CHAPTER 4

RESULTS AND DISCUSSION

4.1 Microbiology Test of Bio-Fermented Products

It was found that the microorganisms from bio-fermented products were general natural microorganisms not the pathogens and total counts were not much about 4,900 CFU/ml. Some types of microorganisms were shown in Table 4.1.

Table 4.1 Microbiological test of bio-fermented products

Type of microorganisms	Count, CFU/ml
Yeast: *Candida Krusei*	1,000
Yeast: *Saccharomyces*	600
Micrococcus	600
Nonfermentative gram-negative bacilli	1,600

4.1.1 Amount of Volatile Oil of Bio-Fermented Products by Hydro Distillation

By hydro distillation, it was found that there was not the volatile oil from bio- fermented products that prior prepared for sample fermentation process.

4.2 Hydro Distillation of Distilled Lime Oil from Sample

Oil of pressed peel of lime called Namhom was 0.15 % and colorless while some research oil from pressed peel of lime called Namhomtoolklao was 0.18 % that

obtained from hydro distillation for 5 hours and color was pale yellow [52] because of distillation for long time that effected on their quality. So this method used only an hour for distillation through all samples. The results were shown in Table 4.2 and Figure 4.1.

Amounts of distilled lime oil after pressed peel was lower than before pressing. But it was presented in Table 4.2 that amounts of distilled lime oil from sample after fermentation were increasing and distilled lime oil was changed in new odor. This method used shot time by hydro distillation for 1 hour so all distilled lime oil was colorless but still changed in odor after fermentation because pH of sample was high acidic solution and heat [53]. Due to this experimental did not add any flavor microorganisms or enzymes to produce or convert aroma. So the most result in changes may be come from reaction in acidic solution during fermentation and the walls of oil cells was broken down and released essential oil during soaking in high acidic solution for a long time.

Table 4.2 Amount of distilled lime oil, appearance and pH value of sample before hydro distillation

Fermented day	% Yield, v/w	Appearance	pH
Fresh peel	0.31	colorless, fresh citrus, sweet	-
Pressed peel	0.15	colorless, fresh citrus, sweet	-
15	0.11	colorless, fresh citrus	3
30	0.16	colorless, fresh citrus	2.9
45	0.17	colorless, fresh citrus	2.9
60	0.19	colorless, fresh citrus	2.9
90	0.21	colorless, fresh citrus	2.9

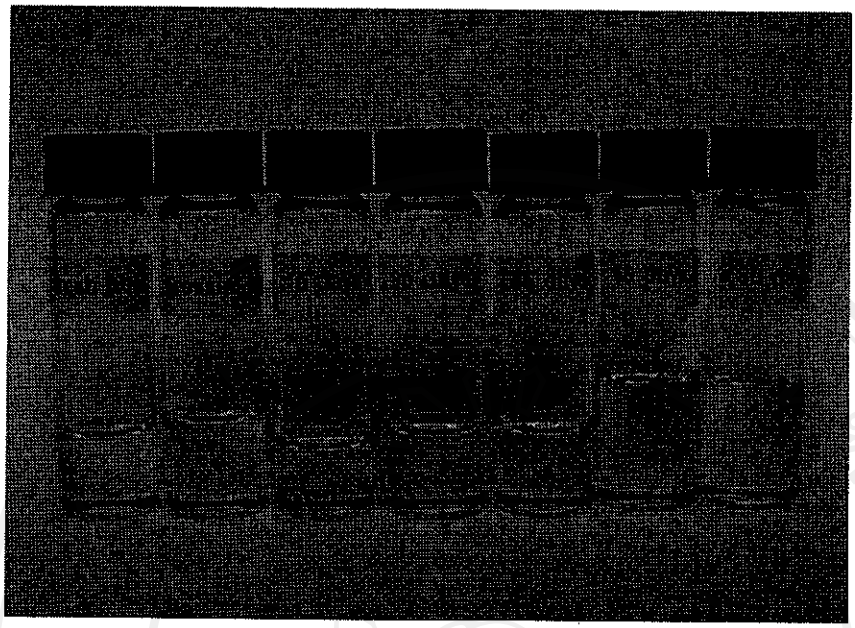


Figure 4.1 Distilled lime oil from fresh peel, pressed peel, fermented Peel

4.3 Physical Properties of Distilled Lime Oil

When compared with distilled lime oil from pressed peel before fermentation and fresh peel, it was found that refractive index (25°C) and specific gravity (25°C) of them from fermented peel were not much different.

