CHAPTER III

EXPERIMENTAL

3.1 Apparatus

A Waters™ Controllers, model 600

A Waters™ Autosampler, model 717

A Waters™ Pump, model 600

A Waters™ Photodiode Array Detector, model 996

A COMPAQ Computer

A LaserJet 4L Printer, Hewlett Packard, USA

A Silicon Power Supply, SR-111

A SHANDON Column, Hypersil , 250 x 4.6 mm I.D., 5 μm,

A Waters Vacuum Pump, model DOA-V130-BN, with Pressure Regulator, Millipore, USA.

A Glass Filter Holder Set (300 mL Funnel, 1L Flask, Glass base and tube cap, and 47 mm Spring clamp) for HPLC mobile phase filtration xx1504700, Millipore, USA.

Membrane Filters, type FH 0.50 μm, Millipore, USA

A Milli-Q, Ultrapure Water Systems, with Millipak®40 Filter Unit 0.22 μm, model Millipore ZMQS5V00Y, Millipore, USA.

A RiOs *8*, Reverse Osmosis System, model Millipore ZROS5008Y, with Automatic Sanitization Module, Millipore, USA.

Helium Gas 99.99% purity, TIG, Thailand

A 744 pH meter, Metrohm, Switzerland.

A microsyringe 250 μL, Unimetrics Corporation, USA.

Graduated pipettes 1.00, 2.00, 5.00, and 10.00 mL

Volumetric pipette 2.00 and 5.00 mL

Cylinders 5.0, 25.0, 250.0, and 500.0 mL

Volumetric flasks 5.00, 10.00, 25.00, 50.00, and 100.00 mL

Beakers 5, 10, 50, 250, 500, and 1000 mL

Vials 1 mL with caps, Millipore, USA.

All glass apparatus were washed in detergent, thoroughly rinsed with purified water and then soaked in acidic cleaning solution overnight. The glass apparatus were then rinsed with purified water and baked in an oven at 150 °C for at least 3 hours, except volumetric flasks, pipettes, and cylinders. In the last step, all glass apparatus were rinsed with water or methanol before use.

3.2 Chemicals

3.2.1 The Standard of Mercury Compounds

Mercuric chloride ($HgCl_2$) was purchased from Merck, Germany and Methylmercury chloride (MeHgCl) was purchased from Farmitala Carlo Erba. They were analytical grade (AR grade). Phenylmercury chloride (PhHgCl) with percent purity > 97% was purchased from Fluka Chemica, Switzerland.

3.2.2 Organic Solvent and Reagents

Methanol was obtained from J.T. Baker, USA. It was analytical grade and was purified by fraction distillation in all glass apparatus and the distilled was checked for purity by high performance liquid chromatography prior to use. Acetic acid glacial and hydrochloric acid were obtained from BDH laboratory supplies, England. Nitric acid was obtained from Ajax Chemicals, Australia. Sodium acetate trihydrate and sodium hydroxide anhydrous pellets were obtained from Farmitala Carlo Erba. They were analytical reagent grade. The purified water with resistance 18.2 MΩ.cm. was obtained from the Milli-Q, Ultrapure Water System.

2-Mercaptoethanol, the complexing agent, was obtained from Merck-Schucharat, Germany. It was synthesis grade for the study of optimization chromatographic condition and analytical grade for the study of linearity, calibration curve, precision, accuracy, and detection limit. Tetrabutylammonium bromide (TBABr) with percent purity > 98%(Br) was obtained from Fluka Chemica, Switzerland. Tetrabutylammonium hydroxide (TBAOH) solution in 10% methanol was HPLC grade and was obtained from TCI, Japan. Sodium hexanesulfonate was ion-pair reagent grade and was obtained from FSA Laboratory Supplied, England. They were used as ion-pairing reagent.

3.3 Preparation of the Standard Solutions

3.3.1 The Stock Standard Solutions of Mercury Compounds

1. The 1000.00 ppm (as Hg) stock solution of inorganic mercury was prepared by dissolving 0.0135 g of mercuric chloride (MW= 271.500) and then diluting it to the mark with the purified water in 10.00 mL volumetric flask.

2. The 500.00 ppm (as Hg) stock standard solution of methylmercury was prepared by dissolving 0.0312 g of methylmercury chloride (MW= 251.101) and then diluting it to the mark with methanol in 50.00 mL volumetric flask.

3. The 250.00 ppm (as Hg) stock standard solution of phenylmercury was prepared by dissolving 0.0195 g of phenylmercury chloride (MW= 313.150) and then diluting it to the mark with methanol in 50.00 mL volumetric flask.

4. The 100.00 ppm (as Hg) stock standard solution of inorganic mercury was prepared by pipetting 1.00 mL of 1000.00 ppm (as Hg) stock standard inorganic mercury solution into 10.00 mL volumetric flask and then diluting it to the mark with the purified water.

5. The standard mixture solution of mercury compounds (5.00, 10.00, and 10.00 ppm (as Hg) of inorganic, methyl, and phenylmercury, respectively) was prepared by pipetting 0.50 mL of 100.00 ppm (as Hg) of inorganic mercury standard solution, 0.20 mL of 500.00 ppm (as Hg) of methylmercury standard solution, and

0.40 mL of 250.00 ppm (as Hg) of phenylmercury standard solution into 10.00 mL volumetric flask and then diluting it to the mark with the purified water.

