Chapter 3

Materials and Methods

3.1 Materials

3.1.1 Plant materials in Part I

Krachai-Dam rhizomes used in this study were selected using 3 intensity levels of internal rhizomes colors which were 'Rom-Klao' cultivar (dark purple color), 'Nam-Juang' cultivar (purple color) and 'Kheg-Noi#2' cultivar (pale-purple color) (Figure 3.1). They were collected from 3 commercial areas in Phitsanulok and Phetchabun provinces and were grown at Phurua Highland Agricultural Experiment Station, Phurua, Loei province (950 m above average sea level; m asl) in May 2003 and harvested in January 2004 (crop season of 2003-2004). These rhizomes were obtained by layout incomplete on the ground in shaded air-ventilated areas.

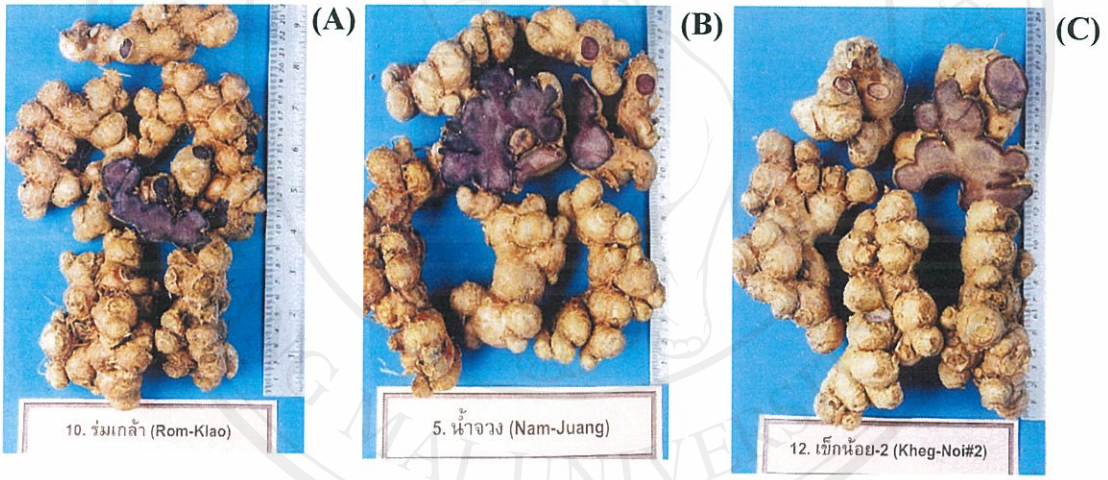

Figure 3.1 The rhizomes of 3 of Krachai-Dam cultivars, which were (A) Phurua-10 (Rom-Klao) (B) Phurua-5 (Nam-Juang) and (C) Phurua-12 (Kheg-Noi#2).

3.1.2 Plant materials in Part II

Krachai-Dam rhizomes used in this study were selected from the best cultivars from experiment Part I. The rhizomes were grown during May to August 2005 and harvested during November 2005 to March 2006 at 4 plantation areas which were Nakhonphanom Horticultural Research Center (Muang, Nakhonphanom province; 150 m asl), Phrae Horticultural Research Center (Muang, Phrae province; 200 m asl), Phurua Highland Agricultural Experiment Station (Phurua, Loei province; 950 m asl) and Royal Chiang Mai Agricultural Research Center at Maechonluang branch (Maecham, Chiang Mai province; 1,350 m asl). These rhizomes were obtained by layout on the ground, in net bags and layout in the cold storage at 13±1°C, at 65% relative humidity.

3.1.3 Honey

Three sources of flowers for honey collection were used in this study, which were Longan (*Euphoria longana* Lam.), Lychee (*Litchi chinensis* Somn.) and Sab-

suea (*Eupatorium odoratum* L.). They were collected from honey farm in Hang-Dong, Chiang Mai Province. These honeys were collected during January to April 2003 for part I, and during March to April 2005 for part II.

3.1.4 Yeast strains

Yeast strains used in this study were *Saccharomyces cereviseae*. Three strains of dry commercial active powders were used which were Lalvin V1116 (Lallemand), Fermivin, and Fermivin PDM (DSM Food Specialities USA, Inc.).

