CHAPTER 3
EXPERIMENTAL

3.1 Apparatus

1. Analytical balance, Mettler Toledo AG 285, Switzerland
2. Filter apparatus, Millipore, USA
3. Filter papers No. 42, Whatman, England
4. High performance liquid chromatograph (HPLC-UV model G 1314A) system, Agilent Technologies, USA, consisting of :
    a. Auto injector, HP 1100
    b. Binary pump, HP 1100
    c. Degasser, HP 1100
    d. UV-Visible absorbance detector, HP 1100
    e. Chromatograph workstation, HP chemstation, by Agilent Technologies, USA.
5. HPLC column (Hyperclone® C18 5µm, 4.6 x 250 mm and Inersil® ODS-3 5µm, 4.6 x 250 mm), USA
6. Nylon membrane filters, Sartorius, Germany
7. pH Meter, Model inolab pH Level 2, Germany
8. Rotary vacuum evaporator, Japan
    a. Digital water bath, SB-651, Japan
    b. Eyela cool all, CA-1111, Japan
9. Syringe, 5 mL, and 10 mL, Terumo (Philippines) corporation, Philippines
10. Solid phase extraction manifold, manufactured J. T. Baker Inc., USA

3.2 Chemicals

1. Acetonitrile, HPLC grade, Fisher chemicals, UK
2. L-ascorbic acid, USP grade, Fisher chemicals. UK
3. Beta-carotene, USP grade, Fluka, Switzerland
4. Ethanol absolute, Merck, Germany
5. Ethanol, HPLC grade, LAB-scan, Ireland
6. Ethyl acetate, Analytical grade, LAB-scan, Ireland
7. Hexane, LAB-scan, Ireland
8. Methanol, HPLC grade, Fisher Chemicals, UK
9. Nicotinamide, USP grade, Sigma, Germany
10. Pyridoxine hydrochloride, USP grade, Sigma, Germany
11. Riboflavin, USP grade, Sigma, Germany
12. Sodium-1-octanesulfonate, Tokyo kasei, Japan
13. Thiamin hydrochloride, USP grade, Sigma, Germany
14. Vitamin E, USP grade, Fisher, Switzerland

3.3 Procedure

Part I Determination of vitamins in *M. citrifolia* and *P. emblica* fruits.

3.3.1 Sample preparations

M. citrifolia and *P. emblica* collected from Chiang Mai were used in this investigation. The samples were cleaned, chopped into small pieces and homogenized.

3.3.2 Extraction for analysis vitamins C, B_1, B_2, B_3 and B_6

About 10 g of the sample was weighed accurately and transferred into a 250 ml erlenmeyer flask, extracted with water 50 ml and mixed by an automatic shaker. The resulting combined extract was filtered through filter paper no. 42 and collected filtrate. The mixture was centrifuged at 9000 rpm for 20 min and then removes interfering components by SPE.

3.3.3 Solid-phase extraction (SPE) for analysis water soluble vitamins (C, B_1, B_2, B_3 and B_6)

The procedure was used the Sep-Pak C_8 cartridges to remove most of the interfering components and pre-concentration of sample. First, we flushed the stationary phase with 2 mL methanol and 2 mL acidified water pH 4.2 to activate the stationary phase; we then loaded a 1 mL of sample extracted. The acidified water was prepared by adding a 0.005 M HCl solution drop by drop with stirring until its pH reaches the predetermined value. The sample was eluted with 1 mL water (pH 4.2) followed by 2 mL methanol at a flow rate of 1 $mL.min^{-1}$. The eluents were collected in a bottle and evaporated to dryness. The residue was dissolved in acidified water (pH 3.7). The solid phase extraction scheme is summarized in Fig. 3.1 [181]

