CHAPTER II

THEORY AND LITERATURE SURVEY

2.1 Electrochemical techniques [9]

Electroanalytical chemistry encompasses a group of quantitative analytical methods that are based upon the redox properties of the analyte when it is made part of an electrochemical cell. Electroanalytical methods have certain general advantages over other types of procedures. First, electrochemical measurements are often specific for a particular oxidation state of the element. For example, electrochemical methods make possible for the determination of each species in a mixture of cerium(III) and cerium(IV), whereas most other analytical methods are able to reveal only the total cerium concentration. Second, electrochemical instrumentation is relatively inexpensive. The third feature of certain electrochemical methods which may be an advantage or disadvantage, is that they provide information about activities rather than concentrations of chemical species. Ordinarily, in physicological studies, activities of ions such as calcium and potassium are of greater significance than concentrations.

Up to now, there are various electroanalytical methods that have been used for wide range applications. Fig. 2.1 shows the flow diagram of electroanalytical methods that are generally used. These methods are divided into bulk methods and interfacial methods which is wider usage than the former ones.

Interfacial methods are the methods in which the reactions occur at the interface between the surface of electrode and the thin layer of solution near the surfaces. On the other hand, bulk methods are the methods that reactions occur in the bulk of the solution that avoid the interfacial effects.

Static and dynamic methods are the major subgroups of the interfacial methods based upon the electrochemical cell being performed in the presence and absence of current. There are a few methods including potentiometric measurement that is the subgroups in the static methods, however, it is quite used until now because of their speed and selectivity.

Dynamic interfacial methods, in which the currents play an important role, are divided into controlled-potential and constant current methods. The potential of the

cell in controlled potential methods is controlled while the other variables are measured. The advantages of these methods are high sensitivity and wide dynamic range, protability, wide range of working electrode that can be used, low consumption of sample volumes, and low limit of detection (LOD).

In the constant-current dynamic methods, the current in the cells is held constant while the data are collected.

In this research, the dynamic methods especially voltammetry and amperometry were used. The details of these methods are described as the following topics.
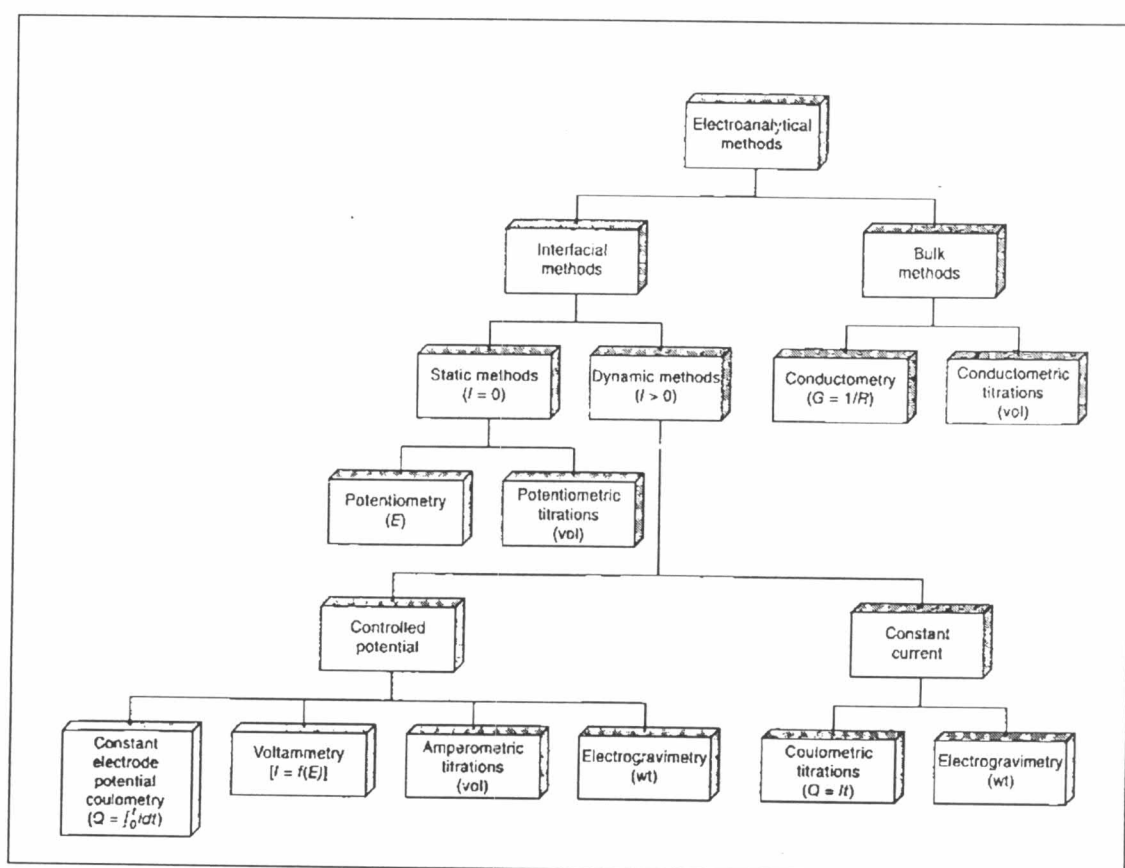

Figure 2.1 Schematic diagram of general electroanalytical methods

2.1.1 Cyclic voltammetry [9,10]

Cyclic voltammetry consists of scanning linearly the potential stationary working electrode (in an unstirred solution) using a triangular potential waveform. While the amount of oxidized species at the electrode becomes depleted

by the reduction, it is of course replaced by reduced species, which diffuses away into the solution. Hence we reverse the potential sweep from the positive side of the peak, we shall observe the reverse effect. As the potential sweeps back towards the redox potential, the reduced species will start to be reoxidized to oxidized state. The current will now increase in the negative (oxidizing) direction until an oxidation peak is reached. The direction of the initial scan may be either positive or negative, depends upon the initial composition of sample. The peak current, i_p, is described by the Randles-Sevcik equation:

$$i_p = (2.69 \times 10^5)\, n^{3/2} A\, C\, D^{1/2} \upsilon^{1/2} \tag{2.1}$$

where n is the number of moles of electrons transferred in the reaction, A is the area of the electrode, C is the analyte concentration (in moles/cm^3), D is the diffusion coefficient, and υ is the scan rate of the applied potential.

The potential difference between the reduction and oxidation peaks is theoretically 59 mV for a reversible reaction. In practice, the difference is typically 70-100 mV. Larger differences, or nonsymmetric reduction and oxidation peaks are an indication of a nonreversible reaction. These parameters of cyclic voltammograms are used for the characterization and mechanistic studies of redox reactions at electrodes.

2.1.2 Chronoamperometry and Amperometry [11]

Chronoamperometry is a technique that clearly shows the diffusion control. Its modified form is particular useful with biosensors. Instead of sweeping the potential it is stepped in a square-wave fashion (Fig.2.2) to a potential just past where peak would be in linear sweep voltammetry. The current is then monitored as a function of time. It decays because of the collapse (or spreading out) of the diffusion layer as shown in Fig. 2.2. The simplest solution to the diffusion equation which can be obtained analytically, shows that the decay is proportional to the reciprocal of the square root of time, as shown in the Conttrell equation:

$$i = \frac{nFADC_{0x}}{\pi^{1/2} t^{1/2}} \tag{2.2}$$

The current-time profile is shown in Fig. 2.2. Chronoamperometry can

be used to determine any of the variables in the equation, knowing the others. Usually it is used to determine n, A or D and sometimes C. The term $i/t^{1/2}$ can be determined from the data which form Fig.2.2. The different variables can then be obtained.

