# CHAPTER I

## INTRODUCTION

Quantitative determination of amino acids usually involves separation by liquid chromatography and various detection systems. A number of these are available commercially : these include refractive index, UV absorption, fluorescence and electrochemical detection(1). Spectrophotometric detection with ninhydrin is widely used (2), but other methods have also developed, such as fluorimetric detection with an o-phthalaldehyde (OPA) reagent (3-4). The main disadvantage of the detection system is associated with the post-column volume introduced by the reaction coil. This can, if excessive, lead to peak broadening and subsequent loss in resolution, which is especially noticeable for rapidly eluting peaks. Another widely used approach is pre-column derivatization of amino acids, whereupon the derivatives are separated by reversed-phase high performance liquid chromatography (HPLC). Commonly used are derivatives with phenylthiohydantion (PTH) (5-8), 1,4-dinitrophenol(DNP)(9), 1-N,N'-dimethylaminonaphthalene-5-sulphonyl (Dns)(10-12) and OPA(13-15). These derivatives can be detected by UV absorbance (PTH,DNP) or fluorimetric (Dns,OPA) measurement.

There have been reported on the selective detection methods for underivatized amino acids such as potentiometry with a copper-selective electrode has been used as a potentiometric detector for amino acids in HPLC. A copper(II) solution was added after column and the loss of copper (II) activity caused by the complexation

reaction was taken as a measure for the amino acid concentration in the column effluent(1). Potentiometric detection of amino acids in HPLC is also possible with copper wire and tubular electrode(16). The main disadvantage of these potentiometric measurements is the fact that, according to theory, the calibration curves are inherently non-linear. Only when a large excess of copper (II) ions is added to the solution,the electrode response can be expected to be proportional to the concentration of amino acid. However, in this case the changes in electrode potential will be small. For direct quantitative measurements, an amperometric method would be preferable. Amperometric detection of amino acids was studied by Kok, et al. (17). Their study was mainly on kinetics reaction at the copper rotating disc electrode.

In this present study, the behaviour of an amperometric detector with copper electrode towards amino acids was investigated in batch system and flow injection system. The application of this electrode to be a detector for HPLC was also examined. Before the aims of this thesis are described in more detail, introduction to amino acids, voltammetry and amperometry, the flow injection analysis and HPLC are reveiwed with some of the theoritical aspects and detection method used.

## 1.1 Introduction to Amino Acids

Proteins are essential components of all living cells. During hydrolysis, proteins break down into amino acids. The significance of amino acids is no limited to their being components of proteins but, in metabolism, they undergo many other reactions and supply precursor for other endogeneous substances (e.g. hemoglobin of blood) (18).

Amino acids are the substances that possess two characteristic functional groups: the amino group($-NH_2$) and the carboxyl group ($-COOH$). All amino acids that occur as components of proteins have their amino group in α-position to the carboxyl group, namely α-amino acids. The α-amino acids are the most significant in the biological world, since they are the fundamental units from which proteins are constructed. The general formula of an α-amino acid is shown in Figure 1.1(a)

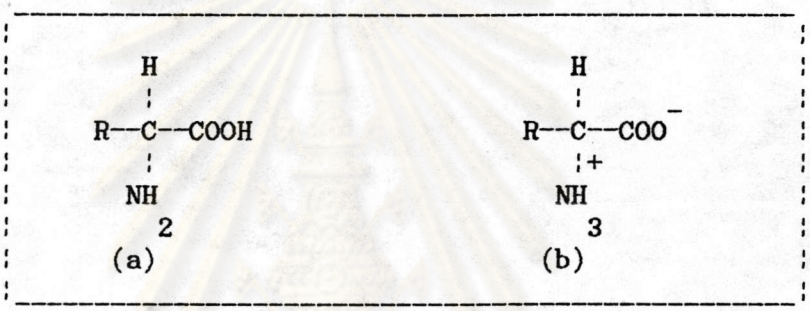
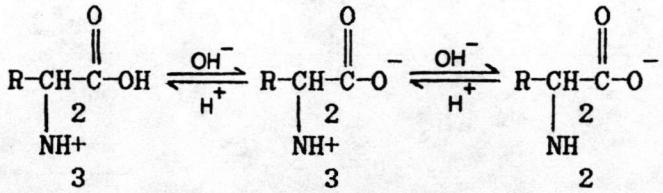



