CHAPTER III

EXPERIMENTAL

3.1 Instruments and equipments

The following were the list of instruments utilized in this work.

3.1.1 pH meter (Metrohm)

3.1.2 Milli-Q water system, model Millipore ZMQS 5 VOOY, Millipore, USA.

3.1.3 Home made high-voltage

3.1.4 Analytical balance (Metler)

3.1.5 Sonicator (USA)

3.1.6 0.45 μm Nylon membrane syringe filter with polypropylene (PP) housing (Orange Scientific filter)

3.1.7 0.2 μm Nylon membrane filter (Altech)

3.1.8 Teflon tubing (1/16 inch o.d., Upchurch)

3.1.9 Autolab Potentiostat (PG-30, Methrom)

3.1.10 Auto pipette and tips (Eppendrof, Germany)

3.1.11 Silicon wafer

3.1.12 Digital microscope (Keyence)

3.1.13 Glassy carbon electrode (0.07 cm^2, Bioanalytical system Inc) was pretreated by polishing with alumina powder (1 and 0.05 micron, respectively) slurries in ultrapure water on felt pads and rinsed thoroughly with an ultrapure water prior to use.

3.1.14 Ag/AgCl electrode (TCI) with a salt bridge

3.1.15 Cutting set (Altech)

3.1.16 Home made glass cell

3.1.17 Home-made platinum wire

3.1.18 Home-made brass holder

3.1.19 Home-made cap cell (plastic bottle cap)

3.1.20 O-ring viton (0.07 cm^2)

3.1.21 Polishing set of 0.05 and 1 micron alumina powder slurry (Bioanalytical System Inc.)

3.1.22 Polishing set of 0.05 micron powder slurry (Element six)

3.1.23 Centrifuge, CENTAURA 2, (Sanyo)

3.1.24 Microcentrifuge (MINI CENTRIFUGE, Cole Parmer)

3.1.25 Mobile phase filter set including 300 mL glass reservoir, glass membrane holder, 1000 mL flask and metal clip, Millipore, USA

3.2 Apparatus for Microchip Capillary Electrophoresis

The borofloat glass chips with simple-cross single-separation channel (16 mm×95 mm ×2.2 mm) was obtained from Micralyne (model MC-BF4-001, Canada). The microchip had a four-way injection cross that was connected to the three reservoirs and the channel. The original waste reservoir was cut off leaving the channel outlet at the end side of the chip, then facilitating the end-column with electrochemical detection. The chip had a 90 mm long separation channel (from injection cross to the channel outlet) and a 10 mm long injection channel (between the sample and buffer reservoir). All channels were etched to a depth of 20 μM and a width at the top of the channel of 50 μM.

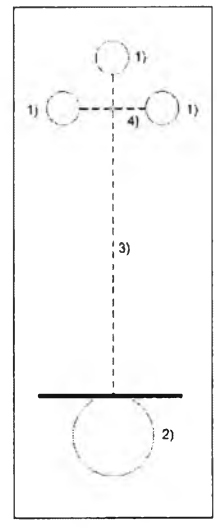

Figure 3.1 Schematic diagram of the glass chip. Top view with (1) buffer and sample reservoirs, (2) waste and detection reservoir, (3) separation channel, (4) injection channel.

The integrated CE-EC microchip system was described previously (43). The CE microchip was placed in a laboratory-built plexiglas holder for housing the separation chip and electrochemical detector. Thus, the setup allowed a convenient replacement. The holder consisted of a sample, running buffer, and unused reservoirs. Short pipette tips were cut and inserted into the fluidic ports of the various reservoirs on the glass chip for providing solution contact between the channel on the chip and the corresponding reservoir on the chip holder. Platinum wires inserted into the compartments provided electrical contact to a high-voltage power supply. A home-made high voltage power supply, with an adjustable voltage range between 0 and +4000 V, was used for controlling the injection and separation. The amperometric detector, placed in the waste reservoir, at the separation channel outlet, consisted of an Ag/AgCl wire reference, a platinum wire counter, and screen-printed carbon working electrode.

3.3 Chemicals and Reagents

All chemicals were of analytical grade or better and were used without further purification. Deionized-distilled water obtained from a Milli-Q-system was used for the preparation of chemical and reagent solutions. List of chemicals and their suppliers was summarized.

