CHAPTER II

EXPERIMENTAL

2.1 Instrument and Apparatus

1. A home-made "Seiney Interface Accessories" (SIA) software (Alpha flow®)
2. A home-made "Pocket SIA" software (Alpha flow®)
3. A home-made selection valve (Alpha flow®)
4. A home-made planar polymethylmethacrylate (PMMA) chip Alpha flow®)
5. A Ten-port selection valve (Valco instruments, USA)
6. Centrifuge
7. Chart recorder (Chessell®, Kipp and Zonen, The Netherlands)
8. Fiber optic cable (Commercial)
9. Glass filter holder with 200 nm and 20 nm membrane filter (Cole palmer)
10. Laptop computer (1.60GHz Centrino PentiumM®, Intel)
11. Light-emitting diode lamp (white light); LED (Commercial)
12. Magnetic stirrer
13. Peristaltic pump (ISMATEC) consisted of 0.13 mm i.d. Tygon tubing
14. Pocket PC with a serial Compac Flash cable interface (Acer N10)
15. Poly(dimethysiloxane); PDMS (Sylgard184, Dow Corning)
16. Photon Correlated Spectrometer (PCS) (Zetasizer 3000, Malvern Instruments)
17. Photomultiplier tube (Electron Tubes Ltd., model 9789QB, Ruislip, London)
18. Scanning Electron Microscope (SEM) (Carl Zeiss SMT, Zeiss EVO-60 SEM)
19. The 0.015 mm i.d. PTFE tubing (Upchurch Scientific, USA)

20. The 0.01 mm i.d PEEK tubing (Upchurch Scientific, USA)
21. Tungsten Lamp, (Ocean Optics Inc., U.S.A.)
22. Ultrasonicator and piezoelectric transducer horn (model XL-2020.)
23. USB2000 spectrophotometer (Ocean Optics Inc.)

2.2. Devices and Interfaces

2.2.1. Home-made selection valve

A home-made 6-port selection valve (Figure 2.1) was made by etching a straight line channel on a small cylinder Teflon (a) and drilling 7-hole on perspex™ plate (b). Subsequently they were assembled together with a stepper motor (M42sp-5 MITSUMI 12V 7.5 deg/step. 100 ohm/coil Stepper motor) (b) which controlled by a computer through a stepping motor driver unit. This unit was developed using IC ULN2803A Darlington array driver on a printed circuit board (PCB) (Figure.2.2)

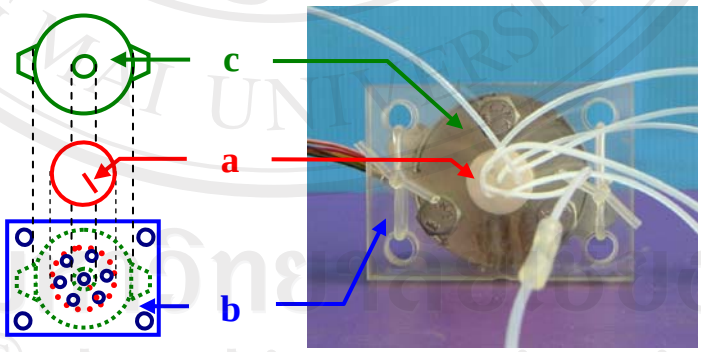


Figure 2.1 A home-made 6-port selection valve

The instrument and the computer command to interface in the FIA system are controlled through a parallel port directly with RS-8255 or PC-8255 which is the

parallel expandable card, the extending port through USB port (for RS8255) or PCI Bus (for PC-8255) of a personal computer. The data are then sent to ULN 2803A to drive the stepping motor by the bit sequence. The input ICL7107 which is high performance 3.5 digit A/D converter and interfaces directly with PC-8255 in order to send the data to the port, where the output as a graph is plotted.

2.2.2. Printed circuit board (PCB)

The designed PCB was supplied with 12V 500mA with direct current driving IC (ULN 2803A). There is a 8 bit common ground Darlington driver to activate the wire of stepping motor in single coil activation technique, half bit step by using 34 pins connector to link TTL signal from PC-8255 or RS-8255 interface card to the IC. Next the 1N4001 is used as a protector to protect back current from the stepping motor's wire. Printed circuit board is designed by Protel 99 with multilayer format, and the CNC Automation is used to make the copper design and vial pads as shown in Figure2.2

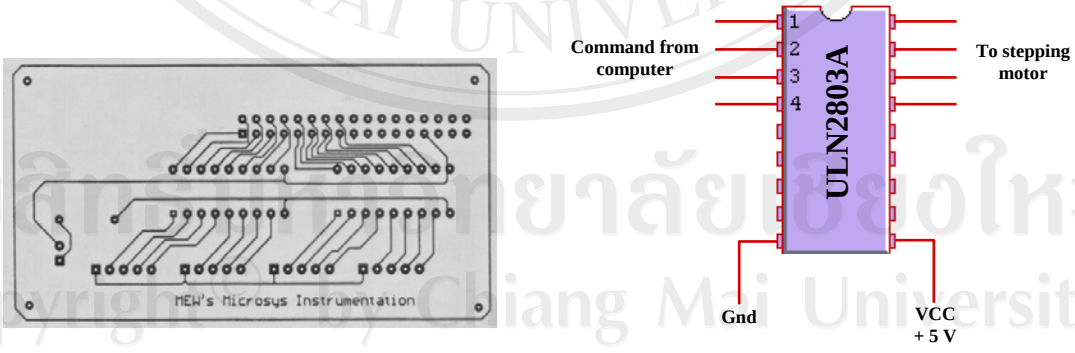


Figure 2.2 PCB for driving a stepping motor (a) and IC ULN2803A Darlington array drivers (b)

2.2.3. Computer interface

An IBM-compatible personal computer or Pocket PC was used. At the beginning, the stepping motor has been driven by setting the RS-8255 or PC-8255 interface card to active at port A for data output from a defined port (such as & HF300). Then send the bit sequence shown in Table 2.1 to drive it clockwise, vice versa send the bit sequence (Table 2.2) to drive it counter clockwise. Changing of bit sequence means that the motor should turn 0.5 x 7.5° from the reference position. (Half bit activation)

Table 2.1 The bit sequences for moving the stepping motor clockwise

Sequence	Bit step (Binary)			
	A0	A1	A2	A3
1	1	0	0	0
2	1	1	0	0
3	0	1	0	0
4	0	1	1	0
5	0	0	1	0
6	0	0	1	1
7	0	0	0	1
8	1	0	0	1

Table 2.2 The bit sequences for moving the stepping motor counter clockwise

Sequence	**Bit step (Binary)**			
	A0	**A1**	**A2**	**A3**
1	1	0	0	1
2	0	0	0	1
3	0	0	1	1
4	0	0	1	0
5	0	1	1	0
6	0	1	0	0
7	1	1	0	0
8	1	0	0	0

By the way, to drive the six-port multi-position valve, each port apart 60° from each other. As turning 360°, it should receive 96 bit step. The bit step of motor to turn 60° segment can be calculated by the equation:

$$\frac{60 \text{ x } 96}{360} = 16 \text{ bit step/60}^{\circ} \text{ segment}$$

Pock PC system has only one serial port available. So more than 1 device needs to be connected to this port. Hence, the RS-232 port switching is used to share the signal to devices under the same baud rate setting. The RS-232 switch box consisted of 1 master (connect to Pocket PC) port and 4 slave ports (connect to the required devices). As retrieving data from the master port, it recognizes whether the data are validly the port changer or not. If the data are the port changer command, the port is changed to the active port number parameter given after the port changer command. Unrecognized data are sent to the active slave port without lagging or hesitating. This communication procedure cannot control all instruments at the same time, but the procedure controls the port and instrument session by session. Each session is one sequence of the RS-232 command.

