CHAPTER II

THEORY

2.1 Tetracyclines [48]

The first tetracycline to be discovered, chlortetracycline, was isolated from *Streptomyces* in 1944. Since 1940s, other tetracyclines have been developed, including oxytetracycline, which was introduced in 1950, tetracycline hydrochloride in 1953, doxycycline in 1962 and minocycline in 1967. Tetracyclines have proved to be effective against both gram-positive and gram-negative bacteria. They are effective against a number of microorganisms and effective in treating the infectious disease syndromes. They are also used for the prevention of malaria caused by mefloquine-resistant *Plasmodium falciparum*. Not only the use of tetracyclines has been reported in clinic but also in livestock is increasing for promoting growth.

2.1.1 Applications of Tetracyclines

2.1.1.1 Human Therapy

Tetracyclines are usually administered orally, although some are also available as parenteral products. Tetracyclines have a role in the treatment of respiratory tract infections and usefulness in the acne. Moreover, tetracyclines have been used as a part of a therapy for management of gastritis and peptic ulcer disease associated with *Helcobacter pylori.* Tetracyclines are also active against malaria.

2.1.1.2 Veterinary Medicine

Tetracyclines have been used for the treatment of infections in poultry, cattle, sheep and fish. The antibiotics are added directly to feed or water or can

be administered in aerosols. Tetracyclines are also used for the treatment of infections in domestic pets.

2.1.1.3 Animal Growth Promoter

Over long periods, tetracyclines are used to improve the rate of growth of animals and feed conversion efficiency. In the United States these antibiotics were approved by Food and Drug Administration as feed additives in 1951 (chlortetracycline) and 1953 (oxytetracycline).

Tetracyclines are used in aquaculture to control infections in fish and shrimp. They are also sprayed onto fruit trees to treat infection by *Erwinia amylovara,* and used to control infection of seeds by *Xanthomonas oampestis.* There are some applications in treatment of insects. Oxytetracycline is used to treat foulbrood disease of the honey bees, which is caused by either *Bacillus larvae* or *Steptococcos pluton.*

2.2 Shrimp Farming

Today there has been a rapid expansion in shrimp farming in southern and eastern Asia, Latin America and other countries. In Thailand shrimp farming started in the early 1980s and expanded in the mid 1980s. For years, shrimp has been one of the leading food exports and is the fifth largest export ($1.7 billion annually) in terms of values. The shrimp export is significant to the Thai economy. Each year the shrimp export increases and brings most foreign currency into the country, shown in Figure 2.1. However, in 1999 the shrimp export decreased because of the diseases and water problems in shrimp aquaculture. Now Thailand is the world's largest exporter of shrimp and other exporters include India, Ecuador, Vietnam, China and Indonesia. The importers of Thai Shrimp are USA, Japan, Singapore, Taiwan, and others (Figure 2.2).

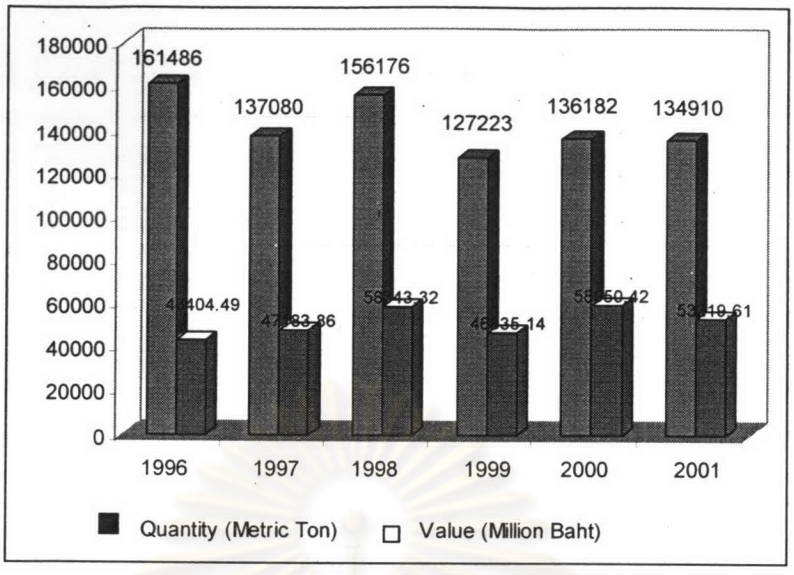


Figure 2.1 Thailand's exports of frozen shrimp in terms of quantity (Metric ton) and values (million baht) (Source from: Thai Frozen Food Association)

