CHAPTER 2

EXPERIMENTAL

2.1 Instrumentation

2.1.1 Voltammetric Instrumentation

Voltammograms and currents were obtained with a PAR (Princeton Applied Research, Inc.) Model 174A Polarographic Analyzer, equipped with a Hewlett-Packard 7040A X-Y recorder for voltammetry, and with a Perkin-Elmer 56 stripchart recorder for amperometry.

2.1.2 Instrumentation for Batch Analysis

The working electrode(5 mm diameter planar copper disc), reference electrode(satuarated calomel electrode) and platinum wire auxiliary electrode joined the cell through holes in its rubber cover(see Figure 2.1). Stirring was accomplished by a magnetic stirrer (Eyela Magnetic RC-2) placed under the cell; a small teflon stirring bar(ca. 5 mm length) was used.

2.1.3 Instrumentation for Flow Injection Analysis

The flow injection system consisted of a mechanical pump (PU 4010 pump) and connected via stainless steel tubing (0.5 mm bore) to a injection port. The outlet of the injector was connected to a flow-through electrochemical cell via teflon tubing (1 mm bore). The cell was coupled to PAR 174A instrument and the out-put was recorded with a stripchart recorder. Figure 2.2 shows a diagram of the

complete system.

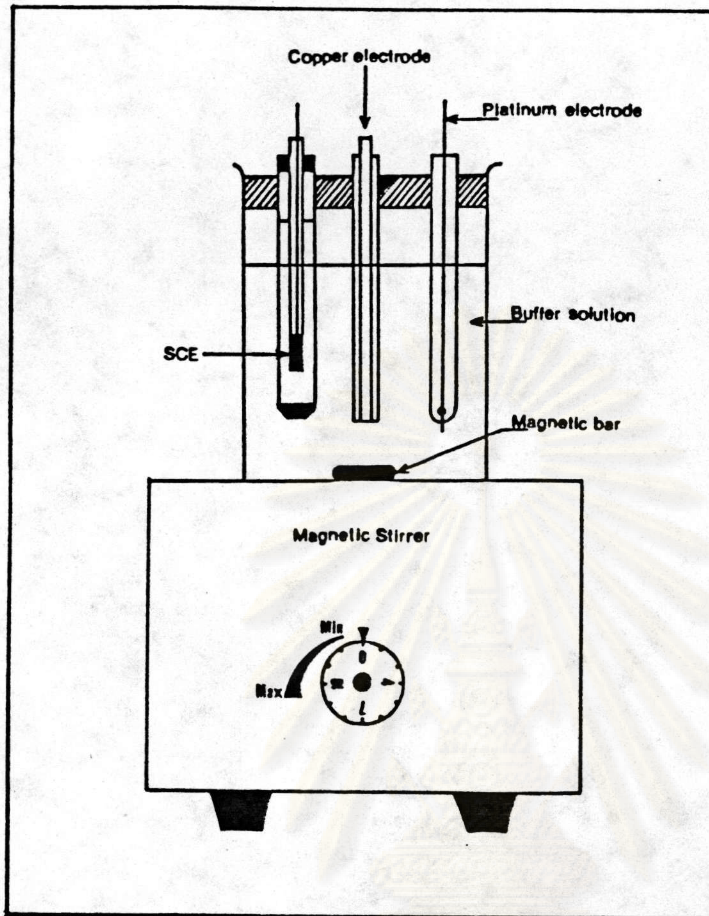

Figure 2.1 Diagram of batch system.

A conventional septum-injection valve from a normal liquid chromatography(Figure 2.3) was used as injection port for FIA.

A flow cell was constructed of high density polyethylene (by Machine Mechanical Workshop, King Mongkut's Institute of Technology North Bangkok), as shown in Figure 2.4, consisting of 2 electrode configuration with a third electrode, a saturated calomel electrode, placed downstream from the cell in a separate flow cup. The two electrodes were metal wires, one of copper(1.5 mm diameter) as a working electrode and the second of platinum(1.0 mm diameter) as the auxiliary electrode with a 0.8 mm flow path separating the two.