Amperometry is the common name of the chronoamperometric technique. With the certain cell and electrode configurations, the decay of the currents reaches an approximately steady state after a certain time. The current has become effectively independent of time, as defined by the equation 2.3

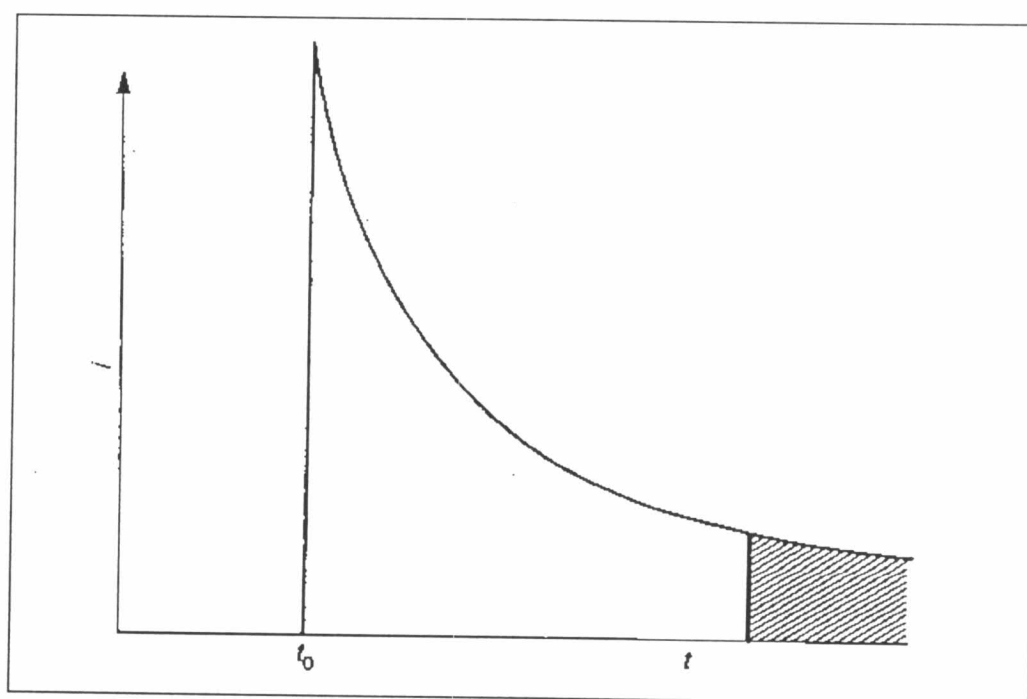$$i = \frac{nFADC_{0x}}{\delta} \qquad (2.3)$$

Figure 2.2 The current-time profile associated with a potential step redox process.

2.2 Working electrodes [10,12]

The heart of electroanalytical methods especially in the voltammetric measurements is the working electrode. The selection of electrode material depends on their properties such as the background current over the working range, the potential window, cost, toxicity, surface reproducibility, and the redox properties of the target molecule on the electrode surface, etc. Among the electrodes, mercury, noble metal, and carbon electrode are most widely used.

2.2.1 Mercury electrode

The mercury electrode is widely used either as working electrode or reference electrode. There are many forms of mercury that can be used include dropping mercury electrode, hanging mercury electrode, static mercury drop electrode, streaming mercury electrode, and mercury film electrode. The reasons hat make them used in many application are the very high overpotential for hydrogen evolution that make them possible to be used in the negative potential, smooth surface, renewable surface in dropping mercury electrode. The main drawbacks of the electrode are their toxicity and the limitation in the positive potential range.

2.2.2 Other metal electrodes

Noble metals particularly gold and platinum are the alternative metals that are widely used. The advantages of these electrodes are wide working range in the anodic potential, and very favorable transfer kinetics. On the other hand, there are many drawbacks including low hydrogen overvoltages limits the cathodic potential window, high background current due to the formation of the oxide on the electrode surface that induce the irreproducible results. The pulse ampromerometric detection including the cleaning and reactivation steps can overcome the problem of the fouling of the electrode surface.

2.2.3 Carbon electrodes

Carbon materials in the form of graphite, glassy carbon (GC), carbon fibers, and high oriented pyrolytic graphite (HOPG) are widely used in electroanalysis because of its important properties including wide variety of form available, cheap, and wide potential window particularly in the positive potential. Glassy carbon and HOPG are composed almost exclusively of sp^2 type carbon. The electronic and electrochemical properties depend on several factors such as surface preparation and the presence of oxygen-carbon functional group and microstructure. The deactivation of GC surface as a function of the time occurred when exposed to the working solution.

2.2.4 Boron-doped diamond thin film (BDD) and modified boron-doped diamond thin film electrodes [1,13,14]

Carbon materials including carbon fiber, graphite, and glassy carbon are used in various electrochemical techniques as electrode materials in electroanalytical applications. These materials have similar microstructures consisting of layers of condensed, six-membered aromatic rings with sp^2-hybridized carbon atoms trigonally bonded to one another. The use of synthetic conductive and semiconductive diamond thin film in electrochemical applications has recently been reported. Diamond possesses several technological important properties including extreme hardness, high electric resistance, chemical inertness, high thermal conductivity, high electron and hole mobilities, and optical transparency. The material is a wide bandgap semiconductor (E_g = 5.5 eV) and offers advantage for electronic application under extreme environment conditions. Each diamond's carbon atom is tetrahedrally bonded to four other carbon using sp^3 hybrid orbitals. Microstructurally, the atom arranges themselves in stacked, six-membered rings, with each ring in a "chair" rather than a planar conformation. In boron-doped films, the boron impurity atoms substitute some of carbon atoms during film growth. Diamond thin films can posse electronic properties ranging from those of an insulator to the semimetal level depending on the boron doping level.

Diamond thin films can be produced synthetically by various established deposition methods. The most popular methods are hot-filament and microwave-assisted CVD. Proper control over the source gas composition, system process, and substrate temperature allows diamond, instead of graphite or other sp^2-bonded carbon microstructures, to be grown preferentially and metastably. Two general sources of gas mixtures, including methane/hydrogen and methane/argon gas mixtures, are commonly used. First, methane/hydrogen gas mixture can be used to produce the microcrystalline morphology. This gas mixture is energetically activated to decompose into the methyl radicals and atomic radicals. These radical species chemisorb on the surface of substrate and react to form sp^3-bonded diamond, through a complex nucleation and growth mechanism. The surface of the film is terminated by hydrogen resulting in the hydrophobic surface. Second, methane/argon gas mixture can be used to produce the nanocrystalline diamond thin films. The smooth-

nanocrystalline morphology results from a very high rate of nucleation in the methane/argon gas mixtrure.