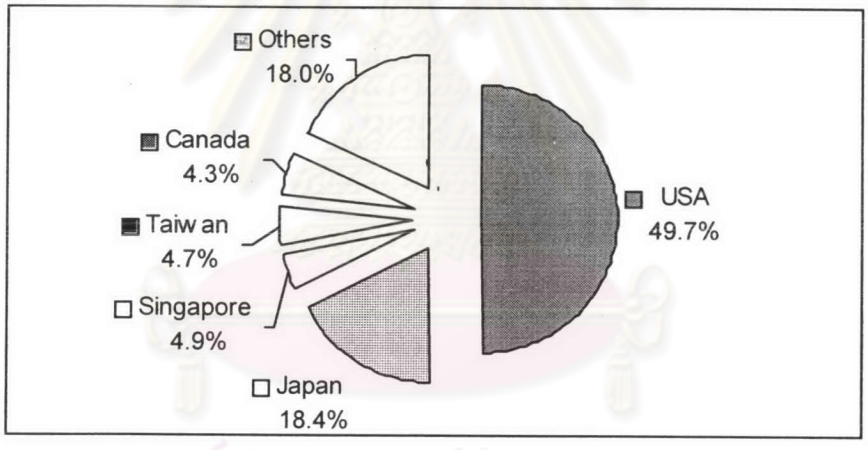


Figure 2.2 Major export markets for Thai frozen shrimp in 2001. (Source from: Thai Frozen Food Association)

Shrimp aquaculture may have some problems from diseases in shrimp [49]. Causative organisms for diseases in shrimp including viruses, parasites, fungi, and bacteria. The most of bacteria that affect shrimps including numerous species of *Vibrio* such as *Vibrio harveyi, Vibrio vulnificus, Vibrio parahaemolyticus, Vibrio anguillarum, Vibrio alginolyticus, Vibrio damsela* and *Vibrio fluvialis*. The diseases have had impact on shrimp farming: white spot syndrome or red body and white spot diseases, yellow head disease, monodon baculo virus disease, heptapancreatic parvo virus disease, and infection hypodermal and hematopoetic necrosis disease. These

bacteria can affect all parts of shrimp production including hatchery, nursery, and grow out facilities. These diseases will affect the quantity of shrimp exports.

In many countries, antibiotics for example macrolides, nitrofurans, chloramphenicol, quinolones, rifampicin, sulfonamides, and tetracyclines, are widely used in shrimp aquaculture for treatment and prevention some diseases in shrimp. Tetracyclines, due to their broad antibacterial spectrum and high potency, are commonly used as antibiotics in shrimp farming.

2.3 High Performance Liquid Chromatography (HPLC)

2.3.1 The HPLC Instrument [50, 51]

In general, the apparatus of HPLC instrument are: (a) mobile phase reservoirs, (b) pump, (c) sample injector, (d) column, (e) detector, and (f) data recorder.

2.3.1.1 Mobile phase reservoirs

A modern HPLC instrument is equipped with one or more glass reservoirs, which contain a solvent as a mobile phase. In addition, the mobile phase reservoirs are consisted of filters and degassers to remove dust and dissolved gases from the solvent.

2.3.1.2 Pump

The requirements for the HPLC pump include (i) pulse-free flows, (ii) flow rates ranging from 0.1-10 mL/min, (iii) accurate flow controled with good reproducibility, (iv) generation of high pressure (up to 6000 psi), and (v) corrosion and solvent resistant components. Reciprocating pumps are currently used in the HPLC systems. There are two options for gradient mobile phase operation: high pressure mixing which requires a pump for each solvent and low pressure mixing which requires only one pump.

2.3.1.3 Sample Injector

A manual sample injector is typically comprised of a 6-port 2-position valve that includes a 20 or 100 μL fixed sample loop is typically used. Many HPLC instruments incorporate an autosample with an automatic injector.