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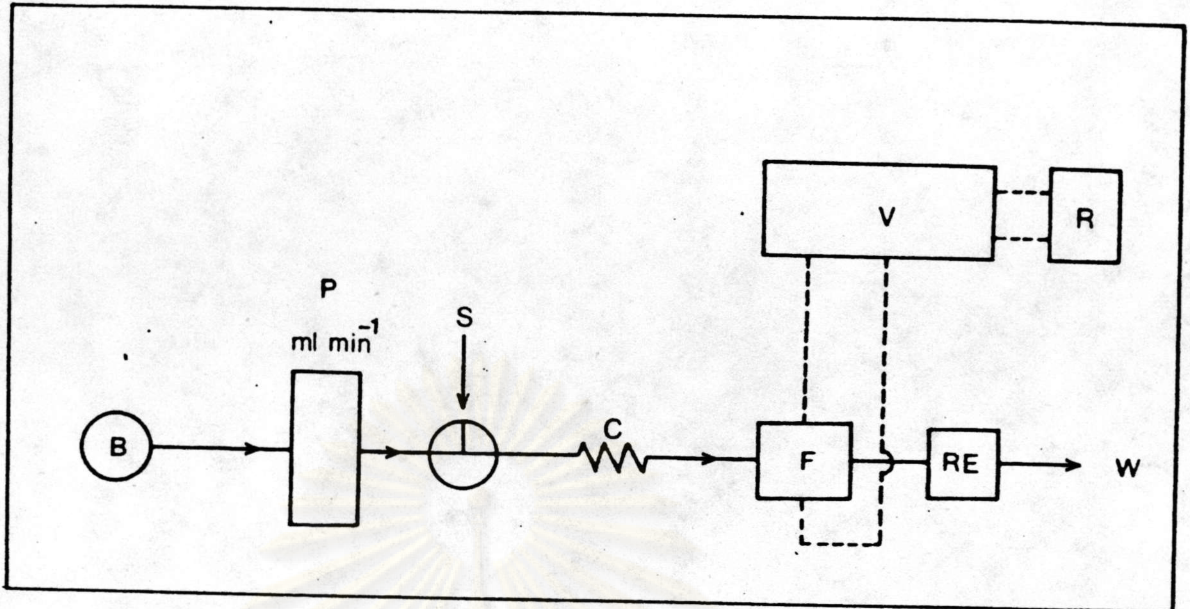

Figure 2.2 Diagram of the flow-injection system. B, buffer solution; P, pump; S, sample injection port; F, flow-cell; RE, reference electrode; W, waste; V, voltammeter; R, recorder.

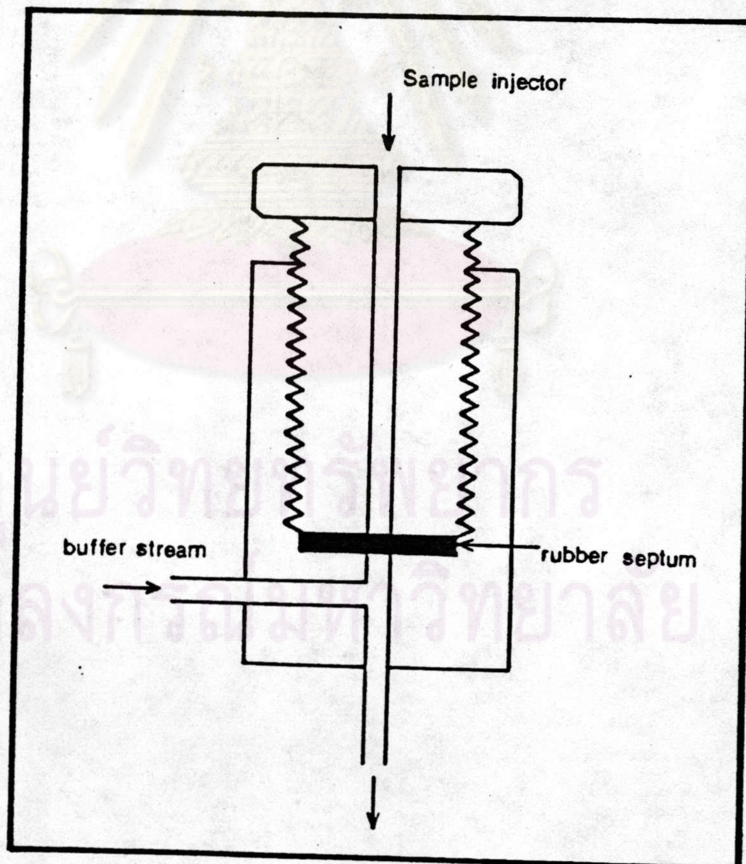

Figure 2.3 Injector for flow-injection analysis.

