliquot to the mark. This solution was used for studying pH dependence and investigating the oxidation of tetracycline by cyclic voltammetry. The 100 μM tetracycline hydrochloride solution for FIA study was prepared by an appropriate dilution.

The 1000 ppm tetracycline hydrochloride solution was prepared by weighing 0.0250 g tetracycline hydrochloride powder and transferring into 25 mL volumetric flask. The 0.1 M phosphate buffer (pH 2.5) was used for diluting this aliquot to the mark. This solution was used for the HPLC study.

3.3.9 Chlortetracycline hydrochloride solutions

Chlortetracycline hydrochloride solutions were prepared in the same manner as described in 3.3.8 except that 0.0515 g chlortetracycline hydrochloride was used instead of the tetracycline hydrochloride.

3.3.10 Doxycycline hydrochloride solutions

Doxycycline hydrochloride solutions were prepared in the same manner as described in 3.3.8

3.3.11 Oxytetracycline hydrochloride solutions

Oxytetracycline hydrochloride solutions were prepared in the same manner as described in 3.3.8 except that 0.0497 g. Oxytetracycline hydrochloride was used instead of the tetracycline hydrochloride.

3.3.12 Tetracycline mixture stock solutions

The stock solution of tetracycline mixture containing 10 ppm each of tetracycline hydrochloride, chlortetracycline hydrochloride, doxycycline hydrochloride and oxytetracycline hydrochloride was prepared by respectively by pipetting 0.25 mL of 1000 ppm standard tetracycline solution (from 3.3.8-3.3.11) into a 25 mL volumetric flask and making up the volume with 0.1 M phosphate buffer (pH 2.5)

3.3.13 Tetracycline mixture standard solutions for calibration

The standard solution of 4-tetracycline mixture at various concentrations, for calibration purpose, were prepared by appropriately diluting either 10 ppm or 1000 ppm stock solution with 0.1 M phosphate buffer (pH 2.5) into 10 mL volumetric flask. The concentration and volumes required for there preparations are shown in Table 3.1

Table 3.1 Compositions of 4-tetracycline mixture standard solutions

Final concentration of 4 standard tetracyclines solution (ppm)	Concentration of 4-tetracycline mixture stock solutions (ppm)	Volume of stock solution (μL)	Volume of 0.1 M phosphate buffer (mL)
0.01	10	10	9.99
0.05	10	50	9.95
0.1	10	100	9.90
0.5	10	500	9.50
1	1000	10	9.99
5	1000	50	9.95
10	1000	100	9.90
20	1000	200	9.80
50	1000	500	9.50
100	1000	1000	9.00

3.4 Procedures

3.4.1 Microwave plasma chemical vapor deposition system

Diamond deposition was carried out using a microwave plasma chemical vapor deposition (MPCVD) system (Figure 3.1) (ASTeX Corp., Woburn, MA), operated under the at hydrogen pressure of 115 Torr and microwave power of 5000 W for 8 h on silicon wafer. Prior to the deposition, the silicon wafer was seeded by ultrasonicating in solution containing suspension of 100 nm diamond particles for 10 min. After polishing, the substrates were rinsed in 2-propranol with ultrasonication. By carrying out the deposition at the microwave power of 3000 W, the diamond obtained had a very low conductivity, whereas with the diamond deposition at microwave power of 5000 W for longer time gave a higher conductivity. The conditions for a deposition of diamond using a microwave plasma CVD system are shown in Table 3.2

Table 3.2 Operating conditions for the deposition of diamond using a microwave plasma CVD system

Substrate:	n-Si (111)
Carbon Source:	Acetone/ Methanol (9:1)
Boron Source:	Boron trioxide
Excitation Source:	Plasma
Substrate Temperature:	Ca. 900-1000°C
Total Pressure:	115 Torr
C/H ratio:	0.03
Time Deposition:	8 hours
Film Thickness:	40-50 μM
Sample Size:	35 x 35 mm