2.3.1.4 Column

Columns for HPLC are often made from stainless steel tubing. The lengths of columns are from 10 to 30 cm and inside diameters of 2 to 5 mm. The common packing for HPLC is prepared from silica particles and these particles are coated with thin organic films that are chemically or physically bonded to the surface. Particle sizes of column packing range from 3 to 10 μm in order to provide high efficiency (40,000 to 60,000 plates/m).

2.3.1.5 Detector

The detector for HPLC is the component that emits a response due to the eluting sample compound and subsequently signals, a peak on the chromatogram. It is positioned immediately posterior to the stationary phase in order to detect the compounds eluted from the column. There are many types of detectors that can be used with HPLC; for example, refractive index detector (RI), ultraviolet detector (UV), fluorescence detector, electrochemical detector, and mass spectrometric detector (MS).

2.4 Electroanalytical Chemistry [52, 53]

2.4.1 Potentiometry

Techniques that measure the potentials of electrochemical cells are called potentiometry. These measurements are performed with a little or no current. Potentiometric methods require a working electrode, a reference electrode and a

device for measuring the potentials. Potentiometry is the most widely used in analytical technique because of its simplicity, versatility and low cost. It is used to detect analyte in titrations, biological fluids, etc. However, the potentiometric methods play a minor role in electrochemical detection for HPLC system.

2.4.2 Voltammetry

Voltammetry is the one method of electroanalytical techniques that measures the current as a function of potential. Voltammetry is typically comprised of three electrodes: (a) working electrode, (b) reference electrode and (c) counter electrode or auxiliary electrode. The potential is applied to the working electrode as a function of time, and then the signal in the form of current as a function of potential obtained is called voltammogram. The most common waveforms used in voltammetry are shown in Figure 2.3.

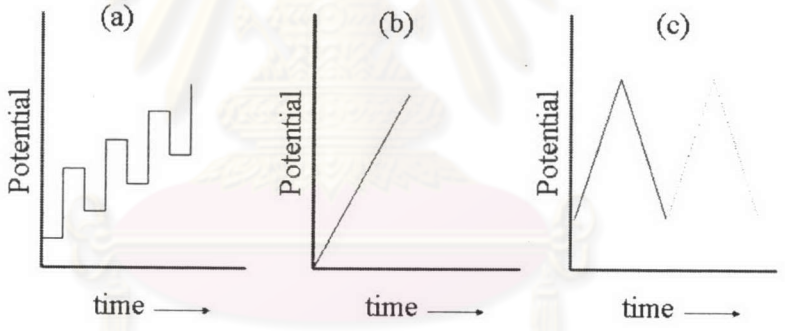


Figure 2.3 Potential-time waveforms are used in various electroanalytical techniques. Waveforms based on (a) square, (b) linear, and (c) triangular potential-time patterns are used in square wave, linear sweep, and cyclic voltammetry, respectively. [54]

2.4.3 Cyclic voltammetry

Cyclic voltammetry is the most widely used technique for the first investigation of electroanalytical study. This method allows mechanistically studying the redox systems, especially the obligation and characterization of redox couples.

Cyclic voltammetry consists of the linearity of scanning potential, using a triangular waveform (Figure 2.3.c). The triangular waveform produces the forward and then the reverse scan. The current-potential (i-E) plot is called a cyclic voltammogram (Figure 2.4). The significant parameters in cyclic voltammogram are the cathodic peak potential (E_{pc}), the anodic peak potential (E_{pa}), the cathodic peak current (i_{pc}), and the anodic peak current (i_{pa}).

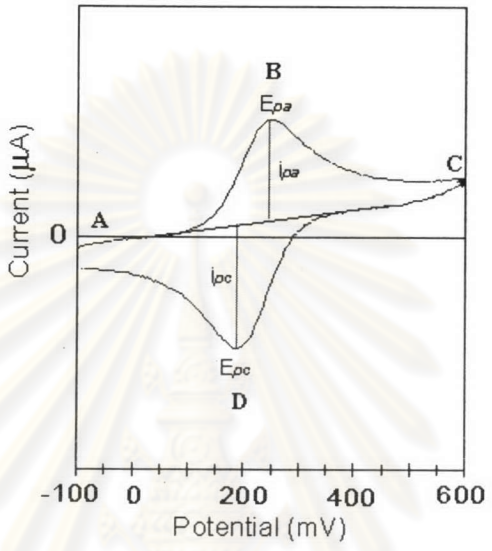


Figure 2.4 The current-potential (i-E) plot is called a cyclic voltammogram.