Figure 1.1 General formula for α-amino acids

(a) un-ionized form , (b) zwitter ion form

The acidic carboxyl group of amino acid can lose a proton by dissociation and the basic amino group can pick up a proton. If both groups are ionized, the result is the "dipolar ion" or "zwitter ion", meaning hybrid ion as shown in Figure 1.1 (b) (19).

All amino acids contain $-COOH$ and $-NH_3^+$ groups that can ionize in aqueous solution, which dependent on pH of the solution The acid-base behaviour of the amino acid is shown below

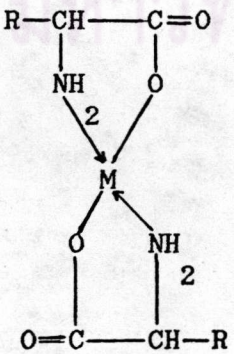


acid solution  zwitter ion (isoelectric point)  basic solution

The zwitter ion form of amino acid has no net charge. The pH at which a molecule has no net charge is called its isoelectric point, and is denoted by pI.

Investigations of the kinetics of complexation reaction of labile metal with amino acids have become apparent that the zwitter ion is a remarkably unreactive form and that, as a result, the reaction of the metal ions with the anionic form of the ligand can be studied at pH's significantly beyond pI of the ligand(20). Next, a more comprehensive introduction about complexation between metal atom and amino acid is given.

The amino acid has two donor atoms, N and O, and may co-ordinate to a metal atom, thus acting as metal chelating reagents. The combination of acidic and amino groups gives rise to salts which are chelated compounds,



where M represents a divalent metal(21).

Amino acids form very stable complexes with copper(II) ions, the stability constants being of the order of $10^{8}$ for the 1:1 complexes and $10^{15}$ for the 1:2 complexes(20). Under suitable conditions, the forward reaction rates can be very high, where as the reverse reaction will obviously be slow, so that the complexation may be regarded as an instantaneous, irreversible reaction.

## 1.2 DC Voltammetry

DC voltammetry may be defined generally as the measurement of current-voltage relationships at an electrode immersed in a solution containing electroactive species. The potential is applied to working electrode with slow voltage scan rate. When voltage scan to positive direction, this process is called anodic voltammetry and oxidation reaction is occurred at working electrode. On the contrary, reduction reaction is occurred with voltage scan to negative direction to give cathodic voltammetry. Current from electrode reactions will be limited by mass transfer. Three mass transfer modes are normally encountered: migration, convection and diffusion. Mass transfer by migration is a result of the force exerts on charged particles by an electric field. Migration effects in laboratory-scale voltammetry serve no useful purpose. In presence of a large excess of background or supporting electrolyte, migration of electroactive material is minimized to an extent where it can be neglected. Convection essentially means stirring of electroactive material to the electrode. It arises from thermal, mechanical, or other disturbances of the electrode surface. Mass transport by convection is important in voltammetry. Processes in stirred solution depend largely on forced convection. Diffusion is perhaps the most widely studied means of mass

transport. In fact, the object of many voltammetric studies is to have only diffusion in the operative process. Diffusion exists whenever concentration differences are established. Since a concentration gradiant develops as soon as electrolysis is initiated, diffusion play a part in every practical electrode reaction (22).

From the application of Fick's law to the electrolysis problem the equation for diffusion leads to the relation (23)

$$i = nFA ( D/\pi t )^{1/2} C \qquad 1.1$$

where i is the electrolysis diffusion current (in μA) flowing at time t(s) from the start of the experiment, n is the number of electrons involved in the electrode reaction, F is the Faraday constant (96487 C per equivalent), A is the area ($cm^2$) of the electrode, D is the Fick's diffusion coefficient ($cm^2 s^{-1}$), and C is the bulk concentration of the electroactive species ($mmol\ l^{-1}$).