2.2.4. A home-made PMMA micro-flow analysis

The T-shape and the T-junction micro-coil (Figure 2.3) on the Polymethylmethacrylate (PMMA) micro-flow analyzer were fabricated using laser ablation (Mercury Laser pro L25, 25 watt laser engraver). The fabrication method was done by first, the channel network is designed using suitable computer drawing software, and then etched on the PMMA plate using laser ablation head. The channel size and depth on the PMMA chip is dependent on the line thickness of the designed

channel network, the power and the velocity of the laser head. The base PMMA plate containing the etched channel network is covered by molding PDMS with small holes which are used for connecting with reagents and samples reservoirs through small tubing (Figure 2.3). The upper plate is aligned with the channel geometry and clamp tightly to protect the solution leak out. The fabricated T-shape and T-junction micro-coil were used for determining iron and copper in water sample, respectively.

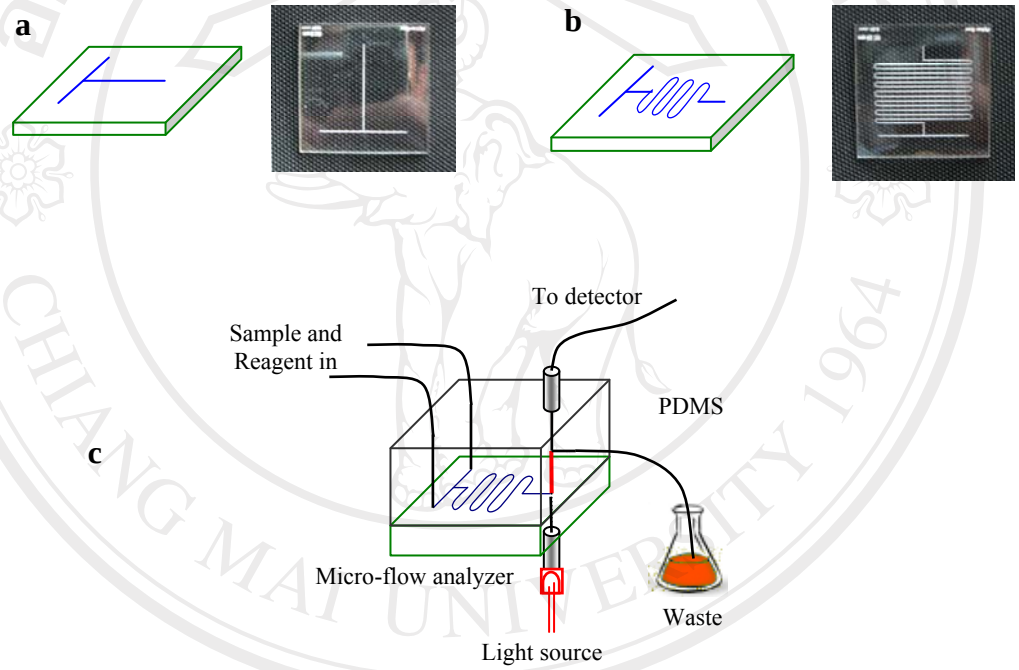


Figure 2.3 The fabricated PMMA chip using laser ablation. a) the T-shape PMMA micro-flow analyzer for iron determination, b) the T-junction micro-coil micro-flow analyzer for copper determination, c) the fabricated micro-flow analyzer topped with PDMS and connected with the light source and detector

2.2.5. Software

2.2.5.1. Software for personal computer

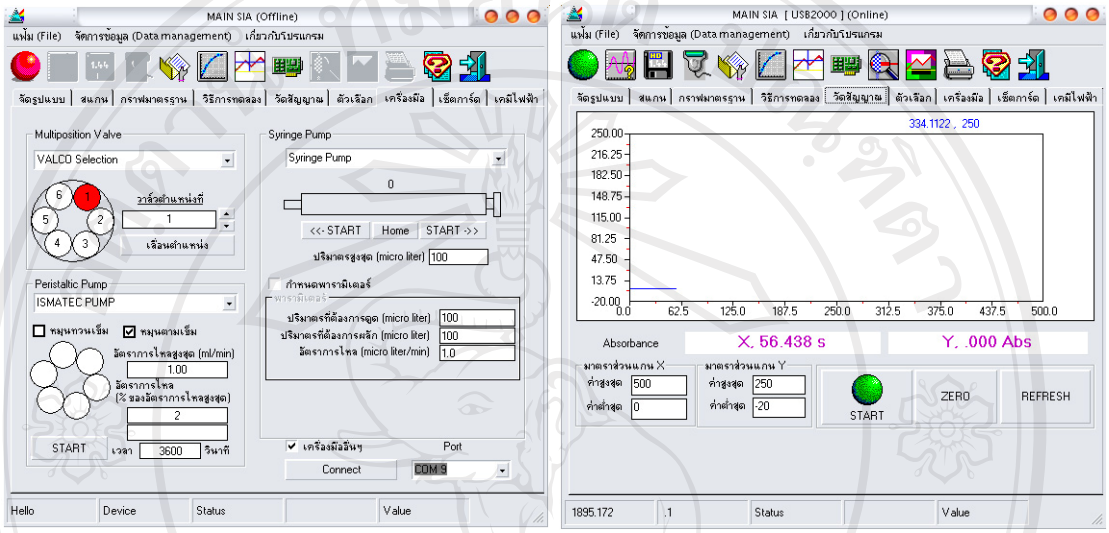
This designed program obeys Object Orientation Programming System (OOPS) named “SIA” as shown in Figure 2.4a. All device controlled modules are designed separately from each other by defined ports of PC-8255 or RS-8255 card through the USB port in case using laptop instead of the desktop computer. The graphic user interface (GUI) tools can be archived by Microsoft Visual Basic™. The data can be saved and exported in many formats, such as SIA format (Sei), comma delimited (Csv) and Text format (txt). This program also designed add-in module to analyze datum in the buffer. It can integrate, find a maximum and minimum, and print out the signal. It can also design for controlling some commercial pump (ISMATEC pump and Syringe pump) and valve (Valco instrument, USA) through the USB port which were used in the μFA system.

2.2.5.2 Software for Pocket PC

Pocket SIA software (Figure 2.4b) designed obeys Object Orientation Programming System (OOPS) is also programmed to control all devices and collect signal from the detector. This software is designed for controlling all modules using pocket PC (PDA) to miniaturize the instrument system and support field analysis. All device modules are controlled through the Compac Flash I/O port or Bluetooth I/O ports, for example pump, valve and detector. The commercial pump (ISMATEC, USA) and valve (Valco instrument, USA) were used in the μFA system and can be controlled by this software. The graphic user interface (GUI) tools can be archived by

the Microsoft eMbedded Visual Tools 3.0™. The data can be saved and exported in Pocket SIA format (Spk) which can be opened by the SIA software on the personal computer subsequent to data analysis.

a



b

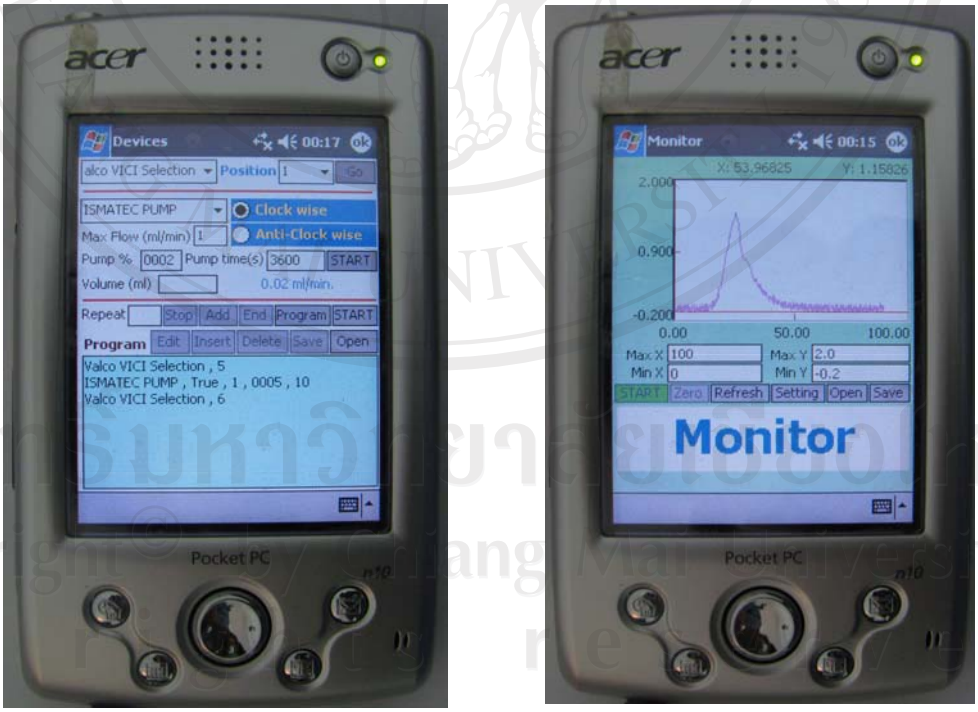


Figure 2.4 Software; a) Seiney Interface Accessories Software (SIA) (Alpha flow Group®, Thailand); b) Pock SIA software (Alpha flow Group®, Thailand)

