CHAPTER III

RESEARCH METHADOLOGY

Materials and Methods

Chemicals

Chitosan with the deacetylation degree (DD) of 95% and the molecular weight (Mw) of 1,000,000 Da was obtained from Aqua Premiere (Thailand). Ethylacetate, chloroform, acetonitrile, trifluoroacetic acid, pentasodium tripolyphosphate, potassium orthophosphate, acetic acid, acetone, hydrochloric acid, ethanol 95%, sodium hydroxide, methanol, 1, 1-diphenyl-2-picryhydrazyl (DPPH), (-)- EGCG, - (-) EC, - (-) ECG and (-) - EGC were purchased from Sigma (Germany). Cyclomethicone (DC 345) and cyclopentasiloxane (and) PEG/PPG-18/18 dimethicone (DC 5225C) were products of Down Corning (America).

Plant materials

Assum green teas (Thidadoi brand) were obtained from Tea Institute, Mae Fah Luang University (Thailand).

Apparatuses

A TSP (Thermo Separation Product) Constametric® 3200 HPLC connected to a UV-visible detector (Spectromonitor®, America) was used. Peak Sample software was used for data acquisition. Separations were achieved using a Platinum EPS C18 100A Rocket Column 3 µm (53mm x 7 mm) (America).

The homogenizer that used for microparticle preparation was Silverson®LM (Germany). Microparticle size analyzer was done using Brookhaven ZetaPALS® (Zeta Potential and Particle Size Analyzer, America).

Tea Extraction

Ten gram of dried green tea leaves were extracted for three times with 140 ml of hot distilled water (80 °C) for 15 minutes. The infusion was then cooled down to room temperature, filtered through Whatman membrane No.1. Then it was mixed with an equal volume of chloroform to remove caffeine and pigments. The remaining aqueous layer was then collect and extracted twice with an equal volume of ethyl acetate. The total green tea extract was obtained after the removal of ethyl acetate using a rotary evaporator [1, 11, 25, 27]. The brown residue was obtained and then kept under reduced pressure in desiccators for 12 hrs. Then, the dried extract was kept in cool place and protected from light until use. The scheme of the extraction procedure is shown in Figure 8.

Determination of antioxidant activity

Green tea extract, Trolox and DPPH were dissolved in methanol unless otherwise stated. The studies were carried out using either a 3 ml cuvette or 96 welled microplate. In a 3 ml cuvette, 1.5 ml of 0.2 mM DPPH was added to 1.5 ml of a sample solution while in 96 welled microplate, 150 µl of 0.2 mM DPPH was added to 75 µl of a sample solution. The mixture was kept at room temperature for 30 min. The absorbance values were then measured at 517 nm using 3 ml cuvette with Cecil model CE1010 spectrophotometer (England) or at 515 nm using a microplate with a Ceres UV900C (Bio-tek Instrument, USA). Percentage radical scavenging was calculated using a following equation:

$$\%\text{scavenging activity} = \left| 1 - \left(\frac{\text{Absorbance of sample}}{\text{Absorbance of blank}} \right) \right| \times 100$$

The solution of 0.2 mM DPPH without a sample was used as blank. The concentration of a sample required to inhibit the formation of DPPH radical at 50% (IC_{50}) was determined by log-probit analysis using 6-8 different concentrations of sample. Data were analyzed by the software package Prism (Graph Pad Inc, Sandiego, USA).