All standard stock solution were kept at 4 ^{0}C

3.3.2 The Sodium Acetate-Acetic Acid Buffer Stock Solution

The 0.20 M sodium acetate solution was prepared by dissolving 27.199 g of sodium acetate trihydrate (MW = 136.08) and then diluting it with the purified water to 1000.0 mL. The 0.20 M acetic acid solution was prepared by diluting the glacial acetic acid (17.4 Formal) 12.0 mL with the purified water to 1000.0 mL. The 0.20 M sodium acetate-acetic acid buffer solution was prepared by mixing 0.20 M sodium acetate solution with 0.20 M acetic acid solution until it was at the desired pH (3.00, 3.50, 4.00, 4.50, 5.00, 5.50, and 6.00). The sodium acetate-acetic acid buffer solution was kept at 4 ^{0}C.

3.4 The Study of Tetrabutylammonium Bromide-2-Mercaptoethanol System

3.4.1 The Study of Various Effect on the Resolution of Mercury Compounds

The various effects on the resolution of mercury compounds including the mobile phase pH, 2-mercaptoethanol concentration, tetrabutylammonium bromide concentration, methanol composition in mobile phase, and mobile phase flow rate were studied in order to determine of the optimum condition for the speciation of mercury compounds. The procedure can be described as follows :

3.4.1.1 The Effect of Methanol composition in Mobile Phase

The procedure for the study of the effect of methanol composition in mobile phase at 10%, 12%, 15%, 20%, 30%, 40%, 50%, 60%, and 70% v/v on the resolution of mercury compounds can be described as follows :

1. 5.00 mL of 0.20M sodium acetate-acetic acid buffer solution pH 3.00 were added into 100.0, 120.0, 150.0, 200.0, 300.0, 400.0, 500.0, 600.0, and

700.0 mL of methanol, and then added with purified water to 1000.0 mL total volume for the methanol composition at 10%, 12%, 15%, 20%, 30%, 40%, 50%, 60%, and 70% v/v, respectively.

2. The mobile phase was prepared by dissolving 2.4928 g of tetrabutylammonium bromide (MW = 332.38) and pipetting 5.00 mL of 2-mercaptoethanol in 995.0 mL of the pH-adjusted mixture methanol-water from step 1.

3. The mobile phase was degassed by purging with helium gas at flow rate 100 mL/min. for 15 minutes and 10 mL/min. during the study.

4. The C_{18} column was equilibrated with the mobile phase at flow rate 1.00 mL/min. for 3 hours and the baseline signal was checked before the study.

5. The standard mixture of inorganic and methylmercury solutions were injected into the HPLC under the HPLC condition in Table 3.1.1

6. The relationships between the retention time, capacity factor, selectivity factor, and resolution of mercury compounds and the methanol composition in mobile phase were shown in Table 4.1.1 and Figure 4.1.1.

7. The comparisons with 2-mercaptoethanol system were shown in Table 4.1.2 and Figures 4.1.4-4.1.6.

The optimum methanol composition in mobile phase found in this section would be used in the next study.

Table 3.1.1: The HPLC conditions for the study of the effect of the methanol composition in mobile phase in tetrabutylammonium bromide-2-mercaptoethanol system

Parameter	Conditions
Analytical Column	Hypersil column, 250 x 4.6 mm. I.D., 5 μm
Mobile Phase	The mixture methanol-water buffered with 1.0×10^{-3}M AcONa-AcOH pH 3.00 containing 0.0075M TBABr and 0.0050% v/v 2-mercaptoethanol
Flow Rate	1.00 mL/min
Injection Volume	10 μL
Detector	Photodiode Array Detector
Data Acquisition	Maximum plot (200-400 nm)

3.4.1.2 The Effect of Mobile Phase Flow Rate

The procedure for the study of the effect of mobile phase flow rate at 1.00, 1.20, 1.50, and 1.80 mL/min on the resolution of mercury compounds can be described as follows:

1. The mixture methanol-water (12:88% v/v) buffered with acetate-acetic acid buffer pH 3.00 was prepared by added 5.00 mL of 0.20M sodium acetate-acetic acid buffer solution pH 3.00 in 120.0 mL of methanol, and then added with the purified water to 1000.0 mL total volume.

2. The mobile phase was prepared by dissolving 2.4928 g of tetrabutylammonium bromide and pipetting 5.00 mL of 2-mercaptoethanol in 995.0 mL of the pH-adjusted mixture methanol-water from step 1.

3. The mobile phase was degassed by purging with helium gas at flow rate 100 mL/min. for 15 minutes and 10 mL/min. during the study.

4. The C_{18} column was equilibrated with the mobile phase at the study flow rate for 3 hours and the baseline signal was checked before the study.

5. The standard mixture of inorganic and methylmercury solutions were injected into the HPLC under the HPLC conditions in Table 3.1.2.

6. The relationships between the retention time, capacity factor, selectivity factor, and resolution of mercury compounds and the mobile phase flow rate were shown in Table 4.1.3 and Figure 4.1.7.

The optimum mobile phase conditions found in this study would be used in the next study.

