CHAPTER II

EXPERIMENTAL

2.1 Chemicals and reagents

All chemicals were analytical grade, otherwise specified, as follows;

2.1.1 Sucrose determination

2.1.1.1 Chemicals

1) Bromoyhymol blue, $C_{27}H_{28}Br_2O_5S$, AR grade, Fluka
2) sodium tetraborate decahydrate, $Na_2B_4O_7 \cdot 10H_2O$, ≥99.0%, (M&B)
3) Hydrochloric acid, HCl, 99.5% (Merck, Germany)
4) Sodium hydroxide, NaOH, 99.0% (Merck, Germany)
5) Sucrose, $C_{12}H_{22}O_{11}$

2.1.1.2 Reagent and solution preparation

All reagent and standard solutions were prepared using ultra pure water (obtained from a Milli-Q system, Millipore, Sweden).

A. Preparation of the standard solutions

The stock standard of sucrose was prepared by dissolving 100 g sucrose in 50 ml deionization (DI) water, (100 g of sucrose in total weight of 150 g DI water as 66.67 degree of brix, ^{o}Bx). The stock solution was diluted with water to give the appropriate concentrations of working standard solutions (10.0-50.0 ^{o}Bx).

B. Preparation of the reagent solution

Bromothymol blue 1.00 %w/v (0.0160 M) was prepared in borate buffer 0.025 M which, was prepared from sodium tetra borate 0.4770 g dissolving in DI water 50 ml and adjusted to pH approximately 9.0 by adding HCl 0.01 molar or NaOH 0.01 molar in a volumetric flask. This buffer could be stored at 4 ^{o}C for two weeks.

C. Preparation of the samples

Syrup samples such as corn, Hale's Blue Boy, cough syrup, etc. They could be injected directly without pretreatment onto a simple LOC.

2.1.2 Fe (II) determination

2.1.2.1 Chemicals

1) Ferrous ammonium sulfate hexahydrate, $Fe(NH_4)_2(SO_4)_2.6H_2O$, 99.0-101.5% (Carlo Erba)
2) Potassium permanganate, $KMnO_4$, 99.0-100.5%, (Merck, Germany)
3) Hydrochloric acid, HCl, 99.5% (Merck, Germany)
4) Acetic acid, CH_3COOH, 99.7% (Analytical Reagent Grade)
5) Ascorbic acid, $C_6H_8O_6$, 99-100.5% (APS)
6) Ammonium acetate, CH_3COONH_4, 97.5% (RANKEM, Analytical Reagent Grade)
7) 1,10-phenanthroline monohydrate, $C_{12}H_8N_2H_2O$, 99.5% (Merck, Germany)

2.1.2.2 Reagents and solution preparation

All reagent and standard solutions were prepared using milli-Q water (Milli-Q, Millipore, Sweden). The glassware was rinsed with concentrated HCl before use, followed by rinsing them thoroughly with milli-Q water to remove deposits of iron oxide.

A. Preparation of standard solution

The stock Fe (II) standard solution was prepared by dissolving 0.7030 g of solid $Fe(NH_4)_2(SO_4)_2.6H_2O$ in 10 ml concentrated H_2SO_4 to 25 ml milli-Q water. Then, the solution was diluted to 500 ml with milli-Q water and mixed well. Concentration of Fe (II) was equivalent to 200 ppm. The stock Fe (II) standard solutions should be stable for several months.

Fe (II) standard solutions for the preparation of calibration graph were prepared daily by diluting a 200 ppm stock Fe (II) standard solution with milli-Q water to the concentration in the range of 0.03-1.00 ppm.

B. Preparation of reagents

Reagents were prepared as follows:

A. Ammonium acetate buffer solution was prepared by dissolving 25.0293 g CH_3COONH_4 in 15 ml milli-Q water. Concentrated CH_3COOH 70 ml was added.

B. Phenanthroline solution was prepared by dissolving 0.1060 g 1,10-phenanthroline monohydrate in 100 ml milli-Q water. Stirring was needed but heating is unnecessary if 2 drops concentrated HCl was added to the milli-Q water.

