CHAPTER 3

MATERIALS AND METHODS

3.1 Equipments and Chemicals

3.1.1 Equipments

1) High performance liquid chromatographic system, (Agilent Technologies, USA.)
2) Scanning electron microscopy, JSM 5910 LV, (JEOL Techniques Ltd., Japan)
3) Ultrasonicator (Cole Parmer, USA)
4) Filter apparatus (Milipore, USA)
5) Shaker (Gallenkamp, USA)
6) Water purification system, Model Mili-Q system (Milipore, USA)
7) Standard Lab Oven, (Binder, Germany)
8) Water bath, (Memmert, Germany)
9) Centrifuge, (Hettich Zentrifugen, Germany)

3.1.2 Chemicals

1) Berberine chloride (98%), AR grade (Sigma-aldrich, Singapore)
2) Methanol, AR grade, (Merck, Germany)
3) Ethanol, AR grade, (Merck, Germany)
4) Formic acid, AR grade, Fulka, (Sigma-Aldrich Co.LLC, Germany)
5) Methanol, HPLC grade, (Fisher, United Kingdom)
6) Acetonitrile, HPLC grade, (Labscan ,Thailand)
7) Sodium hydroxide, Commercial grade (Merck, Germany)
8) Sodium chloride, AR grade (Union Science, Thailand)

3.2 Samples

3.2.1 Stem of *Coscinium fenestratum*

The dry stems of *Coscinium fenestratum* in Fig. 3.1 were collected from Thai northern herbal shop in the local market, Muang, Chiang Mai in February 2012.

Figure 3.1 Dried stem of *C. fenestratum*

3.2.2 Bentonite powder

Commercial bentonite powder was purchased from Union Science Co., Ltd. Chiang Mai, Thailand (Fig. 3.2).

Figure 3.2 Commercial bentonite powder

3.2.3 Pesticides

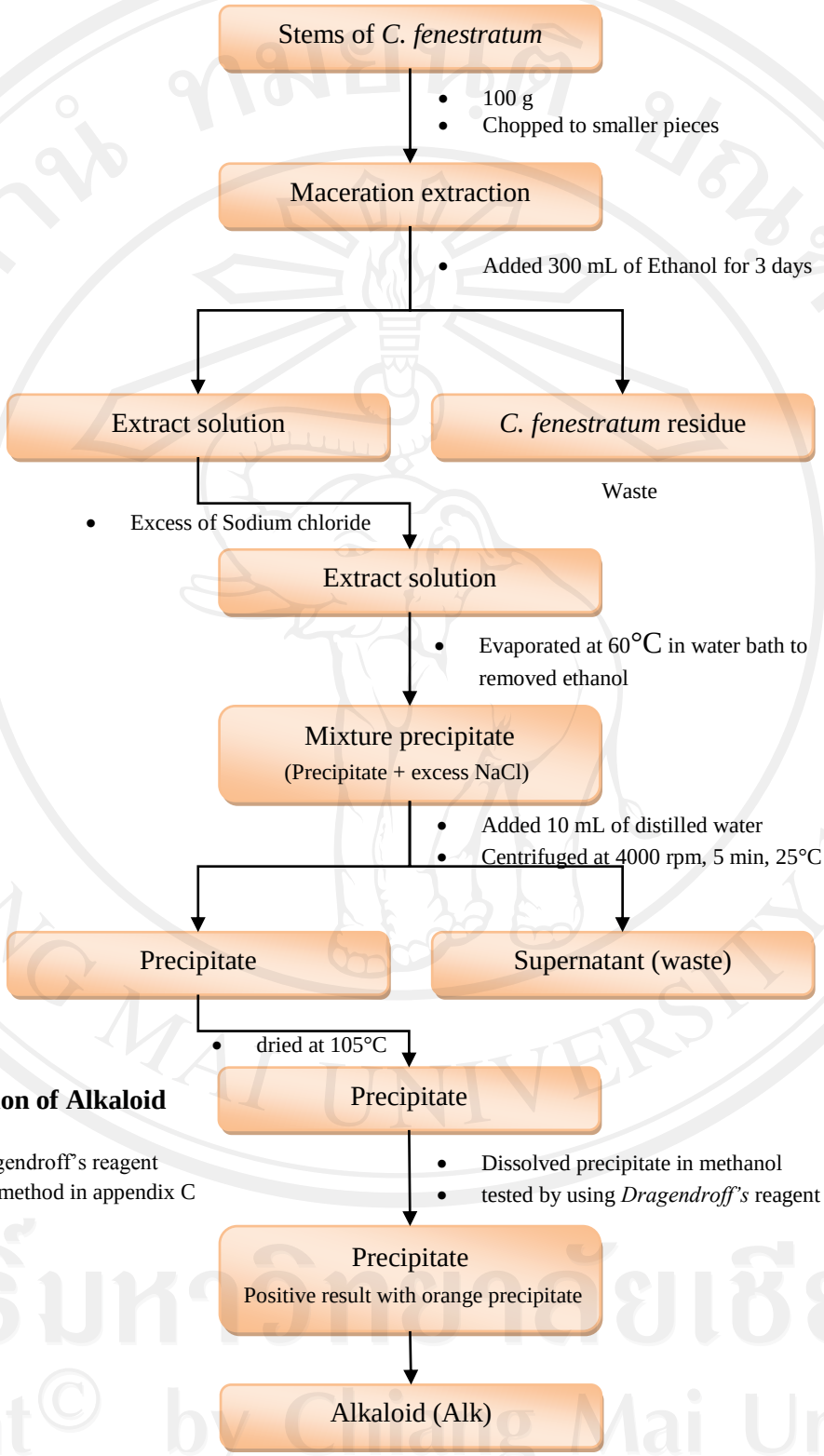
Three standard pesticides (carbaryl, methomyl and methiocarb) were kindly support by Pesticide residue Analytical Laboratory (Royal Project Foundation, Chiang Mai, Thailand). Two pesticides (carbaryl and methomyl) were purchased from Khamtiang market, Thailand.