Table 3.1.2: The HPLC conditions for the study of the effect of mobile phase flow rate in tetrabutylammonium bromide-2-mercaptoethanol system

Parameter	Conditions
Analytical Column	Hypersil column, 250 x 4.6 mm. I.D., 5 μm
Mobile Phase	The mixture methanol-water (12:88% v/v) buffered with 1.0×10^{-3}M AcONa-AcOH pH 3.00 containing 0.0075M TBABr and 0.0050% v/v 2-mercaptoethanol
Flow Rate	variable
Injection Volume	10 μL
Detector	Photodiode Array Detector
Data Acquisition	Maximum plot (200-400 nm)

3.4.1.3 The Effect of Mobile Phase pH

The procedure for the study of the effect of mobile phase pH at 3.00, 3.50, 4.00, and 5.00 on the resolution of mercury compounds can be described as follows :

1. The mixture methanol-water (12:88% v/v) buffered with acetate-acetic acid buffer was prepared by added 5.00 mL of 0.20M sodium acetate-acetic acid buffer solution in 120.0 mL of methanol, and then added with the purified water to 1000.0 mL total volume.

2. The mobile phase was prepared by dissolving 2.4928 g of tetrabutylammonium bromide and pipetting 5.00 mL of 2-mercaptoethanol in 995.0 mL of the pH-adjusted mixture methanol-water from step 1.

3. The mobile phase was degassed by purging with helium gas at flow rate 100 mL/min. for 15 minutes and 10 mL/min. during the study.

4. The C_{18} column was equilibrated with the mobile phase at flow rate 1.50 mL/min. for 3 hours and the baseline signal was checked before the study.

5. The standard mixture of inorganic and methylmercury solutions were injected into the HPLC under the HPLC conditions in Table 3.1.3

6. The relationships between the retention time, capacity factor, selectivity factor, and resolution of mercury compounds and the pH value were shown in Table 4.1.4 and Figure 4.1.8.

The optimum mobile phase pH found in this section would be used in the next study.

Table 3.1.3: The HPLC conditions for the study of the effect of mobile phase pH in tetrabutylammonium bromide-2-mercaptoethanol system

Parameter	Conditions
Analytical Column	Hypersil column, 250 x 4.6 mm. I.D., 5 μm
Mobile Phase	The mixture methanol-water (12:88% v/v) buffered with 1.00×10^{-3}M AcONa-AcOH containing 0.0075M TBABr and 0.0050 % v/v 2-mercaptoethanol.
Flow Rate	1.50 mL/min
Injection Volume	20 μL
Detector	Photodiode Array Detector
Data Acquisition	Maximum plot (200-400 nm)

3.4.1.4 The Effect of Acetate-Acetic Acid Buffer Concentration

The procedure for the study of the effect of acetate-acetic acid buffer concentration at 1.00 x 10^{-3}M, 2.00 x 10^{-3}M, and 5.00 x 10^{-3} M on the resolution of mercury compounds can be described as follows :

1. The mixture methanol-water (12:88% v/v) buffered with varied concentration of acetate-acetic acid buffer solution were prepared by added 0.20M sodium acetate-acetic acid buffer pH 3.00 solution 5.0, 10.0, and 25.0 mL for 1.00 x 10^{-3}M, 2.00 x 10^{-3}M, and 5.00 x 10^{-3} M buffer concentration, respectively in 120.0 mL of methanol, and then added with the purified water to 1000.0 mL total volume.

2. The mobile phase was prepared as step 2 in section 3.4.1.3.

3. The mobile phase was degassed and the column was equilibrated as step 3-4 in section 3.4.1.3.

4. The standard mixture of inorganic and methylmercurysolutions were injected into the HPLC under the HPLC conditions in Table 3.1.4.

5. The baseline signals and the resolution of mercury compounds were measured.

The optimum acetate-acetic acid buffer concentration found in this section would be used in the next study.

Table 3.1.4: The HPLC conditions for the study of the effect of acetate-acetic acid buffer concentration in tetrabutylammonium bromide-2-mercaptoethanol system

Parameter	Conditions
Analytical Column	Hypersil column, 250 x 4.6 mm. I.D., 5 μm
Mobile Phase	The mixture methanol-water (12:88% v/v) buffered with AcONa-AcOH pH 3.00 containing 0.0075M TBABr and 0.0050 % v/v 2-mercaptoethanol.
Flow Rate	1.50 mL/min
Injection Volume	20 μL
Detector	Photodiode Array Detector
Data Acquisition	Maximum plot (200-400 nm)

3.4.1.5 The Effect of 2-Mercaptoethanol Concentration

The procedure for the study of the effect of 2-mercaptoethanol concentration at 0.0040%, 0.0050%, and 0.0060% v/v on the resolution of mercury compounds can be described as follows :

1. The pH-adjusted mixture methanol-water was prepared as step1 in section 3.4.1.2

2. 2-Mercaptoethanol 4.00, 5.00, and 6.00 mL were added in the pH-adjusted mixture from step 1 to total volume 1000.0 mL for 2-mercaptoethanol concentration of 0.0040%, 0.0050%, and 0.0060% v/v, respectively.

3. The mobile phase was prepared by dissolving 2.4928 g of tetrabutylammonium bromide in 1000.0 mL of the solution from step 2.

4. The mobile phase was degassed and the column was equilibrated as step 3-4 in section 3.4.1.3.

5. The standard mixture of inorganic and methylmercury solutions were injected into the HPLC under the HPLC conditions in Table 3.1.5.

6. The relationships between the retention time, capacity factor, selectivity factor, and resolution of mercury compounds and the 2-mercaptoethanol concentration were shown in Table 4.1.5 and Figure 4.1.9.