2.4.4 Amperometry [54]

Amperometry is an electrochemical technique which a constant potential is applied at the working electrode. The simple potential-time waveform is shown in Figure 2.5. At the applied potential, the analytes are underwent oxidation or reduction at the electrode. The current responses are directly proportion to the concentration of the analyte.

The major drawback of amperometry is not reproducibility because of some adsorption of detection products and/or impurities on the electrode surface. To obtain reproducible results, during analysis the electrode surface must often be cleaned by polishing or performing electrochemical process.

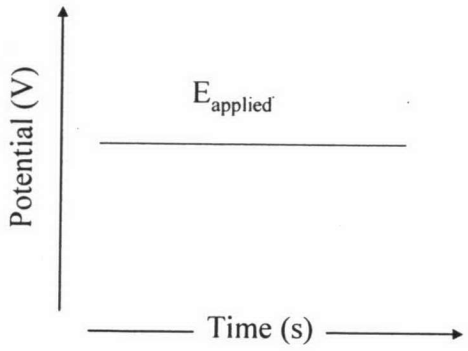


Figure 2.5 Amperometry (E-t) waveform

2.4.5 Pulsed Amperometric Detection (PAD) [54]

Pulsed amperometric detection (PAD) is amperometry that applies multiple potentials. The PAD technique is typically comprised of 3 steps shown in Figure 2.6. The triple-step potential waveform has 7 parameters: the detection potential (E_{det}), the oxidation potential (E_{oxd}), the reduction potential (E_{red}), the integration time (t_{int}), the delay time (t_{del}), the oxidation time (t_{oxd}), and the reduction time (t_{red}). At the detection step, E_{det} is chosen for the oxidation of analytes, and the current is sampled during a short time period (t_{int}) after a delay time (t_{del}). The delay time is essential to overcome double-layer charging currents. The combination of t_{del} and t_{int} constitutes the detection period. Following the detection step, the adsorbed detection products or impurities are removed from the electrode surface at E_{oxd} during t_{oxd}. In this step, the electrode surface is cleaned, but the electrode surface is then generated the oxide layer. To regenerate the oxide free layer, the E_{red} during t_{red} need to be applied to achieve cathodic dissolution of the oxide film prior to the next cycle of the waveform.

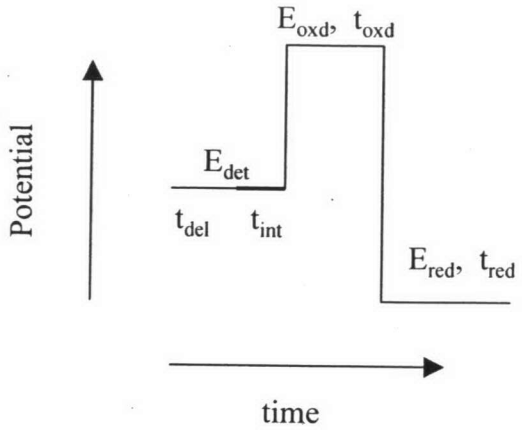


Figure 2.6 Typical PAD waveform

2.5 Solid – Phase Extraction (SPE) [55]

Analytical chemists continue to develop the sample preparation techniques that are fast, easy, safe, and cheap to provide accurate and precise data with reasonable quantitative limits. Solid phase extraction (SPE) techniques have been developed to replace the traditional liquid-liquid extraction method. SPE methods use bonded silica or resin solid sorbents packed into disposable plastic or glass cartridges or imbedded into Teflon or glass fiber disks (Figure 2.7). SPE techniques provide a ways to process sample quickly, use less solvent, isolate analytes from large volumes of samples with minimal or no evaporation losses, and provide good reproducible results.