## 1.3 Amperometry

Amperometry is the technique that measured current-time curves at constant potential. The constant potential is fixed at high enough voltage so that the complete electrode reaction can be obtained. This fixed operating potential can be chosen from the DC voltammogram at the voltage where the current has its limiting value (22).

Amino acids do not produce a reduction wave at the working electrode unless a reducible group is present, e.g., the iodine in thyroxine, amino acid in the aromatic species and their derivatives. Therefore the voltammetric technique is not useful for direct

determination of all amino acids. Amino acids can be determined indirectly by forming a metal complex and then determining this complex(24). The most commonly used complex is the copper complex which is formed by adding the amino acid to a solution of copper(II) ion. However the half-wave potentials of these various comlexes do not appear to differ significantly.

Hernandez Mandez, et al.(25) and Hui and Huber(26) had studied the anodic oxidation of amino acids at dropping copper amalgam electrode and nickel oxide electrode respectively. From these methods the peak height was proportional to the concentration of amino acid being determined.

Indirect method for the determination of complexing agent was studied with a lead /lead dioxide electrode by Yoshimura(27). It has been shown that the metal electrode "passivated" with an oxide layer can respond to complexing agents. Because the current flowing through the lead dioxide electrode at a fixed potential increased proportionally with the concentration of complexing agent, amperometric determination of this concentration was possible.

Electrochemical and x-ray photo-electron spectroscopic studies have shown that when a copper electrode is anodized in a buffer solution, different layers of copper oxide (or hydroxide) are formed (28,29). First, a layer of copper(I) oxide is deposited on the copper surface; its thickness does not depend on the potential. The layer is extremely insoluble and is reduced only at fairly negative potentials. Because such potentials were not normally applied here, the copper electrode in use can be regarded as covered with an "innert" layer of

copper(I) oxide. In the more positive potential region studied, only copper(II) ions are formed and these can diffuse through the copper(I) oxide layer. The solubility of copper(II) ions in weakly acidic or alkaline buffers is limited. Therefore, if the electrode potential is increased, the copper(II) concentration at the electrode surface will increase only upto a certain value. The current at this stage is called background current. If there is amino acid in the solution, it will form complex with copper(II) ion to give more solubility of copper(II) ion than that when the amino acid is absent. Thus the current will be increased from the background current and it will be proportional to amino acid concentration.

## 1.4 Flow System

The basic technique used in continuously flow procedures is that a sample solution is aspirated into a continuously flowing reagent stream and subsequently analyzed in a flow through detector. Continuous flow analysis can be divided into two developed techniques: Segmented Flow Analysis(SFA) and Flow Injection Analysis(FIA). The simpliest form of flow manifold for each continuous flow system is illustrated in Figure 1.2, together with modified techniques called bubble-gating and rapid-flow for comparison.

Segmented flow analysis was originally invented by Skeggs in 1957(30), is characterized by air-segmented flowing stream with operation at steady state signal condition. The original flow system by Skeggs can be represented as shown in Figure 1.2 A. The purpose of segmentation is to minimize longitudinal sample dispersion in the flowing stream. The air introduced in the system is debubbled just

prior to a flow through detector. Improved sampling rates in segmented flow analysis were obtained by a technique of gated-flow analysis(GFA) (31-34). The basic flow diagram of the technique can be represented as shown in Figure 1.2 B. The idea of the technique is to eliminate sample-interection caused by the debubbling system in Figure 1.2 A. Hence the bubbled stream was allowed to pass directly through the flow cell in gated flow, and the signal caused by the bubble was eliminated, e.g., by a comparator system (35).

A more recent development in continuous flow analysis was introduced by Ruzicka and co-worker (36-41). Unlike segmented flow systems, a sample solution is introduced by a discrete injection into a continuously flowing, non-segmented stream. Thus air segmentation is not required in this system and with non-steady state operation. The so-called flow-injection analysis(FIA) can be represented as shown in Figure 1.2 C. In this study, FIA will be employed as a flow system to improve the effect of the uncertain electrode response that can be occurred in batch system.