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2.1.5.3 A Mettler balance,NO.H43, was used for weighing compounds quantitatively.

2.1.5.4 The ultrasonic cleaner(Branson) was used for degassing solvent used for FIA and HPLC.

2.2 Reagent and Stock solution.

The amino acids employed, namely glycine, L-threonine, L-cysteine, L-methionine, L-arginine, L-phenylalanine and L-tryptophan were FLUKA reagents of AnalaR grade. Sodium hydroxide and potassium dihydrogen phosphate were BDH reagents of AnalaR grade. The other chemicals used for this experiment were BDH reagents of AnalaR grade. All solutions were prepared with the double deionized water. The solution, used in FIA and HPLC, were passed through Durapore filters and degassed before use.

2.2.1 Buffer Solution

2.2.1.1 Phosphate Buffer Solution (92)

The stock solution of 0.2 M of each reagent desired, sodium hydroxide and potassium dihydrogen phosphate, was prepared by dissolving the appropriate amounts with the double deionized water. Phosphate buffers, pH 6.2-8.2, were prepared from stock solutions of NaOH and KH_2PO_4, as shown in Table 2.1, to obtain the mixtures of NaOH and KH_2PO_4 with the following respective concentrations and buffer capacities : pH 6.2, 0.006M/0.05M , 0.005 ; pH 7.0, 0.03M/0.05M, 0.02 ; and pH 8.2, 0.05M/0.05M , 0.01.

Table 2.1 Composition of phosphate buffer pH 6.2-8.2

pH of buffer solution	0.2M NaOH solution,mL	0.2M KH_2PO_4 solution,mL	final volume mL
6.2	3.0	25.0	100.0
7.0	15.0	25.0	100.0
8.2	25.0	25.0	100.0

2.2.1.2 Borate Buffer Solution (93)

The stock solution of 0.5M of boric acid and 0.2 M borax were prepared by dissolving its appropriate amounts with the deionized water. The borate buffer pH 8.5 (0.1 M boric acid and 0.1 M borax) was prepared by mixing 20.0 mL of 0.5 M boric acid solution and 50.0 mL of 0.2 M borax solution then made volume to 100.0 mL by double deionized water.

2.2.1.3 Carbonate Buffer Solution (93)

The stock solution of 0.5 M sodium bicarbonate and 0.5 M sodium carbonate were prepared by dissolving its apropriate amount with the double deionized water. The carbonate buffer pH 10.2 (0.1 M $NaHCO_3$ and 0.1 M Na_2CO_3) was prepared by mixing 20 mL of 0.5 M $NaHCO_3$ solution and 20.0 mL of 0.5 M Na_2CO_3 solution then made volume to 100.0 mL by double deionized water.

2.2.2 Stock Standard Solutions

The stock solution of 0.1 M of each amino acid desired was prepared by dissolving its appropriate amounts with 1.0 M

$HClO_4$ and diluted with double deionized water.

2.3 Procedures

2.3.1 Preparation and Conditioning of Working Electrode

A 13-cm copper rod (5 mm diameter, from Machine Machanical Workshop, King Mongkut's Institute of Technology North Bangkok) was inserted in the 12-cm glass tubing (5.5 mm inner diameter) and glued at the tip about 1 cm with cyanoacrylate adhesive. Then it was lightly polished with abrasive paper until the surface smooth to give copper disc electrode (for batch system)

The copper electrode was rinsed with 2 M nitric acid before each series of experiments. Before measurements the electrode was held at a potential of -250 mV for at least 15 min.