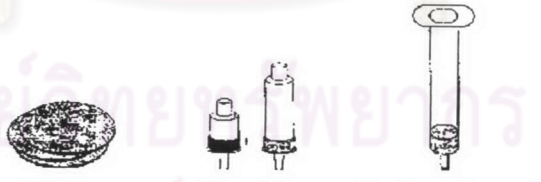


Figure 2.7 Three formats of SPE [55]

2.5.1 Mechanisms of SPE

There are three mechanisms of separation and isolation in SPE (Figure 2.8).

2.5.1.1 Reversed Phase SPE

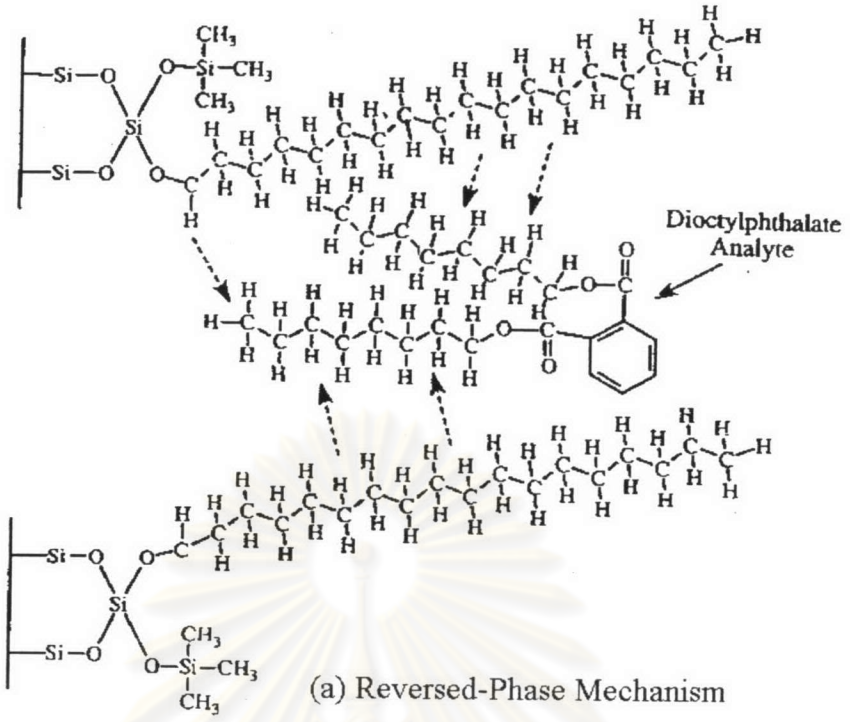
The mechanism of isolation involves a moderately polar or nonpolar analyte and nonpolar stationary phase. The analyte, which is adsorbed on the stationary phase, is due to the attractive forces between the carbon-hydrogen bonds in the analyte and the functional groups of the stationary phase. Many types of functional group of stationary phase in this mode are C_2, C_4, C_8, C_{18}, cyclohexyl and phenyl groups. To elute the analytes from the reversed phase SPE, a nonpolar solvent is used.

2.5.1.2 Normal Phase SPE

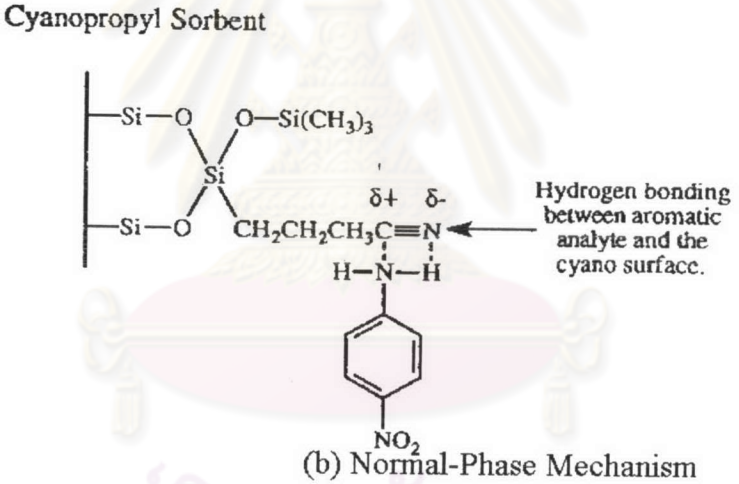
Normal phase SPE involves the sorption of a polar analyte at a polar stationary phase. The mechanism is the opposite of early mode. The types of nonbonded phases in this mode are silica, alumina, and florisil. Normal phase is commonly used as a clean-up procedure for the extraction of organic molecules in water, soil, food, etc.

