Chapter 3
Materials and Methods

3.1 Materials and reagents

3.1.1 Raw materials and hydrocolloids

1. Lime fruits (*Citrus aurantifolia* Swingle) were purchased from fresh markets in Amphoer Muang, Chiang Mai of Thailand
2. Gum acasia : (OV Chemical, Chiang Mai, Thailand)
3. Pectin : (OV Chemical, Chiang Mai, Thailand)
4. CMC : Sodium Carboxymethylcellulose (OV Chemical, Chiang Mai, Thailand)
5. κ– Carrageenan : (OV Chemical, Chiang Mai, Thailand)

3.1.2 Standard reagents

1. Limonin standard from citrus seeds, purity >90% (HPLC-grade) (Sigma, Germany)

3.2 Lime juice sample preparation

Lime fruit was screened and selected for fruit with good qualities, which did not have disease or damaged. The fruit was thoroughly washed with tap water and cut into two halves with a kitchen knife. The half fruits were then squeezed using a stainless steel juice extractor to produce lime juices. The collected juices were filtered using a double

layer filtered cloth to remove pulp, seeds, juice sacs and rags that are separated during the extraction. The filtered lime juices were directly used in any experimental studies or stored at -80^{o}C until further used (Figure 3.1).

3.3 Methodology

3.3.1 Characterization of fresh lime juice

3.3.1.1 Physical, chemical, nutritional and microbiological qualities of fresh lime juice

Lime juice was subjected to different chemical, physical, microbiological and nutritional analyses, including sensory evaluation as well as for its proximate analysis (Figure 3.1). The lime juice samples were prepared in triplicate.

For the bitterness compound of the lime juice, the analysis was :

- D-limonin by doing a chloroform extract of lime juice sample and a measurement using a spectrophotometer method (Vaks and Lifshitz, 1981)

For physical analyses of the lime juice, the analyses include:

- % Yield by weight calculation
- Color by a colorimeter (L*, a* and b*-value)

For chemical analyses of the lime juice samples, the analyses were:

- Total solid followed an AOAC method no. 920.151 (37.1.12) (AOAC, 2000)
- pH value (a pH meter)
- Total Soluble Solid (^{0}Brix) using a hand refractometer
- Total acidity as % citric acid followed an AOAC method no. 942.15 (37.1.37) (AOAC, 2000)

For the nutritional analysis of lime juice, is included:

- Ascorbic acid followed an AOAC method no. 967.21 (45.1.14) (AOAC, 2000)

For the microbiological analysis of lime juice, the analyses were:

- Total Plate Count for total viable microorganisms using Orange Serum Agar (FDA, 2001)
- Yeasts and molds using Potato Dextrose Agar (FDA, 2001)
- *Escherichia coli* using LST broth and EC broth (FDA, 2001)
- Coliform bacteria and fecal coliform bacteria using LST broth, EC broth and Brilliant Green Lactose Bile broth (FDA, 2001)

For the sensory evaluation of the lime juice, the evaluation accessed some sensory parameters which were:

- Color, odor, bitterness, sour and overall acceptability using 9-point hedonic scale (Wiriyajare, 2002)

Proximate analysis for the fresh lime juice included:

- fat / lipid content followed an AOAC method (AOAC, 2000)
- protein content followed an AOAC method no. 920.152 (37.1.35) (AOAC, 2000)
- carbohydrate content followed an AOAC method (AOAC, 2000)
- moisture content followed an AOAC method no. 925.45 (44.1.03) (AOAC, 2000)
- fiber content followed an AOAC method (AOAC, 2000)
- total sugar followed an AOAC method no. 925.36 (37.1.52) (AOAC, 2000)
- reducing sugar followed an AOAC method no. 925.36 (37.1.52) (AOAC, 2000)
- invert sugar followed an AOAC method no. 925.35 (37.1.51) (AOAC, 2000)
- ash content followed an AOAC method no. 920.138 (36.3.14) (AOAC, 2000)

3.3.2 Limonin content of the lime fruit component

Lime fruit was separated for its components, which were flavedo, albedo, segment membranes, seed and juice sacs, and prepared the samples according to McIntosh *et al*., (1987). Analysis of the D-limonin content in each lime juice component was done by a chloroform extraction and a spectrophotometric measurement (Vaks and Lifshitz, 1981) (Figure 3.2).