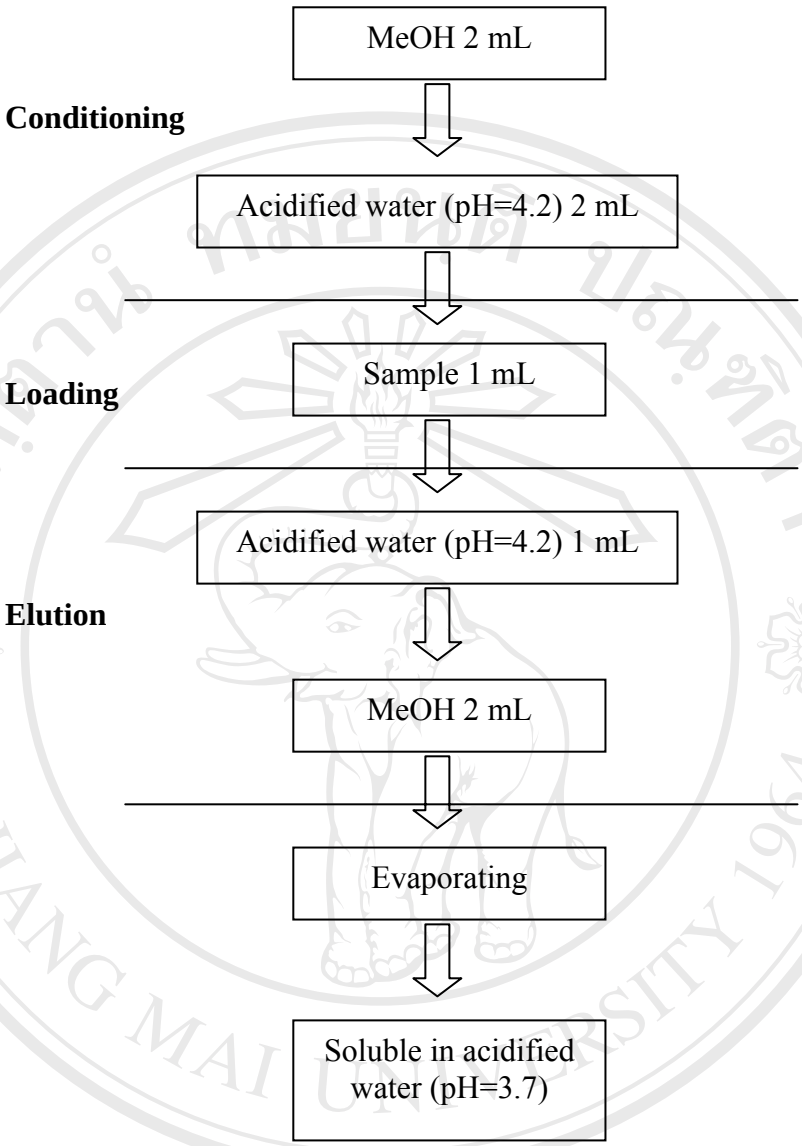

Figure 3.1 The solid phase extraction procedure. [181]

3.3.4 Clean up of sample for the determination of vitamins E and beta-carotene.

The extracts were clean up of sample to degrade fat and transform fat soluble vitamin esters to this corresponding vitamin. The vitamins being studied are sensitive to oxidation. Ascorbic acid was therefore added as an antioxidant, and saponification was performed in a nitrogen atmosphere protected from light. The modification of that reported by Charlotta and Lennart [182] and the procedure is as follow: A 50 g of sample was accurately weight into a 250 ml round bottom flask and then 0.2 g of ascorbic acid, 15 mL of absolute ethanol and 4 mL of 50% potassium hydroxide solution and reflux at 70 °C for 30 min with slow constant stirring.

A 5 mL volume of sodium chloride (25 g/L) was added after cooling and the suspension was extracted three times with 15 mL portions of n-hexane-ethyl acetate (85:15, v/v). The organic phase was evaporated to dryness at 40 °C and the residue was dissolved in 1 mL of methanol. The samples were filtered through nylon membrane filter with a pore size of 0.45 μm. The samples were analysed by RP-HPLC. They were kept at -20 ºC before injection but they only remained stable for a week. The extraction scheme is summarized in Fig. 4.2.