Figure 3.1 A microwave plasma chemical vapor deposition (MPCVD) system

3.4.2 Ni-implanted boron-doped diamond electrode

Ni-implanted boron-doped diamond electrode was prepared by depositing $Ni(NO_3)_2$ solution onto the surface of the boron-doped diamond electrode. This procedures were carried out using a Rutherford backscattering spectroscopic analyzer with particle induced x-ray emission and ion implantation (RAPID). These films were implanted with 750 keV Ni^{2+} with a dose of 5x 10^{14} cm^{-2} (Tandetron 4117-HC, HVEE). The annealing was performed at 850°C for 10 min in an H_2 ambient (80 Torr).

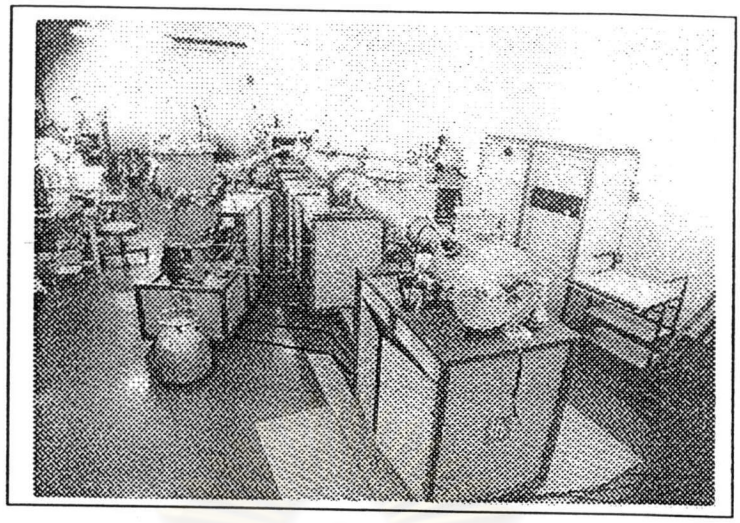

Figure 3.2 The Rutherford backscattering spectroscopic analyzer with particle induced x-ray emission and ion implantation (RAPID)

3.4.3 Characterization of Ni-implanted boron-doped diamond electrode and boron-doped diamond electrode

3.4.3.1 Digital microscope

The surface morphologies of the electrodes were examined by using digital microscope.

3.4.3.2 Raman spectroscopy

Raman spectroscopy was carried out with Ar^+ laser illumination (wavelength=514.5 nm) in a Renishaw Raman imaging microscope system (Renishaw system 2000).

3.4.4 Cyclic voltammetry

The electrochemical measurements were performed in a single compartment glass cell using a potentiostat. Figure 3.3 showed an electrochemical cell for cyclic

voltammetric experiment. An Ag/AgCl was used as the reference electrode and platinum wire was employed as the counter electrode. The Ni-implanted boron-doped diamond and boron-doped diamond electrodes (0.07 cm^2) were used as working electrodes. The working electrodes were pressed against a smooth ground joint at the bottom of the cell isolated by O-ring (area 0.07 cm^2). The exposed geometric area was 0.07 cm^2 Ohmic contacts was made by placing the backside of the Si substrate on a brass plate.