The optimum 2-mercaptoethanol concentration found in this section would be used in the next study.

Table 3.1.5: The HPLC conditions for the study of the effect of 2-mercaptoethanol concentration in tetrabutylammonium bromide-2-mercaptoethanol system

Parameter	Conditions
Analytical Column	Hypersil column, 250 x 4.6 mm. I.D., 5 μm
Mobile Phase	The mixture methanol-water (12:88% v/v) buffered with 1.0×10^{-3}M AcONa-AcOH pH 3.00 containing 0.0075M TBABr and 2-mercaptoethanol.
Flow Rate	1.50 mL/min
Injection Volume	20 μL
Detector	Photodiode Array Detector
Data Acquisition	Maximum plot (200-400 nm)

3.4.1.5 The Effect of Tetrabutylammonium Bromide Concentration

The procedure for the study of the effect of tetrabutylammomium bromide concentration at 0.0060M, 0.0075M, 0.0085M, and 0.0100M on the resolution of mercury compounds can be described as follows:

1. The pH-adjusted mixture methanol-water was prepared as step 1 in section 3.4.1.2

2. 5.00 mL of 2-mercaptoethanol was added in 995.0 mL of the pH-adjusted mixture methanol-water from step 1.

3. The mobile phase was prepared by dissolving tetrabutylammonium bromide 1.9943, 2.4928, 2.8252, and 3.3238 g in the solution from step 2 for the tetrabutylammonium bromide concentration of 0.0060M, 0.0075M, 0.0085 M, and 0.0100M, respectively.

4. The mobile phase was degassed and the column was equilibrated as step 3-4 in section 3.4.1.3.

5. The standard mixture of inorganic and methylmercury solutions were injected into the HPLC under the HPLC conditions in Table 3.1.6.

6. The relationships between the retention time, capacity factor, selectivity factor, and resolution of mercury compounds and the tetrabutylammonium bromide concentration were shown in Table 4.1.6 and Figure 4.1.10.

The optimum tetrabutylammonium bromide concentration found in this section would be used in the next study.

Table 3.1.6: The HPLC conditions for the study of the effect of tetrabutylammonium bromide concentration in tetrabutylammonium bromide-2-mercaptoethanol system

Parameter	Conditions
Analytical Column	Hypersil column, 250 x 4.6 mm. I.D., 5 μm
Mobile Phase	The mixture methanol-water (12:88% v/v) buffered with 1.0×10^{-3}M AcONa-AcOH pH 3.00 containing 0.0050% v/v 2-mercaptocthanol and TBABr
Flow Rate	1.50 mL/min
Injection Volume	20 μL
Detector	Photodiode Array Detector
Data Acquisition	Maximum plot (200-400 nm)

3.4.2 The Study of the Injection Volume on the Response Signals of Mercury Compounds

The procedure for the study of the injection volume at 10, 20, 25, 50, and 100 μL on the response signals of mercury compounds can be described as follows:

1. The mixture methanol-water (12:88% v/v) buffered with acetate-acetic acid buffer pH 3.00 was prepared by added 5.00 mL of 0.20M sodium acetate-acetic acid buffer solution pH 3.00 in 120.0 mL of methanol, and then added with the purified water to 1000.0 mL total volume.

2. The mobile phase was prepared by dissolving 2.4928 g of tetrabutylammonium bromide and pipetting 5.00 mL of 2-mercaptoethanol in 995.0 mL of the pH-adjusted mixture methanol-water from step 1.

3. The C_{18} column was equilibrated with the mobile phase at flow rate 1.50 mL/min. for 3 hours and the baseline signal was checked before the study.

4. The standard mixture of inorganic and methylmercurysolutions were inject into the HPLC system with the volume 10, 20, 25, 50, and 100 μL. The HPLC conditions were shown in Table 3.1.7.

5. The relationship between peak area, peak height, and response signal of each mercury compound and the injection volume were shown in Table 4.1.7 and Figures 4.1.11-4.1.13.

6. The optimum HPLC conditions for 2-mercaptoethanol-tetrabutylammonium bromide system was shown in Table 4.1.8.

The optimum conditions found in this study would be used in the next study.

Table 3.1.7: The HPLC conditions for the study of the effect of an injection volume in tetrabutylammonium bromide-2-mercaptoethanol system

Parameter	Conditions
Analytical Column	Hypersil column, 250 x 4.6 mm. I.D., 5 μm
Mobile Phase	The mixture methanol-water (12:88% v/v) buffered with 1.0×10^{-3}M AcONa-AcOH pH 3.00 containing 0.0075M TBABr and 0.0050% v/v 2-mercaptoethanol
Flow Rate	1.50 mL/min
Injection Volume	variable
Detector	Photodiode Array Detector
Data Acquisition	Maximum plot (200-400 nm)

3.4.3 The Study of Linearity of Standard Mercury Compounds

The procedure for the study of linearity of standard mercury compounds can be described as follows:

1. The concentration of standard mixture solution of inorganic mercury and methylmercury was prepared from 1000.00 ppm (as Hg) stock standard solution of inorganic mercury and 500.00 ppm (as Hg) stock standard solution of methylmercury. The concentration of inorganic mercury in the mixture solutions were 5.00, 10.00, 20.00, 50.00, 100.00, 200.00, 300.00, 400.00 and 500.00 ppm (as Hg) and the concentration of methylmercury in the mixture solutions were 5.00, 10.00, 20.00, 50.00, 100.00, 200.00, 300.00, and 400.00 ppm (as Hg), respectively.