2.3. Chemicals

All chemicals were of analytical reagent grade unless otherwise specified and used without any further purification. Deionized water was used throughout the experiments. The chemicals used in this investigation are listed as below:

1. standard iron solution (AAS standard, 1000 μg mL^{-1} Merck, Germany)
2. standard copper solution (AAS standard, 1000 μg mL^{-1} Merck, Germany)
3. standard cobalt solution (AAS standard, 1000 μg mL^{-1} Merck, Germany)
4. standard nickel solution (AAS standard, 1000 μg mL^{-1} Merck, Germany)
5. Hydrochoric acid, 37% w/w (Carlo Erba, Italy)
6. Nitric acid (Merck, Germany)
7. Glacial acetic acid (Merck, Germany)
8. Sodium acetated (Merck, Darmstadt, Germany)
9. Sodium hydroxide (Lancaster, Eastgate, UK)
10. Ammonium acetate (Merck, Germany)
11. Ammonium hydroxide (Merck, Germany)
12. Nitroso-R salt (Merck, Germany)
13. Deionized water
14. Sulfuric acid, 98.0 % (Fisher, Leicester, UK)
15. Formaldehyde, 37% (Fisher, Leicester, UK)
16. Folic acid (Sigma-Aldrich)
17. Potassium permanganate (Fisher, Leicester, UK)
18. NaCl (Fisher, Leicester, UK)
19. Tetraethyl orthosilicate (Fisher, Leicester, UK)

20. Titron X-100 (Fisher, Leicester, UK)

21. Cyclohexane (Sigma-Aldrich)

22. Acetone and ethanol (Sigma-Aldrich)

23. Sodium formate (Fisher, Leicester, UK)

24. Ammonia (Fisher, Leicester, UK)

25. Phosphoric acid (Fisher, Leicester, UK)

2.4. Preparation of reagents and sample solutions

All solutions used in this research were prepared with deionized water

2.4.1. A standard stock solution of cobalt (II), copper (II), nickel (II) and iron (III) 10 μg mL^{-1}

A standard stock solution of cobalt(II), copper (II), nickel(II) and iron(III) 10 μg mL^{-1} was prepared from a standard cobalt(II), copper (II), nickel(II) and iron(III) solution (AAS standard, 1000 μg mL^{-1} Merck, Germany) by pipetting 1.0 mL of each metals standard solutions in volumetric flask and adjusting volume to 100 mL with deionized water. Working standard solutions were prepared by appropriate dilution of these stock standard solutions.

2.4.2. A stock reagent 2.0 %w/v nitroso-R salt

A stock reagent 2.0 %w/v nitroso-R salt (Merck, Germany) solution was prepared by dissolving 2.0 g nitroso-R salt in deionized water and adjusting volume to 100 mL. Working solution of nitroso-R salt was daily prepared by appropriate dilution of stock nitroso-R salt solution in suitable buffer solution.

2.4.3. Buffer solution

Buffer solutions of pH 3-7 and pH 7-10 were prepared by mixing an appropriate ratio of 0.5 mol L^{-1} acetic acid (28.57 mL HOAc in 1000 mL deionized water) with 0.5 mol L^{-1} sodium acetate (41.0 g NaOAc in 1000 mL deionized water), and 0.5 mol L^{-1} ammonium hydroxide (17.50 g NH_4OH in 1000 mL deionized water) with 0.5 mol L^{-1} ammonium acetate (38.50 g NH_4OAc in 1000 mL deionized water), respectively.

2.4.4. Stock solution of 8.0 mol L^{-1} sulfuric acid

A solution of 8.0 mol L^{-1} sulfuric acid was prepared by pipetting 218.70 ml of 98.0 % sulfuric acid (Fisher, Leicester, UK) into a 500 mL volumetric flask and making to the volume with deionized water. The desired concentrations of this acid were prepared by appropriate dilution of this stock solution with deionized water.

2.4.5. A solution of 1.0 mol L^{-1} formaldehyde

A solution of 1.0 mol L^{-1} formaldehyde was prepared by pipetting 10 mL of 37% formaldehyde into 100 mL volumetric flask and adjusting the volume using 3.0 mol L^{-1} sulfuric acid.

2.4.6. Stock standard solution of 1.0 mmol L^{-1} folic acid

Standard stock solution of 1.0 mmol L^{-1} folic acid was prepared daily by dissolving 0.0110 g folic acid (Sigma-Aldrich) in deionized water and adjusting the volume to 25 mL. The stock solution was kept in the dark cold place. Working standard solutions were prepared by appropriate dilution of stock standard with 1.0 mol L^{-1} formaldehyde acidified with 3.0 mol L^{-1} sulfuric acid.

2.4.7. A solution of 10 mmol L^{-1} potassium permanganate

Potassium permanganate 10 mmol L^{-1} was prepared freshly by dissolving 0.0395 g potassium permanganate (Fisher, Leicester, UK) in deionized water. The solution was kept in the dark bottle to protect from light. The various study concentrations were prepared by appropriate dilution of the stock standard with deionized water or suitable solution.

2.4.8. A solution of 1.0 mmol L^{-1} sodium chloride

A 1.0 mM NaCl solution was prepared by dissolving 0.0850 g of NaCl in deionized water and adjusting volume to 1000 mL.

2.5. Sample collection and pretreatment

Water samples were collected from rain water, Ang-Keaw water reservoir, Ang-Karset water reservoir, Chiang Mai Canal, and Mae Ping River in Chiang Mai and Lumpun provinces (the sampling site see appendix A), Thailand. The water samples were filtered through a 0.45 μm membrane filter at the selected sampling

sites and stored in polyethylene containers that had been previously washed with 10% nitric acid and rinsed with deionized water for several times. After filtration, a 5 ml of concentrated hydrochloric acid was added in each liter of water for iron determination, and a 5 mL of concentrated nitric acid was added in each liter of water for copper determination.

2.5.1. Water pretreatment for iron determination

A 100 mL of water sample was treated with 5.0 mL concentrated hydrochloric acid followed by addition of 2.0 mL 35% v/v H_2O_2 and heated at 200 ^oC. It was allowed to cool to room temperature, filtered and diluted to appropriate concentration. The treated water samples were used for analysis of iron using the proposed method.

2.5.2. Water pretreatment for copper determination

A 25 mL of water sample was pitted into 50 mL beaker; added 2.5 mL concentrated nitric acid and heated (above 200 ^oC) to the lowest volume. Then it was allowed to cool to the room temperature, 5 mL deionized water was added (filtered if necessary), adjusted to 25 mL and diluted to appropriate concentration.

2.6 Methodology

2.6.1. The method for selecting the maximum absorption wavelength

The solutions of 0.4% w/v nitroso-R, with and without 1.0 μg mL^{-1} of each metal ions (Co(II), Cu(II), Ni(II) and Fe(III)) were prepared. Each metal solution (250 μL) were added into 750 μL of 0.4 %w/v nitroso-R salt solution and transferred into the sample cell. Then absorption spectrum of each metal-nitroso-R salt complexes and

the reagent blank solutions was scanned over a range from 400-800 nm using USB2000 spectrophotometer.