Diamond film must be doped with boron concentration of 1×10^{19} cm^{-3} or greater to make a sufficient electrical conductivity for electrochemical applications. This is most often accomplished by adding controlled amounts of diborane or trimethylboron to the source gas mixture, although achieving highly doped films ($\sim 10^{20}$ cm^{-3}) is very difficult. Boron atoms substitutionally insert for some of the carbon atom into the growing diamond lattice. These boron atoms function as electron acceptors, with the activation energy of 0.37 eV or less, depending on the doping level, and, at room temperature, contribute to the formation of free-charge carriers (i.e. holes or electron vacancies). The film's electrical conductivity is directly related to the carrier concentration and the carrier mobility. Typical carrier concentrations are in the range of 10^{18}-10^{20} cm^{-3} with carrier mobilities (holes) of 10-50 cm^2/V-s. The mobility is limited by the defect density within the film.

Several superior properties of boron-doped diamond thin films over conventional carbon electrodes were i) the film exhibit voltammetric background current and double-layer capacitances up to an order of magnitude lower than for glassy carbon leading to enhance signal-to-background (S/B) ratios in voltammetric measurements, ii) it exhibits a wide working potential window for solvent-electrolyte electrolysis in conventional aqueous media (Fig.2.3), and iii) the untreated films exhibit reversible and quasi-reversible electron transfer kinetics for inorganic redox analytes.
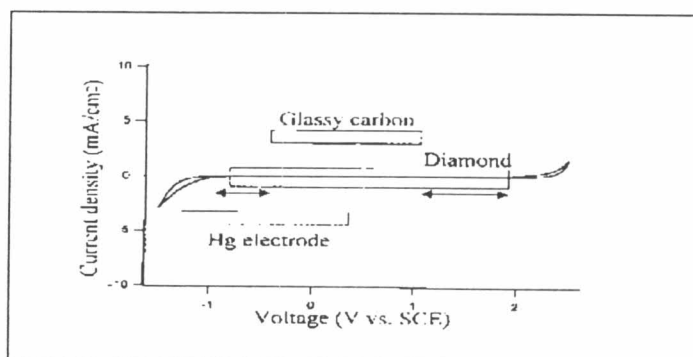

Figure 2.3 Cyclic voltammogram of 0.1 M sulfuric acid at diamond electrode. The range of potential windows for glassy carbon and mercury electrodes are shown for comparison.

For the past few years, some of the chemical modification methods were carried out in order to extend the range of the electroanalytical applications. The simplest technique was the treatment with the oxidizing acid solution such as nitric acid or chromic acid. After the modification, the hydrogen termination changed to the oxygen termination as carbonyl, ether or hydroxyl group. The carbon-oxygen surface functional groups that were produced posses a strong dipole, which the negative end points were outward from the surface. Depending on the details of the surface crystal structure and the coverage with various functional groups, this dipole can be as large as about 3.6 eV (carbonyl group) or 2.6 eV (ether group). The dipole can also affect the electrochemical behavior significantly, leading to a sizable repulsion of anions, resulting in the decrease of the electron transfer.

Oxidation can also be carried out via gas phase reaction with various forms of oxygen, including molecular oxygen, singlet (molecular) oxygen, and atomic oxygen. The reaction with molecular oxygen has been studied extensively. It begins at around 500 ^{0}C and leads to the formation of carboxyl, ether, hydroxyl and carboxylic groups, however, the graphite structure can also be produced.

Electrochemical modifications include i) anodic polarization in aqueous acid or base and ii) radical with reagent are also used. Both of these procedures could provide a surface that can be further functionalized. One of the motivations for using electrochemical oxidation, compared to chemical oxidation, is that oxidizing power could be immediately controlled over a wide potential range. Other motivations, compared to plasma oxidation, were that the process was simple because high kinetic energy was not necessary, leading to negligible surface damage. The potential window of the oxygen-terminated diamond electrodes was wider than that of as-grown electrode however the background currents for the electrochemically polarized and oxygen plasma treated became 1-2 and 3-5 times larger than as-grown electrode, respectively. One additional important advantage is that these anodized BDD electrodes retained the excellent electrochemical properties of hydrogen-terminated BDD electrode. This fact promised to increase the number of the potential applications of the oxidized BDD electrodes. Using of oxidized electrode was applied to a wide range of applications. The first example of the application was the selective determination of uric acid and dopamine in the presence of ascorbic acid [2]. The achievement of this work resulted from the enhancement of selectivity of BDD electrode by using surface oxidation. The other examples were the applications of the

oxidized diamond electrode to determination of phenol derivative [3] and homocysteine [4]. The modification of oxidized BDD electrode was firstly introduced by used of enzymes tryrosinase as the modifier [15]. Due to the hydrophobicity and low chemical conductivity of hydrogen terminated BDD electrode, it was difficult to immobilize the enzyme onto its surface. On the other hand, oxidized BDD electrodes have hydroxyl groups on the surfaces which the amino groups could be introduced by use of subsequent chemical modification with (3-aminopropyl)triethoxysilane (APTES). The covalent linkages between the amino groups on the surface and the amino groups of the enzyme can be made using glutaraldehyde or carbodiimido moieties. Tyrosinase-modified BDD electrode could be applied to detect phenol derivatives including estrogenic derivatives, bisphenol-A and 17β-estradiol, at -0.3 V vs. Ag/AgCl. Interference from direct reduction of oxygen at the electrode surface was almost neglected due to the greater overpotential for the oxygen over the most conventional electrode material. The LOD for bis-phenol-A was 1 μM. These results indicate that by use of surface oxidation and subsequent APTES treatment, any enzymes that contain an amino group can be immobilized onto a BDD electrode in the same way.

The BDD electrodes were also modified by metals and metal oxide on the diamond surface. The advantages of BDD as support of the catalysts are very low background current and large electrochemical stability. The deposition and codeposition of platinum metal on diamond films were the most studied systems due to their high interest in electrocatalysis. The preparation of Pt deposition could be achieved by several methods. Montilla and coworkers [5] reported the two methods for platinum deposition i.e. chemical deposition using thermal decomposition and electrodeposition [15-17]. Electrodeposition in multi-step process lead to well dispersed platinum on the surface in comparison to the chemical deposition. The electrodeposition of platinum followed a mechanism of progressive nucleation, which favors a higher dispersion of the platinum particles, increasing the amount of new nuclei for platinum deposition on the BDD support. Recently, Ivandini and coworkers [16] reported the fabrication of Pt-modified diamond by implantation method. Ion implantation into a material could be used to form near surface composites. The method modified the structure of a target-near surface by bombardment with heavy ions. This electrode could be applied to detection of hydrogen peroxide. The modified electrode exhibited high catalytic activity and

excellent electrochemical stability with a very low background current in comparison to Pt metal electrode.

Other metals, including copper, and nickel, were also interested to study due to their electrocatalytic activities for glucose oxidation [18]. The copper was deposited electrochemically, whereas the nickel was deposited via ion implanation. The behavior of these metals in the form of nanoparticles on BDD electrode was highly attractive in term of glucose determination, because the current for glucose oxidation was increased dramatically without the increasing of the background current.

Besides the metals, metal oxides were also used to modify on the BDD surface. One of the first metal oxides to be examined electrochemically on a diamond substrate was ruthenium dioxide [19-20]. This material was important both for electrochemical capacitor and electrocatalytic applications (chlorine evolution). Recently, vanadium oxide was also supported on the particulate BDD as a catalyst for an organic gas-phase reaction [20].