3.1.5 Other wine production materials

1. Citric acid
2. DAP
3. KMS
4. Bentonite

3.1.6 Other wine production apparatus

1. 20- liter plastic bottles
2. Air-locked apparatuses
3. Silicone tubes
4. Filter cones

3.1.7 Other wine quality measuring devices

1. Folin-ciocalteu apparatuses
2. β-carotene bleaching apparatuses
3. Maceration apparatuses
4. pH-meters
5. Hand refractometers
6. Titratable acidity apparatuses
7. Color-readers of L*a*b* system
8. Ebulliometers
9. Sensory testing apparatuses

3.2 Methodologies

Part I: The optimal method of Krachai-Dam honey wine processing.

Wine processing was studied at the Queen Sirikit Sericulture (Chiang Mai), Hang-Dong, Chiang Mai province. Physical, chemical and pharmaceutical analysis of Krachai-Dam honey wines were studied at the Department of Biology and Chemistry, Faculty of Sciences, Chiang Mai University, whereas sensory testing was studied at the Department of Food Sciences and Technology, Faculty of Agro-Industry, Chiang Mai University, Chiang Mai province.

3.2.1. Krachai-Dam honey wine production

Experiment 1.1: Effects of Krachai-Dam cultivars used as raw materials and yeast strains on Krachai-Dam honey wine qualities.

Experimental design: 3x3 factorial in Randomized Complete Block Design (RCB), 9 treatment combinations, 3 replications (plot)/ treatment.

Factor 1: comprised 3 Krachai-Dam cultivars: Kheg-Noi#2, Nam-Juang and Rom-Klao cultivars.

Factor 2: 3 Yeast strains: Lalvin V1116, Fermivin and Fermivin PDM.

Wine production of these 9 treatment combinations was carried out according to the flow diagram in Figure 3.2 and Table 3.1.

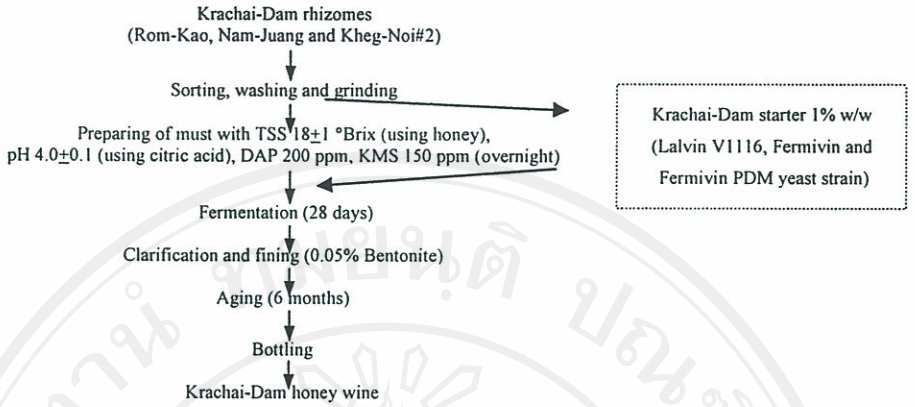

Figure 3.2 Flow diagram of Krachai-Dam honey wine production.

Table 3.1 Treatment combinations in experiment 1.1

Yeast strains	Krachai-Dam cultivars		
	Rom-Klao (Dark purple)	Nam-Juang (Purple)	Kheg-Noi#2 (Pale purple)
Lalvin V1116	Tr37	Tr43	Tr49
Fermivin	Tr38	Tr44	Tr50
Fermivin PDM	Tr39	Tr45	Tr51

Note: All treatments in experiments used a fermentation period of 28 days, a proportion of rhizomes in must of 10% w/w, Longan honey, a pH of must of 4 , and aging periods of 6 months.

Experiment 1.2: Effects of Krachai-Dam cultivar used as raw materials, honey types and proportion of rhizomes on Krachai-Dam honey wine qualities

Experimental design: 3x3x5 factorial in RCB, 45 treatment combination, 3 replications(plot)/treatment.

Factor 1: consisted of 3 Krachai-Dam cultivars; Kheg-Noi#2, Nam-Juang and Rom-Klao cultivars.

Factor 2: 3 honey types; Longan, Lychee and Sab-Suea honeys.

Factor 3: 5 proportions of rhizomes in must; 5, 7.5, 10, 12.5 and 15% w/w.

Wine production of these 45 treatment combination was carried out according to the flow diagram in Figure 3.3 and Table 3.2.