2. The standard mixture solutions from step 1 were injected respectively into HPLC under the optimum conditions.(Table 4.1.8.)

3. The relationships between concentration and peak area and peak height were shown in Table 4.1.9 and Figure 4.1.15-4.1.18.

3.4.4 The Study of Calibration Curve of Standard Mercury Compounds

The procedure for the study of calibration curve of standard mercury compounds can be described as follows:

1. The concentration of standard mixture solution of inorganic mercury and methylmercury was prepared from 100.00 ppm (as Hg) stock standard solution of inorganic mercury and 500.00 ppm (as Hg) stock standard solution of methylmercury. The concentration of each mercury compound in the mixture solution was 1.00, 3.00, 5.00, 10.00, 15.00, and 20.00 ppm (as Hg).

2. The concentration of standard mixture solutions of inorganic and methylmercury were injected respectively in HPLC system under the optimum conditions.(Table 4.1.8)

3. The relationships between concentration and peak area were shown in Table 4.1.10 and Figures 4.1.19-4.1.20.

3.4.5 The Study of Detection Limit of the System

The detection limit was defined as the amount of analyte in standard solution that yields a peak at signal-to-noise ratio equal to three. In this study was triplicate analysis. The procedure for the study can be described as follows:

1. The standard mixture solution of inorganic and methylmercury 10.00 ppm was prepared from 100.00 ppm (as Hg) standard solution of inorganic mercury and 500.00 ppm (as Hg) standard solution of methylmercury.

2. The standard mixture solutions of inorganic and methylmercury 1.00 ppm and concentrations below 1.00 ppm were prepared by diluting 10.00 ppm standard mixture solution form step 1 with purified water.

3. The standard mixture solutions from step 2 were injected into HPLC system under the optimum conditions (Table 4.1.8). The peaks of inorganic and methylmercury were measured from the chromatograms.

4. The detection limits of each mercury compound were found from the concentration that gives the peak signal as high as three times of the baseline signal.

5. The detection limits of each mercury compound were shown in Table 4.2.12.

3.4.6 The Study of Precision of the System

The procedure for the study of precision of 2-mercaptoethanol-tetrabutyl ammonium bromide system can be describe as follows:

1. The concentration of standard mixture solutions of inorganic and methylmercury 4.00, 8.00, 12.00, and 16.00 ppm were prepared from 100.00 ppm (as Hg) standard solution of inorganic mercury and 500.00 ppm (as Hg) standard solution of methylmercury.

2. Standard solutions from step 1 were five replicate injected respectively in HPLC system under the optimum conditions.(Table 4.1.8)

3. The percent relative standard deviations (%RSD) of mercury compounds from the replicate injection of each concentration were calculated and were shown in Table 4.1.11.

3.5 The Study of Sodium Hexanesulfonate-2-Mercaptoethanol System

3.5.1 The Study of Various Effect on the Resolution of Mercury Compounds

The various effects on the resolution of mercury compounds including the mobile phase pH, 2-mercaptoethanol concentration, sodium hexanesulfonate concentration, methanol composition in mobile phase, and mobile phase flow rate were studied in order to determination of the optimum condition for the speciation of mercury compounds. The procedure can be described as follows :

3.5.1.1 The Effect of Mobile Phase pH

The procedure for the study of the effect of mobile phase pH at 3.00, 4.00, 4.50, 5.00, 5.50, and 6.00 on the resolution of mercury compounds can be described as follows:

1. The mixture methanol-water (40:60% v/v) buffered with acetate-acetic acid buffer was prepared by added 5.00 mL of 0.20M sodium acetate-acetic acid buffer solution in 400.0 mL of methanol, and then added with the purified water to 1000.0 mL total volume.

2. The mobile phase was prepared by dissolving 0.9411 g of sodium hexanesulfonate (MW = 188.22) and pipetting 5.00 mL of 2-mercaptoethanol in 995.0 mL of the pH-adjusted mixture methanol-water from step 1.

3. The mobile phase was degassed by purging with helium gas at flow rate 100 mL/min. for 15 minutes and 10 mL/min. during the study.

4. The C_{18} column was equilibrated with the mobile phase at flow rate 1.00 mL/min. for 3 hours and the baseline signal was checked before the study.

5. The standard mixture of inorganic, methylmercury, and phenylmercury solutions were injected into the HPLC under the HPLC conditions in Table 3.2.1.

6. The relationships between the retention time, capacity factor, selectivity factor, and resolution of mercury compounds and the pH value were shown in Table 4.2.1 and Figure 4.2.1.

The optimum mobile phase pH found in this section would be used in the next study.