3.3.1 Lead(II)nitrate (Aldrich)
3.3.2 Cadmium sulfate (Baker Analyzed)
3.3.3 Copper(II) sulphate (BDH)
3.3.4 2-Morpholinoethanesulfonic acid ; MES (Fluka)
3.3.5 (s)-2-Amiono-3-(4-imidazyl)propionic acid ; L-histidine (Fluka)
3.3.6 Potassium hexacyanoferrate(Merck)
3.3.7 Potassium chloride (Merck)
3.3.8 Boron trioxide (Wako)
3.3.9 Acetone (Wako)
3.3.10 Sulfuric acid (Merck)
3.3.11 Acetic acid (Merck)
3.3.12 Methanol (Merck)
3.3.13 Ethylene diamine tetraacetic acid (EDTA, Riedel de Haën)
3.3.14 Potassium dihydrogen orthophosphate (BDH)
3.3.15 Di-sodium hydrogen orthophosphate-dihydrate (BDH)
3.3.16 Sodium hydroxide (Merck)
3.3.17 Hydrochloric acid (Merck)
3.3.18 Phosphoric acid (Merck)
3.3.19 Ethylenediaminetetraacetic acid disodium salt dehydrate (Fluka)
3.3.20 Citric acid monohydrate (Baker analyzed)
3.3.21 A standard buffer solution pH 4 and pH 7 (Metrohm)
3.3.22 Nitric acid (Merck)
3.3.23 Pyroatechol (Fluka)
3.3.24 3,4-Dihydroxyphenethylamine hydrochloride (Dopamine, Wako)
3.3.25 Ethanol (Merck)

3.4 The preparation of supporting electrolyte solution and standard solution

All solutions were prepared using deionized water obtained from a Milli-Q system (Milford, MA, USA). The preparations of buffer solutions are shown below:

3.4.1 The preparation of 0.1 M phosphate buffer

Potassium dihydrogen phosphate 13.60 g and disodium hydrogen phosphate dehydrate 17.60 g were dissolved in 1 L of deionized water to make the solution of 0.1 M potassium dihydrogen phosphate buffer and 0.1 M disodium hydrogen phosphate. The recipes of each pH solution were shown in Table 3.1

Table 3.1 Recipes of phosphate buffer preparation.

pH	0.1 M Potassium dihydrogen phosphate (mL)	0.1 M Disodium hydrogen phosphate (mL)
5	99.2	0.8
6	88.9	11.1
7	41.3	58.7
8	3.7	96.3

The 0.1 M of potassium dihydrogen phosphate was adjusted by phosphoric acid to make the range of pH from 5 to 8.

3.4.2 The preparation of dopamine stock solutions

Dopamine 1.90 mg was weighted and dissolved in 10 mL volumetric flask with 25 mM MES and L-histidine buffer solution to make 1 mM dopamine solution.

3.4.3 The preparation of catechol stock solutions

Catechol 1.1 mg was weighted and dissolved in 10 mL volumetric flask with 25 mM MES and L-histidine buffer solution to make 1 mM catechol solution.

3.4.4 The preparation of 100 mM MES buffer ; (2-morpholinoethanesulfonic acid)

2-Morpholinoethanesulfonic acid 5.33 g was dissolved in 250 mL of deionized water into a 250 mL volumetric flask and making up the volume to make the solution of 100 mM MES buffer.

3.4.5 The preparation of 100 mM L-histidine ;{(s)-2-amiono-3-(4-imidazyl) propionic acid}

(s)-2-Amiono-3-(4-imidazyl)propionic acid) 3.88 g was dissolved in 250 mL of deionized water into a 250 mL volumetric flask and made up the volume to make the solution of 100 mM L-histidine.

3.4.6 The preparation of MES and L-histidine buffer

The buffer for electrolyte in microchip capillary electrophoresis were prepared by appropriately diluting 100 mM MES buffer solution and 100 mM L-histidine with milliQ water into 50 mL volumetric flask. The concentration and volumes required for three preparations are shown in Table 3.2. The buffer pH was adjusted by hydrochloric acid and sodium hydroxide.

Table 3.2 Recipes of MES and L-histidine buffer solution preparation.

Concentration (mM)	MES (mL)	L-histidine (mL)
10	5.0	5.0
15	7.5	7.5
20	10.0	10.0
25	12.5	12.5
30	15.0	15.0
35	17.5	17.5
40	20.0	20.0

3.4.7 The preparation of 20 mM MES and L-histidine buffer

20 mM MES and L-histidine buffer was prepared by pipetting 20 mL of 100 mM MES and L-histidine buffer solution into a 100 mL volumetric flask and made up the volume with milliQ water. This buffer was used for studying the electrochemical characteristic of dopamine, catechol and metal ions by cyclic voltammetry. This solution was used for studying pH dependence and investigating the reduction of metal ions by microchip CE systems.

