CHAPTER 2

EXPERIMENTAL

2.1 Chemicals and materials

2.1.1 Standard chemicals

- Stock solution (1,000 mg L^{-1}) of cadmium (SCP Science, USA)
- Stock solution (1,000 mg L^{-1}) of lead (SCP Science, USA)
- Stock magnesium nitrate (10,000 mg L^{-1}) (Perkin-Elmer, USA)
- Stock ammonium hydrogen phosphate (10%) (Perkin-Elmer, USA)
- Dogfish muscle certified reference material of trace metals; DORM-2 (National Research Council Canada)

2.1.2 General chemicals and solvents

- Nitric acid 69-70% (w /v), AR grade (J.T. Baker, USA.)
- Ammonium hydroxide 28.0-30.0%, A.C.S reagent (J.T. Baker, USA.)
- Ethanol 99.9%, AR grade (Fluka, Switzerland)
- 8-Hydroxyquinoline 99.99 % (Fluka, Switzerland)
- Ultra pure water (ELGA, England)

2.1.3 Samples

Seafood samples from the frozen seafood companies in Trang, Pattani and Songkhla provinces were collected from Central Equipment of Science Faculty, Prince of Songkla University.

2.2 Instruments and apparatus

2.2.1 Graphite furnace atomic absorption spectrometer (GFAAS)

- Atomic absorption spectrometer Model AAnalyst 800 with Zeeman Background correction (GFAAS) (Perkin - Elmer, USA)
- THGA graphite with pyrolytic graphite coated (Perkin-Elmer, USA)
- An auto sampler AS800 (Perkin-Elmer, USA)
- Argon gas, High purity 99.999%, (TIG, Thailand)
- Computer system
- Hollow cathode lamp cadmium and lead (Perkin-Elmer, USA)
- (Perkin-Elmer, USA)
- Inductively couple plasma optical emission spectrometer (Perkin Elmer Optima 4300 DV)

2.2.2 Apparatus

- pH meter Model 15 (Denver Instrument, USA)
- Water bath (SV Medico, Thailand)
- Hot plate (Fisher Scientific, USA)
- Muffle (Cabolite, USA)
- Microlitre pipette model: P20, P100 and P 1,000 $\mu g\ L^{-1}$ (Gilson, France)
- General glassware such as volumetric flask 10, 25, 50, 100, 250, 500 mL; Beaker 50, 100, 500 mL
- Polyethylene bottles
- Polypropylene tube
- Polyethylene bags

2.2.3 Solid phase extraction

- 3M Empore extraction disk, 0.50 ± 0.05 mm., 90% adsorbent particles: 10% PTFE, 60 A^{o}, 12 µm particle size (3M, USA)
- Suction pump (BüiCHI Laboratoriums-Technik AG, Switzerland)
- Standard Millipore apparatus, 47 mm diameter (PYREX, USA)

Figure 2-1 Atomic absorption spectrometer (Perkin Elmer AAnalyst 800 with Zeeman Effect background correction)

2.3 Methodology

2.3.1 Preparation of stock standard solutions

Stock standard solution of 1,000 μg L^{-1}Cd and Pb was prepared by using 1,000 mg L^{-1} Cd and Pb standard solution and diluted for the corresponding stock solutions.

2.3.2 Preparation of glassware and plasticware

The glassware and plasticware were soaked in 10% (v/v) nitric acid for at least 48 h. and then rinsing at least three times with de-ionized water. All the glassware and plasticware were kept in self-seal polyethylene bags (Tuzen, 2003).

2.3.3 Optimization of temperature program for graphite furnace atomic absorption spectrometer (GFAAS)

The temperature programs are the most important part in the graphite furnace atomic absorption spectrometry. The standard furnace temperature program usually includes the following steps:

Drying: the solvent is vaporized during the drying step.

Pyrolysis: the pyrolysis step is used to remove as many matrix components as possible. A matrix modifier can be used to stabilize the analyte or aid in removal of matrix component.

Atomization: the sample is atomized to form ground state atoms in the path of the radiation beam during this step.