C. Preparation of sample

Groundwater and water ponds samples were collected in clean PTFE bottles. Then concentrated HCl was added 2 ml for each 100 ml sample at the time of collection. The samples were filtered through the filter paper. After that, the samples were analyzed for the Fe (II) content only.

2.1.3 Phosphate determination

2.1.3.1 Chemicals

1) Sulfuric acid, $H_2 SO_4$, 98% (Wako Pure Chemical Co., Japan)
2) Potassium antimonyl tartrate, $K(SbO)C_4H_4O_6.1/2H_2O$, ≥99.0%, (M&B)
3) Hydrochloric acid, HCl, 99.5% (Merck, Germany)
4) Ammonium molybdate tetrahydrate, $(NH_4)_6Mo_7O_{24}.4H_2O$, (Fisher scientific, Analytical Reagent Grade)
5) Ascorbic acid, $C_6H_8O_6$, 99-100.5% (APS)
6) Potassium dihydrogen phosphate, KH_2PO_4, 99.9% (Fisher scientific, Analytical Reagent Grade)

2.1.3.2 Reagents and solution preparation

- Preparation of standard solution

The stock phosphate solution 1000 ppm was prepared by dissolving potassium dihydrogen phosphate, KH_2PO_4 1.4334 g in 100 ml of milli-Q water. Preparation of the working standard solution at approximately 0.03-1.00 ppm was done by diluting the stock phosphate standard solution with milli-Q water. Working standard solutions were prepared daily.

The standard addition was used in the experiment. Sample solution, phosphate and their mixture were prepared by adding phosphate standard solution to obtain final added concentration in 10 ml sample in a 25 ml volumetric flask. This procedure was used to prepare the standard addition 0.05-1.00 ppm range for the study of interfering species in pond water.

- Preparation of reagents

A. Sulfuric acid 2.5 M:

A portion of milli-Q water was used to dilute concentrated H_2SO_4 70 ml was added, then adjusting volume to 500 ml with milli-Q water.

B. Potassium antimonyl tartrate solution 0.01 M:

Solution of potassium antimonyl tartrate was prepared by dissolving solid $K(SbO)C_4H_4O_6.1/2H_2O$ 0.1672 g in 40 ml milli-Q water in a 50 ml volumetric flask and diluted to the marked volume. This solution was stored in a glass-stopper bottle.

C. Ammonium molybdate solution 0.03 M:

Solution of ammonium molybdate was prepared by dissolving $(NH_4)_6Mo_7O_{24}.4H_2O$ 1.8360 g in 50 ml milli-Q water. This solution was stored in a glass-stopper bottle.

D. Ascorbic acid 0.01 M:

Ascorbic acid was used as a reducing agent for this method. Ascorbic acid 0.8800 g was dissolved in 50 ml milli-Q water. The solution is stable for about 1 week at 4^oC.

E. Combined reagent

The combined reagent was prepared daily by mixing the amount of each stock reagent in the following proportions to obtain 25 ml of the combined reagent: 12.5 ml (2.5 M) H_2SO_4, 1.25 ml (0.01 M) potassium antimonyl tartrate solution, 3.75 ml (0.03 M) ammonium molybdate solution, and 7.5 ml (0.01 M) ascorbic acid solution. All of reagents were mixed together. If turbidity formed the combined reagent was shaken for a few minute until turbidity disappears period to use. This reagent is stable for 4 hour.

- Preparation of samples

Samples of approximately 1000 ml were collected from ponds around Chiang Mai University. Filtrations of samples were done for the determination of reactive phosphorus content, using ascorbic acid method. This gave the sum of polyphosphate and orthophosphate in the sample. The obtained solution should have a concentration in the range of a calibration graph. Some samples may be diluted for the appropriate analysis range.