3.4.8 The preparation of copper(II) ion solutions

Copper(II) sulphate 15.96 mg was dissolved in 10 mL volumetric flask and made up the volume with electrolyte solution to make the stock solutions of 10 mM Cu(II) ions.

3.4.9 The preparation of cadmium(II) ion solutions

Cadmium(II) sulphate 76.95 mg was dissolved in 10 mL volumetric flask and made up the volume with electrolyte solution to make the stock solutions of 10 mM Cd(II) ions.

3.4.10 The preparation of lead(II) ion solutions

10 mM of Pb(II) ions solutions was prepared in the same manner as described in 3.3.13 except that 33.12 mg Lead(II) nitrate was used instead of the cadmium(II) sulphate.

3.4.11 The Preparation mixture of Metal ions standard solutions for calibration

The standard solution of three-metal ions at various concentrations, for calibration experiment, were prepared by dilution of 1000 μM stock metal ions solution with 25 mM MES and L-histidine buffer solution in 5 mL volumetric flask. The concentration and volumes required for these preparations are shown in Table 3.3.

Table 3.3 Recipes of MES and L-histidine buffer solution preparation.

Concentration (μM)	Metal ions solution (μL)
5	25
10	50
25	125
50	250
100	500
200	1000
300	1500
400	2000
500	2500
600	3000
700	3500
800	4000
900	4500

3.5 Real Sample Analysis

Sample, vegetable juice, 2 mL was pipetted into microcentrifuge tubing and then centrifuged for 5 minute. The filtrate 0.5 mL was pippeted into 0.45 μm Nylon membrane microcentrifuge tube filter. All of samples were prepared daily. Other samples were also prepared in the way as described for the sample preparation. The proposed method was applied to real samples. The standard addition method was used to determine the amount of toxic metal ions in the real samples.

3.6 Procedures

3.6.1 Batch Analysis

3.6.1.1 Cyclic voltammetry

The electrochemical measurements were performed in a single compartment glass cell using a potentiostat. An Ag/AgCl was used as the reference electrode and platinum wire was employed as the counter electrode. The working electrodes, screen-printed carbon electrode.

3.6.1.2 Background current

The experiment was carried out in 25 mM MES and L-histidine buffer using the working electrode at the scan rate of 50 mV s^{-1}.

3.6.1.3 The electrochemical oxidation of dopamine

1 mM solutions of dopamine in the chosen buffer solution from the previous experiment were studied using the working electrode by cyclic voltammetry. A scan rate of 50 mV s^{-1} was used.

3.6.1.4 The electrochemical oxidation of catechol

1 mM solutions of catechol in the chosen buffer solution were studied using the working electrode by cyclic voltammetry. A scan rate of 50 mVs^{-1} was used.

3.6.1.5 The electrochemical oxidation of copper(II) ion

1 mM copper(II) ion in buffer solution was studied using the working electrode by cyclic voltammetry. A scan rate of 50 mV s^{-1} was used.

3.6.1.6 The electrochemical oxidation of cadmium(II) ion

1 mM cadmium(II) ion in buffer solution was studied using the working electrode by cyclic voltammetry. A scan rate of 50 mV s^{-1} was used.

3.6.1.7 The electrochemical oxidation of lead(II) ion

1 mM lead(II) ion in buffer solution was studied using the working electrode by cyclic voltammetry. A scan rate of 50 mV s^{-1} was used.

3.6.1.8 Effect of scan rate

Using 1 mM solutions of analytes, experiments were performed to investigate the adsorption of the analytes on the surface of electrode at various scan rates. The scan rates that were used in these experiments were 10, 20, 50, 100, 200 and 300 mV s^{-1}

3.6.1.9 The analytical performance

Stock solution of 1 mM metal ions were freshly prepared and diluted to a concentration range between 0.005 and 1 mM. A scan rate of 50 mV s^{-1} was used. These studies were carried out to find the linear ranges and detection limits.