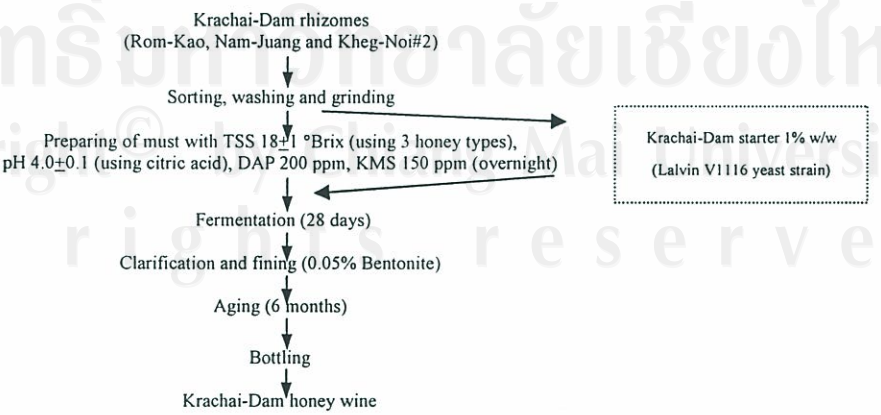

Figure 3.3 Flow diagram of Krachai-Dam honey wine production.

Table 3.2 Treatment combinations in experiment 1.2

Proportion of rhizomes in must	Type of honey	Krachai-Dam cultivars		
		Rom-Klao (Dark purple)	Nam-Juang (Purple)	Kheg-Noi#2 (Pale purple)
5% w/w	Longan	Tr55	Tr64	Tr73
	Lychee	Tr56	Tr65	Tr74
	Sab-seua	Tr57	Tr66	Tr75
7.5 % w/w	Longan	Tr58	Tr67	Tr76
	Lychee	Tr59	Tr68	Tr77
	Sab-seua	Tr60	Tr69	Tr78
10% w/w	Longan	Tr61	Tr70	Tr79
	Lychee	Tr62	Tr71	Tr80
	Sab-seua	Tr63	Tr72	Tr81
12.5% w/w	Longan	Tr163	Tr169	Tr175
	Lychee	Tr164	Tr170	Tr176
	Sab-seua	Tr165	Tr171	Tr177
15.0% w/w	Longan	Tr166	Tr172	Tr178
	Lychee	Tr167	Tr173	Tr179
	Sab-seua	Tr168	Tr174	Tr180

Note: All treatments in experiment 1 used fermentation periods of 28 days, Lalvin V1116 yeast strain, pH of must of 4 and aging periods of 6 months.

3.2.2. Krachai-Dam honey wine qualities

1 Physical, chemical and pharmaceutical qualities

1.1.Wine color in L a* b* system*

A color reader was used to measure wine colors. Within the approximate uniform color space CIELAB, two color coordinates, a* and b*, and lightness, L*, were defined. Coordinate a* represents positive values for reddish colors and negative values for the greenish colors, whereas b* represents positive values for yellowish colors and negative values for the bluish colors. L* is an approximate measurement of luminosity, which is the property according to which each color can be considered as equivalent to a member of the grey scale, between black and white, taking values within the range 0-100.

1.2 Total soluble solids (TSS)

The soluble solids of blueberry juice were dertermined using a refractrometer. Results were reported as °Brix.

1.3 pH

The pH of each sample was measured using an Accument 925 pH meter. Prior to its usage, the meter was calibrated with two buffer solutions, pH 4 and pH 7. Triplicate pH determinations were made for each sample.

1.4 Titratable acidity (TA)

For titratable acidity, 5 ml of each sample were accurately measured and poured into an Erlenmeyer flask, and 100 ml of distilled water was added to each sample. The resulting mixture was titrated, with 0.1 N NaOH to pH 8.1. Titratable acidity was calculated in terms of grams of acid (acetic acid for vinegars and citric acid for juice and wines) per 100 ml of each sample using the formula below, in which the milliequivalents weight of citric acid were 0.07(AOAC, 1990).

$$TA\% = \frac{(\text{mL of NaOH})(0.1\text{N})(\text{milliequivalent weight of acid})(100)}{\text{Sample Volume (ml)}}$$

1.5 Alcoholic percentage

Ebulliometric determination were used to measure alcoholic percentages in Krachai-Dam honey wine samples. Of each sample 50 ml were accurately measured and round into the boiling chamber. This method relied on the depression of the boiling point of water-alcohol mixtures which changed as a function of their concentration. (Patrick *et al.*, 1993).