3.3 Sample preparation

3.3.1 Alkaloid extraction of *C. fenestratum*

The dried stems of *C. fenestratum* (100 g) were chopped to smaller pieces and successively extracted in 300 ml of ethanol for 3 days at room temperature. The

extract solution was collected and excess sodium chloride was added then heated at 60°C for ethanol removal. The mixture were firstly transferred to 50 ml centrifuge tubes and dissolved again with distilled water 10 ml. Then the mixture was centrifuged for 5 min at 25°C. The precipitate was collected and washed twice with distilled water. The precipitate was dried at 105°C and kept in desiccator for the further step (Scheme 3.1).



Detection of Alkaloid

***Dragendroff's reagent preparation method in appendix C

Scheme 3.1 Maceration extraction in ethanol and precipitation of *C. fenestratum*

3.3.2 Identification of *C. fenestratum* extracts using HPLC

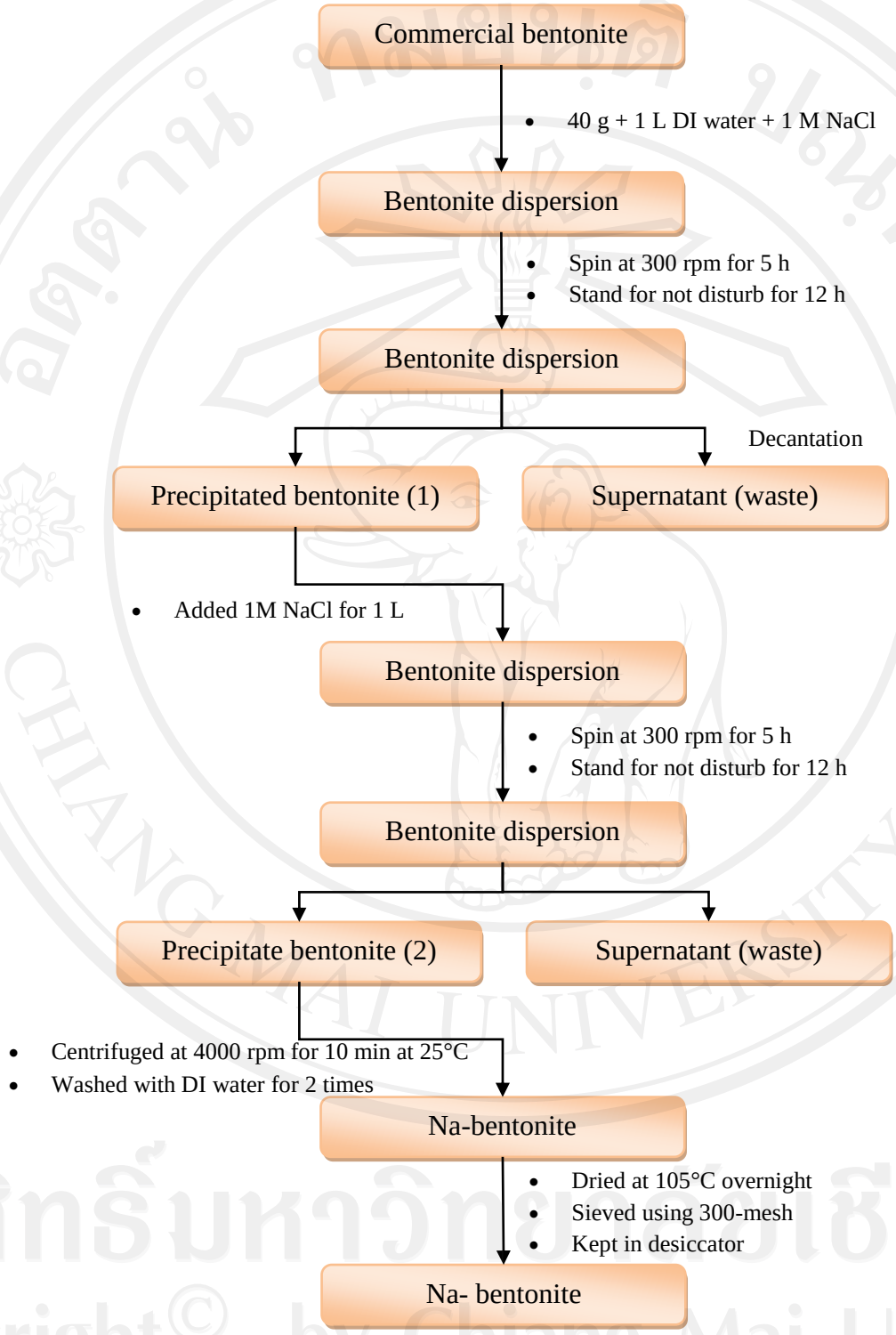
Alk precipitate was further investigated using HPLC. The HPLC condition was followed by Tangpradit (2011) method [88]. Berberine chloride (BbrCl) was used as a standard compound.