2.3 Flow injection analysis (FIA) [21]

Flow injection analysis was firstly described in Denmark by Ruzicka and Hansen in 1975. It is the simple and versatile analytical technique for automating analysis. It is based on the manipulation of a dispersed sample zone from the sample into a flowing carrier stream with detection downstream. The power of FIA as an analytical tool lies in not only the ability to combine these analytical functions in a wide variety of different ways to create a board range of different methodologies but also these methodologies are rapid and automatic, and require low amount of sample consumption.

FIA, the first generation of flow injection techniques, was most widely utilized. In its simplest form, the sample zone is injected into a flowing carrier stream of reagent. As the injected zone moves downstream, the sample solution disperses into the reagent, causing the product to form. A flow through detector placed downstream records the desired physical parameter such as colorimetric absorbance or fluorescence. The modern FIA system usually consists of a high quality multichannel peristaltic pump, an injection valve, a coiled reactor, a detector such as a photometric cell, and an autosample. Fig 2.4 shows the schematic diagram of simple FIA setup.

Additional components may include a flow through heater to increase the speed of chemical reactions, column for sample reduction, debubbles, and filter for particulate removal. FIA has been very successful in simplifying chemical assays. The main reasons for the success are the following advantages of FIA over conventional manual techniques including: i) reduced labor costs, ii) great precision, iii) higher throughput, and iv) smaller sample and reagent consumption and waste generation.
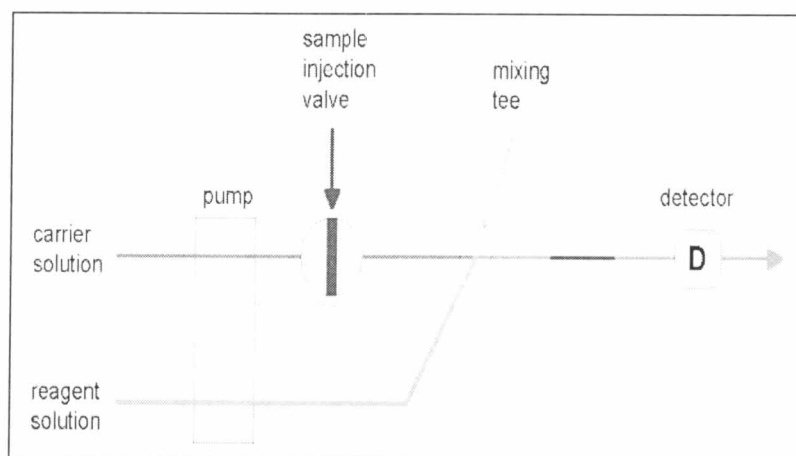

Figure 2.4 A basic flow injection analysis setup.

2.4 High Performance Liquid Chromatography (HPLC) [9,22]

Liquid chromatography is the generic name used to describe any chromatographic procedure in which the mobile phase is liquid. In conventional open column chromatography, solvent is gravity fed onto a column of large particle (about 150-250 μm), and the components of the mixture are then carried through the packed column by the eluent, separation can be achieved by differential distribution of the sample components between the stationary phase and mobile phases.

HPLC is now used in wide range of applications and offer the significant advantages in the analysis of the pharmaceutical formulations, biological fluid, synthetic and natural polymers, environment residues, a variety of inorganic substances and trace element contaminants. High column efficiencies can be achieved comparable to capillary column gas chromatography due to the greater control and choice of the both stationary and mobile phases. A greater advantage over the gas chromatography is that many detectors used in HPLC are non-destructive that the subsequent spectroanalytical studies can be achieved. Moreover, the sample

introduction can be automated and the detection and quantitation can be achieved by the use of continuous flow detectors. This feature leads to enhance accuracy and precision of the analysis. HPLC consists of sample delivery system, sample injection system, column and detector. The details of each component were described as the following:

2.4.1 Solvent delivery systems

The solvent delivery systems consists of solvent reservoirs and inlet filters, solvent degassing facilities, and one or more pumps with associated pressure and flow controls. Most systems are controlled by microprocessor or computer to be selected and monitored during the operation using simple keypad dialogues. A single solvent may be used as the mobile for isocratic elution or mixture of two to four solvent (binary, ternary and quaternary) blended together under computer control for gradient elution, where the composition of the mobile phase is altered during the chromatographic run so as to optimize the separation.

2.4.2 Sample injection system

Sample injection in HPLC is a more critical operation than in gas chromatography. The limiting factor in the precision of liquid chromatographic measurement is the reproducibility of sample introduction. The most widely used method of the sample introduction is based on the sample loop. These devices have interchangeable loops providing the choice of the sample sizes from 5 to 500 µL. Sampling loops allow the introduction of sample with the precision at pressure up to 7000 psi with the precision of a few tenths percent relative standard deviation.

2.4.3 Column

Columns are made from straight lengths of precision bore stainless steel tubing with a smooth internal finish. Typically, columns are 10 to 25 cm long and 4 to 5 mm. i.d. Microbore columns, 20 to 50 cm long with an i.d. of 1 to 2 mm, are sometimes used in order to minimize the sample consumption because the

volumetric flow rate through them is less than a quarter of that through conventional columns. The stationary phase or packing is retained at each end by thin stainless-steel frits or mesh discs of 2 μm porosity or less. Columns are packed by slurry method which involves suspending the particles of packing in a suitable solvent and 'slamming' it into the column rapidly and at pressures in excess of 3000 psi (200 bar). The choice of slurry solvent depends upon the nature of the packing and many solvent have been investigated. HPLC column needs more careful handling and storage than GC column to avoid disturbance of the packed bed. The columns should be kept by sealing at both ending when not in use and flushed with methanol prior to use. The guard column consists of a very short length of column placed between the injection port and the analytical column to trap strong retained species or particulate matter originating in the mobile phase the samples or from wearing of the injection valve. It is packed with relatively large particle (~30 μm) of the same or a similar stationary phase to that used in the analytical column and requires periodic renewal. Scavenger columns are short lengths of tube packed with large particle silica and positioned between the pump and the injection valve to reduce attack on the packing in the analytical column, especially by high or low pH buffers.

2.4.4   Detector

Unlike gas chromatography, HPLC has no detectors that are universally applicable and reliable as the flame ionization and thermal conductivity detector. HPLC detectors are of two basic types, (i) bulk property detection that respond to mobile phase properties and (ii) solute property detector that respond to some property of solute such as UV absorbance, fluorescene, or diffusion current that processed by the mobile phase.

Electrochemical detectors of several types are currently available from instrument manufacturers. These devices are based upon four electroanalytical methods including amperometry, voltammetry, coulometry, and conductometry. The advantages of these methods were high sensitivity, simplicity, convenience and widespread applicability.