2.3.2 Preparation of Reference Electrode (94)

A blank electrode body was constructed of glass tubing (as shown in Figure 2.5 A) by Glass Workshops, Chulalongkorn University. The electrode blank was filled with mercury, Hg-Hg_2Cl_2 paste, and dry calomel (Figure 2.5 B). The solids were lightly compacted with a glass rod and moistened with saturated KCL solution. It was essential that the electrolyte penetrate to the mercury layer. The active end was then sealed with a cotton wool plug moistened with electrolyte and cut flush with the tube end. The complete electrode body was sealed into a salt bridge as shown in Figure 2.5 C

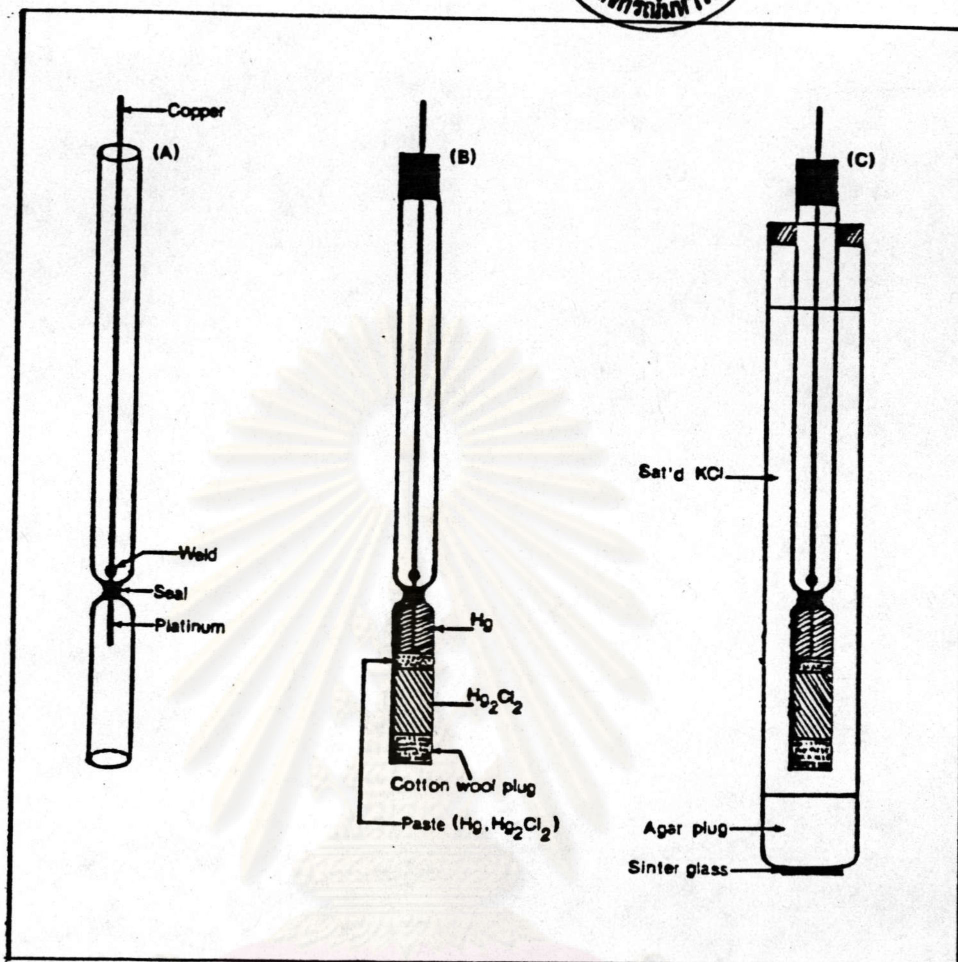

Figure 2.5 (A) A blank electrode body; (B) A complete electrode body; (C) Saturated calomel electrode

2.3.3 Procedure for Voltammetric and Amperometric Analysis

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For voltammetric recordings, the voltage scan was initiated from the PAR 174A instrument and recorded from -0.25 to +0.25V, all potentials with reference to the SCE, the scan rate was set at 5 mV s^{-1} in the d.c operation mode, with current sensitivity 0.02-2.0 A as required, recorder input 10V and chart speed 5 mm min^{-1}. For the amperometric measurements, the voltage was selected at the limiting current region from the voltammogram and this value was set at the initial potential control of the voltammeter while the scan

rate was set at zero.

2.3.3.1 Batch System

The supporting electrolyte solution (buffer solution) was introduced into the voltammetric cell. The solution was stirred and a measurement of the blank solution was then carried out to establish the background current. Sample solution was then added to the blank with a micropipette by the standard addition method and the current measurement was again recorded.