2.6.2. The FIA system for iron determination

2.6.2.1. Instrument setup

The proposed two channel FI manifold for iron determination is shown in Figure 2.5. The system consisted of a home-made 6-port selection valve (V) controlled by a motor driver circuit board (Figure 2.2) (C) and a four-channel peristaltic pump (ISMATEC) (P) with Tygon tubing (0.84 mm i.d. and 1.24 mm o.d.) which were controlled by computer software programmed in our laboratory name "SIA" (using visual basic 6.0, Microsoft) for injecting accurate volume of samples (S) and delivering appropriate flow rate of reagent (R), a PTFE (0.84 mm i.d., 30 cm long) mixing coil was used as reactor, a 10 mm path length with 120 μL flow-through cell (F) in the cell compartment of the UV-Vis spectrophotometer (USB2000, Ocean optics) as detector (D) using two fiber optics. The first probe was connected to flow-through cell and the white light emitting diode (LED) (super bright) as light source. The second one was connected to flow-through cell and spectrophotometer as detector. A home-made computer software name "SIA" was used for collecting the absorption signal and controlling the entire system.

2.6.2.2. The method for iron determination using FIA

The FIA system (Figure 2.5) was integrated with a fiber optic spectrometer to obtain a flow manifold for determining Fe(III). The method involved the injection of 70 μL standard or sample solution containing Fe(III) by switching the

home-made selection valve into a reagent stream of 0.3% w/v nitroso-R salt buffered pH 5 with 0.5 M acetate buffer with an appropriate flow rate of 2.5 ml min^{-1} using peristaltic pump with the specially designed software to control flow system (sample injection volume calculated from aspiration time and flow rate). Nitroso-R salt and Fe(III) solutions were reacted completely in the 30 cm mixing coil (MC) resulting in a green Fe(III)–nitroso-R complex which is passed through the flow through cell (F) of the spectrophotometer using fiber optic probe to measure the absorbance at 720 nm. The amount of Fe(III) content in water samples were calculated by reference to the calibration graph prepared under identical conditions. A comparative determination of the Fe(III) in the sample solutions was carried out by FAAS method

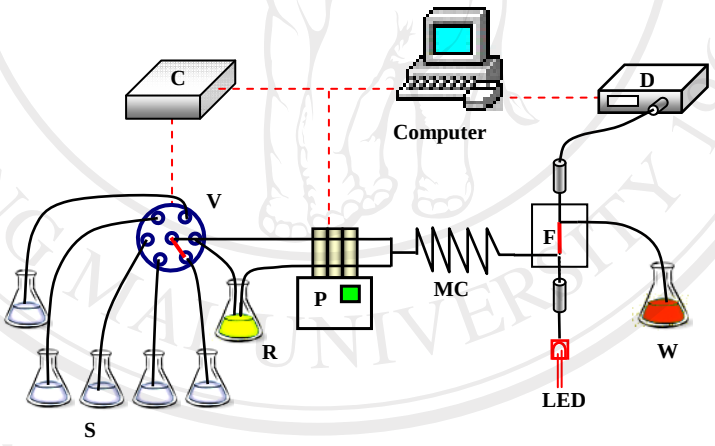


Figure 2.5 The proposed FIA system for iron determination; S is sample, R is reagent, V is a home-made selection valve, C is motor driver unit, P is peristaltic pump, MC is mixing coil, F is flow-through cell, LED is light source, D is spectrometer, W is waste.

2.6.2.3. Optimization of the experimental conditions

The proposed FIA system was optimized by a univariate method, to select the optimum conditions for the highest absorbance with low background and standard deviation of Fe (III)-nitroso-R complex. The value of one variable was changed while keeping the other variables at their constant values. By maintaining that variable at its optimum value, another was studied. The optimization of the experimental conditions was carried out by using standard Fe(III) solution. In all experiments, five replicate measurements were performed for each studied parameter.

2.6.2.3.1. Effect of the reaction coil length

The effect of reaction coil length was examined by measuring the absorbance of Fe(III)-nitroso-R salt complex using 1.5 mm i.d. Tygon tubing and varying coil length in the range from 10 to 50 cm. under the given condition; 1.0 μg mL^{-1} Fe(III), 50 μL injection volume, 2.0 mL min^{-1} flow rate and 0.4 %w/v nitroso-R salt. The coil length which provided the highest absorption signal was selected and subsequently used in all studied experiments.

2.6.2.3.2. Effect of flow rate

The effects of total flow rates on absorption signal of Fe(III)-nitroso-R complex in the FIA system were investigated for determining iron (1.0 μg mL^{-1} Fe(III), 50 μL injection volume, 0.4 %w/v nitroso-R salt and 30 cm coil length). The total flow rate was varied over the range of 1.0 to 5.0 mL min^{-1} (five replicate

measurements) to select the optimum conditions for the highest absorbance with low background and standard deviation of Fe (III)-nitroso-R complex.

2.6.2.3.3. Effect of sample introduction volume

The influence of sample introduction volume on Fe(III)-nitroso-R complex absorption was studied by controlling pump flow rate and changing the switching time of the selection valve at sample line over the range between 50 μL to 100 μL (five replicate measurements) under conditions: 1.0 μg mL^{-1} Fe(III), 2.5 mL min^{-1} flow rate, 0.4 %w/v nitroso-R salt and 30 cm coil length, to select the optimum conditions for the highest absorbance with low background and standard deviation of Fe (III)-nitroso-R complex.

2.6.2.3.4. Effect of pH

To study the effect of pH, the absorbance of Fe(III)-nitroso-R complex was measured at various pH values in the range of 3.0 to 10.0 (five replicate measurements) under conditions; 1.0 μg mL^{-1} Fe(III), 2.5 mL min^{-1} flow rate, 70 μL injection volume, 0.4 %w/v nitroso-R salt and 30 cm coil length. The pH values were adjusted with acetic acid/sodium acetate buffer solution (pH 3-7) and ammonium hydroxide/ammonium acetate buffer solution (pH 7-10) to select the optimum conditions for the highest absorbance of the Fe (III)-nitroso-R complex. The pH of the solutions which gave the highest absorption signal was chosen.

2.6.2.3.5. Effect of nitroso-R salt concentrations

The effects of nitroso-R salt concentrations (0.1 to 1.0 % w/v) on the absorbance of Fe(III)-nitroso-R complex in the FIA system (five replicate measurements) were studied under conditions: 1.0 μg mL^{-1} Fe(III), 2.5 mL min^{-1} flow rate, 70 μL injection volume, 30 cm coil length and pH 7, to select the optimum conditions for the highest absorbance of the Fe (III)-nitroso-R complex. The concentration of nitroso-R salt which exhibited the highest absorption signal was used.

2.6.2.4. The method for studying precision

The precision of the method describes the reproducibility of the results from measurements based on repeatability performed on 11-replicates of three standard solutions covering different concentration levels: low, medium and high (0.1, 1.0 and 4.0 μg mL^{-1}). The peak heights as absorbance were measured under the optimum conditions. Generally, this characteristic is expressed as the relative standard deviation (RSD) and is given by equation 2.1

$$\%RSD = \frac{\text{Standard deviation}}{\text{Mean of replicate measurements}} \times 100 \quad \text{…….. (2.1)}$$

2.6.2.5. The calibration graph and the percent recovery

The calibration graph was accomplished by measuring the absorbance of Fe(III)-nitroso-R salt complexes under the optimum conditions using iron(III) standard concentration of 0.1, 1.0, 2.0, 3.0 and 4.0 μg mL^{-1}. The percentage recoveries were investigated by spiking 1.0 μg mL^{-1} Fe(III) standard solution into

water samples and measuring the absorbance under the optimum conditions. The absorbance of Fe(III)-nitroso-R salt complex obtained from each sample was calculated (using equation 2.2.) referring to the calibration graph.

$$\text{Percentage recovery} = \frac{\text{Concentration of metal ion found}}{\text{Concentration of metal ion added}} \times 100 \quad \text{.....(2.2)}$$

2.6.2.6. Effects of Interfering ions

The effects of some possible interferences (Na^+, K^+, Ca^{2+}, NO_3^-, NO_2^-, SO_4^{2-}, Pb^{2+}, Cr^{3+}, Mg^{2+}, Cd^{2+}, Cl^-, PO_4^{3-}, Mn^{2+}, Zn^{2+}, Co^{2+}, Cu^{2+} and Ni^{2+}) on the determination of Fe(III) in water samples were studied for the maximum concentration ratio of foreign species to Fe(III) up to 200:1. The tolerance is defined as the foreign species concentration causing error smaller than ±10% for determining the analyte of interest.

