CHAPTER I

INTRODUCTION

1.1 Introduction

Many antibiotics are widely used in veterinary for preventing and treating diseases as well as for promoting growth in food producing animals. These antibiotics are aminoglycosides, β-lactams, chloramphenicol, tetracyclines, macrolides, sulphonamides, quinolines, and nitrofurans. Tetracyclines are the one of important antibiotics that we are interested in this study. Tetracyclines produced by *Streptomyces* are broad spectrum antibiotics that are active against both gram-positive and gram-negative bacteria, as well as are especially effective against *staphylococcus, Streptococcus, Pneumococcus, Gonococcus, Cholera, Dysentery bacillis, Pertussis, Rickettsia, Chalmydia, and Mycoplasma.* Because of a broad spectrum antibiotic, commercial availability and low price, the use of tetracyclines is rising in veterinary and aquaculture.

Today the shrimp farming is an important economic section in south-east asian countries, especially Thailand. The most markets of these countries are Japan, the United States and Europe, which have regulations to control quality of imports. The shrimp farming cannot avoid using antibiotics; however, tetracyclines that are the most important ones of antibiotics are generally used in the shrimp farming. The residues of tetracyclines may be found in shrimps and the levels of tetracycline residues may be unacceptable for the international markets. Therefore, the methods of the detection of tetracyclines at low levels are intensively required.

For detecting the tetracycline residues, the microbiological assays, the official methods, are most usually used, but they are complicated, time-consuming and non-specific. Therefore, sensitive and specific analytical methods for identification and

quantitation of tetracyclines are required. High performance liquid chromatography (HPLC) is normally used for this purpose.

HPLC with different detective methods such as spectrophotometry, fluorometry, chemiluminometry, and mass spectrometry has been described to determine tetracyclines. Some drawbacks of these detective methods are complicated due to the derivatization procedure, slow, and expensive. To overcome these problems, the electrochemical methods are more attractive, because they are simple, fast and low cost. One of electrochemical methods generally used as detector in HPLC system is amperometric detection. The major disadvantage of this method is adsorption of impurities and/or matrix at the electrode surface. Therefore, pulsed amperometric detection (PAD) has become one of the most important detection techniques in HPLC.