3.6.2 Microchip CE with amperometric detection

3.6.2.1 Microchip CE layout

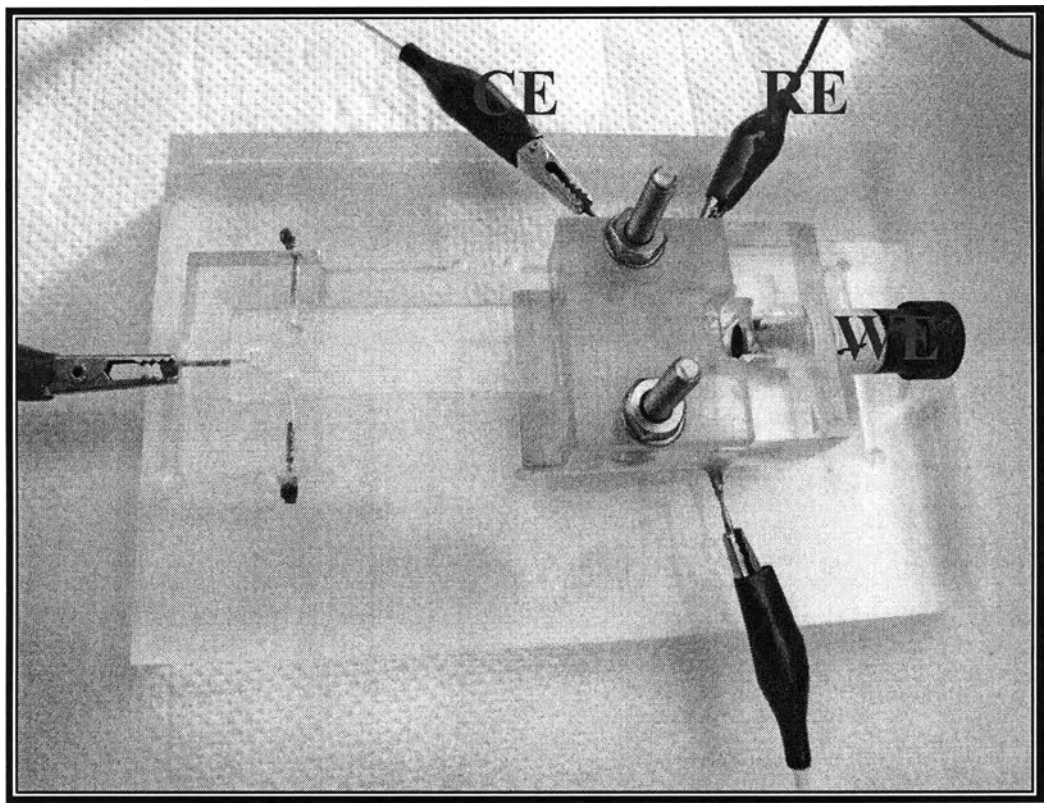

Figure 3.2 The microchip capillary electrophoresis with electrochemical detection systems.

This Figure showed the microchip CE system used in this work. The system consisted of glass chip, chip holder, high voltage power supply, and electrochemical detection. The glass chip was placed in a laboratory-built Plexiglass holder. The holder contained a sample, a buffer, and a detection reservoir. The platinum wires, inserted into each reservoir, served as contacts for the high voltage power supply to create sample injection and separation. A platinum wire and an Ag/AgCl wire were inserted into the detection reservoir, served as the counter and reference electrodes.

3.6.2.2 End-column amperometric detection

The detector performance relies on reproducible positioning of working electrode. This was accomplished by insertion the working electrode in a special groove into which the strip fits exactly. The electrode was further held in place by a plastic screw pressing the strip or disc against the channel outlet. The 20-μm distance from the working electrode to the chip outlet was controlled by the plastic screw for the disk electrode, and the 50-μm distance from the band working electrode to the chip outlet was controlled by a thick tape spacer. The high voltage power supply had switchable voltage ports between the running buffer and sample injections, with an adjustable voltage range of between 0 and 4000 V. Amperometric detection was performed with an Electrochemical Analyzer using the "amperometric i-t curve" mode. The electropherograms were recorded with a time resolution of 0.1 s while applying a desired detection potential versus Ag/AgCl wire. Sample injections were performed after stabilization of the baseline.

3.6.2.3 Electrophoresis procedure

Before electrophoresis, the channels of glass chip were treated by rinsing with deionized water, 0.1 M NaOH, and again with deionized water for 10 min each. The reservoirs were cleaned and the reservoir for sample solution were filled with sample solution, while all other reservoirs were filled with running buffer. Each of the corresponding pipette tips on the micro-channel chip were filled with their respective solution. Injection was carried out by applying the desired potential, 1200 V, between the sample reservoir and the grounded detection reservoir for 3 s, while all other reservoirs floating. Separation was performed by switching the high-voltage contacts and applying the corresponding separation voltages to the running buffer reservoir with the detection reservoir grounded, all other reservoirs floating. As soon as the voltage was switched to perform electrophoresis separation, the electrochemical analyzer was actuated to record signals.