2.3.3.2 Flow System

Establishment of a steady baseline before sample introduction into a flowing stream was necessary for FIA mentioned above, i.e. the buffer solution was continuously passed through the FIA system until the steady baseline was established. A sample was then manually injected through the injection port with a syringe into the flow system. When the sample slug reached the flow cell, the increase in copper dissolution from the working electrode produced a current which was recorded as a bell-shaped peak on a chart recorder.

2.3.4 Procedure for Study of Copper-Amino Acid Ratio in Complex.

2.3.4.1 Anodic Limiting Current Method

The phosphate buffer 20.0 mL was pipetted to the cell and anodic current was then measured in amperometric mode at +150 mV VS SCE. After that 20.0 μL 0.01 M Cu(II) solution was added

and the current was measured again. After added the Cu(II), it was found that the current was decreased due to suppression of the dissolution of Cu(II) from copper electrode(17). The addition was repeated until the recieved current was constant, nearly zero. The above procedure was then repeated again with the solution of 2.00×10^{-4} M amino acid in phosphate buffer.

The difference of the minimum added Cu(II) concentration that suppressed the dissolution of Cu(II) from Cu electrode in phosphate buffer solution and phosphate buffer solution with 2.00×10^{-4} M amino acid was the amount of Cu that formed complex with 2.00×10^{-4} M amino acid. From this result, the ratio of Cu(II)-amino acid complex could be calculated.

2.3.4.2 Cathodic Limiting Current Method

The procedure was similar to the one of anodic limiting current except that the current was measured at -250 mV VS SCE. After Cu(II) was added, The current increased due to reduction of Cu(II) to give cathodic current. The addition was repeated until the constant current was observed because of the occurring of the pricipitation.

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The difference of the minimum added Cu(II) comcentration that begun to give constant current in phosphate buffer solution and phosphate buffer solution with 2.00×10^{-4} M amino acid was the amount of Cu(II) that formed complex with 2.00×10^{-4} M amino acid. Thus, the ratio of Cu(II)-amino acid complex could be calculated.

2.3.5 Measurement of Peak Height

Peak recordings of samples were used to determine peak heights by measurement the distance between the peak summit and the baseline. The height was converted into current units according to the scale expansion factor set on the PAR 174A instrument and recorder.

2.3.6 Measurement of Flow Rate

Flow rates were determined by measuring the time(in seconds) required to fill a 5.00±0.05 mL graduated cylinder with water from the pump tube or the column.

2.3.7 Response Studies

The reponse studies consisted of measurement of dispersion in terms of the factor, Δt, which was defined as the time from baseline to baseline of the peak output signal Δt may also be termed as peak width at the baseline in this work. It was determined by introducing the sample into a flow system with the chart recorder set at high chart speed(e.g. 10 cm/min). The distance between the initial appearance of a peak to the tail of the peak was measured in cm or mm units and then converted into time units according to the chart speed set on the recorder.

Sampling rate obtained for the flow system, was dependent on the time dispension value(Δt), i.e. sampling rate calculated increased with a decrease in time despension(Δt) at a constant sampling time. In flow injection systems, the sampling rates were established by an appropriate timing of injection, e.g. every 30 seconds for 120 samples h^{-1}. The rate of injections that can be

performed, therefore also depends on the value of the time dispersion (Δt).

2.3.8 Measurement of Carryover

The degree of sample interaction between consecutive samples for a chosen sampling rate was expressed in terms of carryover percentage,%CO, which is given by the following relationship:

$$\%CO = \frac{\text{peak height}(l') - \text{peak height}(l)}{\text{peak height}(h)} \times 100\ \%$$

For carryover measurements, test solutions at low concentration(l), followed by a high concentration(h), and then a repeat of the low solution(l) [to give peak height(l′)] were sampled consecutively at the chosen sampling rate.

2.3.9 Measurement of Reproducibility

The degree of reproducibility of n replicate measurements was expressed in terms of the relative standard deviation, given in the following relationship:

$$RSD = \frac{\text{Standard deviation}}{\text{Mean of n replicate measurements}} \times 100\ \%$$

2.3.10 Measurement of Detection Limit

The detection limits for amino acid samples were determined by replicate 10 measurements of the low amino acid concentration then calculated the standard deviation(SD). The detection limits were calculated as 3SD.