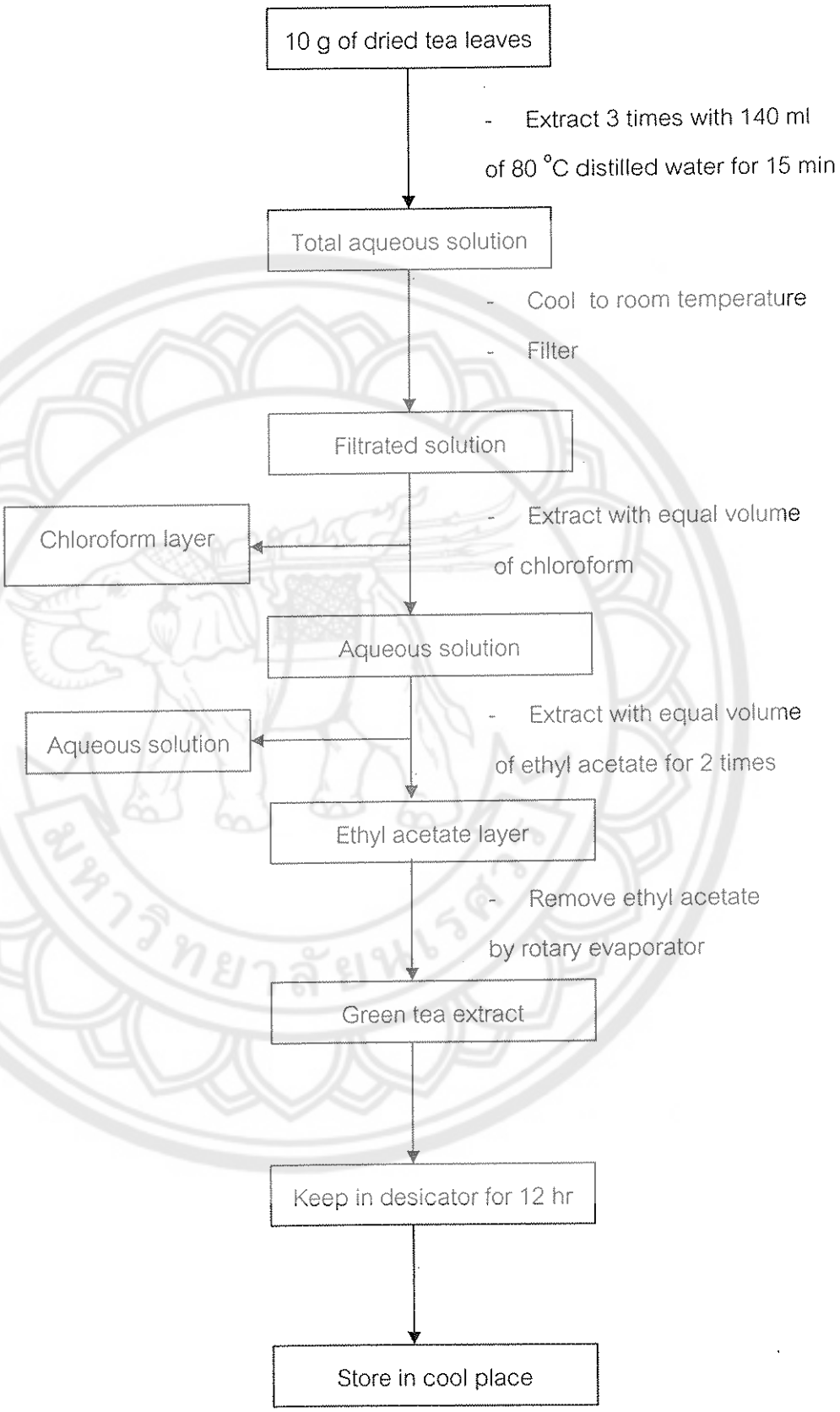

Figure 8 The green tea extraction procedure

HPLC analysis

The analysis of catechins in green tea extract was conducted using TSP a (Thermo Separation Product) Constametric® 3200 HPLC (America). The column used was Platinum EPS C18 100A Rocket Column 3 µm (53mm x 7 mm) (Alltech®, America) connected with Platinum C18 EPS 100A 5 µm, 7.5x4.6 mm All-Guard Cartridge (Alltima, America). The UV-Vis detector (Spectromonitor®, America) was used. Mobile phase consisted of acetronitrile: water (13:87, V/V) containing 0.05% of trifluoroacetic acid. The pH of final solution was 2.0. Elution was performed at a flow rate of 2 ml/min. The chromatogram was detected at 210 nm. The sample injection volume was 20 µl. The column temperature was maintained at 30 C°.

Method validation

The HPLC-method was validated for linearity, limit of detection, accuracy, precision and repeatability.

Linearity

One mg of EGC and ECG as well as ten mg of EC and EGCG were placed in a 10 ml volumetric flask and dissolved in distilled water (stock solution). Six additional calibration levels were prepared by serial dilution with distilled water. Within the range of concentrations injected (1000 –15.6 µg/ml)

Limit of detection and Limit of quantification

The limits of detection (LOD) and quantification (LOQ) were determined by serial dilutions of standard solutions in order to obtain signal/noise ratios of 3:1 for LOD and 10:1 for LOQ [44].

Precision and reproducibility

Intra and inter-day precisions of the assay were verified by analyzing 5 dilutions of samples on 3 consecutive days [45]. Thirty mg of green tea extract was dissolved in 200 ml of distilled water. The green tea solution was diluted to 5 dilutions and analyzed

by using HPLC method. The sample preparation and analysis were repeated for 3 times in each day (for intra-day precision) and 3 consecutive days (for intra-day precision).