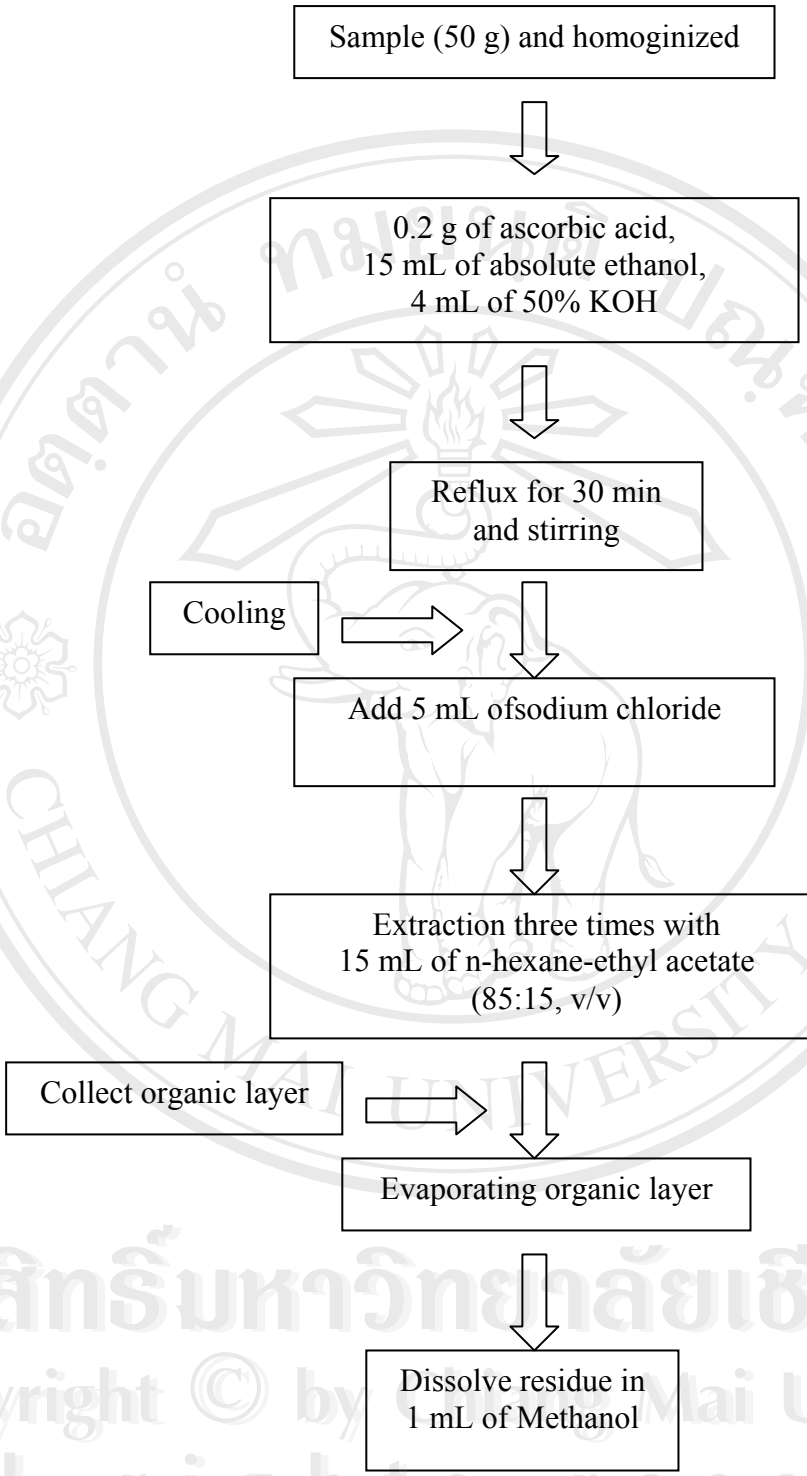

Figure 3.2 Procedure of clean up sample for fruit of *M. citrifolia* and *P. embilca* samples. [182]