Clean out: a high temperature clean-out step after atomization prepares the furnace for subsequent samples (AAnalyst 800, Perkin-Elmer)

In this investigation, the pyrolysis temperature and atomization temperature were optimized. In addition, the type of matrix modifiers was examined and then the effect of using matrix modifier and without matrix modifier was also evaluated.

2.3.3.1 Pyrolysis temperature

The absorbance and peak shape of standard solutions of Cd 2.0 µg L^{-1} and Pb 50.0 µg L^{-1} were measured by GFAAS. Other parameters were set as the recommended conditions by AAnalyst 800 instrument manual. Optimum pyrolysis temperature was investigated by varied temperature at 400-1,200 ^{o}C and 400-1,300 ^{o}C for Cd and Pb respectively. The optimum pyrolysis temperature was obtained from the point which has high absorbance and good peak shape (Chuang *et al.,* 1999).

2.3.3.2 Atomization temperature

The absorbance and peak shape of standard solution of Cd 2.0 µg L^{-1} and Pb 50.0 µg L^{-1} were measured by GFAAS. The pyrolysis temperature was set at the optimum condition obtained from 2.3.3.1 and other parameters were set as the recommended conditions by AAnalyst 800 instrument manual. The optimum atomization temperature was investigated by varied temperature at 1,000-2,200 ^{o}C and 1,000-2,100 ^{o}C for Cd and Pb respectively. The optimum atomization temperature was obtained from the point which has high absorbance and good peak shape (Chuang *et al.,* 1999).

2.3.3.3 Type of matrix modifier

The effect of using modifier and without modifier on the absorbance of Cd 4.0 µg L^{-1} and Pb 100.0 µg L^{-1} were performed by using GFAAS. Pyrolysis temperature and atomization temperature were set at the optimum condition obtained from 2.3.3.1 and 2.3.3.2. The optimum type of matrix modifier was investigated by varied type of modifiers as follows in Table 2-1. The optimum type of matrix modifier was obtained from high absorbance and good peak shape (Correira *et al.,* 2000).

Table 2-1 The type of matrix modifiers used in this investigation

No.	Type of matrix modifier
1	0.06 % (w/v) $Mg(NO_3)_2$
2	1 % (w/v) $NH_4H_2PO_4$
3	0.06 % (w/v) $Mg(NO_3)_2$ + 1% (w/v) $NH_4H_2PO_4$

2.3.3.4 The effect of utilizing matrix modifier and without matrix modifier for determination of Cd and Pb in seafood sample

Fish sample 0.2 g (dry weight) was digested using 5.0 mL of concentrated nitric acid in polypropylene vessel in water bath for 2-3 h. The digested sample solution was diluted to 25.0 mL with de-ionized water and extracted by using solid phase extraction.

The absorbance and peak shape of these metals was measured when using with and without matrix modifier from 2.3.3.3 by GFAAS. The integrated absorbance of Cd and Pb was evaluated when using matrix modifier and without matrix modifier.

2.3.3.5 Linear range

The standard stock solutions of Cd and Pb were diluted with de-ionized water to various concentrations in the range of 0.0-24.0 µg L^{-1} and 0.0-240.0 µg L^{-1} respectively. The 20.0 µL of each concentration were analyzed by GFAAS at the optimum conditions from 2.3.3.1-2.3.3.3. The linear dynamic range obtained from plotting the absorbance versus the concentration. The linearity of response was considered by the correlative coefficient value of the linear curve (Ingle and Crouch, 1988).

2.3.3.6 Detection limits (DL)

The detection limit (DL) is defined by IUPAC as the concentration which will give an absorbance signal three times the magnitude of the baseline noise. The base line noise may be statistically quantitated typically by making 10 or more replicate measurements of the baseline absorbance signal observed for an analytical blank, and determining the standard deviation of the measurements (Beaty and Kerber, 1993).