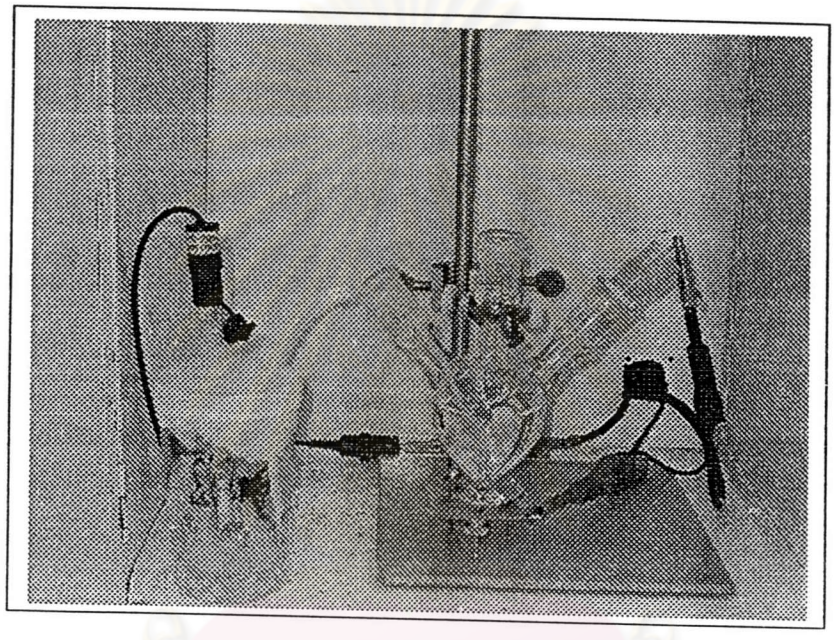

Figure 3.3 The electrochemical cell for cyclic voltammetric study

3.4.4.1 Background current

The experiment was carried out in 0.1 M phosphate buffer using the Ni-implanted boron-doped diamond electrode at the scan rate of 50 mV s^{-1}. The results were compared with those using the boron-doped diamond and glassy carbon electrodes.

3.4.4.2 pH dependence

These experiments were performed to obtain the optimum pH for tetracycline at the scan rate of 50 mVs^{-1}. Tetracycline was used as a model in this study. 1 mM tetracycline solutions were prepared in pH 2, 3, 4, 5, 6, 7, 8, 9 and 10 phosphate buffer, respectively. These solutions were studied by cyclic voltammetry.

3.4.4.3 The electrochemical oxidation of tetracyclines

The 1 mM solutions of tetracyclines in the chosen buffer solution from the previous experiment (Section 3.4.4.2) were studied using the Ni-implanted boron-doped diamond electrode by cyclic voltammetry. The experiments were repeated with the boron-doped diamond and glassy carbon electrode and the results were compared. A scan rate of 50 mVs^{-1} was used.

3.4.4.4 Effect of scan rate

Using 1 mM solutions of analytes, experiments were performed to investigate the adsorption of the analytes on the surface of electrode at various scan rates. The scan rates that were used in these experiments were 10, 20, 50, 100, 200 and 300 mV s^{-1}

3.4.4.5 The analytical performance

Stock solution of 3 mM tetracyclines were freshly prepared and diluted to a concentration range between 0.005 and 3 mM. A scan rate of 50 mV s^{-1} was used. These studies were carried out to find the linear ranges and detection limits.

3.4.5 Flow injection with amperometric detection

The flow injection analysis system used in this experiment consistes of a thin layer flow cell, an injection port with a 20 µl injection loop, a reagent delivery module or a peristaltic pump and an electrochemical detector. The mobile phase was regulated by a reagent delivery module (N_2 gas flow) or a peristaltic pump at a flow rate of 1 ml

min^{-1}. Figure 3.4 shows a thin layer flow cell for the flow injection system. The thin layer flow cell consisted of a silicon rubber gasket as a spacer, an Ag/AgCl electrode as the reference electrode and a stainless steel tube as an auxiliary electrode and an outlet of the flow cell. The experiments were performed in a copper faradaic cage to reduce electric noise.