Accuracy

Accuracy was determined by comparison of the theoretical concentrations of standard added to sample solution and the concentration obtained from the chromatographic analysis. Each sample solution was injected in triplicate [45]. Ten mg of green tea extract was dissolved in 200 ml of distilled water. Standard of catechins at concentration of 0.025 – 0.0005 mg/ml were spiked into green tea extract solution. The analysis was done for 3 times.

Preparation of chitosan microparticle

The chitosan microparticle was prepared using solvent evaporation technique as previously reported with some modification [38, 46, 47]. Chitosan solution (2%, w/v) was prepared by dissolving chitosan in dilute acetic acid (1%, v/v) at room temperature and filtered through nylon cloth to remove any insolubles. The green tea extract was dissolved directly into the chitosan solution to a final concentration of 10% (w/w of chitosan polymer) and stirred for 1 hr to completely soluble. The chitosan-green tea extract solution was mixed with the same amount of acetone. The mixture was then emulsified into DC 345 containing 5% DC5225C (w/w) with a ratio 10:100 under mechanical stirring (3000 rpm). The system was maintained under mechanical stirring for 1 hr to allow complete evaporation of the non-oil solvent. Thereafter, the ionic gelation of the chitosan microspheres in the oily suspension medium was achieved by addition of 2% tripolyphosphate (TPP) aqueous solution with equal volume of chitosan polymer. After a prefixed cross-linking time of 12 hrs the microspheres were isolated by centrifugation with 10,000 rpm for 5 minutes. The silicone residue was removed by washing with 95% of ethanol for three times. The particles were stored at room temperature for 1 hr and kept in cool place.

Some parameters that might influence the particles forming were also studied. Tripolyphosphate at varying concentration of 2%, 6% and 12%; chitosan concentration of

1%, 2%, 4% (w/v) were used. Acetone concentration also varied from 50% to 75 %(v/v of chitosan solution). The last investigated parameter was the speed of homogenizer which was varied as 1000, 2000 and 3000 rmp.

Morphological characterization of microparticles

The morphology of microsphere was examined using scanning electron microscopy (SEM). The microspheres were dried, coated with gold palladium to achieve a film of 20 nm thicknesses and observed with a scanning electron microscope.

Microparticle size determination

The size and size distribution of microspheres were evaluated by ZetaPALS® (Zeta Potential and Particle Size Analyzer) using distilled water as a liquid phase.

Determination of encapsulation efficiency

The drug-loaded microspheres (10 mg) were extracted with 1 ml of 1N HCl for 48 hours at room temperature following with centrifugation at 10,000 rpm for 5 min and then filtered (0.2 mm PTFE filters, Whatman,UK). The filtrate was assayed for catechins content using HPLC. Encapsulation efficiency was calculated from the amount of catechins found in microparticles and the amount catechins loading. All samples were analyzed in triplicate. The encapsulation efficiency was calculated from the following expression:

$$\text{Encapsulation Efficiency (\%)} = \frac{\text{(amount of catechins in particle) X 100}}{\text{Amount of catechins loaded}}$$

Evaluation of the in vitro release

Ten mg of microparticles were placed into centrifuge tube containing 1 ml of dissolution media under agitation. The pH of buffer dissolution media were pH 7.4, 5.5 and 2.0. After centrifugation of the samples (10,000 rpm, 10 min) for 0.5, 1, 1.5, 2, 4, 6

and 8 hour, 1 ml samples were withdrawn and replaced by fresh medium. The amount of drug released from the microparticles was analyzed using HPLC [48].

Green tea extract solutions were incubated in varying pH solutions and simultaneously measured their concentration with the same predetermined time as those of microparticles. This investigation was to observe the catechins recovery under different pH.

Evaluation of stability of chitosan-green tea microparticle

Ten milligram of chitosan-green tea microparticles were dispersed in buffer pH 5, 7.4 and 9. The mixture was incubated at 45, 65 and 80 C° in hot air oven for 24 hr [49] The mixture was then assayed for catechins using HPLC method.

Evaluation of photo-stability of chitosan-green tea microparticle

Ten milligram of chitosan-green tea microparticles were dipersed in 1 ml buffer pH 5.5. The mixture was kept under light and dark condition for 24 hrs. Then, the mixture was assayed for catechins using HPLC. The study was done in both dispersion and powder forms.