CHAPTER 3

EXPERIMENTAL

3.1 Samples

1. Fresh lime peel and pressed lime peel of Namhom lime (*Citrus aurantifolia* Swingle) collected on October 25th 2004 from Lung Nuad garden, Ban Tauhai, Tambol Sansai, Amphur Sansai, Chiang Mai, Thailand.
2. Banana and brown sugar from Muang Mai market, Amphur Muang, Chiang Mai, Thailand.
3. Old bio-fermented products from fermentation for 3 years of fruits: brown sugar: water in formula 3: 1: 10 (w/w/w).

3.2 Standards and Reagents

1. (+)-Limonene stabilized 97% Lot: A019082201 C.A.S.: 5989-27-5, ACROS ORGANICS Co.Ltd. U.S.A.
2. β- Myrcene 90% Lot: 062K0881EC No. 204-622-5, SIGMA® Co.Ltd. U.S.A.
3. *(1S)*-(-)-α -Pinene 219433 383 Fabrik CH-9470 Buchs, Fluka AG Chem Co.Ltd. Switzerland.
4. α-Terpineol 99% mixture of isomers C.A.S.: 98-55-5/361610250, ACROS ORGANICS Co.Ltd. USA.
5. Acetone AR Code No. A 3501 Batch No. 02.02.1006, import by Labscan Asia Co.Ltd. Thailand.
6. Ethanol 95%, Liquor Distiller Organization Excise Department Thailand.
7. Ethyl acetate AR Code no. A3511 Batch no. 03060106, import by Labscan Asia Co.Ltd.Thailand.

8. Methanol 9073-03 Lot. K12689 C.A.S. No.: 67-56-1, J.T.Baker Co.Ltd., U.S.A.
9. Silica gel 60 GF_{254} Lot. TA1168830 405, Merck Co.Ltd. Germany.
10. Sodium sulphate anhydrous GR for analysis 1.06649.1000 Lot A565749 437, Merck Co.Ltd. Germany.
11. Sulphuric acid 96% Code 410306 Lot: 2489L 100 C.A.S.No.:7664-93-9 No.016-020-00-8, FARMITALIA CARLO ERBA S.p.A. Italy.
12. Toluene GR 8325.2500 Lot 038 k14591625, E. Merck Co.Ltd. Germany.
13. Vanillin 1346140 Product no.30569, The British Drug House Ltd., Pool, England.

3.3 Apparatus and Instruments

1. Analytical balance, Mettler Model AE 200, Mettler Co.Ltd. Switzerland.
2. Clevenger-type apparatus 5 L.
3. Capillary column, Alltech 15897 AT-1MS (100% dimethylpolysiloxane) 30 m x 0.25 mm ID x 0.25 μm film thickness for GC/MS of faculty of science, Chiang Mai University.
4. Gas Chromatograph (GC 6890 Agilent Technologies) Mass Spectrometer (MSD 5973 Hewlett Packard) of faculty of science, Chiang Mai University.
5. Capillary column, DB-1 (100% dimethylpolysiloxane) Length 30 m diameter 0. 53 mm (Megabore®) film thickness 1.5 μm P/N 125-1032 Serial # 4378726 Catalog # 125-1032, J & W Scientific Co.Ltd. USA.
6. Gas Chromatograph P/N 9 Model GC-14BPFE No. C10733305495 SA, Shimadzu Corporation, Japan.
7. Micro blue tip 1000 μL T 1000BR, Axygen Scientific, Inc. USA.
8. Microtip 0.5-10 μL T 300R, Axygen Scientific, Inc. USA.
9. Micro-Haematocrit tubes, Soda lime glass 87402 MH412GB, MODULOHM A/S Co.Ltd. Denmark.
10. Microliter ™ syringe 5.00 μL, Halmilton Co.Ltd., Switzerland.
11. Micropipettes 0.5-10 μL 4022375 BIS CO 20 and 100-1000 μL 4049111 BIS CO 26, Biohit PLC. Helsinki, Finland.
12. Polarimeter tube, Type A OT 100 (100mm) Parts no. RE 6705 No.931642, Japan.