1.6 Total phenolics (TP)

Total phenolics content (TPH) was measured by using the Folin-Ciocalteu method (Saura-Calixto, 2003). Five milliliters of each sample were placed into a 100 ml volumetric flask, and 70 ml of distilled water were added. The Folin-Ciocalteu reagent (5 ml) was added to the flask and mixed. After standing for three minutes, 2 ml of saturated Na_2CO_3 aqueous solution were also added and mixed. The content of the flask was then made up to 100 ml with distilled water. The absorbance was measured in a Perkin Elmer UV-V spectrophotometer (Perkin Elmer, Norwalk, CT) at 725 nm against a reagent that turned blank after two hours of reaction at room temperature. Gallic acid was used as a phenolic standard. A standard curve of gallic acid was generated under identical experimental conditions. A standard curve of gallic acid was generated under identical experimental conditions. Five different concentrations of gallic acid solutions were chosen to generate the standard curve. All experiments were performed in triplicate. Results were expressed as mg gallic acid equivalents (GAE) per gram of sample (mg GAE/g) or mg GAE per 100 ml of liquid sample (mg GAE/100ml).

1.7 Antioxidant index (AOI) assays

Antioxidant activity was determined by heat-induced oxidation of an aqueous emulsion system of β-carotene and linoleic acid assay. All measurements were performed was triplicate. The following is a description of methods applied (Chanwitheesuk *et al.*, 2005);

One milliliter of β-carotene (0.2 mg/ml) dissolved in chloroform was added to an erlenmeyer flask containing 0.02 ml of linoleic acid and 0.02 ml of Tween 20. The flask was covered with aluminum foil. Next, 0.1 ml of a wine product, or distilled water (control) was added to the mixture, and the mixture was taken to dryness with nitrogen. Fifty milliliters of distilled water, saturated for 15 minutes with oxygen, were then added to the flask. The resulting mixture was shaken and the absorbance was measured on a spectrophotometer (Perkin Elmer UV-V, Norwalk, CT) at 470 nm immediately (t = 0 min). The solution was then incubated in a water bath at 50 °C for 120 minutes to induce autoxidation. The absorbance of the samples were measured again at the end of the experiment (t = 120 min). The antioxidant index (AOI) was calculated as inhibition of oxidation versus control. The bleaching rate of β-carotene was determined by the difference in the spectral absorbance reading between the initial and last reading of bleaching that remained essentially linear divided by time. The antioxidant index was the ratio of the bleaching rate of the control element (system with no added test compound) to the bleaching rate when a test compound was in the system.

Statistical analysis

Data from experiment 1.1 and 1.2 were analysed by the Multiple-Factor Analysis of Variance (MANOVA) was used to analyze significant differences of the treatments studied. A p-value if less than 0.05 was considered statistically significant. The unweighted pair group method cluster analysis (UPGMA) was also used to groupe the treatment combinations by using 9 physical, chemical and pharmaceutical parameters of Krachai-Dam honey wines using a similarity index. A dendrogram was created to show the groups and subgroups of these treatment-combinations which must be selected by sensory testing to find the most optimal Krachai-Dam honey wine processing of this study.

2. Sensory evaluation

2.1 Choosing trained panelists

The tasting panel was comprised of at least 15 trained panelists who were students, staff and authorities of the Faculty of Agro-industry, Chiang Mai University. In training sessions, the panelists were trained to recognize and evaluate red wines and Krachai-Dam wines on appearance, aroma, flavor, mouth feel and after taste quality (Figure 3.4). Moreover, training was given on the descriptive terms used in this study and standards that best represented each term to be used. After these sessions, every panelist was tested on evaluating their sensory attributes. The trained panelists had to have scores of more than 60%.

2.2 Sensory testing of wine samples

Krachai-Dam honey wines were evaluated various times using a randomized complete block design (RCB), a panelist was a block (replication), more than 15 panelists (blocks)/ treatment.

Thirty to forty milliliter wine samples were rated at room temperature (~25°C) in clear and randomly coded ISO glasses. Samples were poured into wine glasses prior to the arrival of panelists. Sensory testing was conducted in isolated booths illuminated with fluorescence light in the sensory testing room at the Department of Food Sciences and Technology, Faculty of Agro-industry, Chiang Mai University. The wine appreciation chart used to evaluate Krachai-Dam honey wines was adapted from the wine appreciation chart used for grape wines of Margalit (1996). The wine attributes, which were evaluated by panelists, comprised of wine appearance, color, varietal aroma and bouquet, flavor, acidity, defects and general quality. Each attribute was weighted with a difference factor according to their important upon wine qualities. The parameters after weighing were 5 scores for wine color, 10 scores for wine appearance, acidity and defects, 15 scores for flavor, 20 scores for general quality and 30 scores for varietal aroma and bouquet. The total of wine appreciation scores was 100.