2.6.3. The micro flow analysis system for iron determination

2.6.3.1. Instrument setup

The micro-flow analysis (μFA) system for determining iron (Figure 2.6) consisted of a planar T-shaped poly methyl methacrylate (PMMA) μFA topped with a poly dimethyl siloxane (PDMS) plate (MF), a computer controlled a ten-port with zero dead volume selection valve (VICI, Valco Instruments) (V) and a computer controlled peristaltic pump (ISMATEC) (P). The PMMA μFA was fabricated by using laser ablation (Mercury Laser source 25 watt); the channel was 200 μm wide, 50 μm deep and 20 mm long. The PDMS top plate was prepared by mixing the ratio

9:1 of silicone elastomer and silicone curing agent (Sylgard 184, Dow Corning, USA), subsequently the mixture was poured into the mold with appropriate design and cured at 60 ^{o}C for 6 hrs. The PDMS top plate obtained was clear cubic with three small channels. The first and the second channel are used for delivering sample and reagent to the μFA. The third one is used as flow cell (5 μL) where the absorbance of the complex was measured, and the solution passed out. The μFA flow cell was integrated with two fiber optic probes. The first fiber optic probe is connected to USB2000 spectrophotometer, Ocean Optics, as detector (D) and the second one is connected to tungsten light source. FIAlab Software Version 5.0 (FIAlab Instruments) and/or home-made software was used to control fiber optic spectrometer and also used for data collection. A ten-port with zero dead volume selection valve, VICI, Valco Instruments, USA (V) and a peristaltic pump, ISMATEC peristaltic pump 0.001–68 mL min^{-1} per channel with Tygon pump tubing 0.19 mm i.d. (P), controlled by using computer program were used for injecting accurate volume of samples (S) and delivering appropriate flow rate of reagent (R). PTFE tubings (0.19 mm i.d.) were used as flow lines connected with the μFA.

2.6.3.2. The method for Fe(III) determination using μFA

The μFA system (Figure 2.6) was integrated with a fiber optic spectrometer to obtain a micro-flow manifold for determining of Fe(III). The method involved the injection 5 μL of standard or sample solution containing Fe(III) by switching the selection valve into a reagent stream of 0.4% w/v nitroso-R salt adjusted to pH 5 with 0.5 mol L^{-1} acetate buffer with an appropriate flow rate of 30 μL min^{-1}

using peristaltic pump with the specially desired software to control flow system (sample injection volume calculated from aspiration time and flow rate). Nitroso-R salt and Fe(III) were reacted in the T-junction of the μFA (MF) resulting in a green Fe(III)–nitroso-R complex and then passed through the flow cell (5 μL) fixed in PDMS top plate, where the fiber optic probe was placed to measure the absorbance at 720 nm. The amount of iron(III) in each water sample was calculated by reference to the calibration graph prepared under identical conditions. A comparative determination of the iron(III) in the sample solutions was carried out by FAAS method.

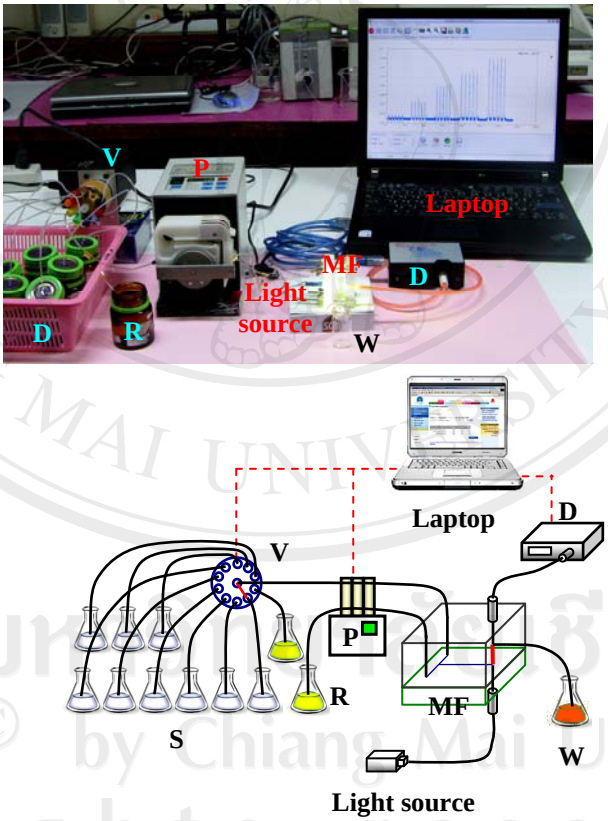


Figure 2.6 The μFA system for Fe(III) determination. S, sample; R, nitroso-R salt reagent; V, selection valve; P, peristaltic pump; MF, a PMMA T-shaped micro-flow analyzer; D, UV-Vis spectrophotometer; W, waste

2.6.3.3. Optimization of the experimental conditions

The proposed μFA system was optimized by a univariate method, to select the optimum conditions for the highest absorbance with low background and standard deviation of Fe(III)-nitroso-R complex. The value of one variable was changed while keeping the other variables at their constant values. By maintaining that variable at its optimum value, another was studied. The optimization of the experimental conditions was carried out by using standard Fe(III) solution. In all experiments, five replicate measurements were performed for each studied parameter.

2.6.3.3.1 Effect of flow rate

The effects of total flow rates on absorption signal of Fe(III)-nitroso-R complex in the μFA system were investigated for determining iron under condition: 1.0 μg ml^{-1} Fe(III), 5.0 μL injection volume and 0.3 %w/v nitroso-R salt. The total flow rate was varied over the range of 10.0 to 100.0 μL min^{-1} (five replicate measurements) to select the optimum conditions for the highest absorbance with low background and standard deviation of Fe (III)-nitroso-R complex. The total flow rate which provided the highest absorption signal was chosen.

2.6.3.3.2 Effect of sample introduction volume

The influence of sample introduction volume on absorbance of Fe(III)-nitroso-R complex absorption in the μFA system was studied by controlling pump flow rate and changing the switching time of the selection valve at the sample line over the range between 2.0 μL and 10.0 μL (five replicate measurements), under the

condition of 1.0 μg ml^{-1} Fe(III), 30 μL min^{-1} flow rate and 0.3 %w/v nitroso-R salt, to select the optimum conditions for the highest absorbance with low background and standard deviation of Fe (III)-nitroso-R complex. The sample volume which exhibited the highest absorbance was used.

2.6.3.3.3. Effect of pH

To studying the effect of pH, the absorbance of Fe(III)-nitroso-R complex was measured at pH values in the range of 3.0 to 10.0 (five replicate measurements) under conditions: 1.0 μg mL^{-1}, 5.0 μL injection volume, Fe(III), 30 μL min^{-1} flow rate and 0.3 %w/v nitroso-R salt. pH values of 3-7 were adjusted with acetic acid/sodium acetate buffer solution and pH 7-10 were adjusted with ammonium hydroxide/ammonium acetate buffer solution to select the optimum conditions for the highest absorbance of the Fe (III)-nitroso-R complex.

2.6.3.3.4. Effect of nitroso-R salt concentrations

The effects of nitroso-R salt concentrations (0.1 to 1.0 % w/v) on the absorbance of Fe(III)-nitroso-R complex in the μFA system (five replicate measurements) were studied under the given conditions (1.0 μg ml^{-1}, 5.0 μL injection volume, Fe(III), 30 μL min^{-1} flow rate and pH 5) to select the optimum conditions for the highest Fe (III)-nitroso-R complex absorbance. The concentration of nitroso-R salt which provided the highest absorbance was selected.