Table 3.1 Optimization condition for HPLC analysis

HPLC condition:	
Column	HP hypersil C18 column, Agilent Technologies, USA (125 mm x 4.6 mm i.d., 5 μm)
Mobile phase	A: 5% acetonitrile in 0.1% formic acid B: 95% Acetonitrile in 0.1% formic acid Note: 0.1% formic acid adjusted to pH 4.5 before use
Gradient system	0-5 min, 0-35 %B 6-15 min, 36-60 %B 16-20 min, 61-100 %B 20-30 min, 100 %B
Detector	UV Detector (wavelength 350 nm)
Injection volume	20 μL
Total time	30 min

3.3.3 Saturated Na-bentonite preparation

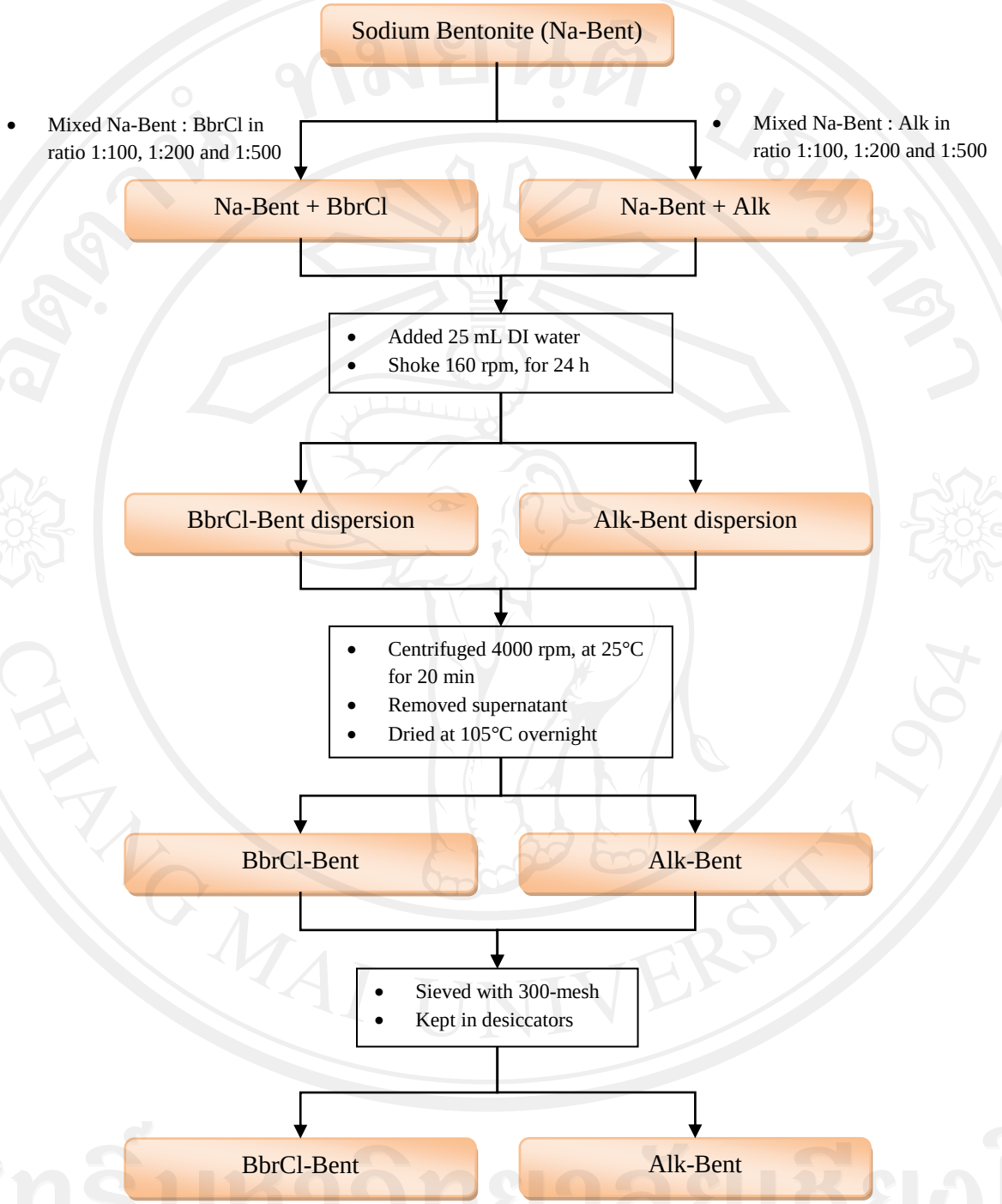
40 g of bentonite powder was dispersed in 1 M of sodium chloride solution for 1,000 mL and mixed for 5 hrs at 300 rpm. Afterwards, the mixture was completely dispersion and standing out of disturbing for 12 h to settle down bentonite clay. NaCl (1 M) solution in the dispersion was decanted and only bentonite dispersion was left in the bottle. New solution of NaCl was filled into bentonite cake to repeat the procedure again. Only bentonite where of settle was further centrifuged at 4000 rpm, 25°C for 10 min to remove sodium chloride solution and washed twice with Deionized (DI) water. After centrifugation, Na-rich-bentonite was heated at 105°C overnight. Bentonite mass was ground with mortar to get smaller particle mass, filtered by 300-mesh sieve and kept in desiccator before use in pesticide adsorption experiments (Scheme 3.2).