2.5 Tetracycline antibiotics

Tetracyclines (TCs), produced by *Streptomyces*, are broad spectrum activities against aerobic and anaerobic including gram positive and gram negative bacteria and act by inhibit protein synthesis. Among the tetracycline families, chlortetracycline is the first member that was discovered in 1948. To date, eight TCs. are commercially available. Tetracycline (TC), Chlrotetracycline (CTC), Oxytetracycline (OTC), and Doxycycline (DC) (Fig. 2.5) are four members of this antibiotic group that are commonly used for the past forty years as therapeutic agent in human, veterinary medicine as well as a feed additive in farming animal and food producing animals because of their broad spectrum activity and low production cost. This raises the possibility that the residue is remained in animal products. For this reason, the EU has laid down maximum residue limit (MRLs) for OTC, TC and CTC which have been set at 100 ng/g in muscle, 200 ng/g in egg, 300 ng/g in liver and 600 ng/g in kidney, in order to protect humans to exposure to TCs in edible products of animal origin. The MRLs for DC has been temporary set at the same limit and is still under consideration [23].
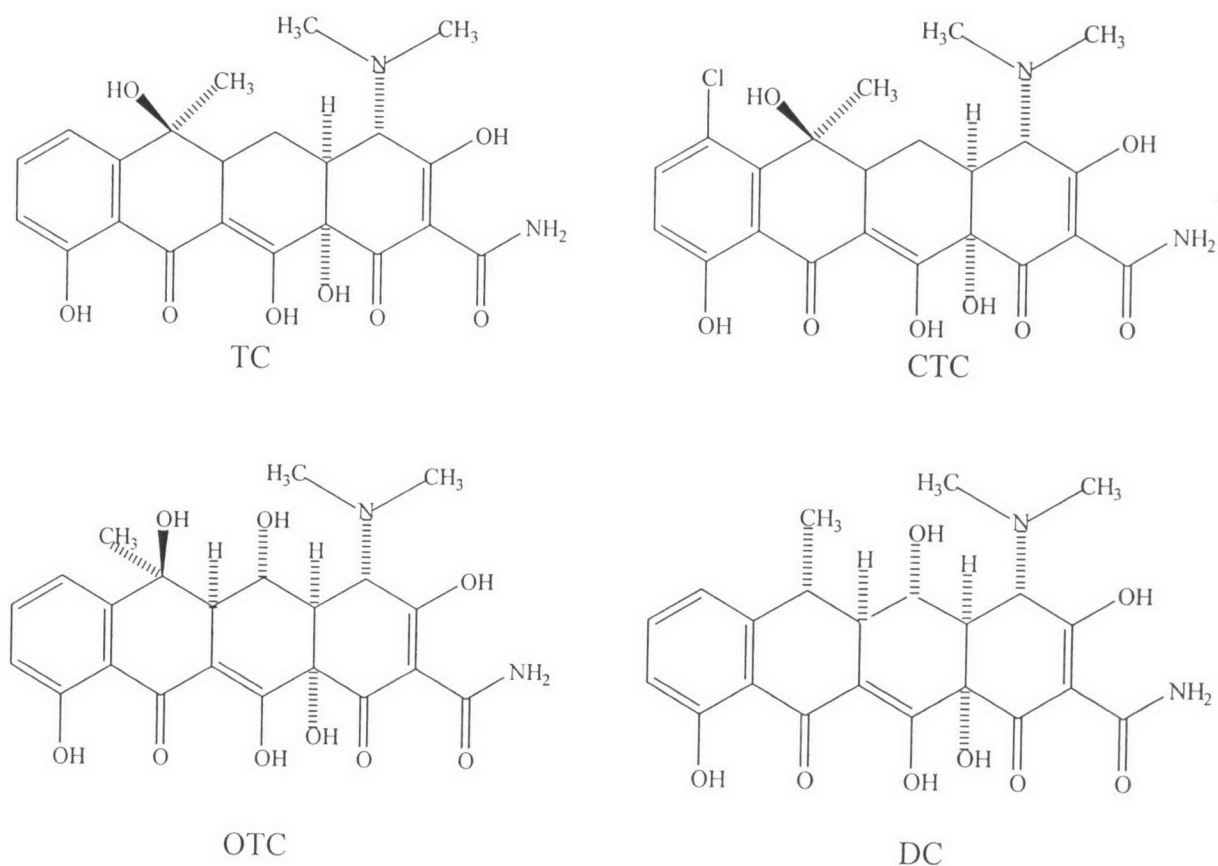
Figure 2.5 TCs structures

Among the analytical methods, microbiological methods were the most commonly used for screening of TCs [24-25]. However, these methods were non-specific and time consuming, relying on inhibition of a microorganism (usually *Bacillus cereus* or *Bacillus subitils*) in special culture medium as a mean of detection. The inhibition of the growth could occur with any antibiotic in the test solution. Therefore, the specificity of the methods is very low.

Fluorimetric and spectroscopic techniques were the alternative methods that were sensitive for the determination of TCs [28-30]. Various methods employing chelation with metal ions and fluorescene detection were developed. In 1961, Kohn [27] developed a fluorimetric method for the determination of TCs in tissues and plasma based on the formation of a calcium-barbital complex. Procedures were developed based on this phenomenon, which combine the use of fluorometry and complexometric extraction to yield high sensitive and specific method for the TCs (with the exception of OTC). The approach of Ibsen and coworkers [28] was based on the extraction of OTC from aqueous acid into amyl alcohol in biological media. The concentration of OTC was proportional to the change in fluorescence that

caused by the addition of excess ethylene diaminetetraacetate to the Mg complex of OTC. Recently, Georges and Ghazarian [29] were presented the europium sensitized fluorescene of TC in a micellar solution of Triton X-100 via the formation of an organic chelate, was used for the sensitive detection of TC. The fluorescene enhancement obtained in the micellar medium was correlated with an increase in the fluorescene lifetime of europium emission. The LOD for TC are 10 nM and 3 nM in the absence and presence of Triton X-100, respectively.

Methods based on the generation of chemiluminescene (CL) had the advantage features for the determination such as high sensitivity, selectivity and simple sample preparations. Several CL methods were also exploited for the determination of TCs [31-37]. For example, Han and coworkers [31] developed the CL method for the determination of TC with Tris(2'2-bipyridien)ruthenium(II) (Ru(bby)$_3^{2+}$). The LOD of this method was 2.0×10^{-8} g mL^{-1} for TC, 1.0×10^{-8} g mL^{-1} for CTC, and 2.0×10^{-8} g mL^{-1} for OTC, but the Ru(bby)$_3^{2+}$ was quite expensive. Lau and coworkers [33] developed the CL method based on the reaction between hydrogen peroxide and acetonitrile that its ultra-weak CL could be enhanced by TC. To speed up the measurement, simple and continuous flow injection chemiluminescene methods were developed for the determination of TCs. Alwarthan and Townshend [34] reported a bromine-based CL method for the determination of TC in a flow system. The LOD of this method was 4.3×10^{-5} M. In this method, a complex procedure was needed for the preparation of the bromine standard addition. Zheng and coworkers [35] employed in situ electrogenerated bromine as the oxidant for the determination of TC and found that the LOD was as low as 2.3×10^{-8} M. However, the CL-set-up was comparatively complicated. Syropoulos and Calokerinos [36] presented the continuous flow CL that CL was generated by the reaction between lucigenin or hexacyanoferrate and TC, allows the development of method for the determination of 0.100–1.20 µg mL^{-1} for TC, 0.100–10.0 µg mL^{-1} for CTC and 1.00–10.0 µg mL^{-1} for OTC and DC. However, these methods were sensitive for the determination of TCs in various samples, the interference from other materials cannot always be excluded. To avoid these problems, extraction cleanup methods as well as a large number of chromatography based on thin layer chromatography (TLC), gas chromatography and liquid chromatography were used.