13. Polarimeter, Type A JE-BB Model POLAX-L Serial No.950113, ATAGO Co.Ltd. Japan.
14. Pycnometer, 10.0 mL.
15. Refractometer, Model 3T Serial No.921, ATAGO Co.Ltd. Japan.
16. Scintillation vials with screw caps 20 mL 986541, Wheaton Science Products U.S.A.
17. Spraying flask 100 mL, SHOTT-DURAN Germany.
18. Thermometer, 0-50°C.
19. TLC aluminum sheets 20x20 cm coated with Silica gel 60 F_{254} 1.05554.0001 Lot OB476400, Merck Co.Ltd., Germany.
20. TLC tanks DESAGA 120100, Heidenburg, Germany.
21. Tube with screw cap 16x100 mm No.9825, PYREX®, U.S.A.
22. UV chromatographic viewer Model CHROMATO-VUE®C-70G 5491UV UVP, Inc., U.S.A.

3.4 Methods

3.4.1. Preparation of Bio-Fermented Products (BFP)

1. Blend brown sugar 10 kg with water 100 kg in plastic tank.
2. Add banana 30 kg and old bio-fermented products 10 kg then close the lid of tank.
3. Left 3 months for fermentation and release excess gas from bottle of sample during fermentation after that filter bio-fermented products.
4. Fill in clean 5-liter PET bottle.

3.4.2. Fermentation of Pressed Lime Peel with Bio-Fermented Products

1. Cut lime peel 2 kg in small size and fill in PET bottle.
2. Add bio-fermented products 2 kg in PET bottle then close the lid.
3. Left sample to ferment for 15, 30, 45, 60 and 90 days before hydro distillation.
4. Release excess gas from bottle of sample during fermentation.

3.4.3. Microbiology Test of Bio-Fermented Products

Fill bio-fermented products (3.4.1.) in 100 mL sterilized brown bottle and suddenly sent to analyze at Clinical Services Center, Faculty of Associated Medical Sciences, Chiang Mai University, Chiang Mai, Thailand.

3.4.4. Hydro Distillation of Volatile Oil

3.4.4.1. Hydro Distillation of Volatile Oil from Bio-Fermented Products

1. Pour bio-fermented products (3.4.1.) 2 L in round bottom flask 5 L.
2. Set and open Clevenger-type apparatus.
3. Adjust suitable temperature of heating mantle about 100°C.
4. Collect volatile oil in tube screw cap and add enough sodium sulphate anhydrous for dehydration of volatile oil.
5. Measure volume of volatile oil, fill in brown bottle and store over silica gel in a suitable box then keep in refrigerator.

3.4.4.2. Hydro Distillation of Distilled Lime Oil from fresh lime peel or pressed lime peel

1. Weigh fresh lime peel or pressed lime peel and record
2. Pour sample in round bottom flask 5 L.
3. Add water 2 L.
4. Set and open Clevenger-type apparatus.
5. Adjust suitable temperature of heating mantle about 100°C.
6. Collect volatile oil in tube screw cap and add enough sodium sulphate anhydrous for dehydration of volatile oil.
7. Measure volume of volatile oil, fill in brown bottle and store over silica gel in a suitable box then keep in refrigerator.

3.4.4.3. Hydro Distillation of Distilled Lime Oil from Sample

1. Measure pH of sample and pour sample in round bottom flask 5 L.
2. Rinse with water 250 mL and pour in round bottom flask 5 L.
3. Set and open Clevenger-type apparatus.
4. Adjust suitable temperature of heating mantle about 100°C.
5. Collect volatile oil in tube screw cap and add enough sodium sulphate anhydrous for dehydration of volatile oil.
6. Measure volume of volatile oil, fill in brown bottle and store over silica gel in a suitable box then keep in refrigerator.

3.4.5. Physicochemical Properties Test of Distilled Lime Oil

3.4.5.1. Appearance

Observe appearance of distilled lime oil, for example status, color and odor and record.

3.4.5.2. Determination of Refractive index (25°C) by USP 27<831> p. 2385

1. Switch on digital thermometer button.
2. Switch on lamp button for sample no.1and for scale of refractometer.
3. Lift up the illuminating prism and clean the illuminating prism and the measuring prism with 95% ethanol and dry with tissue.
4. Use Pasteur pipette to drop distilled lime oil on the measuring prism.
5. Lift down illuminating prism.
6. Look through eyes piece while turning color compensator knob until the sharp borderline is present in field of vision.
7. Turning measurement scale knob until the borderline reach the cross-section of the cross hair.
8. Record refractive index value and temperature.
9. Clean the measuring prism with 95% ethanol and dry with tissue.