3.3.3 Different quality parameters of lime juice during storage at 4-6^{o}C and at ambient temperature for 1 month

Lime juice samples were prepared according to the section 3.2 and kept in sterilized amber bottles at 2 different storage temperatures of 4-6^{o}C and ambient temperature for 1 month period. During this storage period, representative samples were taken on 0, 7, 14, 21 and 28 days to be analyzed according to the section 3.3.1, excepted for the proximate analysis, total solid and percentage of yield.

The analysis results of lime juice during storage at 2 different temperatures for 28 days were collected and statistically analyzed using an Analysis of Variance by applying a Completely Randomized Design (CRD) experiment. All treatment were carried out in 3 replicates. The statistical program of SPSS v.11.5 (SPSS Inc., Chicago, USA) was used for the data analysis and Duncan's multiple range test was used for comparing differences between means (Hanmhongkolphipat, 2004).

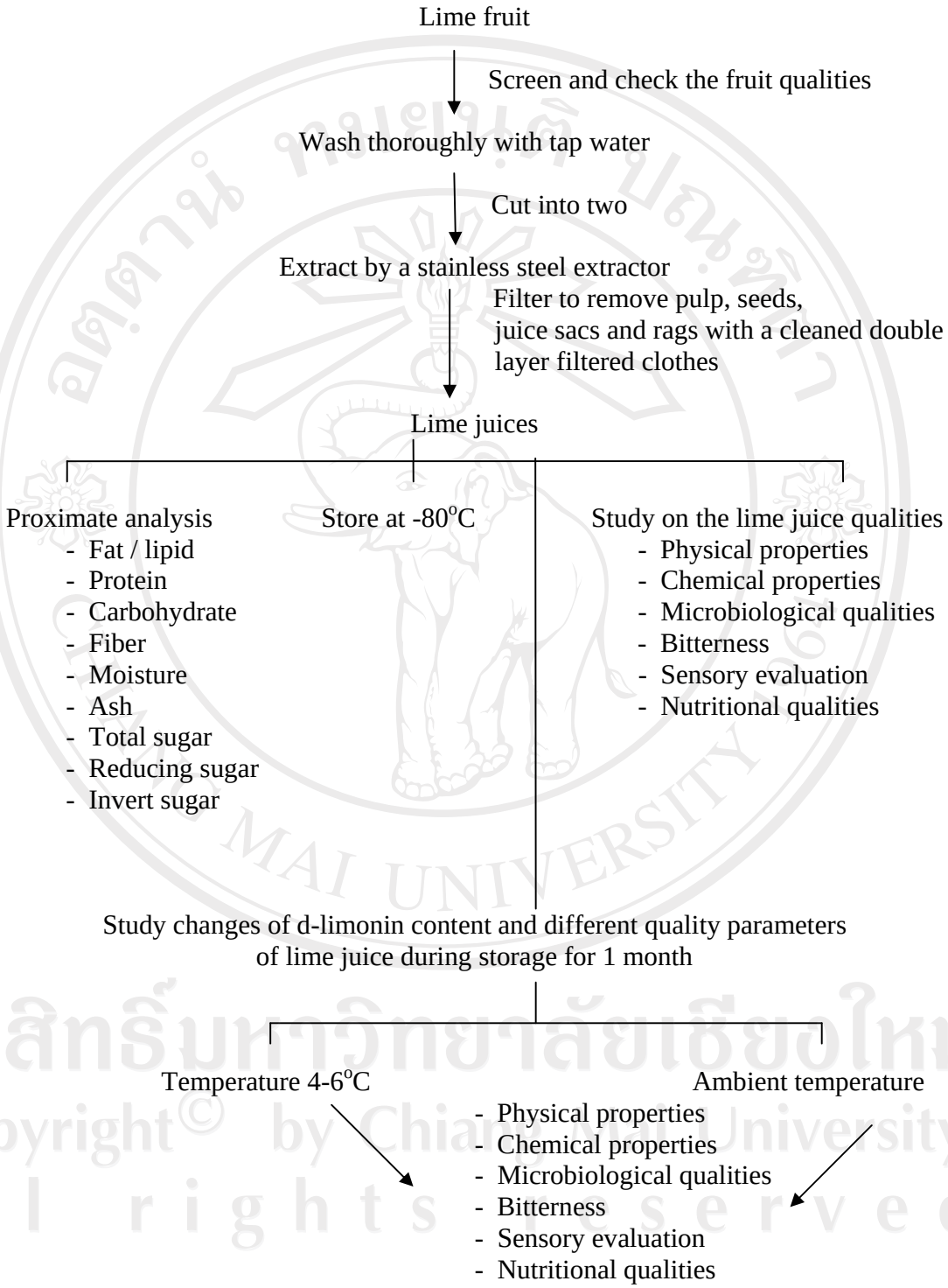

Figure 3.1: A diagram for the production process of lime juice, storage of the juice and analyses of the juice samples (sections 3.2, 3.3.1 and 3.3.3)

3.3.4 The effect of different types and levels of hydrocolloids on the d-limonin content and qualities of lime juice during storage at ambient temperature

Samples of lime juice which were prepared according to the section 3.2. Into this lime juice, 4 types of hydrocolloids, including gum acacia, pectin, κ-carageenan and sodium carboxymethylcellulose (CMC) were added at 3 different concentrations of 0.5, 1.0 and 1.5 g/l (w/v) and kept in sterilized clear bottles at ambient temperature for 1 month. All the hydrocolloids used in this experiment were in powder form and had a food grade standard.

During the storage period, representative samples were taken on 0, 7, 14, 21 and 28 days to be analyzed and monitored for their quality changes, including bitterness (d-limonin), color, ascorbic acid, total soluble solid (^{0}Brix), pH value and total acidity as % citric acid. Collected data of the hydrocolloid added lime juices was analyzed statistically using a 4x3x5 factorial in CRD. All treatments were carried out in 3 replicates. The statistical program, SPSS v.11.5 (SPSS Inc., Chicago, USA), was used for the data analysis and Duncan's multiple range test was used for comparing differences between means (Hanmhongkolphipat, 2004). The statistical analysis from the collected data would show the best hydrocolloid type at a specific addition level that could maintain the quality of lime juice during storage (Figure 3.2).