Scheme 3.2 Na-bentonite preparation

3.4 Experimental of organoclays

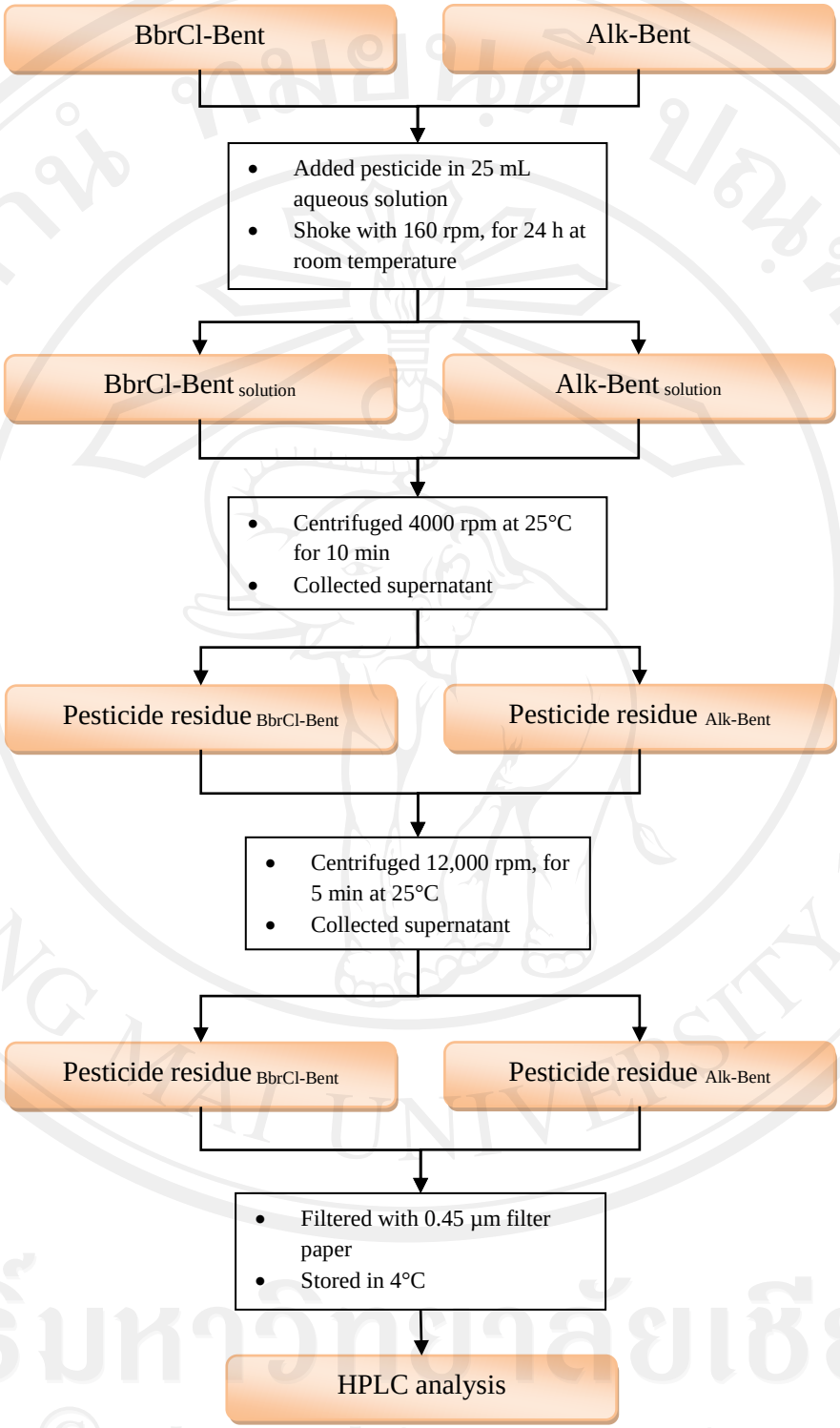
Na-bentonite was modified by BbrCl and Alk through cation exchange method. Standard BbrCl or Alk precipitate (from 3.3.1) modified with bentonite clay weight ratio 1:100, 1:200 and 1:500- denoted as BbrCl-Bent$_{1:100}$, BbrCl-Bent$_{1:200}$, BbrCl-Bent$_{1:500}$ and Alk-Bent$_{1:100}$, Alk-Bent$_{1:200}$ and Alk-Bent$_{1:500}$, respectively, were prepared. The mixtures were dispersed in 25 mL DI water under continuous mechanical shaker for 24 h, 160 rpm speed. Then, the dispersions were centrifuged at 4,000 rpm, 25°C for 10 min. The modified clay was collected and dried overnight at 105°C in a laboratory oven. Finally, dried samples were through 300-mesh sieve and stored in a desiccator before using in sorption experiments, which is shown in Scheme 3.3.



Scheme 3.3 Modification of Na-bentonite using standard berberine chloride and Alk precipitate

3.5 Adsorption of carbamate pesticides

Preparation of pesticides were dissolve in DI water (carbaryl; 200 ppm, methomyl; 200 ppm, and methiocarb; 25 ppm). Two types of organoclay (BbrCl-Bent and Alk-Bent) were prepared to disperse in each pesticide solution. 500 mg each of organoclay was dispersed under pesticide aqueous solution for 25 ml in triplicates and stirred at 160 rpm for 24 h. afterward, separation of organoclay which transferred into centrifuge tube; 10 min for 4000 rpm was conditioned at 25°C. Supernatant was collected to investigate the pesticide residues using HPLC, followed the optimized condition with Table 3.1 at detection wavelength at 280 nm for carbaryl residue detection and wavelength 230 nm for methomyl and methiocarb residue detection (Scheme 3.4).



Scheme 3.4 Pesticide adsorption method

3.6 Adsorption analysis

HPLC analysis was used to determine the amount of adsorption by calculating the difference concentration between initial and final concentration of each pesticides. The condition program was also adjusted by Tangpradit procedure. Three carbamate pesticide standards were prepared in methanol at concentration 5, 10, 15, 20 and 25 ppm. HPLC conditions were followed as section 3.3.2.

3.7 Scanning electron microscopy (SEM)

SEM was used to examine the morphology of all prepared materials including, commercial bentonite, Na-bentonite, BbrCl-bent and Alk-bent samples. The specimens were prepared by place samples on a copper tap which set up on each stub. Gold was used as conductive material for samples coating. Accelerating voltage at 15 kV and 10,000 time magnification were applied.