In this study, the absorption of blank was measured and then the calibration curve of Cd and Pb were established by GFAAS. Therefore the detection limit in this investigation was evaluated by equation (2.1) (Ingle and Crouch, 1988).

$$\text{Detection limit} = (3*SD)/m \qquad \text{……..(2.1)}$$

When, SD = standard deviation of blank

m = slope of calibration graph

2.3.3.7 Accuracy and precision

The accuracy and precision of analytical method was assessed from certified reference material and %RSD. The accuracy of the studied method was performed by analysis the certified reference material (DORM-2). The certified reference material (DORM-2) 0.2 g was digested by using 5 mL of concentrated nitric acid in polypropylene vessel and heated on water bath. Sample solution was diluted to 25.0 mL and preconcentrated by using solid phase extraction. The concentration of Cd and Pb was evaluated by using standard addition method with GFAAS. The results from experimental and certified values were compared and then %RSD and percent recovery were also examined.

The precision of the solid phase extraction method was performed by analysis the standard solution of Cd 1.0 µg L^{-1} and Pb 20.0 µg L^{-1}. These metals were adsorbed on the modified solid phase extraction disks. The concentration of metals was evaluated by using calibration graph and then %RSD and percent recovery were calculated.

2.4 Sample preparation using solid phase extraction

2.4.1 Preparation of octadecyl silica membrane disks

The solid phase extraction procedure in this study is shown in Figure 2-2. First, place the octadecyl silica membrane disk (C18-Disk) in the standard millipore filtration apparatus. The C18-silica membrane disk was washed with 10.0 mL ethanol to remove all contaminants. After all of the solvent has passed through the disk, it was dried by passing air through it for a few minutes. The disk conditioning was then begun by pouring 10.0 mL deionized water and 10.0 mL ethanol onto the disk. A solution of 10.00 mg of 8-hydroxylquinoline in 2.0 mL ethanol was introduced onto the disk and was drawn slowly through the disk by applying vacuum. Finally, the disk was washed with 25.0 mL de-ionized water and dried by passing air through it. The membrane disks modified by 8-hydroxyquinoline is now ready for sample extraction. The pH of 25.0 mL sample solution was adjusted to 6.0 using 0.1 M ammonium hydroxide and then passed through the modified membrane disk. After the extraction, the disk was dried by passing air through it for a few minutes. The analytes were retained on the disk and eluted with 5.0 mL of 1 M nitric acid (Shamsipur *et al.,* 2000).