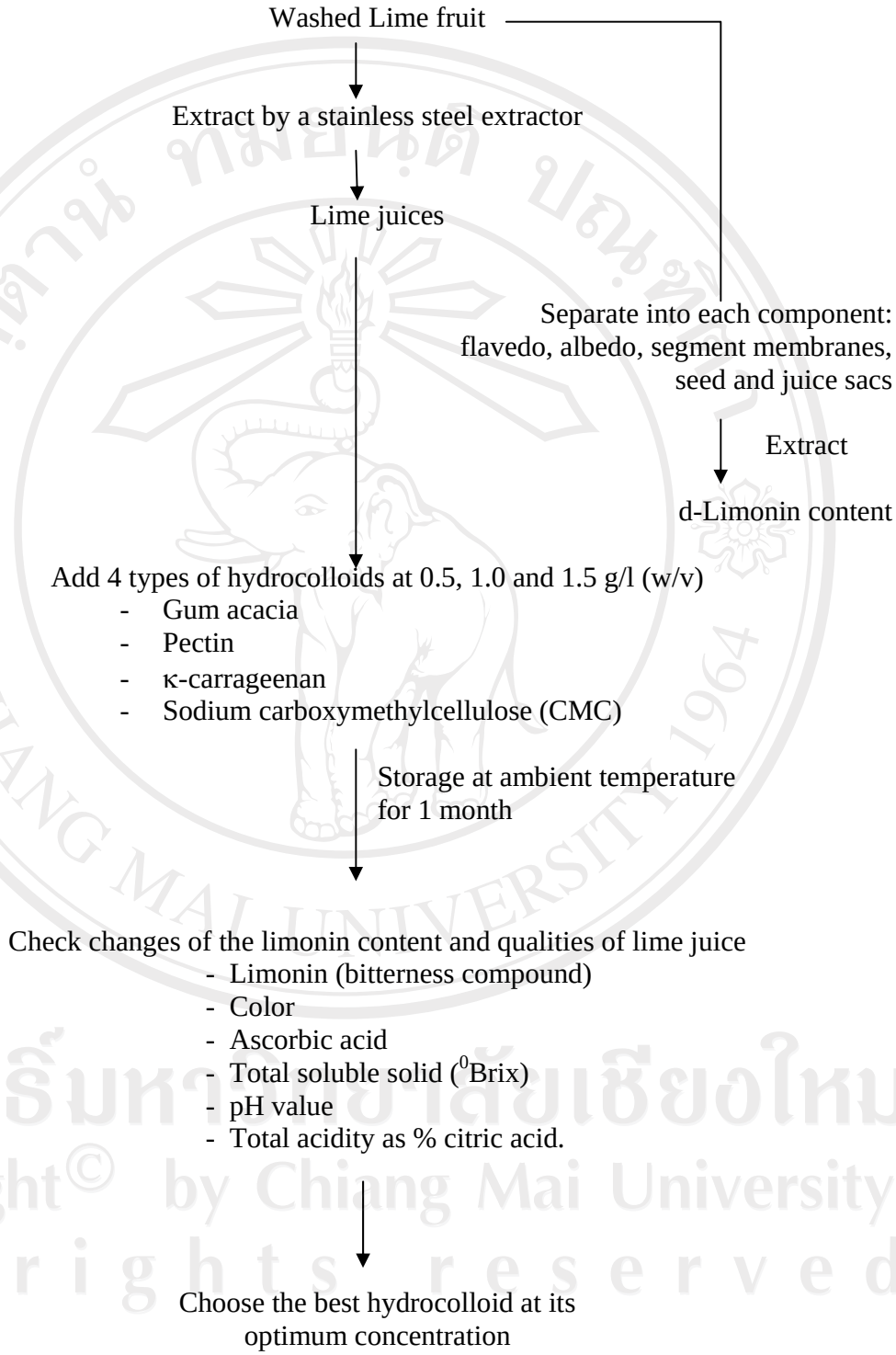

Figure 3.2 A diagram for d-limonin content of each lime fruit component and hydrocolloid treated-lime juice (sections 3.2 and 3.3.4)

3.3.5 The effect of High Pressure Processing (HPP) on the limonin content and the quality of lime juices during storage at 4-6^{o}C and at ambient temperature

3.3.5.1 Preparation of lime juice

Fresh lime juice was prepared according to the section 3.2. Into this lime juice, the best hydrocolloid type at a specific addition level according to the result in the section 3.3.4 was added and mixed throughly. Then the hydrocolloid added lime juice was filled into transparent polyethylene pouches. After expelling as much air as possible, the pouches were sealed and subjected into HPP using a prototype Stansted "Food Lab" model 900 high pressure rigs. In the HPP treatment, the lime juice pouches were submerged in ethanol containing oil, which acted as a hydrostatic fluid medium during pressurization. Samples of lime juice were subjected into 3 different pressure treatments of 400, 500 and 600 MPa for 15 minutes holding time at 25 ± 2^{o}C (Figure 3.3).

3.3.5.2 The quality of hydrocolloid and HP processed– lime juice during storage at 4-6^{o}C and at ambient temperature

Hydrocolloid-treated lime juices and the control without hydrocolloid addition by a HPP treatment were stored at 4-6^{o}C and at ambient temperature for 1 month. During this storage time, representative samples were taken on 0, 7, 14, 21 and 28 days to be analyzed and monitored for the lime juice quality changes according to the section 3.3.1.1, excepted for the proximate analysis, total solid and percentage of yield. The experiment was carried out in triplicate. In this section, the applied statistical analysis was a 2x4x5 factorial in CRD. The statistical program, SPSS v.11.5 (SPSS Inc.,

Chicago, USA), was used for the data analysis and Duncan's multiple range test was used for comparing differences between means (Hanmhongkolphipat, 2004).