For metal ions detection: the running buffer for the separation of the metals ions, copper(II), cadmium(II), lead(II) ions, were 25 mM MES containing 25 mM L-histidine pH 7.0. Each of the reservoirs in the chip holder and corresponding pipette tips on the micro channel chip were filled with their respective solutions. The sample reservoir was filled with a mixture of three metal ions. The potential of 1200 V was applied to the sample reservoir for 3 s with the detection reservoir grounded and other reservoirs floating. The separation performed by applying 1200 V to the running buffer reservoir. By switching the high voltage contacts, the separation potential was subsequently applied to the running buffer reservoir for the separation of the metal ions. All experiments were carried out at room temperature.

3.6.2.4 Safety considerations

The high voltage power supply and associated open electrical connections should be handled with extreme care to prevent electrical shock. Metal ions are toxic/ irritant/ dangerous for the environment, and should be handled in the fume hood. Skin and eye contact, and accidental inhalation or ingestion should be avoided. The stock solutions must be stored in closed small glass containers, and isolated from any reducing reagents, These metals are also toxic and in part carcinogenic and mutagenic. Therefore, special care must be taken to despose of waste solution.

3.6.2.5 Buffer pH dependence

The Buffer pH dependence was carried out by injecting flow concentrations of analyte solutions under the various pH of buffer. These experiments were done to obtain the optimum pH for separation of analytes. The current signal was defined the good sensitivity and separation that was obtained the optimum buffer pH for analysis system.

3.6.2.6 Buffer concentration dependence

The Buffer concentration dependence was carried out by injection flow concentrations of analyte solutions under the optimum buffer pH from the previous experiment (Section 3.6.10.3) using various concentration of buffer. These experiments were done to obtain the optimum buffer concentration for separation of analytes. The current response was defined the high sensitivity as well as separation that was obtained the optimum buffer concentration for analysis system.

3.6.2.7 Detection Voltage dependence

These studies were done under the optimum condition of previous experiment (Section 3.6.10.3 - 3.6.10.4) then various detection voltage in range -0.7 to -0.9 V. The current response was investigated the high sensitivity as well as separation that was obtained the optimum detection voltage for analysis system.

3.6.2.8 Separation Voltage dependence

The analytes solution was studies using the chosen buffer solution from the previous experiment (Section 3.6.10.3 - 3.6.10.5) then various the separation voltage. These experiments were performed to obtain the optimum separation voltage that was investigated the highest response.

3.6.2.9 Injection time dependence

Using the optimum condition from the previous experiment (Section 3.6.10.3 - 3.6.10.6) injection time were varied using microchip/CE systems. These experiments were performed to obtain the optimum injection time that defined the highest response of both current response and resolution of analysis.

3.6.2.10 Calibration and linear range

2 mM stock solutions of each analyte were freshly prepared and then diluted to a concentration range from 5 μM to 2 mM. The experiments were carried out by injection of three replicates of each concentration. The results were used to plot the calibration curve and to obtain the linear range.

3.6.2.11 Limit of detection (LOD)

The limit of detection (LOD) was carried out by injecting the concentrations of analyte solutions for three replicates under the optimal potential. The detection limit was defined as the concentration that provided a current response three times higher than the noise (S/N $\geq$3)

3.6.2.12 Limit of quantitation (LOQ)

The limit of quantitation (LOQ) was carried out by injecting the concentrations of analyte solutions for three replicates under the optimal potential. LOQ was defined as the concentration that provided a current response ten times higher than the noise (S/N $\geq$10).

3.6.2.13 Repeatability

The repeatability was studied by injecting ten replicates of analyte solutions. The repeatability is assessed in terms of the relative standard deviation (%RSD), using the following formula:

$$\%RSD = \frac{\text{standard deviation}}{\text{Mean}} \times 100$$

3.6.2.14 Accuracy

The accuracy of the developed method was assessed by calculating the average of % recovery that was obtained from each spiking level on two different days.

3.6.3 Appilcations

3.6.3.1 Real sample analysis

A sample solution was blended for 300 s with a homogenizer. 2 mL of sample solution was placed in capped centrifuge tubes then centrifuged for 10 min at 3500 rpm. The supernatant 0.5 mL was poured into the second filter-centrifuge tube (0.45 μ m Nylon membrane) then centrifuged for 5 min. The filterate was placed in capped tubes and repeated filter. 50 μl filterate sample solution were spiked with 50 to 500 μL of 2 mM mixed metal ions stock solution (lead(II), cadmium(II) , and copper(II) ions) and further diluted with 25 mM MES and L-histidine buffer (pH 7.0) to give the final concentration of 100, 200, 400, 600, 800 and 1000 μM , respectively. Finally, the solutions were analysed by Microchip CE.