2.2 Equipment/Apparatus

2.2.1 Sucrose determination

1) A simple chips were made of a 2cm×3 cm×1.2 cm and 2.5cm×5 cm ×1.2 cm of acrylic pieces
2) Fiber-optic UV-VIS diode array detector USB 2000 (Ocean Optics Inc., Dunedin, USA)
3) Analytical balance, Model BP210s (Sartorious, Germany)
4) pH meter, model 744 (Metrohm, USA)
5) Nuts and Ferrules (Upchurch)
6) Three-way valves (MEDITOP)
7) Syringes
8) Stopwatch
9) PTFE tubing i.d. 1.6 mm (Elkay Hanpshire, England)
10) Flow through cell (BAS, USA)
11) Peristaltic pump (Ismatec)
12) Injection valve (Upchurch, USA)
13) eDAQ Chart software (PowerChrom 280)
14) A data recorder by the program FIA lab for windows 5.0.

2.2.2 Fe (II) determination

1) A simple chip was made of a 2 cm×3 cm×1.2 cm acrylic piece
2) Fiber-optic UV-VIS diode array detector USB 2000 (Ocean Optics Inc., Dunedin, USA)
3) Analytical balance, Model BP210s (Sartorious, Germany)
4) pH meter, model 744 (Metrohm, USA)
5) Nuts and Ferrules (Upchurch)
6) PTFE tubing i.d. 1/4" (Elkay Hanpshire, England)
7) Solenoid pumps (Biochem valve, USA)
8) Solenoid valves (Takasago, Japan)
9) eDAQ Chart software (PowerChrom 280)
10) A data recorder by the program FIA lab for windows 5.0.
11) UV-VIS spectrometer, Series 1700 (Shimadzu)

2.2.3 Phosphate determination

1) A simple chip was made of a 2 cm×3 cm×1.2 cm acrylic piece
2) Fiber-optic UV-VIS diode array detector USB 2000 (Ocean Optics Inc., Dunedin, USA)
3) Analytical balance, Model BP210s (Sartorious, Germany)
4) pH meter, model 744 (Metrohm, USA)
5) Nuts and Ferrules (Upchurch)
6) PTFE tubing i.d. 1/4" (Elkay Hanpshire, England)
7) Solenoid pumps (Biochem valve, USA)
8) Solenoid valves (Takasago, Japan)
9) eDAQ Chart software (PowerChrom 280)
10) A data recorder by the program FIA lab for windows 5.0.
11) UV-VIS spectrometer, Series 1700 (Shimadzu)

2.3 Manifold/Procedure

2.3.1 Sucrose determination

2.3.1.1 Simple LOC with naked eyes

Simple LOC with naked eyes was designed, as depicted in **Figure 2.1 (a)**. This system consists of a chip that was made of an acrylic piece of 2.50 cm × 5.00 cm × 1.2 cm (width × length × thickness) by drilling channels through the acrylic piece using 1.00 mm drill bit. Total volume of the channels was approximately 60 µl. Detection was done by naked eyes with a simple stop-watch based on migration time of the reaction zone.

The preliminary operation step of a simple LOC with naked eyes detection was described as follows. Sucrose standard solution was injected into the vertical channel. After that, bromothymol blue 1.00% w/v in borate buffer was injected into the horizontal channels Detection time was recorded using a stop-watch from the channels crossing to the detection mark. A calibration graph was constructed from concentrations of sucrose and migration times. Reproducibility of the response was tested by injecting of sucrose concentration 3 times repeatedly.

2.3.1.2 Simple LOC with fiber optic spectrophotometer

Simple LOC system coupled with fiber optic spectrophotometer is shown in **Figure 2.1 (b)**. This system consists of a peristaltic pump (Ismatec) through which, a carrier stream was injected into a chip. The size of a chip was 2.00 cm × 3.00 cm × 1.2 cm (width × length × thickness) acrylic plastic piece and channels were drilled through a chip using a 1.2 mm drill bit. Total volume of the channels was approximately 50 µl. The time based detection point was set by placing a fiber optic spectrophotometer (two probes) with USB 2000 at 1.50 cm from the crossing of channels on the chip. The most efficient wavelength 300-1100 nm range was used.