The last technique in continuous flow analysis, rapid flow analysis(RFA), is characterized by a rapid flow velocity, and the concept can be represented by the flow manifold as shown in Figure 1.2 D. The flowing stream is non-segmented except for intersample air bubbles, and therefore the debubbling system is required. Both internal bore diameter and length of sample flow path must be minimized in this flow system.

A more comprehensive discussion on FIA in this study, is given in the following section.

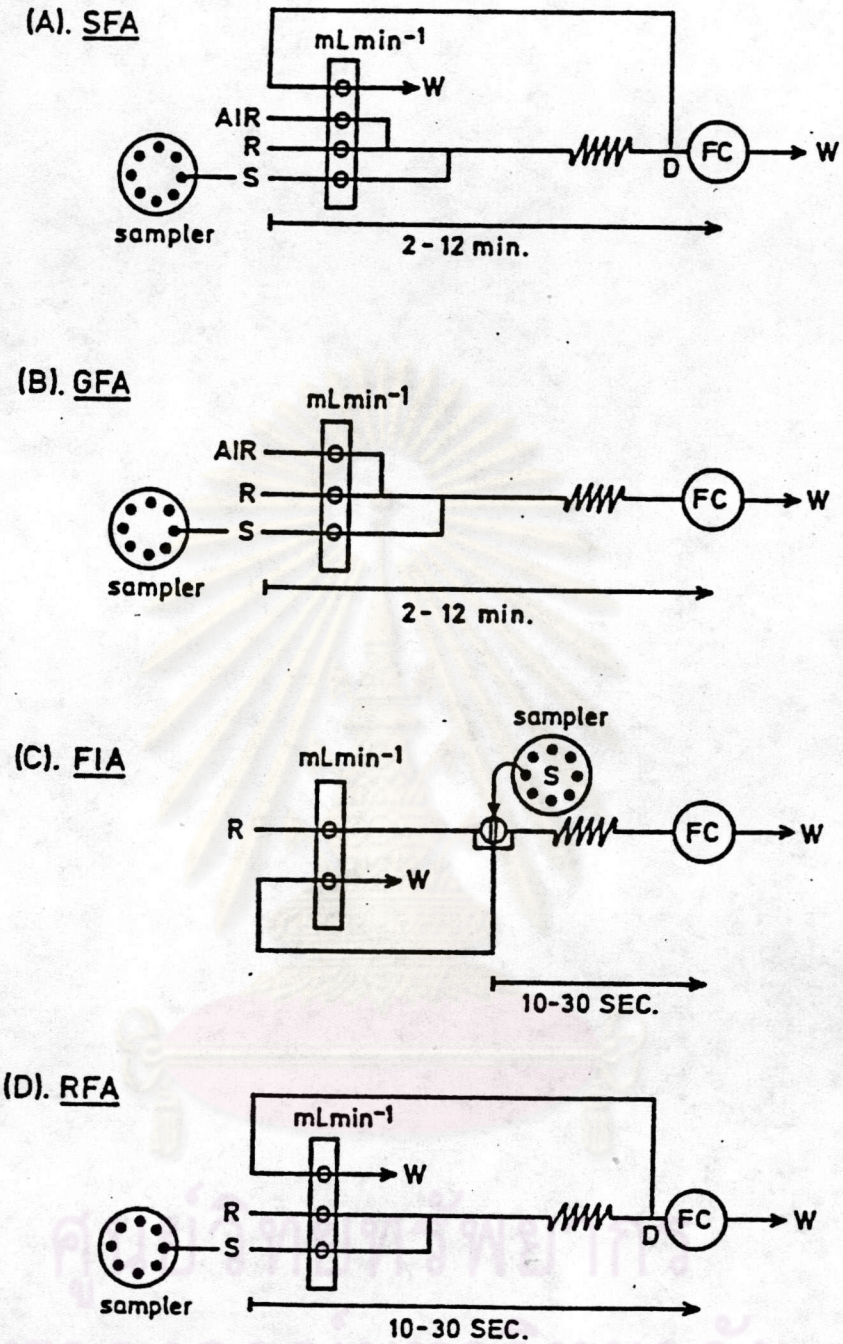



Figure 1.2. Simple automated flow analysis systems.