Several sample extraction and cleanup procedures were used to isolate the TCs from the samples. Literature surveys and published reviews revealed that aqueous

based extraction with McIlvaine/ethylenediaminetetraacetic acid (EDTA) (pH 4) were mostly used as the sample preparation of TCs in many biological and tissue samples [38-39]. Aqueous based systems provided greater solubility for TCs over many organics, excluding alcohols, and are miscible with the biological matrices.

Besides aqueous systems, the organic based extractions were also established with methanoic trichloroacetic acid (TCA) [40], acetonitrile [41], ethyl acetate [42], and dichloromethane [43]. Iwaki and coworkers [40] found that TCA was more effective than other organic based extraction of TCs in serum. Acetonitrile was also used to conjugate with citrate buffer to the successive extraction of TCs from the egg samples without further extraction cleanup. Ethyl acetate was also used to defatted/cleanup samples. Chemical deproteination within biological matrices were also important and were accomplished via acid, organic solvent and heat. For example, Furasawa [44] presented the simple deproteination of milk sample with 20% TCA and acetonitrile.

Some sample preparation procedures needed to cleanup samples after the extractions. Solid phase extraction (SPE) was commonly employed to accomplish the sample cleanups. TCs can be applied to a wide range of SPE types. However, TCs were mainly performed with reversed-phase such as C_{18} [45,46] or polymer sorbent material [47]. Metal chelate affinity chromatography (MCAC) was also used for cleanup the TCs based on the TCs metal complexing properties [23,48]. These sample cleanups were time-consuming and labor-intensive. To avoid these disadvantages, the automated sample cleanup based on the on-line MCAC coupled LC system was used [42].

A number of workers reported TLC methods to separate TCs using different sorbent layers such as silica gel [49-51], kieselguhr [52],and cellulose[53]. Although, TLC was a simple equipment, excessive time was required for the preparation of TLC plate to avoid the metal binding with TCs by adding the EDTA in the absorbent [54-55]. Several detection techniques have been used for TCs determination including fluorescene and fast atom bombardment mass spectrometry (FAB-MS) [56. Oka et.al. [56] established the successive separation of eight TCs on silica gel high performance TLC using fluorescene as detection method. This method provided the LOD at 0.1 ppm. To confirm the kind of TCs on a TLC plate, TLC coupled with fast atom bombardment mass spectrometry was used to investigate the TCs species and the residues in food samples.

A few researchers established the gas chromatography to separate TCs. One of the examples was the use of gas liquid chromatography, developed by Tsuji and Robertson [57], for the separation and quantitation of TC antibiotics, their isomer and derivatives. This method was quite complicate due to the requirement of derivatization process under certain condition. The degradation compounds of TCs, formed in the derivatization step, were facilitated for the determination by gas liquid chromatography.

Capillary electrophoresis (CE) has gained increasing interest as a powerful analytical tool because of its extensively high sensitivity and resolution, its short analysis times and its low sample and solvent consumption relative to HPLC. CE was used to analyze drug residue at the ppb levels due to the low sample employed (< 100 nL). Among the publications, only a few reports dealed with the residual analysis of TCs in food. Chen and Gu [58] developed CE for the simultaneous determination of OTC, TC, CTC, and DC in milk, serum, and urine by deproteinized using succinate buffer. The sample was further cleaned up by MCAC. This cleanup procedure was highly effective to eliminate the interference substance in sample. Recoveries of TCs from sample were 40-84% with a relative standard deviation of 3.3-9.1%. Non-aqueous CE was developed by Tjonelund and coworkers [59] to separate of the TCs in milk and plasma. This method was based on metal complexation in non-aqueous and also improved the detection of TCs using laser induced fluorescene detection. Magnesium was the most suitable ions for the separation of TCs and relatively low current was generated making it applicable at high concentrations of complexing metal ion resulting in achieving of the LOD low down to the ppb level. Recently, a new procedure of non-aqueous CE procedure based on the addition of anionic carboxylic surfactant into the basic amphiprotic organic solvent in order to form the neutral surfactant aggregate was performed [60]. The neutral surfactant aggregate acted as a new pseudostationary phase. The new pseudostationary phase improved the electrophilic resolution of charge substances. The sensitivity of this method was improved using a flow manifold coupled on-line to the CE system in order to preconcentrate the analytes. The LOD ranged from 50 to 90 ppb, recoveries ranged from 97 to 104%, and precision ranged from 5.4 to 7.0% were obtained.

Many HPLC methods were described for the determination of TCs using silica gel, silica based and polymer based stationary phase. One of the main interests was to find the robust systems for the evaluation of TCs in many samples. Among of these

materials, C_8 and C_{18} were most widely used to separate TCs in many analytical applications [61-62]. C_8 modified silica gel column was suitable for analyzing the parent TCs while C_{18} modified silica gel column was suitable for the impurities of TCs. However, these columns encountered a serious problem that TCs bind irreversibly to the silanol groups on silica material resulting in the peak tailing and low column effeciency. Use of the oxalic acid containing mobile phase to suppress the dissociation of TCs molecules have been overcome the problems and also enabled a good separation [64-65]. The use of end-capped modified silica gel synthesized from 99.99% purity silica gel column was also enabled to separate TCs without adding the oxalic acid [66]. However, the reversed phase silica based columns were unstable when the pH less than 2, at which highest column efficiencies obtained. Thus, the use of polystylene divinyl benzene copolymer was the alternative ways to used because this stationary phase was very stable in a wide range of pH condition (pH 1-13) [67].

Not only the column material, but detector was the heart of the HPLC to accomplish high sensitivity and accuracy for the determination of TCs. Many of detectors including UV-Vis [68.69], fluorescence [70-72], chemiluminescence [73], mass spectrometry [74] have been used in HPLC system. Some of drawbacks of these methods were the complication of the methods due to the requirement of derivatization before the measurement and expensive instrument. Electrochemical method was an alternative method that can be used as the detector in many systems because it is cheap, simple and sensitive. A few publications used the electrochemical as the HPLC detectors. One of the examples was the method developed by Karemifard coworkers in 1997 [75]. The technique permitted a simultaneous determination of trace amount of TCs and their common contaminants in pharmaceutical formulations. The working electrode was glassy carbon electrode. However, one of the drawbacks of glassy carbon electrode was large background current. To overcome these problems, many researches tried to find the new electrode materials. The synthetic boron-doped diamond (BDD) have been used as electrode material for the past 20 years with their unique properties including low background current, slight adsorption of polar molecule and wide working potential window. BDD have been used for detection of several organic molecules such as acetaminophen [76], penicillamine [77], tiopronin[78], captropril [79], and lincomycin [80]. To extend and improve the sensitivity of BDD electrode, the

electrodes were pretreated by oxidation with acidic or alkaline solutions. The anodized BDD electrode retained the excellent properties of as-deposited BDD electrode. The anodized BDD electrodes have been employed for the detection of several compounds such as homocysteine [4], uric acid [2], polyaromatic hydrocarbon [81], and glutathione and glutathione disulfide [82].

2.6 Hydrogen peroxide

Hydrogen peroxide is a naturally occurring water-like liquid that has many practical applications due to its strong oxidizing properties and is therefore powerful bleaching agent. Hydrogen peroxide is commonly used (in very low concentrations, typically around 5%) to bleach human hair. It burns the skin upon contact in sufficient concentration. In lower concentration (3%), it is medically used for cleaning wounds and removing dead tissue. The Food and Drug Administration has approved 3% hydrogen peroxide ("Food Grade", or without added chemical stabilizers) for using in mouthwash.