The desired volume of the carrier solution (DI water) was introduced via the peristaltic pump to fill the vertical channel. After that, the sample/standard solution was injected into the horizontal channel and recorded by the program FIA lab for window 5.0. Absorbance spectra and time were recored. Each concentrations of standard/sample were injected for three replicates.

2.3.1.3 Flow injection analysis (FIA) with fiber optic spectrophotometer

The schematic diagram of the FIA is given in **Figure 2 (c)**. Single stream manifold consists of a peristaltic pump (1/16 // I.D. of the peristaltic tubing). Each sucrose solution was introduced to the FI system via a six port injection valve. Total volume is 100 µl (Teflon tubing 1/16 // I.D.), connecting to a flow through cell whereon to be point of detection at 1.00 cm on a chip. Fiber optic spectrophotometer with USB 2000 was used as a detector for the FI system. Recording and evaluating data were with FI program, Microsoft Excel and E-DAQ.

Generally three injections were done for each standard/sample. The results, the data of turning point or time based analysis and absorbance spectra were obtained. Standard solutions 10, 20, 30, 40, and 50 ^{o}Bx were prepared in DI water and used to constructed calibration graph. The set-up was as represented in **Figure 2.1.**

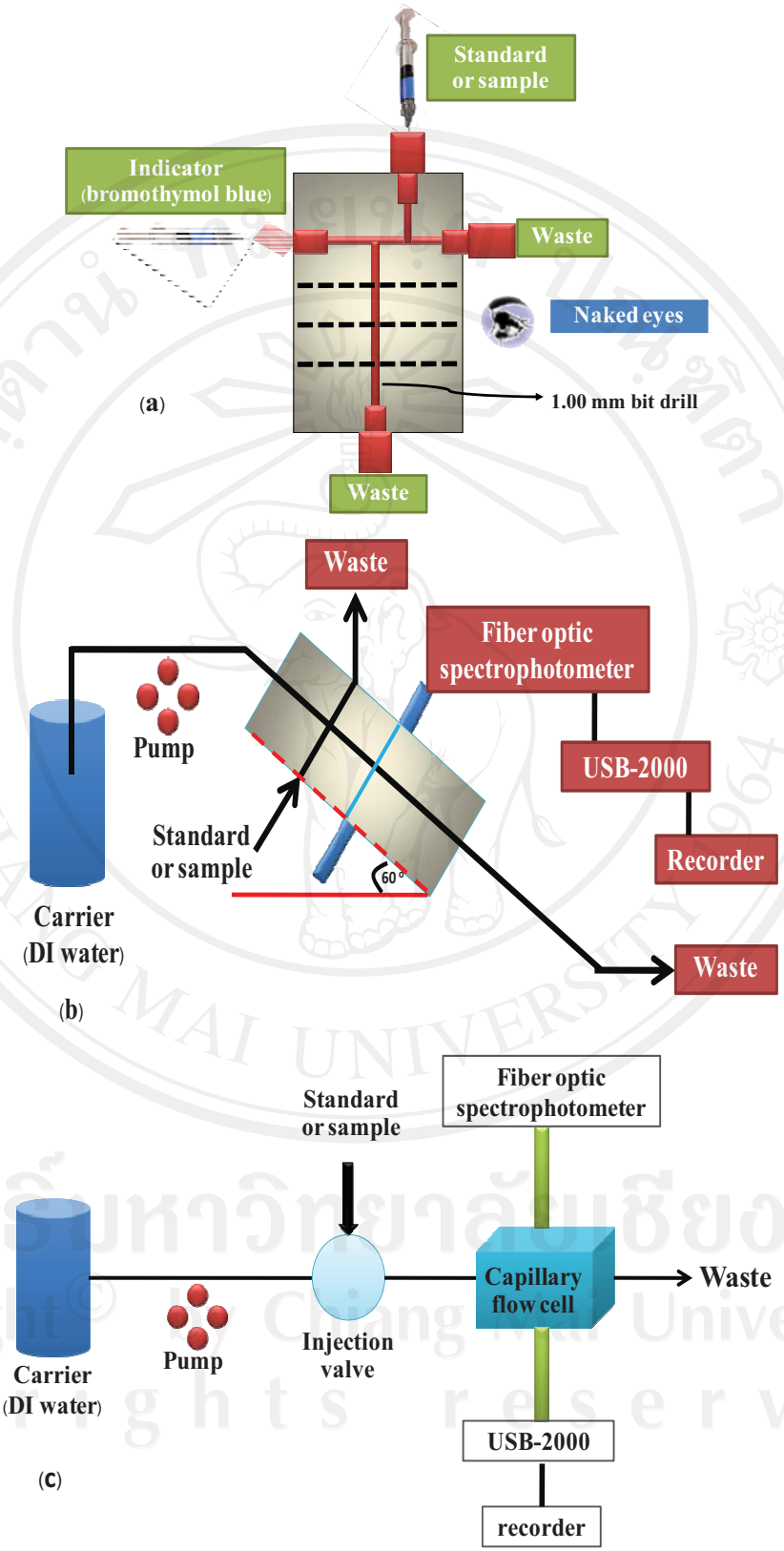

Figure 2.1 (a) Simple LOC with naked eyes (b) Simple LOC with USB 2000 (c) A normal FI manifold

2.3.2 Fe (II) determination

2.3.2.1 Simple LOC with fiber optic spectrophotometer

A manifold of a simple LOC with fiber optic spectrophotometer used in this work is shown in **Figure 2.2.**

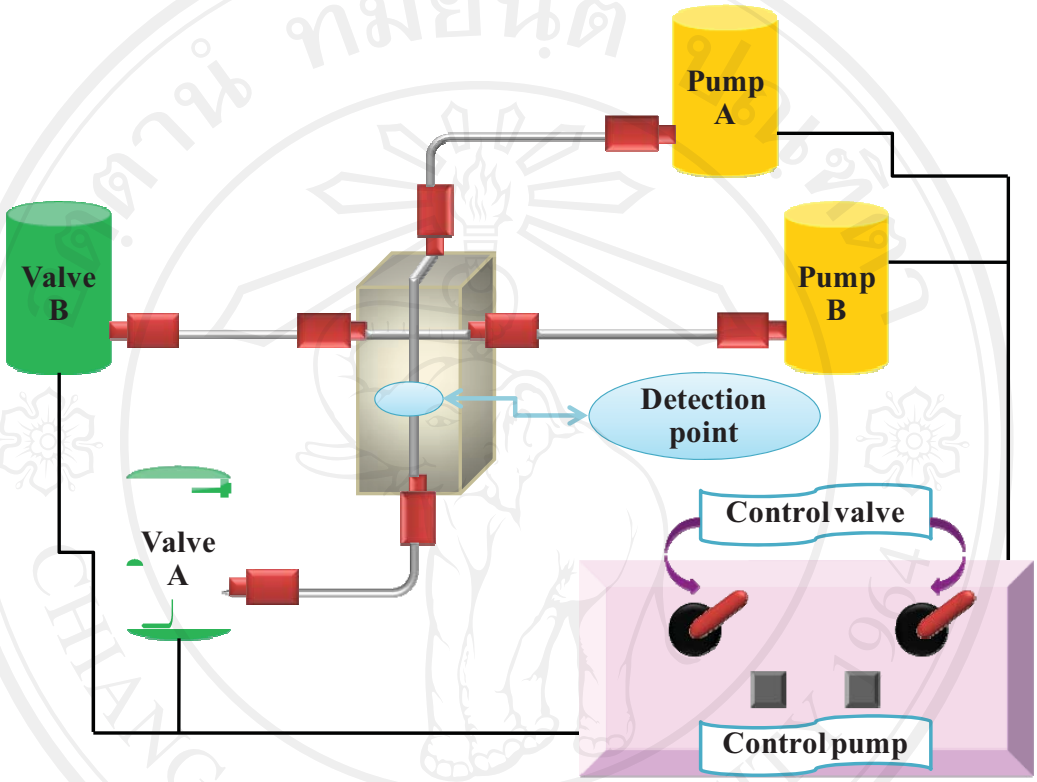

Figure 2.2 Simple LOC with fiber optic spectrophotometer for assay Fe (II)