Table 3.2.1: The HPLC conditions for the study of the effect of mobile phase pH in sodium hexanesulfonate-2-mercaptoethanol system

Parameter	Conditions
Analytical Column	Hypersil column, 250 x 4.6 mm. I.D., 5 μm
Mobile Phase	The mixture methanol-water (40:60% v/v) buffered with 1.00×10^{-3}M AcONa-AcOH containing 0.0050M sodium hexanesulfonate and 0.0050 % v/v 2-mercaptoethanol.
Flow Rate	1.00 mL/min
Injection Volume	20 μL
Detector	Photodiode Array Detector
Data Acquisition	Maximum plot (200-400 nm)

3.5.1.2 The Effect of Acetate-Acetic Acid Buffer Concentration

The procedure for the study of the effect of acetate-acetic acid buffer concentration at 1.00×10^{-3}M, 2.00×10^{-3}M, and 5.00×10^{-3} M on the resolution of mercury compounds can be described as follows:

1. The mixture methanol-water (40:60% v/v) buffered with varied concentration of acetate-acetic acid buffer solution were prepared by added 0.20M sodium acetate-acetic acid buffer pH 5.00 solution 5.0, 10.0, and 25.0 mL for 1.00×10^{-3}M, 2.00×10^{-3}M, and 5.00×10^{-3} M buffer concentration, respectively in 400.0 mL of methanol, and then added with the purified water to 1000.0 mL total volume.

2. The mobile phase was prepared as step 2 in section 3.5.1.1

3. The mobile phase was degassed and the column was equilibrated as step 3-4 in section 3.5.1.1.

4. The standard mixture of inorganic, methylmercury, and phenylmercury solutions were injected into the HPLC under the HPLC conditions in Table 3.2.2.

5. The baseline signals and the resolution of mercury compounds were measured.

The optimum acetate-acetic acid buffer concentration found in this section would be used in the next study.

Table 3.2.2: The HPLC conditions for the study of the effect acetate-acetic acid buffer concentration in sodium hexanesulfonate-2-mercaptoethanol system

Parameter	Conditions
Analytical Column	Hypersil column, 250 x 4.6 mm. I.D., 5 μm
Mobile Phase	The mixture methanol-water (40:60% v/v) buffered with AcONa-AcOH pH 5.00 containing 0.0050M sodium hexanesulfonate and 0.0050 % 2-mercaptoethanol.
Flow Rate	1.00 mL/min
Injection Volume	20 μL
Detector	Photodiode Array Detector
Data Acquisition	Maximum plot (200-400 nm)

3.5.1.3 The Effect of 2-Mercaptoethanol Concentration

The procedure for the study of the effect of 2-mercaptoethanol concentration at 0.0020%, 0.0040%, 0.0060%, 0.0080%, and 0.0100% v/v on the resolution of mercury compounds can be described as follows:

1. The mixture methanol-water (40:60% v/v) buffered with acetate-acetic acid buffer pH 5.00 was prepared by added 5.00 mL of 0.20M sodium acetate-acetic acid buffer solution pH 5.00 in 400.0 mL of methanol, and then added with the purified water to 1000.0 mL total volume.

2. 2-Mercaptoethanol 2.00, 4.00, 6.00, 8.00, and 10.00 mL were added in the solution from step 2 to total volume 1000.0 mL for 2-mercaptoethanol

concentration of 0.0020%, 0.0040%, 0.0060%, 0.0080%, and 0.0100% v/v, respectively.

3. The mobile phase was prepared by dissolving 0.9411 g of sodium hexanesulfonate in 1000.0 mL of the solution from step 2.

4. The mobile phase was degassed and the column was equilibrated as step 3-4 in section 3.5.1.1.

5. The standard mixture of inorganic, methylmercury, and phenylmercury solutions were injected into the HPLC under the HPLC condition in Table 3.2.3

6. The relationships between the retention time, capacity factor, selectivity factor, and resolution of mercury compounds and the 2-mercaptoethanol concentration were shown in Table 4.2.2 and Figure 4.2.2.

The optimum 2-mercaptoethanol concentration found in this section would be used in the next study.

Table 3.2.3: The HPLC conditions for the study of the effect of 2-mercaptoethanol concentration in sodium hexanesulfonate-2-mercaptoethanol system

Parameter	Conditions
Analytical Column	Hypersil column, 250 x 4.6 mm. I.D., 5 μm
Mobile Phase	The mixture methanol-water (40:60% v/v) buffered with 1.0×10^{-3}M AcONa-AcOH pH 5.00 containing 0.0050M sodium hexanesulfonate and 2-mercaptoethanol.
Flow Rate	1.00 mL/min
Injection Volume	20 μL
Detector	Photodiode Array Detector
Data Acquisition	Maximum plot (200-400 nm)

3.5.1.4 The Effect of Sodium Hexanesulfonate Concentration

The procedure for the study of the effect of sodium hexanesulfonate concentration at 0.0000M, 0.0010M, 0.0025M, 0.0040M, 0.0050M, 0.0075M, 0.0100M, and 0.0150M on the resolution of mercury compounds can be described as follows :

1. The pH-adjusted mixture methanol-water was prepared as step 1 in section 3.5.1.3.

2. 4.00 mL of 2-mercaptoethanol was added in 996.0 mL of the pH-adjusted mixture methanol-water from step 1.

3.The mobile phase was prepared by dissolving sodium hexanesulfonate 0.0000, 0.1882, 0.4705, 0.7529, 0.9411, 1.4116, 1.8822, and 2.823 g in solution from step 2 for the sodium hexanesulfanote concentration of 0.0000M, 0.0010M, 0.0025M, 0.0040M, 0.0050M, 0.0075M, 0.0100M, and 0.0150M, respectively.