Hydrogen peroxide has been used as antiseptic and anti-bacteria agent for many years. Hydrogen peroxide is also used by doctors and dentists in many hospitals for sterlising and cleaning. Advocates of the product claim that it can be diluted and used for "hyper-oxygenation therapy" to treat AIDS and cancer

Hydrogen peroxide is a molecule interest in many fields of applications. A numerous reports have appeared on several analytical methods for its determination including iodometric titration, fluorimetry, chemiluminescene and electrochemical methods.

In 1986, Kieber and Helz [83] developed iodometric titration based on the reaction between iodide and hydrogen peroxide for determination hydrogen peroxide in natural waters. Iodide could be oxidized quantitatively to iodine at pH 4 by aqueous hydrogen peroxide in the presence of $(NH_4)_6Mo_7O_4.4H_2O$. The generated iodine was further reacted with phenylarsine oxide. The remaining of phenylarsine oxide was titrated with standardized iodine titrant to an amperometrically determination end point. The difference between titrations with and without the enzyme catalase was proportional to the hydrogen peroxide concentration. The LOD of the method was 0.02 μM. However, this method was not sensitive.

Several techniques including fluorometry and chemluminescence (CL) were used to determine hydrogen peroxide in both gas and liquid sample with a high sensitivity and selectivity. Fluorimetry based on hourseadish peroxide (HRP)-scopoletin method was most widely used because the method was sufficiently sensitive to determine hydrogen peroxide at the nanomolar level. Holm, et.al [84] modified the fluorometric HRP method for the field determinations of hydrogen peroxide in groundwater. The LODs from 3.6 to 44.6 nM were obtained. However, the storage period more than 1 hr caused serious errors and irreproducible results. The immobilization could improve enzyme stability that made the enzyme easier to handle and used small amount of an expensive enzyme. Recently, Li and Townshead [85] reported the simple procedure of immobilization of HRP on the inner wall of teflon tubing by physical adsorption. The HRP immobilized tubing was used to form a reaction coil that was inserted into the flow system. The immobilized reaction coil was stable for at least one month by kept in phosphate buffer pH 5.8 in refrigerator. Linear range from 4 to 80 ppb and LOD of 3 nM were obtained. In the same year, Sakuragawa and coworkers [86] established the immobilized enzyme which consisted of chitosan beads as a carrier and HRP as enzyme. This method could be applied for detection of microamount of hydrogen peroxide in environment samples. The LOD of this method was about 50 ng cm^{-3}.

Chemiluminescence (CL) is one of the luminescent phenomena and it can be defined as the light emission produced by chemical reaction. Most of chemical sensors were based on the CL with luminol and peroxyoxalate as CL reagent [87].

Kok et al developed the method based on luminescene CL technique [88]. The oxidation of luminol was catalyzed by Cu(II) ions that generated light with the maximum wavelength about 450 nm. The concentrations of hydrogen peroxide in the atmosphere were measured by several researchers using this technique. Yoshizumi et.al [89] developed the method of Kok et al. using haemin as a catalyst for the determination of hydrogen peroxide in a blood component. The CL response to hydrogen peroxide provided a good linearity, the LOD lower than 2.94 nM, and the sensitivity higher than the original method by about 10 times. The interference from SO_2 could be avoided by collection solution above pH 10, however, the ozone was shown the significant interference at about 2% of concentration in terms of molar ratio. This method was applied to measure the hydrogen peroxide in rain water collected in Tokyo. In 1998, Janasek and cowork [90] presented the novel CL sensors

for the selective flow injection analysis in the presence of luminol at cobalt and copper foils. The CL can also be induced electrochemically and provided the linear range of 0.1-200 µM on cobalt and 5-2000 µM on copper. To avoid the interference in the samples, the detector was combined with a thin layer gas dialysis cell. The obtained linear range was 0.5-100 µM. The interference from a more than 100 fold excess of EDTA, α-ketocaboxylic acid and peroxodisulfate could be excluded however peroxomonosulfate concentration greater than 10 fold excess of hydrogen peroxide cause a significant bias which resulted from the hydrolysis of peroxomonosulfate. .

Recently, a reagentless CL flow biosensor was developed by Zhou and coworkers [91] based on the immobilization of HRP on biocompatible chitosan which was formed on the glass coil to construct a transparent sensing flow cell. The CL reagent luminol was immobilized on anion-exchange resins and packed in a glass to determine hydrogen peroxide. The result indicated that HRP retained a native structure in the biocompatible chitosan film. This method provided the sample throughput of 120 hr^{-1} with small sample volumes (30 µL), two working linear region from 1.0×10^{-7} to 1.0×10^{-5} M and 1.0×10^{-5} to 2.0×10^{-4} M, the LOD of 4.0×10^{-8} M, a standard deviation of 1.1% for the 4.0×10^{-6} M hydrogen peroxide in 31 repeated measurements, and the storage period of 3 months.

A CL detection system for flow injection determination of hydrogen peroxide was established by Ding and coworkers [92] based on the reaction of peroxyoxalate and Rhodamine B. The LOD of 50 nM was obtained In the same way, Katayama and coworkers [93] described the hydrogen peroxide determination by flow injection analysis with bis[4-nitro-2-(3,6,9-trioxadecyloxycarbanyl)phenyl]oxalate and sulphorhodamine 101 and measuring the CL intensity at 550 nm. This method provided the LOD of 3 nM. Recently, Quabβ and Klockkow [94] established the procedure based on the reaction of oxygen with bis(2,4,6-trichlorophenyl)oxalate (TCPO) in the presence of Fe(II) for the determination of Fe(II) and hydrogen peroxide in atmospheric rain with high sensitivity and the LOD lower than 100 nM Fe(II). This method was performed a new static fibre optic luminometer with a flow injection system and applied to rain water sample. In order to avoid Fe(II) interfere the determination of hydrogen peroxide, two masking agents 2,2'-bipyridine and 1,10-fenantroline were used, resulting in a higher stability of the formed complex.

Besides the TCPO that were mentioned above, there were other CL reagents in the peroxalate groups that were also used for the determination of hydrogen peroxide. For example, in 1994, Stigbrand and coworkers [95] presented the CL based on the reaction of 1,1'-oxalydiimidazole (ODI) and hydrogen peroxide applied to a flow system to detect hydrogen peroxide in water. The flow system was simplified by the use of an immobilized fluorophore (3-aminofluoranthene) on an acrylate polymer. The results could be concluded that ODI is about 10 times more sensitivity than TCPO (catalyzed by imidazole) with the LOD of 10 nM. In the next two year, the same workers [96] developed the method based on ODI reaction using flow injection method. The atmospheric samples were collected into a diffusion scrubber and detection. This method was selective for the hydrogen peroxide in the sample that presence of the organic peroxides interference especially methyl hydroperoxide and hydroxymethyl hydroperoxide that were the most abundant in atmospheric sample. The LOD was 23 ppt and linear range up to 3.37 ppb. These methods were the traditional methods that used for the accurate determination of hydrogen peroxide in a wide range of sample. Many of these procedures were often time-consuming and were unreliable for some food and biological samples because the final solutions of the measurements were not clear. Among the analytical techniques, the electrochemical methods could overcome these drawbacks, especially an amperometric biosensor due to its high sensitivity and simplicity.