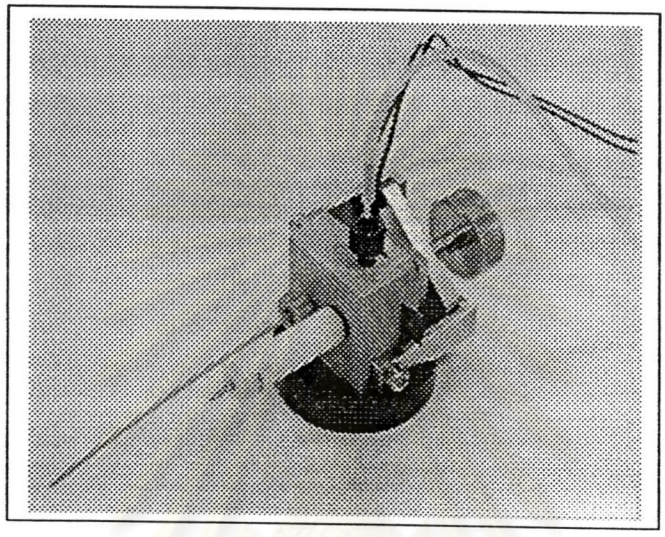

Figure 3.4 The thin layer flow cell

3.4.5.1 Hydrodynamic voltammetry

Hydrodynamic voltammetry were performed for each analyte before the amperometric determination to find the optimum operating potential. The data were obtained by recording the background current at each potential and then injecting a series of three replicate of 20 µl of 100 µM analyte solutions, respectively. The peak current after each injection was recorded, together with the corresponding background current. These data were plotted as a function of applied potential to obtain hydrodynamic voltammograms.

3.4.5.2 Calibration and linear range

5 mM stock solutions of each analyte were freshly prepared and then diluted to a concentration range from 10 nM to 3 mM. The experiments were carried out by

injection of three replicates of each concentration. The results were used to plot the calibration curve and the linear range can be obtained.

3.4.5.3 Limit of detection (LOD) and Limit of quantitation (LOQ)

The limit of detection (LOD) and the limit of Quantitation (LOQ) were carried out by injection flow concentrations of analyte solutions for three replicates under the optimal potential. The detection limit was defined as the concentration that provided a current response three times higher than the noise (S/N $\geq$3) for LOD and ten times (S/N $\geq$10) for LOQ.

3.4.5.4 Repeatability

The repeatability was studied by injecting ten replicates of analyte solutions. The repeatability is assessed in terms of the relative standard deviation (%RSD), using the following formula:

$$\%RSD = \frac{\text{standard deviation}}{\text{Mean}} \times 100$$

3.4.5.5 Real sample analysis

The proposed method was applied to real samples. The real samples were drug capsules. The standard addition method was used to determine the amount of drug in the real sample.

(1) Tetracycline hydrochloride capsule

Tetracycline hydrochloride capsule 250 mg (Vesgo, USA) was used in this study. The sample preparation is described below.

A mass of powder of twenty capsules of tetracycline hydrochloride (TC-Mycin, 250 mg) were pooled. An accurately weighed portion of the powder, equivalent to the antibiotic content of one capsule, was transferred to a 100 ml volumetric flask and dissolved in 0.1 M phosphate buffer (pH 2), filtrated through 0.45 μM Nylon membrane syringe filter. Then, the filtrated was further diluted with 0.1 M phosphate buffer (pH 2) to obtain a final concentration of 50 .00 μg/ml. (0.1 mM).

A stock solution of 0.0481 μg/ml (10 μM) of tetracycline hydrochloride in 0.1 M phosphate buffer (pH 2) and a set of six 10 ml volumetric flasks were prepared. 2.0 ml of sample solution was pipetted in each flask and then 0, 2.0, 3.0, 4.0, 5.0 and 6.0 ml of a stock solution of tetracycline hydrochloride was added to give the final concentration of standard solution equivalent to 0, 29.34, 35.82, 40.63, 44.88, 50.77 μg/ml, respectively.

3.4.6 High performance liquid chromatography (HPLC)

A HPLC equipped with a C18 column and phosphate buffer and acetonitrile as mobile phase were used to develop optimum separation. The HPLC system consists of a thin layer flow cell, an injection port with a 20 μl injection loop, a reagent delivery module or a peristaltic pump and an electrochemical detector. The mobile phase was regulated by peristaltic pump at a flow rate of 1 ml min^{-1}. Figure 3.5 shows a thin layer flow cell for the HPLC system. The flow cell is assembled with a silicon rubber gasket as a spacer, a Ag/AgCl electrode as the reference electrode and a stainless steel tube as an auxiliary electrode and outlet of the flow cell. The experiments were performed in a copper faradaic cage to minimize electric noise. The separation of all compounds was first tested with standard mixture and the condition was later tested with shrimp spiked matrix standard. Table 3.3 summarizes this optimal HPLC condition.