(A): Segmented flow analysis (SFA)

(B): Gated-flow analysis (GFA)

(C): Flow-injection analysis (FIA)

(D): Rapid flow analysis (RFA)

### 1.4.1 Flow Injection Analysis (FIA)

Ruzicka and Hansen(36) and Stewart,et al.(42) reported a new concept of continuous flow analysis. They termed it flow injection analysis (FIA) which was based on injection of a liquid sample into a continuous, flowing, unsegmented reagent stream. The purpose of sample injection is to insert a discrete volume of sample into a continuously moving carrier stream in such a way that the movement of the stream is not disturbed. The volume of sample need not be known accurately, but it must be introduced into the carrier stream precisely, so that both the volume and the length of the sample zone can be reproduced with great precision from sample to sample. Since any imprecision in the residence time will be reflected in the readout, highly reproducible timing is essential. The signal is read on the transient peak, rather than on the flat peak. In early work, samples were injected with a syringe through a septum(36) or a flap valve(43). Later developments included rotary valves with bores as sample containers and various forms of loop valves of the type used in liquid chromatographic system(44-46). Thus the special feature of FIA system is the absence of air-segmentation, the sample injection, the controlled dispersion after injection, and the reproducible timing.

In 1970, a similar concept was also introduced by Nagy, et al. (47). A sample solution was injected into a non-segmented stream of an electrolyte with a silicon-rubber based graphite electrode as a detector.

The theoretical basis, techniques, applications, and trends occurring in FIA have been extensively described by Ruzicka and Hansen(44-48). According to the publication of Betteridge's review(43) on FIA, the system has received steadily increasing recognition. A monograph(49), several reviews(50-56), and more than 400 papers(56) have been published on FIA.

FIA has been considered as a hybrid of SFA and high performance liquid chromatography(HPLC)(50). The success of the method stems from the use of flow controlled sample dispersion for mixing of sample and reagent. It allows high sampling rates with almost instant availability of the analytical readout due to short dwell times. In addition Ruzicka and Hansen(44) point out that the most important feature of the technique may well be the concept of controlled dispersion. The dispersion ,D, defined in FIA (48) as the ratio of the concentration of sample solution before and after the dispersion process has taken place (equation 1.2)

$$D = C / C^{max} = \text{const } H^{\circ}/H \qquad 1.2$$

where D is the dispersion, C is the original of the injected sample solution (mol $L^{-1}$), $C^{max}$ is concentration of sample solution at peak maximum (mol $L^{-1}$),$H^{\circ}$is peak height recorded with flow cell filled with solution of concentration, C (mm) and H is peak height at time equals mean resident time(s)

The actual flow conditions obtained in FIA have been the subject of a number of investigations. While it was originally thought that turbulent flow occurred(36-37), it was subsequently determined

that FIA operates only in laminar flow regions(43,48,50). In laminar flow the dispersion takes place by convection, diffusion or both(48), and the signal recorded is a response of the detector to the sample zone reflecting both dispersion and chemical reactions in the flow system(43).

The dispersion of the sample occurring in a stream is partly by longitudinal dispersion and partly by radial dispersion. Thus the extent of dispersion has been shown to be dependent on certain experiment flow parameters including the length and diameter of the tubing, flow rate, sample size, dwell time and diffusion coefficient(43). Longitudinal diffusion is affected by the flow pattern, where as radial diffusion is always important in narrow tubes, and it may even be the major mechanism for dispersion at low flow rates(57).

Generally, many laboratory instruments can be employed as detectors in flow injection systems provided that the instruments can be equipped with flow through cells. A wide range of detection methods have been successfully used in FIA including flame photometry(58-61), AAS(62-64), ICP(65), spectrophotometry (66-69), fluorometry (70), chemiluminescence (71-72), potentiometry(73), polarography/voltammetry (74-75), and ASV(76). These detection methods are commonly coupled with strip chart recorders, and analytical results are calculated from sample peak heights or areas via calibration curves.