3.3.5 Preparation of standard solutions

Water soluble vitamins (vitamin C and B-groups)

Stock standard solution

The standard stock solutions of the vitamins for analyzing samples were prepared to a concentration of approximately 25 mg of ascorbic acid in water, 12.5 mg of riboflavin in 2.4% (v/v) aqueous acetic acid, 25 mg of thiamin hydrochloride, nicotinamide and pyridoxine hydrochloride in 2.4% (v/v) aqueous acetic acid.

Working standard solutions: 200.0 to 1000.0 $\mu g.mL^{-1}$ for ascorbic acid, 10.0 to 100.0 $\mu g.mL^{-1}$ for thiamin hydrochloride, 10.0 to 70.0 $\mu g.mL^{-1}$ for riboflavin, 30.0 to 300.0 $\mu g.mL^{-1}$for nicotinamide and 0.5 to 7.0 $\mu g.mL^{-1}$ for pyridoxine hydrochloride, all of them in 2.4% (v/v) aqueous acetic acid. All standard solutions should be filtered through a 0.45 µm nylon membrane, protected from light and stored at 4 ºC.

Fat soluble vitamins (vitamin E and Beta-carotene)

Stock standard solution

The standard stock solutions of the vitamin E and β-carotene were prepared by dissolving 2.50 mg of α-tocopherol and 2.50 mg of β-carotene in a separated 25 ml volumetric flasks and the solutions were made up to the mark with absolute ethanol.

Working standard solutions of α-tocopherol and β-carotene were prepared from stock solutions: 1.0, 3.0, 5.0, 7.0 and 10.0 $\mu g.mL^{-1}$ for α-tocopherol and 1.0, 3.0, 5.0 and 10.0 $\mu g.mL^{-1}$ for β-carotene, all for them in mobile phase. All standard solutions should be filtered through a 0.45 µm nylon membrane, and stored at 4 ºC.

3.3.6 Determination of vitamin C

A mobile phase was 2% acetic acid and the elution was performed at a flow rate of 0.8 ml min^{-1}. The analytical column was kept at 20 °C. Detection was performed at 210 nm. The mobile phases were filtered through a 0.45 µm nylon membrane to remove the impurities that might be present and degassed by sonication prior to use.

3.3.7 Determination of vitamins B-group

The mobile phase containing 5 mM octanesulfonic acid, in water pH 2.5 and acetonitrile (75:25, v/v) was prepared as follows: preparation of 5 mM octanesulfonic acid. About 1.01 g of octanesulfonic acid was accurately weighed and transferred into 1-litre volumetric flask, dissolved with ~800 mL of distilled water, than adjusted to pH 2.5 with glacial acetic acid made up to volume with distilled water. The mobile phases were filtered through a 0.45 µm nylon membrane to remove the impurities that might be present and degassed by sonication prior to use.

The experiment was performed with isocratic HPLC. Sample and standard solutions was analysed by C_{18} column using 5 mM octanesulfonic acid (pH 2.5) : acetonitrile (75:25%) as mobile phase with flow rate of 1.0 $mL.min^{-1}$, while the UV detector was set at 280 nm.

3.3.8 Determination of vitamin E and beta-carotene

Vitamins E and beta-carotene were analysed by injection 10 µl sample into a reversed-phase HPLC system, using a mobile phase of ethanol (solvent A)-methanol (solvent B) gradient that follows: in the range 0-6 min, 35 : 65; at 9 min, 100% B flow rate 1.2 ml min^{-1}; at 15 min, 35 : 65. The mobile phase flow rate was 1 ml min^{-1}. Column effluents were monitored at 295 and 450 nm for vitamin E and beta-carotene, respectively. The mobile phases were filtered through a 0.45 µm nylon membrane to remove the impurities that might be present and degassed by sonication prior to use.