4. The mobile phase was degassed and the column was equilibrated as step 3-4 in section 3.5.1.1.

5. The standard mixture of inorganic, methylmercury, and phenylmercury solutions were injected into the HPLC under the HPLC condition in Table 3.2.4

6. The relationships between the retention time, capacity factor, selectivity factor, and resolution of mercury compounds and the sodium hexanesulfonate concentration were shown in Table 4.2.3 and Figure4.2.3.

The optimum sodium hexanesulfonate concentration found in this section would be used in the next study.

Table 3.2.4: The HPLC conditions for the study of the effect of sodium hexanesulfonate concentration in sodium hexanesulfonate-2-mercaptoethanol system

Parameter	Conditions
Analytical Column	Hypersil column, 250 x 4.6 mm. I.D., 5 μm
Mobile Phase	The mixture methanol-water (40:60% v/v) buffered with 1.0×10^{-3}M AcONa-AcOH pH 5.00 containing 0.0040% 2-mercaptoethanol and sodium hexanesulfonate
Flow Rate	1.00 mL/min
Injection Volume	20 μL
Detector	Photodiode Array Detector
Data Acquisition	Maximum plot (200-400 nm)

3.5.1.5 The Effect of the Methanol Composition in Mobile Phase

The procedure for the study of the effect of methanol composition in mobile phase at 10%, 20%, 30%, 40%, 50%, 55%, 60%, and 70% v/v on the resolution of mercury compounds can be described as follows :

1. 5.00 mL of 0.20M sodium acetate-acetic acid buffer solution pH 5.00 were added into 100.0, 200.0, 300.0, 400.0, 500.0, 600.0, and 700.0 mL of methanol, and then added with purified water to 1000.0 mL total volume for the methanol composition at 10%, 20%, 30%, 40%, 50%, 60%, and 70% v/v, respectively.

2. The mobile phase was prepared by dissolving 0.7529g of sodium hexanesulfonate and pipetting 4.00 mL of 2-mercaptoethanol in 996.0 mL of the pH-adjusted mixture methanol-water from step 1.

3. The mobile phase was degassed and the column was equilibrated as step 3-4 in section 3.5.1.1.

4. The standard mixture of inorganic, methylmercury, and phenylmercury solutions were injected into the HPLC under the HPLC condition in Table 3.2.5.

5. The relationships between the retention time, capacity factor, selectivity factor, and resolution of mercury compounds and the methanol composition in mobile phase were shown in Table 4.2.4 and Figures 4.2.4-4.2.6.

6. he comparisons with 2-mercaptoethanol system were shown Figures 4.2.7-4.2.9.

The optimum methanol composition in mobile phase found in this section would be used in the next study.

Table 3.2.5: The HPLC conditions for the study of the effect of the methanol composition in mobile phase in sodium hexanesulfonate-2-mercaptoethanol system

Parameter	Conditions
Analytical Column	Hypersil column, 250 x 4.6 mm. I.D., 5 μm
Mobile Phase	The mixture methanol-water buffered with 1.0×10^{-3}M AcONa-AcOH pH 5.00 containing 0.0040M sodium hexanesulfonate and 0.0040% 2-mercaptoethanol
Flow Rate	1.00 mL/min
Injection Volume	20 μL
Detector	Photodiode Array Detector
Data Acquisition	Maximum plot (200-400 nm)

3.5.1.6 The Effect of Mobile Phase Flow Rate

The procedure for the study of the effect of mobile phase flow rate at 0.80, 1.00, 1.20, and 1.50 mL/min on the resolution of mercury compounds can be described as follows:

1. The mixture methanol-water (40:60% v/v) buffered with acetate-acetic acid buffer pH 5.00 was prepared by added 5.00 mL of 0.20M sodium acetate-acetic acid buffer solution pH 5.00 in 400.0 mL of methanol, and then added it with the purified water to 1000.0 mL total volume.

2. The mobile phase was prepared by dissolving 0.7529g of sodium hexanesulfonate and pipetting 4.00 mL of 2-mercaptoethanol in 996.0 mL of the pH-adjusted mixture methanol-water from step 1.

3. The mobile phase was degassed by purging with helium gas at flow rate 100 mL/min. for 15 minutes and 10 mL/min. during the study.

4. The C_{18} column was equilibrated with the mobile phase at the study flow rate for 3 hours and the baseline signal was checked before the study.

5. The standard mixture of inorganic, methylmercury, and phenylmercury solutions were injected into the HPLC under the HPLC condition in Table 3.2.6

6. The relationships between the retention time, capacity factor, selectivity factor, and resolution of mercury compounds and the mobile phase flow rate were shown in Table 4.2.5 and Figure 4.2.10.

The optimum mobile phase condition found in this study would be used in the next study.

Table 3.2.6: The HPLC conditions for the study of the effect of mobile phase flow rate in sodium hexanesulfonate-2-mercaptoethanol system

Parameter	Conditions
Analytical Column	Hypersil column, 250 x 4.6 mm. I.D., 5 μm
Mobile Phase	The mixture methanol-water (40:60% v/v) buffered with 1.0×10^{-3}M AcONa-AcOH pH 5.00 containing 0.0040M sodium hexanesulfonate and 0.0040% 2-mercaptoethanol
Flow Rate	variable
Injection Volume	20 μL
Detector	Photodiode Array Detector
Data Acquisition	Maximum plot (200-400 nm)

3.5.2 The Study of the Injection Volume on the Response Signals of Mercury Compounds

The procedure for the study of the injection volume at 10, 20, 25, 50, and 100 μL on the response signals of mercury compounds can be described as follows:

1. The mobile phase was prepared and was degassed as step 1-3 in section 3.5.1.6.

2. The C_{18} column was equilibrated with the mobile phase at flow rate 1.20 mL/min. for 3 hours and the baseline signal was checked before the study.