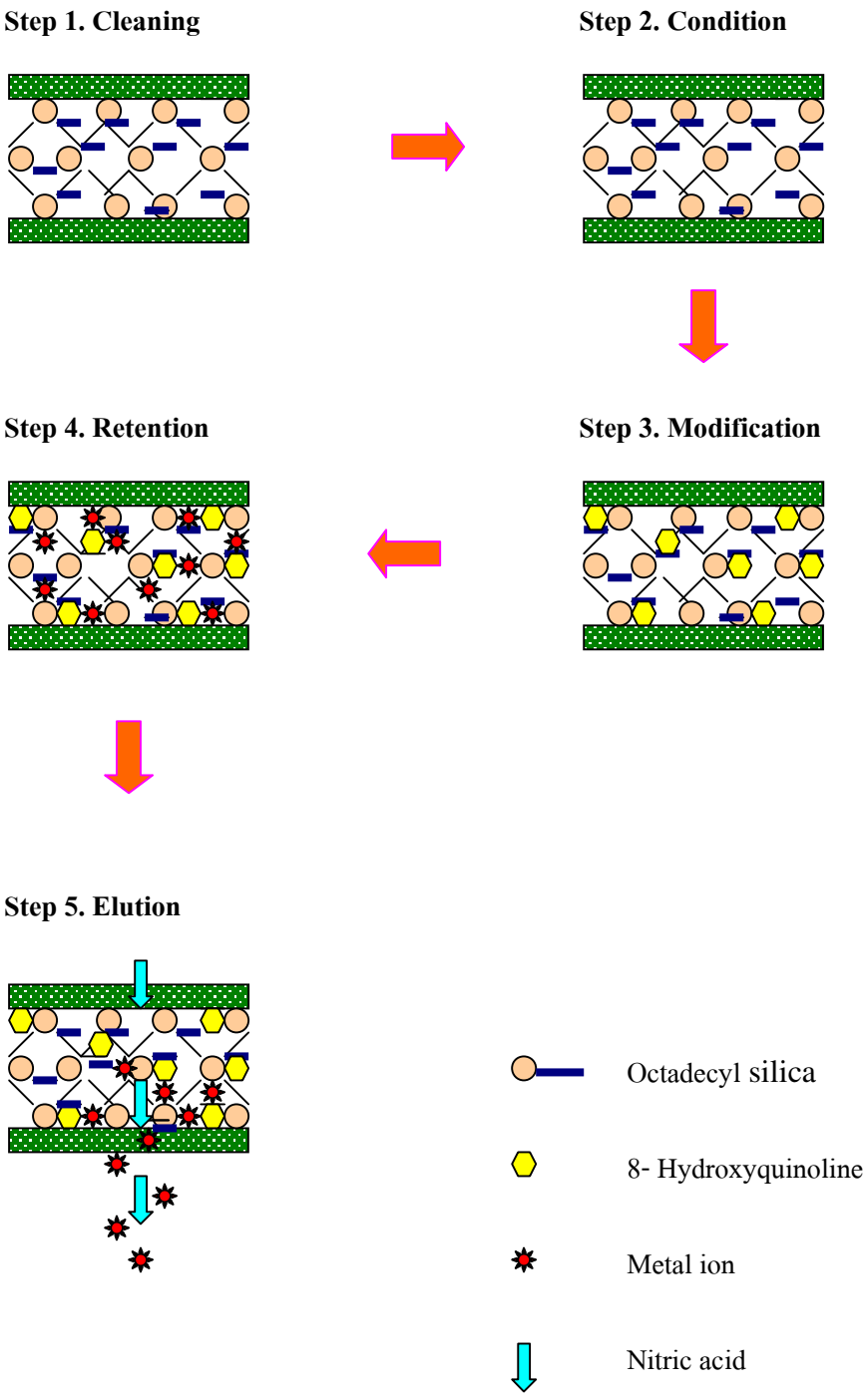

Figure 2-2 The solid phase extraction procedure by using 8-hydroxyquinoline modified octadecyl silica membrane disks.

2.4.2 Effect of pH of sample solution on adsorption of Cd and Pb on octadecyl silica membrane disks

The 25.0 mL mixed standard solution containing 1.0 μg L^{-1} of Cd and 20.0 μg L^{-1} of Pb was adjusted pH varied from 1.0-8.0 and adjusted by using 0.1 M nitric acid and ammonium hydroxide. The mixed standard solution was extracted using the 15.0 mg of 8-hydroxyquinoline modified octadecyl silica membrane disks and performed by using the procedure in 2.4.1. The absorbance of extracted solution was measured by GFAAS.

2.4.3 Effect of amount of 8-hydroxyquinoline on adsorption of Cd and Pb on octadecyl silica membrane disks

The optimum pH of 6.0 found in 2.4.2 was then used to study the effect of amount of 8-hydroxyquinoline. The amount of 8-hydroxyquinoline was set to 1.0, 5.0, 10.0, 15.0, 20.0, 25.0 and 40.0 mg (in 2 mL ethanol) and the procedure used in section 2.4.1 -2.4.2 was repeated.

2.4.4 Effect of eluent type and concentration on desorption of Cd and Pb on octadecyl silica membrane disks

The optimum pH and amount of 8-hydroxyquinoline found in the previous experimental was then used to study the effect of eluent type and concentration on desorption of Cd and Pb. The 25.0 mL of eluent type and concentration were 0.1, 0.3, 0.5, 0.7, 1.0, 2.0 M nitric acid and 0.1, 0.3, 0.5, 0.7, 1.0 M EDTA and the procedure used in section 2.4.1-2.4.3 was repeated

2.4.5 Effect of eluent volume on desorption of Cd and Pb on octadecyl silica membrane disks

The optimum pH, amount of 8-hydroxyquinoline, eluent type and concentration found in previous experiment was then used to study the effect of eluent volume on desorption of Cd and Pb on the modified octadecyl silica membrane disks. The eluent volume was set to 3.0, 5.0, 8.0, 10.0, 15.0 and 20.0 mL and the procedure used in section 2.4.1-2.4.4 was repeated.