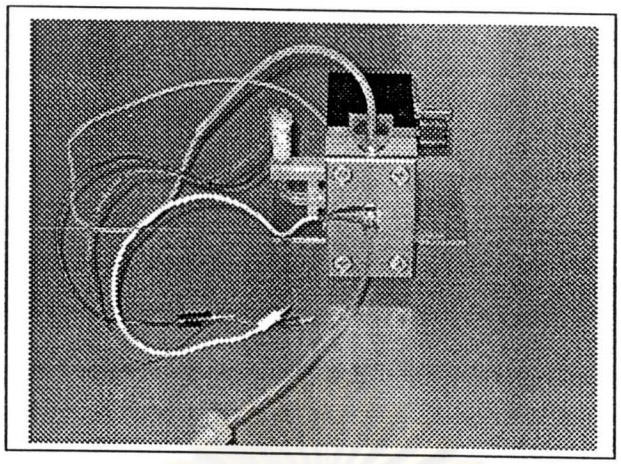

Figure 3.5 The thin layer flow cell for HPLC system

Table 3.3 Parameters of the HPLC condition

Parameter	Condition
Column	ODS-3 Inertsil C18, 5 μM 4.6 x 250 mm i.d.
Mobile phase	Isocratic elution at 0.1 M. phosphate buffer(pH 2.5) (A) and 20% acetonitrile (B)
Flow rate	1 min/mL
Injection volumn	20 μL
Detector	Electrochemical detector

3.4.6.1 Optimum potential for HPLC

Optimum potential was carried out for the 4-tetracycline mixture standard solution prior to the amperometric determination to find the optimum operating potential to be used to set the instrument for HPLC system. The data were obtained by recording the background current at each potential and then injecting a series of two replicate of 20 μl of 10 ppm tetracycline mixture standard solution using the condition in Table 3.3. The peak current after each injection was recorded, together with the corresponding background current. These data were plotted as a function of applied potential to obtain hydrodynamic voltammograms.

3.4.6.2 Calibration curve and linear range

Each concentration of mixed 4 standard tetracyclines solution in Table 1 was measured. The peak areas were plotted as a function of concentration. Each point was the average of three replicates. The Value of Slope, Retention Time, Intercept and Correlation Coefficient (R^2) of each tetracycline in the mixed standard were obtained using the conditions in Table 3.3.

3.4.6.3 Limit of detection (LOD) and limit of quantitation (LOQ)

The limit of detection (LOD) and the limit of Quantitation (LOQ) were determined by injection of analyte solution. The analytes were injected into HPLC system under optimum condition listed in Table 3.3. The detection limit was defined as the concentration that provided a current response three times higher than the noise ($S/N \geq 3$) for LOD and ten times ($S/N \geq 10$) for LOQ.

3.4.6.4 Sample preparation technique

Retail control shrimp was kept in frozen. Shrimp were sliced, blended and homogenized until the contents had the consistency of a fine powder. The homogenate was degassed overnight and kept in frozen until use.

A 2.50 g shrimp samples were placed in 15 mL capped centrifuge tubes. Fortify 2.50 g known blank shrimps were spiked with 50, 100, 500 and 1000 μL of 100 ppm tetracyclines mixed stock solution (tetracycline hydrochloride, oxytetracycline hydrochloride, chlortetracycline hydrochloride and doxycycline hydrochloride) and further diluted with each tetracycline to give the final concentration of 0.5, 1, 5 and 10 ppm , respectively.