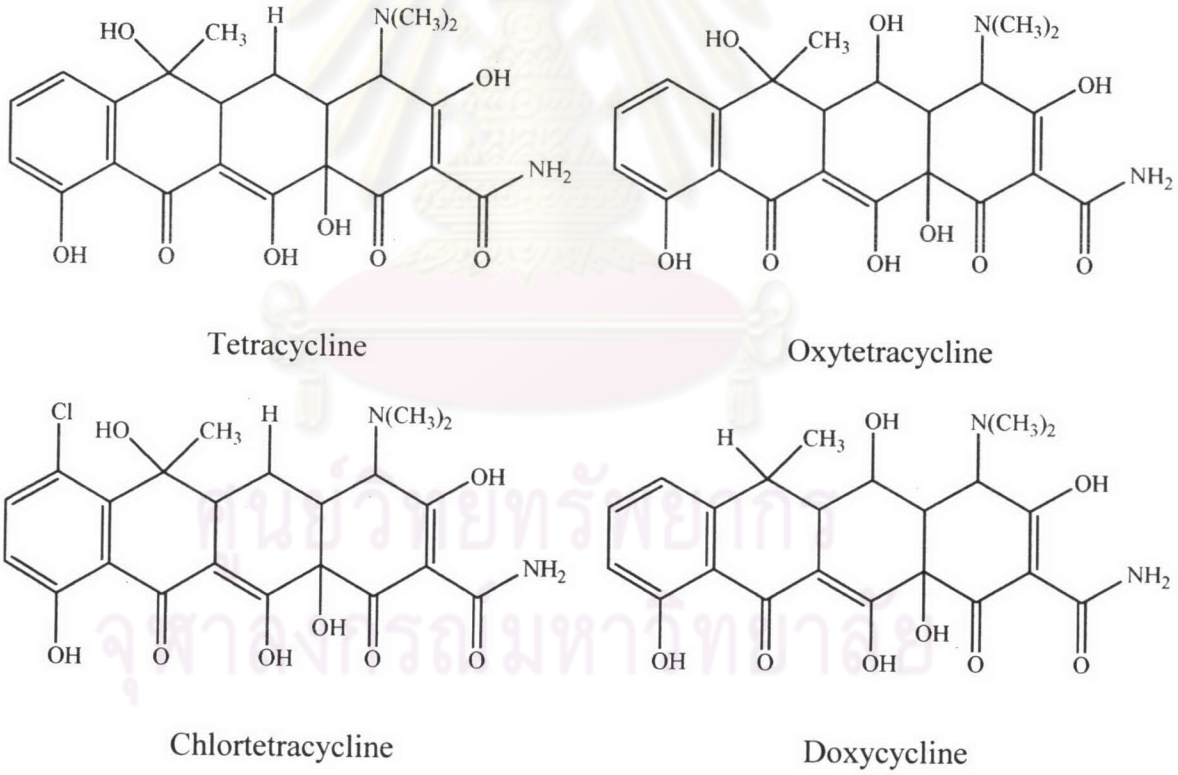

Figure 1.1 Structures of 4 tetracyclines

1.2 Literature reviews

Tetracyclines are now widely used as medicine in animals such as cows, fish, and shrimps including honeybees. Because of the broad spectrum antibiotics, tetracyclines are not only used as medicine but also as feed additive to promoting growth in animals. The use of tetracyclines may result in the presence of residues in animal tissues as well as animal product.

Early microbiological assay was used to detect tetracyclines in animal tissue and food. Microbiological assays for the detection of chlortetracycline and oxytetracycline residues in milk began in 1951 by Schipper and Peterson. The reduction of methylene blue was used as an indicator of bacterial growth. Several microbiological assays for the detection of tetracyclines residues have been described in many publications[1-3]. However, main drawbacks of these methods are non-specific, poorly sensitive, laborious, expensive and slow.

Therefore, many methods have been developed for the detection of tetracyclines in food, animal tissues as well as environment. To overcome problems from the microbiological methods, the chromatographic methods are commonly used with different detectors. Not only the chromatographic methods are important but also the sample preparation methods must work together with the procedures of separation.

Isolation of tetracyclines from various tissues has followed by homogenization of samples in the presence of extracting solvent. Then the supernatant is put through a series of manipulations to remove interferences while keeping the target tetracyclines. There is an enormous variety of extraction methods for tetracycline analysis. The most one of these methods is usually using the aqueous solutions containing masking agents to reduce the binding of tetracyclines with cations in the matrix. EDTA, oxalic acid and citric acid are commonly used as the masking agents. The use of EDTA-McIlvaine buffer, combined with the solid phase extraction (SPE) using alkyl-bonded silica cartridges for clean-up, was began in 1985 by Oka et al.[4] and also used in the current standard method for the extraction of tetracyclines from animal tissues.

In 1973, Tsuji and Robertson[5] began the use of gas-liquid chromatography for the detection of tetracyclines. Tetracyclines were derivatized with trimethylsilylation for obtaining sufficiently volatile and stable derivatives for packed-column gas chromatography-flame ionization detector. However, the derivatives of tetracyclines are unstable and sensitive to variables. There are only a few publications in this field.

A number of publications have been reported using thin-layer chromatography (TLC) to detect tetracyclines with various adsorbent plates, such as cellulose[6-8], silica gel[9-11] and kieselguhr[12, 13]. To avoid binding of tetracyclines with metals in adsorbent, EDTA[8, 12] or Na_2EDTA[9-11, 14] was been added to the adsorbent when preparing TLC plates and also in the solvent systems. In the reversed-phase TLC (RP-TLC), the solvent systems containing oxalic acid to obtain no tailing spots of tetracyclines have been reported. Several methods for the detection of tetracyclines on a TLC plate, fluorescence detection[6-8, 14], UV detection[9-11, 15] and fast atom bombardment mass spectrometry (FAB-MS)[16-18] have been reported. For the use of fluorescence or UV detector, magnesium chloride[10], diazotized *p*-nitroaniline, diazonium salt[11, 12] and diphynylpicrylhydrazyl reagents have been used as spray reagent or detection reagent. In general, TLC is simple but this method for the detection of tetracyclines need special reagents for detection as well as excessive time for TLC plate preparation.