Figure 3.4 The panelists were trained to recognize and evaluate red wines and Krachai-Dam honey wines (A), and wine testing techniques which comprised look, smell, taste and aftertaste (B and C).

All statistical analyzes were performed using the one-way analysis of variance (ANOVA) was carried out to establish wine appreciation scores of each attribute and total scores to indicate the significant differences between the treatments studied.

Part II: Study on the effects of certain factors on the qualities of Krachai-Dam rhizomes used as raw materials for Krachai-Dam honey wine production.

Wine production in this part used the most optimal Krachai-Dam honey wine processing as referred to in part I. Wine production of these 45 treatment combinations was carried out according to table 3.3. The Krachai-Dam cultivar used was selected cultivar. This part consisted of 4 experiments as follows;

Experiment 2.1 Effects of plantation areas and harvesting months of raw materials on wine qualities.

Experimental design:4x5 factorial in RCB, 20 treatment combinations, 3 replications (plots) per treatment, plot sizes of 24 m^2 per replication (plot), spacing of 20x30 cm.

Factor 1: 4x4 factorial in Randomized Completely Block Designs (RCB), 16 treatments, 3 replications (plots) per treatment, plot sizes of 24 m^2 per replication (plot), spacing of 20x30 cm.

Factor 2: 5 harvesting month: 6, 7, 8, 9 and 10 months after growing which were November, December 2005, January, February and March 2006.

All treatments were undertaken at the same time in May 2005.

Experiment 2.2 Effects of plantation areas and planting months of raw materials on wine qualities.

Experimental design: 4x4 factorial in RCB, 16 treatment combinations, 3 replications (plots) per treatment, plot sizes of 24 m^2 per replication (plot), spacing of 20x30 cm.

Factor 1: comprised 4 plantation areas; Nakhonphanom (150 m asl), Phrae (200 m asl), Phurua (950 m asl) and Maechonluang (1,350 m asl).

Factor 2: 4 plantation times; May, June, July and August 2005 (every 1^{st}-4^{th} of each month).

All treatments were harvested at the same time, in January 2006.

Experiment 2.3 Effects of plantation areas and number of year crops of raw materials on wine qualities.

Experimental design: 4x2 factorial in RCB, 8 treatment combinations, 3 replications (plots) per treatment, plot sizes of 24 m^2 per replication (plot), spacing of 20x30 cm.

Factor 1: 4 plantation areas (the same as experiment 2.1).

Factor 2: 2 crop cycles: 1 crop cycle (rhizomes grown since May 2005 were harvested in January 2006) and 2 crop cycles (rhizomes grown since May 2004 were harvested in January 2006).

All treatments were harvested at the same time, in January 2006.

Experiment 2.4 Effects of (a) plantation area, (b) storage time and (c) storage method of raw materials on wine qualities.

Experimental design: 4x3x3 factorial in RCB, 36 treatments, 3 replications (plots) per treatment, plot sizes of 24 m^2 per replication (plot), spacing of 20x30 cm.

Factor 1: 4 plantation areas (as the same of experiment 2.1)

Factor 2: 3 storage time; 0, 3 and 6 months after harvested

Factor 3: 3 storage methods; layout on the ground, keeping in a net bag and cold storage with $13\pm1^{\circ}C$ and a relative humidity of 65%

All treatments were carried out in May 2005 and harvested in January 2006.

Table 3.3: Treatment combinations of Krachai-Dam honey wines processing in crop season of 2005-2006 (part II).