2.6.3.4. The method for studying precision

The precision of the method was determined by 11-replicates of three standard solutions covering different concentration levels: low, medium and high (0.1, 1.0 and 3.0 μg mL^{-1}); and the peak heights as absorbance were measured under the optimum conditions and the %RSD calculated as given by equation 2.1

2.6.3.5. The calibration graph and the percent recovery

The calibration graph and the percentage recovery were accomplished using the method as section 2.6.2.5 under the optimum conditions of the μFA system.

2.6.3.6. Effects of Interfering ions

The method for studying the effects of some possible interferences on the determination of Fe(III) in water samples was done as section 2.6.2.6 under the optimum conditions of the proposed μFA system.

2.6.4. The micro flow analysis system for copper determination

2.6.4.1 Instrument setup

The micro flow analysis (μFA) system for determining copper (Figure. 2.7) consisted of the same devices as iron system excepted a planar T-junction with micro coil channels on a poly(methylmethacrylate) (PMMA) plate was fabricated using laser ablation (Mercury Laser source 25 watt); the channel was 200 μm wide, 50 μm deep and 400 mm long covered with a poly dimethyl siloxane (PDMS) plate (M) containing a flow-cell (4 μL) as micro chip, a micro peristaltic pump as

delivering device, Light emitting diode (LED) super bright (white light) as light source and a pocket PC with Pocket SIA software as controlling and data collecting unit instead. Pocket SIA software (programming in our laboratory) was used to control the USB2000 spectrophotometer for measuring signal and collecting data. A ten-port selection valve with zero dead volume (VICI, Valco Instruments, USA) (V) and a micro peristaltic pump with small pump tubing (0.19 mm i.d.) (P) controlling by Pocket PC software was used for injecting accurate volumes of samples (S) and delivering appropriate reagent (R) flow rate.

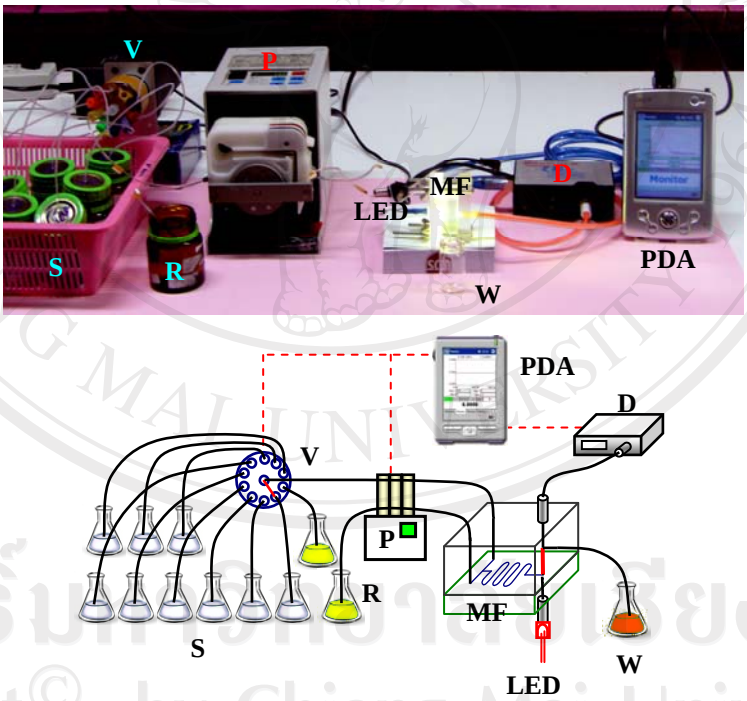


Figure 2.7 The μFA system for Cu(II) determination. S, sample; R, nitroso-R salt reagent; V, selection valve; P, peristaltic pump ; MF, a PMMA T-junction with micro-channel on the micro-flow analyzer; D, UV-Vis spectrophotometer; W, waste; PDA, pocket PC.

2.6.4.2. The method for copper determination using μFA

A micro-flow analysis system with fiber optic spectrometer for determination of copper was shown in Figure 2.7. The auxiliary peristaltic pump was used to transport the reagent and sample solutions to the system. The method included the aspiration of standard or sample solution containing copper into the reagent stream of nitroso-R salt buffered at pH 7 with 0.5 mol L^{-1} acetated buffer flowing at an appropriate total flow rate of 20 μL min^{-1} controlled using a peristaltic pump (time injection, sample volume calculated from aspiration time and flow rate). Nitroso-R salt and copper(II) were reacted in a micro reactor producing a red Cu(II)-nitroso-R complex then passed through the small volume flow-through cell (4 μL) fixed in PDMS top plate where the fiber optic probe was placed to measure the absorbance. The amount of copper(II) in each water sample was calculated by reference to the calibration graph prepared under identical conditions. A comparative determination of the copper(II) in the sample solutions was carried out by FAAS method.

2.6.4.3. Optimization of the experimental conditions

The proposed μFA system was optimized by a univariate method, to select the optimum conditions for the highest absorbance with low background and standard deviation of Cu(II)-nitroso-R complex. The value of one variable was changed while keeping the other variables at their constant values. By maintaining that variable at its optimum value, another was studied. The optimization of the experimental conditions was carried out by using standard Cu(II) solution. In all experiments, five replicate measurements were performed for each studied parameter.

2.6.4.3.1 Effect of flow rate

The effects of total flow rates on absorption signal of Cu(II)-nitroso-R complex in the μFA system were investigated for determining copper (1.0 μg ml^{-1} Cu(II), 5.0 μL injection volume and 0.4 %w/v nitroso-R salt). The total flow rate was varied over the range of 10 to 100 μL min^{-1} (five replicate measurements) to select the optimum conditions for the highest absorbance with low background and standard deviation of Cu(II)-nitroso-R complex. The total flow rate which provided the highest absorbance was chosen.

2.6.4.3.2 Effect of sample introduction volume

The influence of sample introduction volume on Cu(II)-nitroso-R complex absorption was studied by controlling pump flow rate and changing the switching time of the selection valve at sample line over the range between 2.0 μL and 10.0 μL (five replicate measurements) under the given conditions (1.0 μg ml^{-1} Cu(II), 20 μL min^{-1} and 0.4 %w/v nitroso-R salt), to select the optimum conditions for the highest absorbance with low background and standard deviation of Cu(II)-nitroso-R complex.

2.6.4.3.3. Effect of pH

The absorbance of Cu(II)-nitroso-R complex was studied at various pH values in the range of 3.0 to 10.0 (five replicate measurements), condition; 1.0 μg mL^{-1} Cu(II), 20 μL min^{-1}, 4 μL injection volume, and 0.4 %w/v nitroso-R salt. The pH values were adjusted with acetic acid/sodium acetate buffer solution (pH 3-7) and

ammonium hydroxide/ammonium acetate buffer solution (pH 7-10) to select the optimum conditions for the highest absorbance of the Cu(II)-nitroso-R complex.

2.6.4.3.4. Effect of nitroso-R salt concentrations

The effect of nitroso-R salt concentrations (0.1 to 1.0 % w/v) on the absorbance of Cu(II)-nitroso-R complex in the μFA system (five replicate measurements) were studied under the conditions: 1.0 μg mL^{-1} Cu(II), 20 μL min^{-1}, 4 μL injection volume, and pH 7, to select the optimum conditions for the highest absorbance of the Cu(II)-nitroso-R complex.

2.6.4.3.5. Effect of the micro-coil length

The effect of micro-coil length on the absorbance of the Cu(II)-nitroso-R salt complex was studied by varying the coil length of the PMMA micro-flow analyzer over the range of 200 to 500 mm, then they were used for analysis of 1.0 μg mL^{-1} standard Cu(II) under the optimum conditions (20 μL min^{-1}, 4 μL injection volume, pH 7 and 0.4 %w/v nitroso-R salt). The coil length that provided the highest absorbance was chosen.