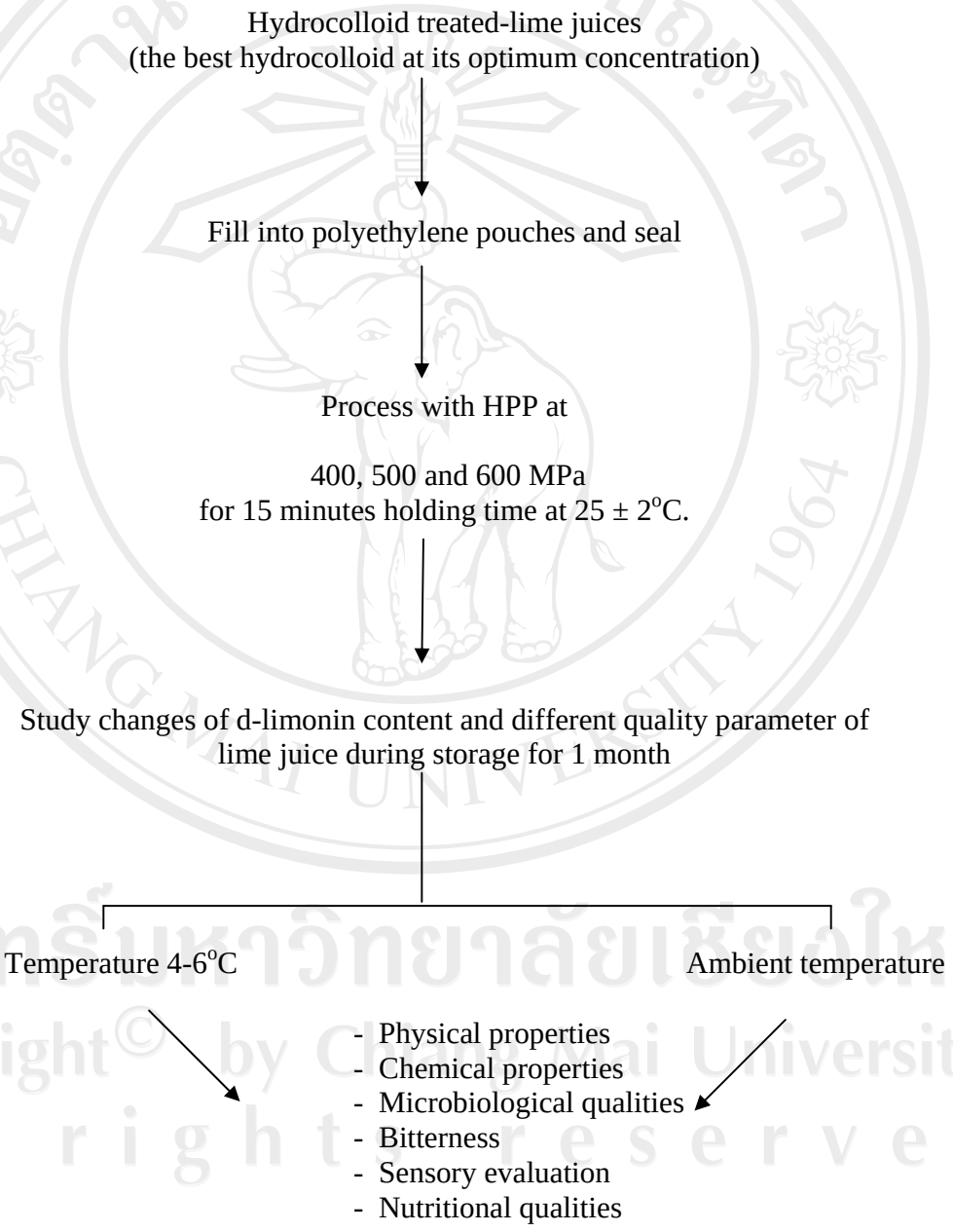

Figure 3.3 A diagram for hydrocolloid and HPP treated-lime juice during storage (section 3.3.5)

3.4 Sensory evaluation

From lime juice and HPP treated lime juice with and without hydrocolloid addition that were kept at a different storage temperatures were separated on 0, 7, 14, 21 and 28 days of storage and subjected to a sensory evaluation. A number of 45 untrained panelists consisted of housewives and students in the Department of Food Science and Technology, Department of Biotechnology and Department of Biochemistry, Chiang Mai University was participated in the sensory test. The panelists were requested to rating the intensity of bitterness, color, aroma, sour and overall acceptability of lime juice samples using a 9-point hedonic scale technique (Wiriyajare, 2002).

Three or six lime juice samples were served randomly in plastic cups that had specifically three digit numbers on a tray together with a cup of water and a piece of non-salted cracker. The water and the cracker were suggested to be consumed between any testing of the lime juice samples.

Data resulting from different experiments was analyzed statistically for their interpretation using an Analysis of Variance. The statistical program, SPSS v.11.5 (SPSS Inc., Chicago, USA), was used for data analysis and Duncan's multiple range test was used for comparing differences between means (Hanmhongkolphipat, 2004).