10. Carry out at least 3 measurements and determine the mean value.
11. Check frequently the temperature control and cleanliness of the instrument by determining the refractive index of distilled water, which is 1.3325 at 25°C.

3.4.5.3. Determination of Specific Gravity (25°C) by USP 27<841> p. 2385

1. Select a scrupulously clean, dry pycnometer that previously has been calibrated by determining its weight and the weight of recently deionized water contained in it at 25°C for 5 times and determine the mean value.
2. Weigh the clean, dry pycnometer.
3. Adjust the temperature of distilled lime oil to about 23-24°C.
4. Fill the pycnometer with distilled lime oil.
5. Adjust the temperature of the filled pycnometer to 25°C, remove any excess of distilled lime oil and weigh.
6. Subtract the tare weight of the pycnometer from the filled weight of the pycnometer.
7. Carry out at least 3 measurements and determine the mean value.
8. The specific gravity is the quotient obtained by the follow equation:

$$\text{Specific gravity (25°C)} = \frac{\text{Weight of lime oil contained in the pycnometer (25°C)}}{\text{Weight of water contained in the pycnometer (25°C)}}$$

3.4.5.4. Determination of the Optical Rotation (25°C) by USP 27<781> p. 2234

1. Switch on the light source about 10-15 min.
2. Set the zero point of the polarimeter with the dry empty polarimeter tube.
3. Fill the polarimeter tube with 10% distilled lime oil in 95% ethanol previously brought to a temperature of 25 ± 1°C; ensure the absence of air bubbles.
4. Place the tube in the polarimeter, adjust button up or down while looking through the color until dull color is bright yellow equal both left and right side.

5. Read the optical rotation value of the oil on the scale of the instrument then record temperature.
6. Carry out at least 3 measurements and determine the mean value.

3.4.6. Chemical Components of Distilled Lime Oil

3.4.6.1. Determination of Chemical Components of Distilled Lime Oil by Thin Layer Chromatography (TLC) by USP 27<621> p. 2274

(A) Preparation TLC Plate

1. Dry the 20 x 20cm glass plates at 60°C for 15-20 min and clean it with acetone.
2. Arrange the plates on the aligning tray.
3. Mix 1 part of silica gel 60 GF_{254} with 2 part of water by shaking vigorously for 30 seconds in a glass-stopper conical flask, and transfer the slurry to the spreader that fixed thickness 0.25 mm.
4. Draw the spreader smoothly over the plates toward the raised end of the aligning tray.
5. Allow the plates to remain undisturbed for 5 min, then transfer the square plates to the storage rack, and dry at 105°C for 30 min.
6. When the plates are dry, allow them to cool to room temperature and store them over silica gel in a suitable chamber.

(B) Preparation of Vanillin–Sulphuric Acid Spraying Reagent [51]

Solution I: 5% (v/v) Sulphuric acid in 95% ethanol

Pour conc. sulphuric acid 5.0 mL in spraying flask and adjust to 100 mL with 95% ethanol.

Solution II: 1% (w/v) Vanillin in 95% ethanol

Weigh 1.0 g of vanillin in spraying flask and adjust to 100 mL with 95% ethanol.

(C) Preparation of Developing Solvents

1. Mix toluene 94 mL and ethyl acetate 6 mL in beaker.
2. Place 2-filters paper into TLC tank and add the solvent 100 mL.
3. Seal the cover to the top of the tank and allow the solvent system to equilibrate for 1 hour before TLC analysis.