2.6.4.4. The method for studying precision

Precision describes the reproducibility of the results from measurements based on repeatability performed on 11-replicates of three standard solutions covering different concentration levels: low, medium and high (0.1, 1.0 and

3.0 μg mL^{-1}). The peak heights of absorbance were measured. This characteristic is expressed as the relative standard deviation (RSD) and is given by equation 2.1

2.6.4.5. The calibration graph and the percentage recovery

The calibration graph was accomplished by measuring the absorbance of Cu(II)-nitroso-R salt complexes under the optimum conditions using Cu(II) standard concentration of 0.1, 0.5, 1.0, 2.0 and 3.0 μg mL^{-1}. The percentage recoveries were investigated by spiking 1.0 μg mL^{-1} copper(II) standard solution into water samples and measuring the absorbance under the optimum conditions. The absorbance of Cu(II)-nitroso-R salt complexes obtained from each sample was calculated (using equation 2.2.) referring to calibration graph.

2.6.4.6. Effects of interfering ions

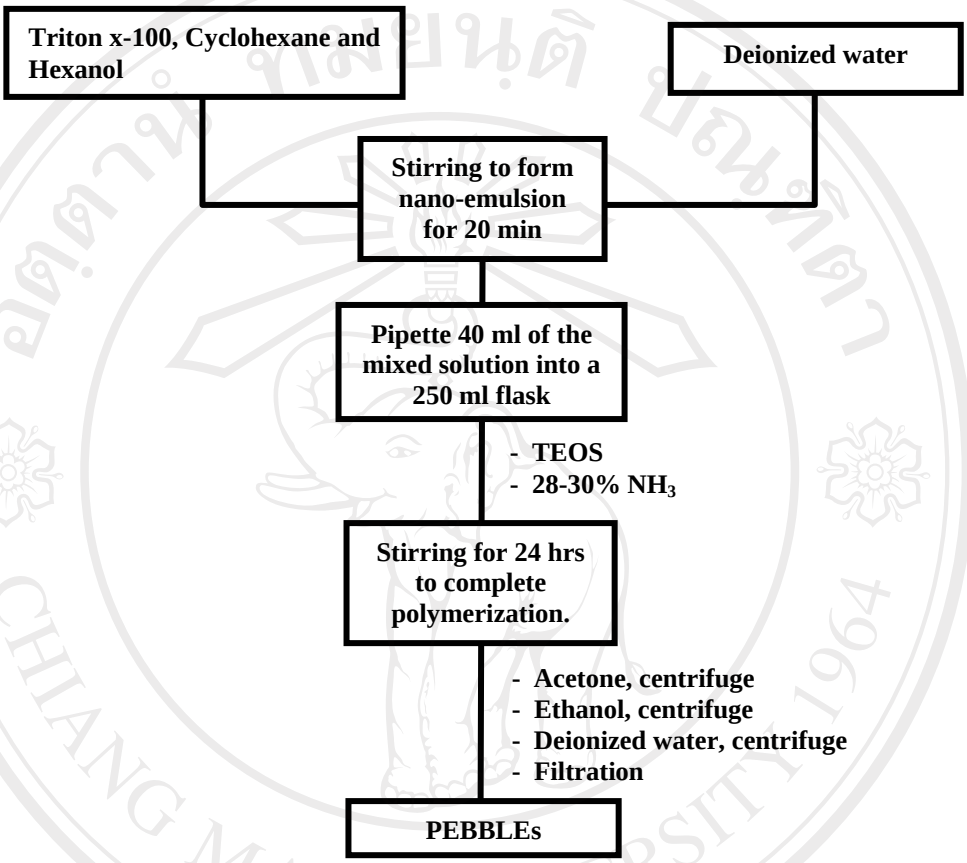
The effects of some possible interferences (Na^+, K^+, Ca^{2+}, NO_3^-, NO_2^-, SO_4^{2-}, Pb^{2+}, Cr^{3+}, Mg^{2+}, Cd^{2+}, Cl^-, PO_4^{3-}, Mn^{2+}, Zn^{2+}, Co^{2+}, Fe^{3+} and Ni^{2+}) on the determination of Cu(II) in water samples were studied for the maximum concentration ratio of foreign species to Cu(II) up to 100:1. The tolerance is defined as the foreign species concentration causing error smaller than ±10% for determining the analyte of interest.

2.6.5. Probes Encapsulated by Biologically Localized Embedding (PEBBLEs)

2.6.5.1 Preparation of Blank PEBBLEs

Blank PEBBLEs were prepared in three stages (scheme 1). Firstly, formation of the core PEBBLEs were produced by water in oil nano-emulsion. It was done by mixing of 8.00 ml triton X-100, 33.60 ml cyclohexane, 8.00 ml, hexanol and 1.232 ml DI water into a 250 ml Erlenmeyer flask placed on the magnetic stirrer. Then they were mixed together by stirring for 20-30 min to form water in oil (w/o) nano-emulsion. Secondly, the core PEBBLEs were coated with polymerization of tetraethyl orthosilicate (TEOS) in basic solution. It was done by pipetting 40.00 ml of the solution from the first stage into a 250 erlenmeyer flask placed on the magnetic stirrer, 1.6 ml TEOS and 0.944 ml 28-30% NH_3 were added into the solution and continued fast stir for 24 hrs to complete polymerization of TEOS. After 24 hrs, the crude blank PEBBLEs were obtained. Thirdly, the prepared PEBBLEs were then cleaned several times by using acetone, ethanol and deionized water, respectively. This was carried out by adding acetone, stirred for 30 min by using magnetic stirrer and centrifuge at 4000 rpm for 30 min several times (at least 5 times) to remove oil, surfactant, monomer, and unreacted chemicals. Acetone from this stage was removed by washing with ethanol, stirred by using vortex mixer for 1 min and centrifuge at 4000 rpm for 30 min several times (at least 5 times), followed by washing with deionized water, using the same washing method as with acetone and ethanol. The prepared blank PEBBLEs were filtered by using 200 nm and 20 nm membrane filter, respectively to remove the PEBBLEs size greater than 200 nm and collect the

prepared PEBBLEs size between 20 nm to 200 nm. Finally the prepared PEBBLEs were then dried and kept in a desiccator under acetone atmosphere.



Scheme 1 The method for preparation of Blank PEBBLEs.

2.6.5.2 Preparation of PEBBLEs containing silica nanoparticles modified with functional group for holding Mn(IV)

PEBBLEs containing silica nanoparticles modified with functional group for holding Mn(IV) were prepared in three stages similar to the method for preparing blank PEBBLEs but using silica nanoparticles modified with functional group for holding Mn(IV) (Figure 2.8) instead of deionized water in the first stage.

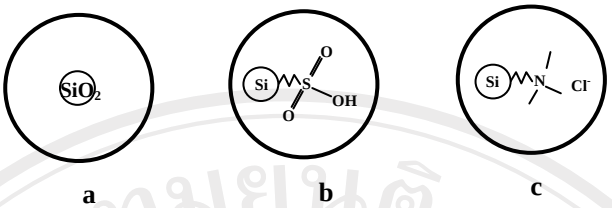


Figure 2.8 The PEBBLEs containing 15 nm silica nanoparticle modified with various functional groups; **a)** unmodified (SiO_2), **b)** modified with sulfonic acid groups, **c)** modified with quaternary ammonium chloride groups.

2.6.5.3 Method for characterization of prepared PEBBLEs

2.4.5.3.1 Characterization of the prepared PEBBLEs by Photon Correlation Spectrometer (PCS)

The surface charge and size of the prepared PEBBLEs were characterized by measuring zeta potential and size distribution via Photon Correlation Spectrometer. The surface charge of the prepared PEBBLEs is related to the suspension efficiency of them in the solution. The method for measuring the zeta potential and size distribution were done by suspending 1.0 mg mL^{-1} of the prepared PEBBLEs in 1 mM NaCl solution in ultrasonic bath for 1 hr. The air bubble in the suspension was removed by purging with nitrogen gas for 15 min. Then 1.0 ml of the suspension was transferred into the PCS sample cell.