2.5.1.3 Ion Exchange SPE

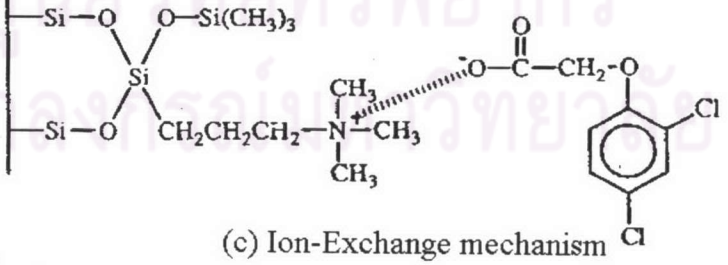
Ion exchange SPE is used for the analyte, which is charged in a solution. The sorbents must have strong cation or anion exchangers and have permanent fixed charges. In case of the isolation of cation compounds, the bonded phases are the aliphatic sulfonic acid groups or aliphatic carboxylic groups. Anion compounds are isolated on the aliphatic quaternary amine groups as the bonded phases.



(a) Reversed-Phase Mechanism



(b) Normal-Phase Mechanism



(c) Ion-Exchange mechanism

Figure 2.8 Mechanisms of SPE systems; (a) Reversed-phase, (b) Normal-phase and (c) Ion-exchange SPE [55]

2.5.2 Steps of SPE

There are four steps in the process of SPE, as shown in Figure 2.9.

Step I

The solid sorbent is conditioned or activated with the organic solvent or mixture of organic solvents and reagent water. The conditioned solvent is generally methanol and then is followed by water or buffers.

Step II

This is retention or loading step. The sample is applied to the SPE system. It is important that the mechanism of retention holds the analyte on the column while the sample is added. The mechanisms of retention include van der Waals interactions, hydrogen bonding, dipole-dipole forces, size exclusions and cation and anion exchanges. In this step, the analyte is concentrated by the sorbent. Some interferences in matrix may pass through, which give some purification of analyte, but others may also be retained on the sorbents.

Step III

To remove interferences and retain the analyte, the sorbent is washed by the appropriate solutions. If sample matrix is dissolved in an aqueous solution, an aqueous buffer or a water-organic solvent mixture may be used. If the sample is dissolved in an organic solvent, the rinsed solvent could be the same solvent.

Step IV

The last step is eluting of the analyte from the sorbent. The chosen solvent removes only the analyte, but any impurities do not remove. The analyte is collected and detected directly.

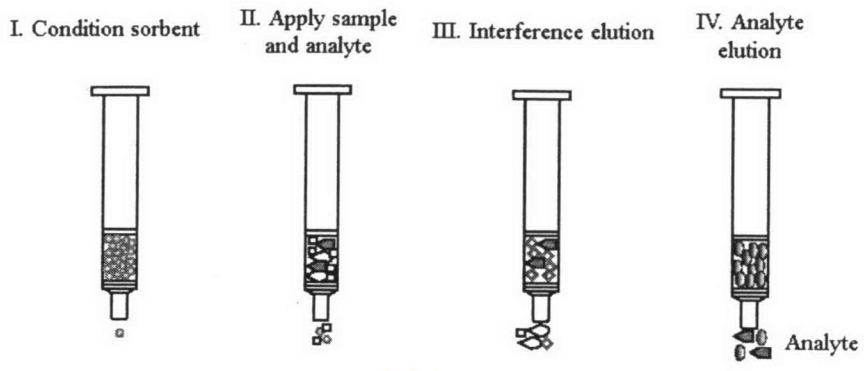


Figure 2.9 Steps of solid-phase extraction [55]