(D) Monograph TLC Analysis

1. Apply the standard and distilled lime oil, as directed in the individual monograph, at point 2 cm apart and 2 cm from the lower edge of the plate, and allow drying.
2. Place a mark 10 to 15 cm above the spot point, introduce the plate into TLC tank and do not allow the spot points to be immersed.
3. Put the cover in place, and main the system until the solvent ascends to a point 10 to 15 cm above the initial spots, this usually requires about 15 minutes to 1 hour.
4. Remove the plate from TLC tank, mark the solvent front and air-dry the plate.
5. Observe first under shot-wavelength UV light (254 nm) and then under long-wavelength UV light (365 nm).
6. Measure and record the distance of each spot the wavelength under which it was observed.
7. In hood, spray the spots homogeneously with the solution I about 10-15 mL and then with the solution II about 5-10 mL after that allow the plate to dry in oven 10-15 minutes.
8. Take a photograph, observe, measure and record the distance and color of each spot.
9. Determine the R_f values of each substance and compare the test chromatogram with the standard chromatogram.

3.4.6.1. Determination of Chemical Components of Distilled Lime Oil by Gas Chromatography (GC)

(A) Preparation of 1% (v/v) Distilled Lime Oil in Methanol

1. Pipette distilled lime oil 10 μL and methanol 990 μL into vial 10 mL.
2. Close the lid and shake gently.
3. Wrap vial with aluminum foil and keep in refrigerator.

(B) Preparation of 0.5% (v/v) Standard in Methanol

1. Pipette standard 5 μL and methanol 995 μL into vial 10 mL.
2. Close the lid and shake gently.
3. Wrap vial with aluminium foil and keep in refrigerator.

(C) Operation of GC

1. Connect capillary column to injection port and detector port.
2. Open N_2 gas at required flow rate.
3. Switch on GC power and CBM power, wait for CBM online then switch on power guard.
4. Turn on computer power and monitor then click the program GLASS-GC 10 > Real Time Analysis.
5. Open method file and click to load set file then set temperature of column oven, injection port, detector and stop time.
6. Click system on >GC monitor and wait temperature of injector to reach 120°C.
7. Open H_2 gas and air about 50 kPa, press AIR 2 button for release some O_2 while ignite flame at channel 2.
8. Check the set temperatures from GC monitor then press log in (report on) after bluish ready word was shown.
9. Use cleaned microsyringe introduced distilled lime oil 1 μL to injection port then press the start switch on.
10. After separation of substance was finished, GC chromatogram is shown.
11. Turn printer on, check slope from file print before print and copy GC chromatogram to diskette.

12. When ready word is blue, continue to inject the next distilled lime oil and standard. (Determine each component by external standard analysis-not do)
13. After GC analysis was finished, close flow control, H_2 gas and air.
14. Open method file>cool.met to decrease temperature of column oven, injection port and detector to 25°C.
15. Switch off computer, power guard, CBM and GC power. Close flow control and N_2 gas.

(D) GC conditions

Some research studied constitutes of distilled lime oil from various pressed lime that obtained by hydro distillation. The conditions of GC were following [52].

Capillary column	DB-5, 30m x 0.32mm ID x 0.25μm film thickness
Carrier gas	Helium
Pressure of carrier gas in column	50 psi
Sample concentration	10 % (v/v) in absolute ethanol
Volume of sample	1μL
Injection port	Split 20:1, 230°C
Detector	FID, 230°C
Column temperature	70°C (5 min)--4°C /min--220°C /min (5 min)
Stop time	47.5 min

3.4.6.3. Determination of Major Components of Distilled Lime Oil by Gas Chromatography/Mass Spectrometry (GC/MS)

Send to Science and Technology Service Center, 30th year Science Building, Faculty of Science, Chiang Mai University, Chiang Mai,Thailand.

(A) GC/MS conditions

Some research studied compositions of distilled lime oil from various pressed lime that obtained by hydro distillation. The conditions of GC/MS were following [52].

Capillary column	DB-1, 30m x 0.25mm ID x 0.25μm film thickness
Carrier gas	Helium
Sample concentration	10 % (v/v) in absolute ethanol
Volume of sample	0.05 μL
Inlet	Split 20:1, 250°C
Column temperature	35°C (3 min)--4°C /min→225°C /min (10 min)
Stop time	35.5 min
EI	70 eV

3.4.6.3. Determination of α-terpineol of Distilled Lime Oil by Gas Chromatography (Internal Standardization)

The conditions of GC were the same as 3.4.6.1. Send to Science and Technology Service Center, 30th year Science Building, Faculty of Science, Chiang Mai University, Chiang Mai, Thailand.