Optical rotation was decreased after fermentation and distillation, it was shown that amount of most components of oil were changed to more laevorotatory. Physical properties of distilled lime oil were shown in Table 4.3.

From study of physic properties of distilled lime oil of pressed peel of lime called Namhomtoolklao, it was found that refractive index (20°C) was 1.4759, specific gravity (20°C) was 0.8715 and optical rotation (20°C) was 37.25 [52] that the results were close to other research in lime oil. It was the same as this research result except optical rotation was much different from other research in lime oil but close to lemon oil which the range of optical rotation (+57° to +65.6°) was higher than lime oil (+34° to +47°).

Table 4.3 Physical properties of distilled lime oil from sample before and after fermentation

Fermented day	Refractive index (25 °C)	Specific gravity (25 °C)	Optical rotation* (25 °C)
Fresh peel	1.4711	0.8487	5.95°
Pressed peel	1.4705	0.8445	6.63°
15	1.4707	0.8383	6.28°
30	1.4710	0.8372	6.25°
45	1.4712	0.8454	6.25°
60	1.4711	0.8436	6.25°
90	1.4699	0.8412	6.05°

* Optical rotation from 10% distilled lime oil in 95% ethanol

4.4 Chemical Components of Distilled Lime Oil

4.1 Determination of Chemical Components of Distilled Lime Oil by TLC Analysis

Results of TLC analysis of distilled lime oil before and after fermentation were shown in Figures 4.2-4.3 and Tables 4.4-4.5 (Tables 4.11-4.13 see in Appendix A). It was presented that chemical components of distilled lime oil were changed after fermentation that could clearly observed at 45-60 days (substance no.6-7). It was shown in Table 4.4. The violet colors under UV light at point no.2, which disappear at 15 and 30 day, have appeared again after 45-day fermentation and the colors after spraying were darker again. When compared fermented lime oil with lemon oil, fresh peel lime oil and unfermented pressed peel lime oil from Table 4.5, it was found that there were different chemical component.

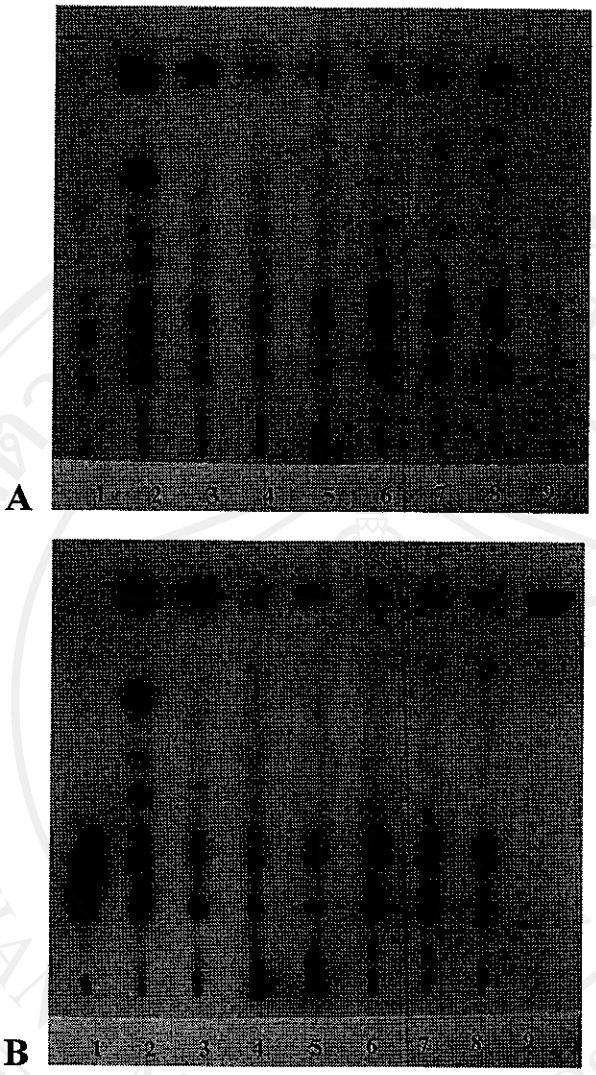


Figure 4.2 TLC chromatograms of distilled lime oil and standards.
DVS; toluene: ethyl acetate (96: 4, v/v) and; DA; Vanillin–Sulphuric Acid spraying reagent