## 1.5 High Performance Liquid Chromatography

The classical liquid chromatographic method employed a glass tube with a diameter of 10 to 50 mm to hold a 50 to 500 cm column of solid particles of the stationary phase. To assure resonable flow rates, the particles of the solid were kept larger than 150-200 μm in diameter. Ordinarily, the head of liquid above the packing sufficed to force the mobile phase down the column. Flow rate were, at best a few tenths of a milliliter per minute; thus, separations tended to be time consuming(77).

Early in the history of liquid chromatography, it was realized that large increases in column efficiency could be expected to accompany decreases in the particle size of packings. It was not until the late 1960S, however, that the technology of producing and using packings with particle diameters as small as 10 μm was developed. This technology required sophisticated instruments that contrasted markedly with the simple devices employed in classical liquid chromatography. The name high performance liquid chromatography(HPLC) is often employed to distinguish these newer procedures from the classical methods, which still find considerable use for preparative purposes(77).

By HPLC the separation efficiency is greatly enhanced at low particle diameters. Reasonable flow rates with packings consisting of such particles can only be realized, however, by pumping at high pressure. The high pressure that must be applied to the mobile liquid to force it through the column at a satisfactory rate can be obtained by a pump. As a consequence, the equipment required for HPLC is

considerably more elaborate than the simple gravity-fed column used in classical liquid chromatography(77).

Liquid chromatogaphy now embraces a variety of processes which are based on differential distributions of the sample components between two phases. Then the common features of the types of liquid chromatography will be discussed in the following sections.

1.5.1 Types of Liquid Chromatography

Several type of LC are distinguished by their predominant mechanism of separation e.g. ion-exchange chromatography, exclusion chromatography, adsorption chromatograhpy and partition chromatography(78).

Ion-exchange chromatography is based on the affinity of ions in solution for oppositely charged ions on the stationary phase. For exclusion chromatography, referred to as gel-permeation or gel-filtration chromatography, the stationary phase should be chemically inert. Exclusion chromatography involves selectivety diffusing solute molecules into and out of mobile-phase filled pores in a three-dimentional network, which may be a gel or porous inorganic solid.

Adsorption chromatography, often referred to as liquid-solid chromatography(LSC), is based on interactions between the solute and fixed actived sites on the solid adsorbent used as the stationary phase. In partition chromatography, also referred to as liquid-liquid chromatography(LLC), the solute molecules distribute themselves between two immiscible liquid phases, the stationary phase and the mobile phase, according to their relative solubilities. Partition

chromatography is termed "normal phase" if the stationary phase is more polar than the mobile phase and "reversed phase" if the mobile phase is more polar than the stationary phase. Often, the stationary phase is chemically bonded to the support material rather than mechanically applied to it. This is referred to as bonded-phase chromatography(BPC).

In reversed phase chromatography the selectivity achieved is significantly different from that achieved with either LSC or normal phase LLC. In this technique the most polar components elute first. Therefore the elution order is generally related to the increasing hydrophobic nature of the solute.

Winspear and Oaks(13) and Kim(5) used reversed phase HPLC to analyse amino acids by spectrophotometer detectors. Some applications of this method were the determination of amino acids in plasma and other biological meterial(3,79-80) and the determination of amino acid in human serum and blood for diagnosis of inborn errors of metabolism(10).

Loscombe ,et al.(1) and Alexander ,et al.(16) used reversed phase HPLC to analyse amino acids by potentiometric detectors with copper ion-selective electrode and copper tubular electrode, respectively. The copper tubular electrode has been applied to the analysis of urine and intravenous amino acid preparation(16).

### 1.5.2 Detection Methods in HPLC

Two types of LC detectors are in use: the bulk property or selective detectors. Bulk property detectors measure a

change in some overall physical properties of the mobile phase plus solute. The solute property detectors are sensitive only to the solute(81). Thus specific detection system must be employed, depending upon the nature of the sample.