Many publications have been reported using capillary electrophoresis (CE) for the determination of tetracyclines[19-23]. Comparison with HPLC, CE has many advantages: using a little organic solvent, short run time for separation, high efficiency, etc. Huang et al.[19] have applied CE for the detection of oxytetracycline in fish at the level of 0.1-25 ppm. Recently, the use of non-aqueous solvents has been widely used in CE. J. Tjornelund and S. H. Hanson [22] have reported that the use of metal complexation in non-aqueous CE was evaluated the separation of tetracyclines and improved detection of tetracyclines using florescence detection.

HPLC is commonly used for the detection of tetracyclines in various matrices[24]. The tetracyclines have been analyzed via separation on reversed-phase (C_8- C_{18}) modified silica solid supports, polymer- or resin-based noninorganic solid

supports and ion-exchange solid support as the stationary phase. The reversed-phase systems, especially C_8, and C_{18}, have many applications for the detection of tetracyclines in some pharmaceuticals, animal tissues, milk, and environment. A variety of buffers such as EDTA, phosphate, citric acid, oxalic acid, imidazole buffer and glycine buffer in mobile phase systems have been described for the separation of tetracyclines.

Recently, W. C. Andersen et al.[25] have reported that the determination of tetracycline residues in shrimp and whole milk by using C_8 column with 75:15:10 of 0.1% formic acid: acetonitrile: methanol and ultraviolet detection at 370 nm. The detection limits of this method in shrimp and milk were 0.025 and 0.05 mg/kg, respectively. Several detective methods for HPLC have been described for the detection tetracyclines, for example: ultraviolet (UV) spectrometry, fluorescence spectrometry, electrochemistry, and mass spectrometry.

From the literature review, there is only one research using HPLC with electrochemical detection for the detection of tetracyclines. In 1997, A. G. Kazemifard and D. E. Moore[26] reported the use of amperometric detection at the glassy carbon electrode for the detection of tetracyclines in pharmaceutical formulations, following liquid chromatography. Although amperometric detection provided high sensitivity, the major drawback was the deposition of the detection products or impurities on the electrode surface. In 2003, Palaharn et al.[27, 28] reported the use of the pulsed amperometric detection (PAD) at a gold electrode for the detection of tetracycline and applied to a flow injection system. The advantage of PAD offers cleaning and reactivating the electrode surface after a measuring cycle. A number of publications have been reported that the use of HPLC coupled with PAD could detect amino acids[29-31], carbohydrates[32], aminoglycosides[33], sugars[34, 35], creatine[36], etc. in any foods, drugs, and tissues. Moreover, PAD can be used as the detector in FIA[27, 28], CE [37, 38] and microchip[39] systems.

The boron-doped diamond (BDD) electrode is an alternative electrode that has the unique electrochemical properties [40-41] for example, (i) wide potential windows in aqueous and non aqueous media, (ii) very low background current and (iii) long-term stability of the response. BDD electrodes have been used for the detection of

several analytes such as, tiopronin[42], captopril[43] and acetaminophen[44]. Recently, BDD electrode has been chemically pretreated by electrochemical oxidation. The anodized BDD electrode retains the excellent properties of the as-deposited diamond electrode. The anodized BDD electrodes are used for the detection of many analytes such as chlorophenol[45], homocysteine[46] and tetracyclines[47].

1.3 The Objective of The Study

The main objective of this study is to develop an analytical method for the determination of tetracyclines at low levels in shrimps. The use of high performance liquid chromatography (HPLC) with pulsed amperometric detection (PAD) is proposed.

In the amperometric detection, two electrodes, namely, a gold (Au) electrode and an anodized boron-doped diamond thin film electrode are used. In the process of developing the above mentioned method the following specific objectives of the study should therefore be described.

(1) To investigate the oxidation of tetracyclines using cyclic voltammetry at both Au and anodized BDD electrode.
(2) To obtain the optimal waveform parameters for PAD through optimization at both Au and anodized BDD electrodes under HPLC system.
(3) To establish the optimal conditions for HPLC system.
(4) To study the validation of the method.
(5) To compare the developed method to the AOAC Official method.