2.4.6 The comparison of the calibration and standard addition method for determination of Cd and Pb in seafood sample

The aim of this experiment was to study the determination method between calibration and standard addition method for analysis of Cd and Pb in seafood sample. Seafood sample were digested using water bath digestion method (2.6.3.2) and extracted by the previous optimum conditions that obtained from 2.4.1-2.4.5 and then determined Cd and Pb by using calibration and standard addition method.

2.4.7 The study of percent recovery of Cd and Pb in seafood sample

The aim of this experiment was to establish the validation of this method and then applied for determination of Cd and Pb in seafood samples. The percent recovery was assessed by spiking Cd 1.0 μg L^{-1} and Pb 20.0 μg L^{-1} in seafood sample solution. The metals concentrations add and the percent recovery was calculated from standard addition graph.

2.5 Analytical performance of the modified octadecyl silica membrane disks

2.5.1 Maximum capacity of the modified octadecyl silica membrane disks

The aim of this experiment was to investigate the maximum amount of Cd and Pb can be retained on the modified octadecyl membrane disk. The 25.0 mL standard solution of 1,000 mg $L^{-1}$of Cd and Pb was passed through the modified octadecyl silica membrane disk and the procedure used in section 2.4.1-2.4.5 was repeated. The absorbance of extracted solution was measured by GFAAS (Hashemi *et al.,* 2001).

2.5.2 Breakthrough volume of the modified octadecyl silica membrane disk on adsorption of Cd and Pb

The volumes of 50.0 and 100.0 mL of standard solution of Cd 1.0 μg $L^{-1}$and Pb 20.0 μg L^{-1} were prepared. The pH of standard solution was adjusted to 6.0 using 0.1 M ammonium hydroxide. The solution was passed through the modified membrane disks and the procedure used in section 2.4.1 was repeated. The absorbance of extracted solution was measured by GFAAS. In addition, the percent recovery was calculated (Hashemi *et al.,* 2001).

2.5.3 Interference of coexist ions on adsorption of Cd and Pb by using the modified octadecyl silica membrane disks

The mixed standard solution of Cd 1.0 µg $L^{-1}$and Pb 20.0 µg L^{-1} were spiked various ions such as Na^{+} 15,000 $mg.L^{-1}$, K^{+} 1,000 $mg.L^{-1}$,Ca^{2+} 50 $mg.L^{-1}$,Mg^{2+} 50 $mg.L^{-1}$, Mn^{2+} 25 $mg.L^{-1}$, Zn^{2+} 20.0 mg L^{-1} and Cl^{-} 15,000 mg L^{-1} respectively. The solution was extracted by using the modified octadecyl silica membrane disk and the procedure used in section 2.4.1-2.4.5 was repeated. The effect of coexist ions were investigated by comparing the integrated absorbance of Cd and Pb with and without various ions (Lemos *et al.,* 2001).

2.6 Application of this investigation method in seafood samples

2.6.1 Sampling

The frozen seafood samples such as tunafishes (Skipjacks), squids, cuttlefishes, octopuses and prawns from the frozen seafood companies in Trang, Pattani and Songkhla provinces were supported from Central Equipment of Science Faculty, Prince of Songkla University. The seafood samples were passed the manufacturing process and kept in the clean packages. The seafood samples were stored in polyethylene bags and then frozen at -5 ^{o}C until further analysis (Monteiro *et al.,* 2003).

2.6.2 Sample pretreatment

The frozen seafood products were defrosted and cut in small pieces by stainless steel knife and then homogenized using a blender. The homogenized tissue samples were contained in Petri disk and dried to obtain constant weight at 80 ^{o}C for 24 h. in oven. The dried homogenized samples were grinded to be powder with plastic spatula and collected in polyethylene bags until analysis (Monteiro *et al.,* 2003).