Plantating monts		Plantation areas			
		Nakhonphanom (150 m asl)	Phrae (200 m asl)	Phurua (950 m asl)	Maechonluang (1,350 m asl)
May		**Tr.2-1**	**Tr.2-2**	**Tr.2-3**	**Tr.2-4**
June		Tr.2-5	Tr.2-6	Tr.2-7	Tr.2-8
July		Tr.2-9	Tr.2-10	Tr.2-11	Tr.2-12
August		Tr.2-13	Tr.2-14	Tr.2-15	Tr.2-16
Harvesting months					
November		Tr.2-17	Tr.2-18	Tr.2-19	Tr.2-20
December		Tr.2-21	Tr.2-22	2 Tr.2-3	Tr.2-24
January		**Tr.2-1**	**Tr.2-2**	**Tr.2-3**	**Tr.2-4**
February		Tr.2-25	Tr.2-26	Tr.2-27	Tr.2-28
March		Tr.2-29	Tr.2-30	Tr.2-31	Tr.2-32
Number of year crops					
1-year crop		**Tr.2-1**	**Tr.2-2**	**Tr.2-3**	**Tr.2-4**
2-year crop		Tr.2-33	Tr.2-34	Tr.2-35	Tr.2-36
Storage times	Stroge methods				
0 month	Layout	**Tr.2-1**	**Tr.2-2**	**Tr.2-3**	**Tr.2-4**
	Net bag	**Tr.2-1**	**Tr.2-2**	**Tr.2-3**	**Tr.2-4**
	Cold storage	**Tr.2-1**	**Tr.2-2**	**Tr.2-3**	**Tr.2-4**
3 months	Layout	Tr.2-37	Tr.2-38	Tr.2-39	Tr.2-40
	Net bag	Tr.2-41	Tr.2-42	Tr.2-43	Tr.2-44
	Cold storage	Tr.2-45	Tr.2-46	Tr.2-47	Tr.2-48
6 months	Layout	Tr.2-49	Tr.2-50	Tr.2-51	Tr.2-52
	Net bag	Tr.2-53	Tr.2-54	Tr.2-55	Tr.2-56
	Cold storage	Tr.2-57	Tr.2-58	Tr.2-59	Tr.2-60

The rhizomes of experiment 2.1 to 2.4 were used as raw materials for Krachai-Dam honey wine production by using the most optimal processing from part 1. The physical, chemical and pharmaceutical qualities of Krachai-Dam honey wines were analyzed in the same way as in part 1. The sensory testing by trained panelists was also studied by grouping of treatments in experiment 2.1 to 2.4 according to 9 physical, chemical and pharmaceutical parameters, using UPGMA.

Part III: Factors that influenced Krachai-Dam rhizomes used as raw materials for Krachai-Dam

The agricultural characteristics of Krachai-Dam were studied as followed;

1. A study on quantitative yields of Krachai-Dam focusing on production per rai, weight of rhizomes in crop year of 2005-2006, comparing among the 4 plantation areas studied.

2. A study on qualitative yields of Krachai-Dam focusing on internal rhizomes color, total phenolics and antioxidant indexes of ethanolic extracts from Krachai-Dam rhizomes.

3. A study on soil types, pH, organic matters and major elements (N, P, K, Ca and Mg) in planting soils, comparing between the 4 plantation areas in the crop year of 2005-2006

4. Meteorological data of 4 plantation areas in the crop year 2004-2006.

Krachai-Dam rhizomes, which were used as raw materials for Krachai-Dam honey wine processing in experiments 2.1 to 2.4 in part II were also studied with

regard to their qualitative yields. The experiments in part III had 4 experiments as followed;

Figure 3.5 The Krachai-Dam plants which were grown in the field at Phrae area (200 m asl) in crop season of 2005-2006.

Experiment 3.1 Effects of plantation areas and harvesting months on qualitative yields of Krachai-Dam.

The same as experiment 2.1.

Experiment 3.2 Effects of plantation area and planting month on qualitative yields of Krachai-Dam.

The same as experiment 2.2.

Experiment 3.3 Effects of plantation areas and number of year crops on qualitative yields of Krachai-Dam.

The same as experiment 2.3.

Experiment 3.4 Effects of (a) plantation areas, (b) storage times and (c) storage methods on qualitative yields of Krachai-Dam

The same as experiment 2.4.

Data from experiments 3.1 to 3.4 were collected and compared with the qualitative yields of Krachai-Dam rhizomes in each treatment. The internal rhizomes color, total phenolics and antioxidant indexes of extracts were analysed. MANOVA was performed to compare the treatment combinations..

Part IV: To Summarise and to draft the Good Agricultural Practices (GAP) of Krachai-Dam for Krachai-Dam honey wines purposes.

1. Summarizing all the data in this study to show the most optimal Krachai-Dam honey wine process and factors influencing Krachai-Dam rhizomes used as raw materials for wine processing, including wine qualities.
2. Drafting the Good Agricultural Practices (GAP) of Krachai-Dam growing for Krachai-Dam honey wine production.

3.3 Time for study

Three years, from April 2004 to March 2007.