3. The standard mixture of inorganic, methylmercury, and phenylmercury solutions were inject into the HPLC system with the volume 10, 20, 25, 50, and 100 μL. The HPLC conditions were shown in Table 3.2.7

4. The relationships between peak area, peak height, and response signal of each mercury compound and the injection volume were shown in Table 4.2.6 and Figures 4.2.11-4.2.13.

5. The optimum HPLC conditions for 2-mercaptoethanol-sodium hexane-sulfonate system was shown in Table 4.2.7.

The optimum conditions found in this study would be used in the next study.

Table 3.2.7: The HPLC conditions for the study of the effect an injection volume in sodium hexanesulfonate-2-mercaptoethanol system

Parameter	Conditions
Analytical Column	Hypersil column, 250 x 4.6 mm. I.D., 5 μm
Mobile Phase	The mixture methanol-water (40:60% v/v) buffered with 1.0×10^{-3}M AcONa-AcOH pH 5.00 containing 0.0040M sodium hexanesulfonate and 0.0040% 2-mercaptoethanol
Flow Rate	1.20 mL/min
Injection Volume	variable
Detector	Photodiode Array Detector
Data Acquisition	Maximum plot (200-400 nm)

3.5.3 The Study of Linearity of Standard Mercury Compounds

The procedure for the study of linearity of standard mercury compounds can be described as follows:

1. The concentration of standard mixture solution of inorganic mercury, methylmercury, and phenylmercury was prepared from 1000.00 ppm (as Hg) stock standard solution of inorganic mercury, 500.00 ppm (as Hg) stock standard solution of methylmercury and 250.00 ppm (as Hg) stock standard solution of phenylmercury, respectively. The concentration of inorganic mercury in the mixture solutions were 5.00, 10.00, 20.00, 50.00, 100.00, 200.00, 300.00, 400.00, and 500.00 ppm (as Hg). The concentration of methylmercury in the mixture solutions were 5.00, 10.00, 20.00, 50.00, 100.00, 200.00, 300.00, and 400.00 ppm (as Hg) and the concentration of phenylmercury in the mixture solutions were 5.00, 10.00, 20.00, 50.00, 100.00, and 200.00, respectively.

2. The standard mixture solutions from step 1 were injected respectively into HPLC under the optimum condition. (Table 4.2.7)

3. The relationships between concentration and peak area and peak height were shown in Table 4.2.8 and Figures 4.2.15-4.2.20.

3.5.4 The Study of Calibration Curve of Standard Mercury Compounds

The procedure for the study of calibration curve of standard mercury compounds can be described as follows:

1.The concentration of standard mixture solution of inorganic mercury, methyl mercury, and phenylmercury was prepared from 100.00 ppm (as Hg) stock standard solution of inorganic mercury, 500.00 ppm (as Hg) stock standard solution of methylmercury, and 250.00 ppm (as Hg) standard solution of phenylmercury, respectively. The concentration of each mercury compound in the mixture solutions was 1.00, 3.00, 5.00, 10.00, 15.00, and 20.00 ppm (as Hg).

2. The concentration of standard mixture solutions of mercury compounds were injected respectively in HPLC system under the optimum conditions. (Table 4.2.7)

3. The relationships between concentration and peak area were shown in Table 4.2.9 and Figures 4.2.21-4.2.23.

3.5.5 The Study of Detection Limit of the System

The detection limit was defined as the amount of analyte in standard solution that yields a peak at signal-to-noise ratio equal to three. In this study was triplicate analysis. The procedure for the study can be described as follows:

1. The standard mixture solution of mercury compounds 10.00 ppm was prepared from 100.00 ppm (as Hg) standard solution of inorganic mercury, 500.00 ppm (as Hg) standard solution of methylmercury and 250.00 ppm (as Hg) standard solution of phenylmercury.

2. The standard mixture solutions of mercury compounds 1.00 ppm and concentrations below 1.00 ppm were prepared by diluting 10.00 ppm standard mixture solution form step 1 with purified water.

3. The standard mixture solutions from step 2 were injected into HPLC system under the optimum conditions (Table 4.2.7). The peaks of inorganic, methylmercury and phenylmercury were measured from the chromatograms.

4. The detection limits of each mercury compound were found from the concentration that gives the peak signal as high as three times of the baseline signal.

5. The detection limits of each mercury compound were shown in Table 4.2.12.

3.4.6 The Study of Precision of the System

The procedure for the study of precision of 2-mercaptoethanol-tetrabutyl ammonium bromide system can be describe as follows:

1. The concentration of standard mixture solutions of inorganic and methylmercury 4.00, 8.00, 12.00, and 16.00 ppm were prepared from 100.00 ppm (as Hg) standard solution of inorganic mercury, 500.00 ppm (as Hg) standard solution of methylmercury and 250.00 ppm (as Hg) standard solution of phenylmercury.

2. The standard solutions from step 1 were five replicate injected respectively in HPLC system under the optimum conditions.(Table 4.2.7)

3. The percent relative standard deviations (%RSD) of mercury compounds from the replicate injection of each concentration were calculated and were shown in Table 4.2.10