First, the system was cleaned with milli-Q water by pumping milli-Q water with pump B (horizontal channel) through valve B. After that, valve A was opened and the vertical channel was cleaned by pumping milli-Q water through with pump A. Then, the standard solution was pumping into vertical channel through an opened valve A. A 5 ml of 1, 10-phenanthroline was mixed with 2.5 ml ammonium acetate buffer solution and the mixture was shaken until homogenous. This solution was pumped into the horizontal channel via valve B. Finally, the reaction zone started at the channel crossing migrated passing the detection point (1.00 cm) from the crossing equipped with a fiber optic spectrophotometer. Absorbance spectra were obtained.

2.3.2.2 Visible spectrophotometry

The determination of the Fe (II) was based on phenanthroline method. The batchwise operation of this visible spectrophotometric detection was used as a standard method.

12.5 ml portion of acidified sample was added into volumetric flask. After that, a 5 ml 1,10-phenanthroline and 2.5 ml CH_3COONH_4 solution were added with vigorous stirring. Finally, the mixture was diluted to 25 ml with milli-Q water and its colored intensity was measured within 5 to 10 min at wavelength 510 nm, using quartz cells with the light path of 1.00 cm and a visible spectrophotometer.

2.3.3 Phosphate determination

2.3.3.1 Simple LOC with fiber optic spectrophotometer

A simple chip systems presented here was made of a 2 cm ×3 cm ×1.2 cm acrylic piece (width × length × thickness). Channels were created by drilling through the side of the acrylic piece using a 1.20 mm drill bit. Total volume of the channels was approximately 50 µl. A detection port was made by drilling at the top of acrylic plastic piece approximately 1.00 cm from the cross. The fiber optic spectrophotometer (one probe) was connected to the chip at this port. The chip was placed in a black box made of plastic to protect light from outside. This chip was installed on a stand that can be tilted to appropriate angles for different reactions. Two solenoid pumps (Biochem valve, USA) were connected to the vertical channel (Pump A) and the horizontal channel (Pump B). In addition, two solenoid valves (Valve A and Valve B, Takasago, Japan) were connected to the waste lines. Solenoid pumps and valves in the closed box were controlled by a control switches of a 12 volt DC power supply placed outside the box, see **Figure 2.2.**

The operation step, this system was cleaned with milli-Q water by pumping milli-Q water with pump B (horizontal channel) through the opened valve A and valve B. Phosphate standard solution was pumped into the vertical channel (Valve A) with pump A. Then, valve A was closed and valve B was opened. The combined reagent was pumped into the horizontal channel (Valve B) with pump B. Valve B was then closed. The migration time of the reaction zone was recorded using a fiber optic spectrophotometer with USB 2000. The reaction zone was detected at the mark (at 1.00 cm downstream of the cross) by following the blue color of

phosphoantimonyl molybdenum blue complex. Law data were obtained as absorbance spectra via time. Each of phosphate concentration was repeatedly analyted three times within one day.

2.3.3.2 Visible spectrophotometry

The standard/sample solutions were pipetted 12.5 ml into a clean 25 ml volumetric flask. After that, 2 ml of the combined reagent was added and mixed thoroughly. Absorbance of this solution was measured at 880 nm using reagent blank as the reference solution. The mixture solution could be measured after at least 10 min of mixing but no more than 30 min for the best color development.

2.3.4 Refractometry for sucrose measurement

A refractometer was employed. (The experiments were kindly carried out by Mr. Wasin Wongwilai.)

2.4 Optimization

2.4.1 Sucrose determination

2.4.1.1 Simple LOC with naked eyes

A. Linearity of calibration curve

Different solutions of sucrose with various degrees of brix (^{o}Bx) for were injected into a chip. A calibration graph was obtained by plotting of migration time against concentrations of sucrose.