The most common detector for HPLC are based upon absorption of ultraviolet or visible radiation. The former usually employ the 254 and 280 nm lines from a mercury source; at these wavelengths many important classes of compounds absorb(77). To be more generally useful in terms of selectivity, a spectrophotometer, with its continuously variable wavelength, is desirable, even though there will be some loss of sensitivity. A number of spectrophotometers are available with the necessary condensing optics to give a narrow intense beam of radiation. They provide an additional degree of selectivity not possessed by other LC detectors, in being able to discriminate between various components of the column effluent(82).

The second most widely used LC detector is the differential refractometer. This device continuously the difference in refractive index(RI) between a reference mobile phase and the mobile phase containing the sample as it elutes from the column. Since this is a bulk property and thus it will be a general detector, it responds to all solutes under the proper operating condition(81).

A detector related to UV absortion is based on fluorescense measurements following excitation with the 254 nm line of mercury arc. This highly selective and sensitive detector is especially important in biochemical applications. However the possibility of quenching by other solution components or by dissolved

oxygen must always be aware of (81).

Yet another technique for identifying and measuring column eluate is by a reaction detector. The liquid stream from the column is mixed continuously with a substance that will react with the expected species to form an absorbing or fluorescing product. The combined flow is then passed through an appropriate optical detector (82). Joseph and Davies(3) and Kim(5) used the application of this principle for the determination of protein and amino acids by UV absorption and fluorescence detectors. In their works reversed phase HPLC with ODS(octadecyl silane) column was used to separated amino acids. After chromatographic separation, amino acids were reacted with the reagent to give derivatives that were responded by UV detector or fluorometric detector.

Many electro - reducible and electro - oxidizable substances have been detected in column effluents by polarography (83). Amperometric detection, voltammetry at fixed potential, has been most widely used over the past several years for applications involving the trace analysis of organic components in complex biological media(84-86). The active region of most amperometric detector consists either of a tubular electrode or a thin-layer cell. Some applications of this method were the determination of trace amounts of tyrosine metabolites in tissue samples (esp.brain)(87) and body fluid(88-89).

Some compounds are not intrinsically electrochemically active within currently useful potential range such as amino acids, with the exception of tryptophan, tyrosine and cysteine(90). Usually

amino acids after chromatographic separation react with OPA reagent to give OPA-amino acids that can be determined by fluoremetric detector(3-4). From the structure of OPA-amino acids contains an isoindole grouping(91), which has the same electrochemical activity as indoles (such as tryptophan), therefore OPA derivatives can be detected electrochemically(3).

Another method to detect amino acids is indirect electrochemical detector. In this detection system a solid-state copper ion selective electrode is used as potentiometric detector in a flow through cell to monitor the loss of free copper ion activity after the post-chromatographic reaction between an eluted ligand such as amino acids and an added copper ion solution(1).

In this thesis reversed phase HPLC was used to separate mixture of standard amino acids and the amperometric detector with the anodized copper electrode was employed as an indirect electrochemical detector for the column eluate.

## 1.6 Aims of The Thesis

The aims of this thesis were to study characteristics of an anodized copper electrode as amperometric detector for determination of amino acids in both static and flowing solution. Although electrodes are used most widely for analysis in static solution, they can also be used as detectors in flow system at construction of a suitable flow through cell. The flow system that used for the study in flow analysis is FIA.

Firstly, in static solution, the suitable conditions such as buffer solution, stirred speed and choice of fixed operating potential for amperometry were investigated. The ratio of copper(II)-amino acid was determined to confirm that the reaction is fast and thus this detection method can be applied for amino acid analysis. After that the characteristics of copper electrode including linear range, sensitivity, reproducibility and detection limit, were determined.

Secondly, the suitable conditions such as flow rate and tube length, for amperometric detector in FIA were examined. Characteristics of this detector in FIA were performed on sensitivity, linear range, reproducibility and detection limit, and the results were then compared with those in static solution.

Finally, application study was performed on the use of this flow through cell detector as the detector of HPLC for qualitative and quantitative analysis of amino acids.