2.6.3 The study of digestion method for Cd and Pb determination in seafood samples

The aim of digestion method is to eliminate organic matrix from seafood samples such as protein and lipid. Wet digestion and dry ashing method are commonly used. The wet digestion methods using in this study were hot plate and water bath digestion. The digested

sample solution was diluted to 25.0 mL with de-ionized water. The pH of sample solution was adjusted to 6.0 using 0.1 M ammonium hydroxide prior to extraction using procedure in section 2.4.1. The suitable digestion method was obtained from the comparison of experimental data and certified values. In addition %RSD and percent recovery of the metals in certified reference material were also studied (Sheppard *et al*., 2004).

2.6.3.1 Hot plate digestion method

Certified reference material (DORM-2) 0.2 g (dry weight) was accurately weighted into beaker and 5.0 mL of concentrated nitric acid was added. The beaker was covered using watch glass and heated on the hot plate at 65 ^{o}C for 8 h. to obtain the clear solution and then diluted to 25.0 mL with de-ionized water. The pH of sample solution was adjusted to 6.0 using 0.1 M ammonium hydroxide. The procedure in section 2.4.1 was repeated (Acar, 2001). The hot plate digestion method is shown in Figure 2-3.

Figure 2-3 Hot plate digestion

2.6.3.2 Water bath digestion method

Certified reference material (DORM-2) 0.2 g (dry weight) was accurately weighted into polypropylene vessel and 5.0 mL of concentrated nitric acid was added. The vessel was covered with spiral cap and heated on the water bath at 65 ^{o}C for 2-3 h. to obtain the clear solution and diluted to 25.0 mL with de-ionized water. The pH of sample solution was adjusted to 6.0 using 0.1 M ammonium hydroxide. The procedure in section 2.4.1 was repeated (Acar, 2001). The water bath digestion method is illustrated in Figure 2-4.

Figure 2-4 Water bath digestion

2.6.3.3 Dry ashing method

Certified reference material (DORM-2) 0.2 g (dry weight) was accurately weighted into crucible and was ashed at 720 ^{o}C about 3 h. and cooled overnight in muffle before the further procedure. The ash was dissolved in 2.0 mL of 5.0 M nitric acid and heated on the hot plate until dryness and 5.0 mL of 2 M nitric acid was added. The solution was diluted to 25.0 mL with de-ionized water. The procedure in section 2.4.1-2.4.5 was repeated (Tuzen, 2003). The dry ashing method is shown in Figure 2-5.

Figure 2-5 Dry ashing

2.6.4 Determination of Cd and Pb in seafood samples using GFAAS

The studied method was used for Cd and Pb determination in several seafood samples such as tunafishes, squids, cuttlefishes, octopuses and prawns. The 0.2 g of seafood samples were digested using water bath digestion (2.6.3.2) and extracted by using solid phase extraction with 8-hydroxyquinoline modified octadecyl silica membrane disk. The procedure in section 2.4.1-2.4.5 was repeated.

2.6.5 Determination of Cd and Pb in seafood samples using ICP-OES

The seafood samples were prepared before analysis by using ICP-OES as the following procedure. The 0.2 g (dry weight) of seafood samples were accurately weighed into crucible and ashed at 720 ^{o}C for 3 h. and cooled overnight in the muffle before the further procedure. The ash was dissolved in 2.0 mL of 5 M nitric acid and heated on the hot plate until dryness and 5.0 mL of 2 M nitric acid was added. The solution was diluted to 25.0 mL with de-ionized water before analysis by ICP-OES. The experimental in this section was carried out at Central Equipment of Science Faculty, Prince of Songkla University.

2.6.6 Statistical analysis

Statistical analysis of data was carried out with the SPSS version 11.0 statistical program. The effect of matrix interferences on Cd and Pb determination was evaluated by using two- way ANOVA. In addition, the coexist ion interferences were studied in section 2.5.3 and the comparison of determination method between GFAAS and ICP-OES were evaluated by using Student t-test. All the data analysis was evaluated at the 95% confidential level (Significant (P) = 0.05)