B. Sucrose analysis in real samples

The method was applied for syrup samples such as, cough syrup, and fruit syrup.

C. Precision

The precision of all the proposed method was studied by injecting three replications of each sucrose concentration and sample solution.

2.4.1.2 Simple LOC with fiber optic spectrophotometer

A. Wavelength

The main factor that affects the signals was wavelength. The wavelengths of 470, 500, 600, and 800 nm were selected for the study of the signals of turning point.

B. Calibration curve

Standard solutions (10-50 ^{o}Bx) were used for calibration graph.

2.4.1.3 Flow injection analysis (FIA) with fiber optic spectrophotometer

A. Wavelength

The wavelengths of 470, 500, 600, and 700 nm were selected for the study of the signals of turning point.

2.4.1.4 Refractometry for sucrose measurement

Standard/sample solutions were prepared as described previously.

2.4.2 Fe (II) determination

A. Spectrum study

Concentration of Fe (II) standard solution was selected at 1.00 ppm for the study of the colored complex formation.

B. Linearity of calibration curve

This calibration graph was constructed using Fe (II) standard and a visible spectrophotometer. A series of Fe (II) standard solution with different concentrations (0.03-1.00 ppm) from fresh working standard solutions were injected into a simple LOC at optimum conditions. The resulting absorbance was measured. A typical calibration graph was plotted between the absorbance against various concentrations of Fe (II).

C. Recovery assay and Interference study

The investigation of percentage recoveries and interfering species of Fe (II) concentrations (0, 0.10, 0.50, 1.00 ppm) in groundwater and pond water were done through spiked and un-spiked samples. The results were considered as standard addition data and were compared with the calibration graph.

D. Analysis of real samples

The method was applied for the determination of Fe (II) in groundwater and pond water samples. The optimum conditions used for this experiment was adjusted using a test manifold. When necessary, samples were diluted manually before analysis.

2.4.3 Phosphate determination

A. Spectrum study

The standard phosphate solution was injected into the vertical channel. Then, the combined reagent was injected into the horizontal channel. The absorbance

of the blue color product and correspond wavelength were recorded. The wavelength that offered maximum absorbance was selected for determination of phosphate.

B. Elevation of the chip

The suitable elevation of the chip was investigated by comparing the differences of the signals between absorbance against time when tilting. The chip at 0, 10, 15, and 30 degree as respected to the horizontal plane.

C. The effect of the injection into vertical and horizontal channels

The optimum effect of the injection to monitor the phosphoantimonyl molybdenum blue complex has been studied by varying reagent added. This studied has investigated the need to optimize the different signals of blue color formation between phosphoantimonyl molybdenum blue complexes. The optimizations of the experiment are listed in **Table 2.1**.

Table 2.1 The conditions for preliminary injection of phosphate concentration

The conditions	The vertical channel	The horizontal channel
Condition 1	Phosphate concentration 0, 0.05, 0.50, and 1.00 ppm	- H_2SO_4 2.5 M: 12.5 ml - Potassium antimonyl tartrate solution 0.01 M: 1.25 ml - Ammonium molybdate solution 0.03 M: 3.75 ml - Ascorbic acid 0.01 M: 7.5 ml
Condition 2	- H_2SO_4 2.5 M: 12.5 ml - Potassium antimonyl tartrate solution 0.01 M: 1.25 ml - Ammonium molybdate solution 0.03 M: 3.75 ml - Ascorbic acid 0.01 M: 7.5 ml	Phosphate concentration 0, 0.05, 0.50, and 1.00 ppm

D. The effect of potassium antimonyl tartrate

The effect of potassium antimonyl tartrate solution was studied by adding this solution into 0.50 ppm of phosphate standard solution. Absorbance via time was recorded.

E. Calibration curve

Calibration graph was constructed from a series of six/seven standards (0.03-1.00 ppm). The quantitative of analyzes of phosphate/phosphorus in pond water can be done using this calibration graph.