Different electrode systems, which included either simple metals such as platinum, gold, iridium and palladium or electrodes modified with enzymes and Prussian Blue analogues have been investigated. Platinum was commonly used because of its high reactivity toward hydrogen peroxide oxidation. The use of platinum offered a better reproducibility of electrochemical response, compared to gold electrode. However, a great drawback of this approach was the high overvoltage of hydrogen peroxide (about 500 – 700 mV vs. Ag/AgCl) that tend to interfere with many electroactive substrates (eg. ascorbic acid and uric acid), which were presented in real samples.

One of the most common ways to overcome this problem was the use of the bioelectrocatalytic reduction of hydrogen peroxide such as peroxidase, namely horseradish peroxidase (HRP) which catalyzed the reduction of hydrogen peroxide. The HRP allowed the direct electron transfer between its active site and the electrode surface. Although this approach exhibited good sensitivity and accuracy, it suffered

from some important shortcomings such as high cost, low stability and the limited binding of HRP to solid surfaces [97]. The finding of other mediators to fabricate novel hydrogen peroxide sensor was quite important. For this perspective, Myoglobin (Mb) which was known to have some intrinsic peroxidase activity due to its close similarity to peroxidase, was used. Therefore, it might be possible to employ Mb containing a heme group that serve as the active center to catalyze the reduction of hydrogen peroxide. However, electron transfer between Mb and bare solid electrodes is usually slow and the protein was irreversibly denatured. Thus, the searching for the way to develop a Mb-based electrode with well behaved electrochemistry and good stability was necessary. Recently, Yang and coworkers [98] were presented the hydrogen peroxide biosensors based on Mb/colloidal gold nanoparticles immobilized on glassy carbon electrode using nafion film. This electrode system provided excellent electrocatalytic responses to the reduction of hydrogen peroxide. The linear range from 1×10^{-6} to 9×10^{-5} M and the LOD of 5×10^{-7} M (S/N = 3) were obtained.

Other biosensors were also used for detection of hydrogen peroxide including microbial peroxidase, hemenonapeptide as a peroxidase substitute, liver tissue, pineapple and bacteria based biosensors.

Mascini and coworkers [99] presented a liver-tissue-based biosensor in which the electrode consisted of a 0.1 mm thick slice of bovine liver. The biosensor was held by a nylon net over the membrane of a standard oxygen electrode. The catalase in the liver decomposed hydrogen peroxide in the solution to give oxygen, which was sensed by the electrode. The LOD was 10 μM. This sensor decreased rapidly in response after use for 8 days.

Rechnitz and coworkers [100,101] presented grape-tissue-based, and tobacco callus tissue biosensors for the determination of hydrogen peroxide. The electrode response remained stable more than 17 days for grape based biosensor. The calibration graph of grape-tissue electrode was linear from 10 to 500 μM hydrogen peroxide.

Bacterial based biosensor for determination of hydrogen peroxide has been presented by Tai and coworkers [102]. This electrode was immobilized the living bacteria strain *Bacillu subtills* AS 1.398 coupled to a clark type oxygen electrode. The hydrogen peroxide was catalyzed by catalase (EC1.11.1.6) from the bacteria to produce hydrogen peroxide and O_2. The calibration graph was rectilinear

in the range 10 to 2000 μM hydrogen peroxide. The bacterial sensor provided a lifetime more than 2 months, and there was no significant interference from substances such as amino acid, metal ions, polyphenols, glucose, and ascorbic acid. This sensor provided the advantages in term of linearity, lifetime, and selectivity, especially as the bacteria could be used as a catalase enzyme membrane that could reproduce itself.

Using of inorganic mediators which catalyzed the oxidation or reduction of hydrogen peroxide was alternative mediators that was preferred over HRP. The methods were used for the assembling of oxidase-based biosensors. The sensor decreased the applied potential in order to avoid many electrochemical interferences. In this perspective, hexacyanoferrates, particularly Prussian Blue (ferric hexacyanoferrate-PB) have been found a large use. In 1978, Neff [104] firstly introduced the electrochemical behavior and the successively deposite of thin film on a platinum foil. Up to now, there were many publications developed the method to prepare the PB as well as the many electrodes used as substrates. For example, Itaya and coworkers [104] presented the simple preparation of PB modified electrode based on electrochemical reduction of ferric-ferricyanide on different electrode materials (SnO_2, platinum, gold and glassy carbon).

The most important disadvantages encountered in the use of PB in amperometric biosensors were poor working stability and sensitivity to pH-changes. The increase of solubility of PB at pH > 6, due to the formation of $Fe(OH)_3$, restrained its use in bioassys coupled with enzymes which have optimum pH at slight acidic to neutral region.

Similar to ferric hexacyanoferrate, transition metal (i.e. chromium, cobalt, vanadium and copper) hexacyanoferrate (MHCF), a class of polynuclear inorganic compounds, were also used as electrocatalysts for hydrogen peroxide. The most significant advantage of a mixed-valence based biosensor was its low operational potential at cathodic region. This low overvoltage of the cluster resulted from the proper selection of the two metal centers as well as the bridging ligand in the mixed-valence cluster. Thus, the properly designed molecular cluster limited interference from the easily oxidizable compounds such as ascorbic acid, catecholamines, or uric acid. Chromium (III) hexacyanoferrate (II) was the one example that could fulfill these advantages. The first design based on glassy electrode

was presented by Lin and Shih [106]. The cluster was prepared by cyclic voltammetry in the solution containing chromium nitrate and potassium hexacyanoferrate on glassy carbon electrode. The LOD of the sensor was 3.0×10^8 M and linear range up to 1.3 mM. Recently, the same workers also used chromium (III) hexacyanoferrate (II) as glucose a biosensor [107]. The same workers also demonstrated the feasibility of utilizing a chromium (III) hexacycanoferrate (II) for the biosensing applications. The cluster was generated by cycling potential in a solution containing enzyme and a pair of carefully selected metal ions and the cyanide as a bridging ligand. This process introduced the formation of chromium (III) hexacyanoferrate (II) on the glassy carbon electrode surface which provided a suitable energy surface and was proper for the catalysis of hydrogen peroxide with limited number of interferences.

The finding of new electrode based material was an interest topic to investigate. BDD was the new carbon based material that have been emerged to use as electrode material for the past few years for many electroanalytical applications due to its unique electrochemical properties such as low background current, slight adsorption of molar molecular and wide working potential window.

Because BDD was found completely inactive for the catalytic reaction, the electrode was highly supposed that BDD films could be the best support for the deposition of metal electrocatalysts. Modification of BDD electrode with metal has attracted much attention since it has been reported that the dispersion of metallic particles within organic polymer and inert surface caused a drastic increase of the catalytic activity and sensitivity of the electrodes. There were many methods for the preparation of some metal-modified BDD electrode for electrochemical applications such as chemical precipitation, electrochemical deposition and metal implantation. Recently, Tereshima and coworkers [108] presented the hydrous iridium oxide modified BDD electrode for catalytic sensor application. This sensor could be applied for use as hydrogen peroxide and pH sensors.