2.6.5.3.2. Characterization of the prepared PEBBLEs by Scanning Electron Microscope (SEM)

The prepared PEBBLEs were also characterized via Scanning Electron Microscope (SEM). It was done by suspending of 1.0 mg mL^{-1} of the prepared

PEBBLEs in 1 mM NaCl solution in ultra sonic bath for 1 h. The air bubble in the suspension was removed by purging with nitrogen gas for 15 min. The suspension was transferred into SEM the sample cell.

2.6.5.4 Method for testing prepared PEBBLEs

2.6.5.4.1. Method for holding Mn(IV)

The method for holding Mn(IV) was done by incubating 0.1000 g of the blank PEBBLEs or the prepared PEBBLEs in 10.00 ml of 1.0 mM $KMnO_4$. The suspension was stirred for 24 hrs to complete holding of Mn(IV) (Figure 2.9).

Figure 2.9 Incubation of prepared PEBBLEs in the $KMnO_4$ solution.

2.6.5.4.2 Method for folic acid analysis by the prepared PEBBLEs

Suspending 0.1000 g of the blank PEBBLEs or the prepared PEBBLEs holding Mn(IV) in 2 M H_2SO_4 by using ultrasonic bath for 1 h. A 500 µL of the mixture solution (10^{-4} M folic acid, 1 M HCHO acidified with 2.0 M H_2SO_4) was pipetted and transferred into the sample cell positioned against the PMT window. A 150 µl of the suspended PEBBLEs was injected into the sample cell. The CL emitted

from the reaction was detected using PMT and recorded using chart recorder (Figure 2.10).

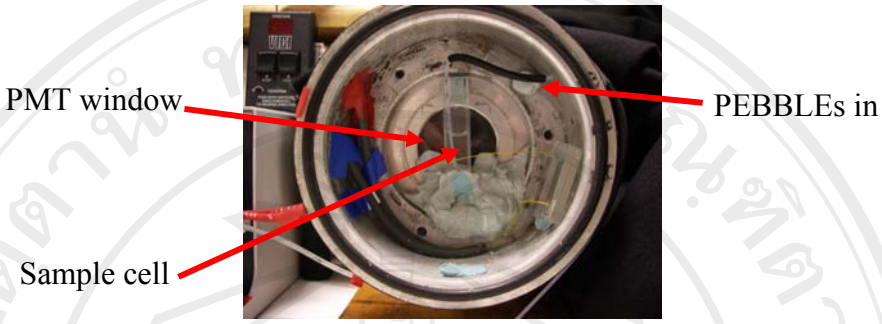


Figure 2.10 Analysis of standard folic acid using the prepared PEBBLEs.

2.6.5.5 Optimization of the prepared PEBBLEs

2.6.5.5.1. Incubation time for holding Mn(IV)

The optimization of the incubation time for holding Mn (IV) was done by weighing 0.1000 g of the prepared PEBBLEs containing silica nanoparticles modified with sulfonic group in 10.0 ml of 1.0 mM $KMnO_4$ placed on the magnetic stirrer. The mixture was stirred by varying the stirring time of 5, 10, 15, 20, 22, 24, 25, 26 28 and 30 hrs. After that 500 µL of each of the incubated PEBBLEs were injected into the sample cell (placed against the PMT window) containing 500 µL of 0.1 mM folic acid, 1.0 M formaldehyde as enhancer and acidified with 2.0 M sulfuric acid. Then CL signal detected by the PMT was recorded using chart recorder. The incubation time which provided the highest CL signal was selected.

2.6.5.5.2 Optimization of formaldehyde concentration

The method for optimizing formaldehyde concentration for enhancing the CL signal provided by the prepared PEBBLEs was done by adding various formaldehyde concentrations of 0.1, 0.3, 0.5, 0.8, 1.0, 1.3, 1.5, 2.0, 2.5 and 3.0 M into 0.1 mM folic acid acidified with 2.0 M sulfuric acid. Then each mixture was pipetted into sample cell placed against the PMT window. After that 150 μL of the suspended PEBBLEs containing Mn(IV) was injected into the sample cell. The CL signal detected by the PMT was recorded using chart recorder. The concentration of formaldehyde which provided the highest CL signal was chosen.

2.6.5.5.3. Optimization of potassium permanganate concentration

The method for optimizing potassium permanganate concentration for incubating the prepared PEBBLEs was done by weighing 0.1000 g of the prepared PEBBBLEs containing silica nanoparticles modified with sulfonic group in 10.0 ml various concentrations of potassium permanganate (0.1 to 1.0 mM), placed on the magnetic stirrer, stirred for 24 hrs. A 150 μL of each of the incubated PEBBLEs were injected into the sample cell (positioned against the PMT windows) containing 500 μL of 0.1 mM standard folic acid acidified with 2.0 M sulfuric acid and 1.0 M formaldehyde as enhancer. The CL signal provided from the PMT was recorded using chart recorder. The concentration of potassium permanganate which provided the highest CL signal was chosen.

2.6.5.5.4. Effect of various acid on the CL signal

The Effect of various acid on the CL signal obtained from the prepared PEBBLEs was done by incubating 0.1000 g of the prepared PEBBLEs containing silica nanoparticles modified with sulfonic group in each 10.0 ml of 2.0 M of sulphuric acid, nitric acid, hydrochloric acid, phosphoric acid and acetic acid. The mixture was placed on the magnetic stirrer and stirred for 24 hrs. A 150 μL of the incubated PEBBLEs acidifyied with each acid was injected into the sample cell (positioned against the PMT windows) containing 500 μL of 0.1 mM standard folic acid acidified with 2.0 M sulfuric acid and 1.0 M formaldehyde as enhancer. The CL signal detected by the PMT was recorded using chart recorder. The acid which provided the highest CL signal was selected.

2.6.5.5.5. Optimization of sulfuric acid concentration

The method for optimizing sulfuric concentration was done by incubating 0.1000 g of the prepared PEBBLEs containing silica nanoparticles modified with sulfonic group in each 10.0 mL of various sulphuric acid concentrations (1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0 and 5.0 M). The mixture was placed on the magnetic stirrer and stirred for 24 hrs. A 150 μL of each incubated PEBBLEs were injected into the sample cell (positioned against the PMT windows) containing 500 μL of 0.1 mM standard folic acid acidified with 2.0 M sulfuric acid and 1.0 M formaldehyde as enhancer. The CL signal detected by the PMT was recorded using a chart recorder. The concentration of sulfuric acid which provided the highest CL signal was chosen.

2.6.5.6. Studying of the stability of the prepared PEBBLEs

The method for studying the stability of the prepared PEBBLEs was done by incubating 0.1000 g of the PEBBLEs containing silica nanoparticles modified with sulfonic group in 10.0 ml of 0.7 mM potassium permanganate for 24 h., washed several times with deionized water, filtered and dried. The incubated PEBBLEs were left for 1 to 5 days in desiccator. After that each incubated PEBBLEs were acidified in 10.0 ml of 3.0 M sulphuric acid. A 150 μL of each acidified PEBBLEs was injected into sample cell (left against the PMT window) containing 0.1 mM folic acid acidified with 3.0 M sulphuric acid and 1.0 M formaldehyde as enhancer. The CL signal detected by the PMT was recorded using a chart recorder. The incubated PEBBLEs which provided the highest CL signal was selected.

2.6.5.7. Studying interferences

The method for studying interferences was done by suspending 0.1000 g of the PEBBLEs containing silica nanoparticles modified sulfonic group with holding Mn(IV) in 3 M H_2SO_4 using ultrasonic bath for 1 h. Each 0.5% of albumin, glutathione, γ-globulin and cysteine were added in each mixtures solution of 0.10 mM folic acid and 1.0 M HCHO acidified with 3.0 M H_2SO_4. 500 μL of each mixture was pipetted into each sample cell positioned against the PMT window, after that a 150 μl of the suspended PEBBLEs was injected. The CL emitted from the reaction was detected using the PMT and recorded using chart recorder. The CL signals obtained from all solutions were compared.