A 12.5 mL of McIlvaine buffer-EDTA solution was added to each tube portion and blended for 30 s with a homogenizer. The resulting homogenates were shak for 10 min on flat-bed shaker at high speed. The tube was removed from shaker

and centrifuged for 30 min at 3500 rpm. The supernatant was poured into the second centrifuge tube and filterate into filter paper.

SPE cartridges and SPE apparatus were prepared. Each cartridge was conditioned with 5 mL methanol followed by 5 mL water.

12.5 mL of the filtrates were added to the columns. The column was each sequentially washed with 20 mL of water. The tetracyclines were gravity eluted with 10 mL of methanol. The shrimp extracts were evaporated under room temperature and then transferred into a 10 mL volumetric flask and the volume was brought up with 0.1 M phosphate buffer (pH 2.5). The solutions were analysed by HPLC.

3.4.6.5 Precision

Two types of precision were evaluated, namely, the intra-day precision obtained by repeat analysis of spiked sample in one day and the inter-day precision, which is obtained by analysis on different days. The study was carried out at the spiking level of 5 ppm. Three injections of each of the 3 extractions were analysed in the same day to assess the intra-day precison and were then repeated on a different day to obtain the inter-day precision.

3.4.6.6 Accuracy

The accuracy of the developed method was assessed by calculating the average %recovery, SD and %RSD that were obtained from each spiking level on 2 different days.

3.4.6.7 AOAC standard method

The method in 3.4.6.3 was used to compare with AOAC standard method 995.09 for edible animal tissues. A HPLC equipped with a C18 column and oxalic acid solution, acetonitrile and methanol as mobile phase were used for separation. The

injection volume was 20 µl and the detector was UV-Visible type. The separation of all compounds was first tested with standard mixture and the condition was later tested with shrimp spiked matrix standard. Table 3.4 list the HPLC condition for AOAC standard method 995.09.

Table 3.4 HPLC condition for AOAC standard method

Parameter	Condition
Column	ODS-3 Inertsil C18, 5 µM 4.6 x 250 mm i.d.
Mobile phase	Isocratic elution at 60% 0.1 M. oxalic acid (A) 30% acetonitrile (B) and 10% methanol
Flow rate	1.0 min/mL
Injection volume	20 µL
Detector	UV detector at 350 nm.

Sample preparation technique for AOAC standard method

Retail control shrimp was kept in frozen. Shrimp were sliced, blended and homogenized until the contents had the consistency of a fine powder. The homogenate was degassed overnight and kept in frozen until use.

A 5.00 g shrimp samples were placed in 15 mL capped centrifuge tubes. Fortify 5.00 g known blank shrimps were spiked with 50, 100, 500 and 1000 µL of 100 ppm tetracyclines mixed stock solution (tetracycline hydrochloride, oxytetracycline hydrochloride, chlortetracycline hydrochloride and doxycycline hydrochloride) and further diluted with each tetracycline to give the final concentration of 0.5, 1, 5 and 10 ppm , respectively.

A 12.5 mL of McIlvaine buffer-EDTA solution was added to each tube portion and blended for 30 s with a homogenizer. The resulting homogenates were shak for 10 min on flat-bed shaker at high speed. The tube was removed from shaker and centrifuged for 10 min at 2500 rpm. The supernatant was poured into the second

centrifuge tube. Add 12.5 mL McIlvaine buffer-EDTA solution and repeat all steps, until supernates from all 4 extractions are collected into the second tube and filterate into filter paper.

SPE cartridges and SPE apparatus were prepared. Each cartridge was conditioned with 20 mL methanol followed by 20 mL water.

50.0 mL of the filtrates were added to the columns. The column was each sequentially washed with 20 mL of water. The tetracyclines were gravity eluted with 6 mL of methanolic oxalic acid. The shrimp extracts were evaporated under room temperature and then transferred into a 10 mL volumetric flask and the volume was brought up with 0.1 M phosphate buffer (pH 2.5). The solutions were analysed by HPLC.

In this method, was obtained the study linear range, LOD, LOQ and accuracy and comparisons result of 3 method in 2 shrimp sample.