Part II Determination of vitamins in fermented juice containing *M. citrifolia* and *P. emblica*.

3.3.9 Preparation of fermented juice products containing *M. citrifolia* and *P. emblica*.

Commonly edible and abundantly available fresh fruits of *M. citrifolia* and *P. emblica* were purchased from local markets. Fresh fruits were cleaned with water. Four most popular production processes and control were selected for this study. The formulas are different in the reduction of microorganisms in raw materials (potassium metabisulfite (KMS), the starter (with and without *Lactobacillus casei* spp.), and component ratio. Biologically fermented products containing *M. citrifolia* and *P. emblica* fruits were produced with the explanation of conditions as follows:

1. Fermentation of herbs and raw cane-sugar with *L. casei*.
2. Fermentation of herbs and honey with *L. casei*
3. Fermentation of herbs and raw cane-sugar
4. Fermentation of herbs and honey
5. Fermentation of crushed *M. citrifolia,* water and raw cane-sugar in solid state fermentation during the first period (added water on the 15th day of fermentation period)
6. Fermentation of crushed *M. citrifolia,* water and raw cane-sugar. Added potassium metabisulfite (KMS) about 250 mg/L before inoculated with the starter culture in concentration of 10% (w/w).
7. Fermentation of herbs and raw cane-sugar (pasteurize at 72.5 °C for 15 sec and *L. casei*)

Control processes

1. Control blank, fermentation of raw cane-sugar and *L casei.*
2. Control blank, fermentation of raw cane-sugar
3. Control blank, fermentation of honey and *L casei.*
4. Control blank, fermentation of honey

The formulas are composed of herbs, raw cane-sugar or honey and water at ratio of 3: 1: 10

3.3.10 Samples preparation

The samples were taken on day 0^{th}, 7^{th}, 15^{th}, 30^{th}, 45^{th}, 60^{th} and 90^{th} of fermentation times. The samples were filtered through No. 42, filter paper and kept in freezer at –20 °C until used.

3.3.11 Analysis of Fermented juices products

The stability of fermented juices products was also investigated. Vitamin C and Vitamin B group in each fermented juice products were determined by HPLC, using the experimental conditions as described in section 3.2.1.6 and 3.2.1.7.

3.3.12 Stability study

The stability of vitamin C and vitamins B in fermented juices was studies by HPLC method. Fermented juice of vitamin C and vitamins B with concentration of 500 $\mu g.mL^{-1}$ and 100 $\mu g.mL^{-1}$ was prepared in triplicate and stored in the dark without air (ambient temperature) and were periodically analyzed over a 3 month period following the method described above.

3.4 Method validation

3.4.1 Precision and accuracy

Precision was studied by determining vitamins content in Fruits of *M. citrifolia* and *P. emblic*a from the same source by the same analyst on the same and different days. Five injections were made for each sample. Precision was expressed as % relative standard deviation (%R.S.D.). The accuracy calculated from recovery was determined using standard addition method. Vitamins in the added juice sample were determined by HPLC method as described in the previous section. Five injections were made for each standard added solution. The recoveries were calculated using the expressions:

$$\% \text{ Recovery} = \frac{\text{Amount of standard recovered}}{\text{Amount of standard added}} \times 100$$

3.4.2 Linear range

The linearity of the method was determined by adding standard vitamins in the juice samples. Linearity was obtained in the range of the standard concentration for each vitamin. A series of solution at low and high concentration levels were prepared, each solution was injected three times and the regression was calculated by the method of least-squares. Peak areas were calculated and the results interpolated on the calibration graph for each vitamin.

3.4.3 Limit of detection and quantitation

The limit of detection (LOD) was determined by decreasing the concentration of standard vitamins, at a signal-to-noise ratio (S/N) of 3, and the limit of quantitation was calculated at an signal-to-noise ratio (S/N) at 10 for all three compounds.