1= (A) (1S)-(-)-α-pinene (B) α-terpineol
2= fresh peel lime oil
3= pressed peel lime oil
4= 15 day fermented peel lime oil
5= 30 day fermented peel lime oil
6= 45 day fermented peel lime oil
7= 60 day fermented peel lime oil
8= 90 day fermented peel lime oil
9= (A) (+)-limonene (B) β-myrcene

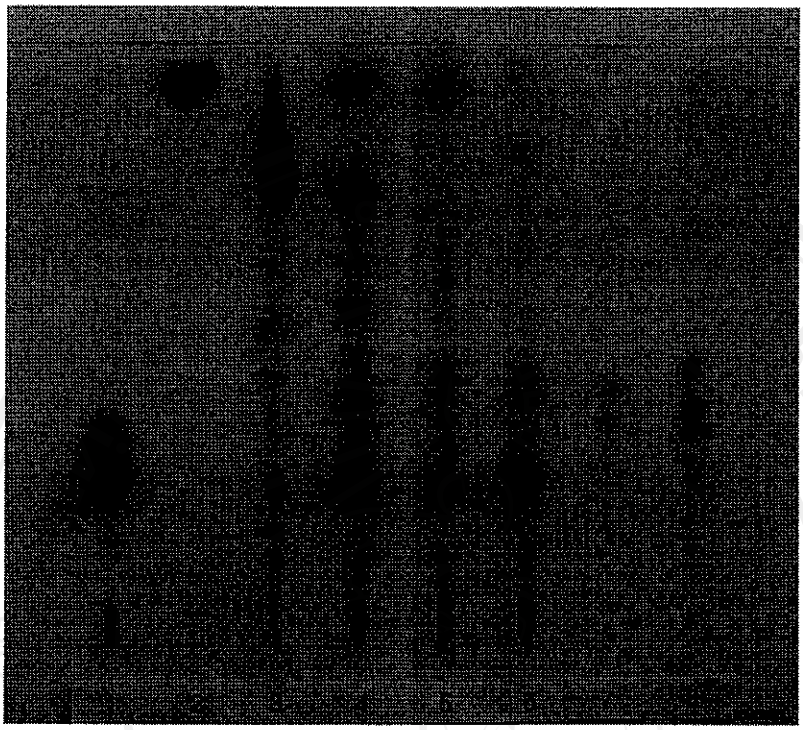


Figure 4.3 TLC chromatograms of lemon Oil , distilled lime oil before and after fermentation and standards

DVS; toluene: ethyl acetate (96: 4, v/v) and DA; Vanillin–Sulphuric Acid spraying reagent

1= α-terpineol

2= β-myrcene

3= lemon oil

4= fresh peel lime oil

5= pressed peel lime oil

6= 45 day fermented peel lime oil

7= (+)-limonene

8= (1S)-(-)-α-pinene

Table 4.4 Some interested points of R_f values from TLC chromatograms of Figure 4.2-4.3

Substance	Spot no.	Color under UV light at		Color after use spraying reagent	R_f
		254 nm	365 nm		
1.(1S)-(-)-α-pinene	4	dull	absent	dark grey	0.26
1. α-terpineol	1	greenish yellow	absent	red brown,dark blue rim	0.32
2. fresh peel lime oil	2	greenish yellow	bright blue	violet blue	0.26
	3	dull	absent	dark grey	0.32
	5	dull	absent	bluish violet	0.45
	7	dull	absent	violet grey	0.53
	9	greenish yellow	violet	violet	0.72
	10	dull	absent	dark violet	0.91
3. pressed peel lime oil	2	greenish yellow	violet	violet blue	0.26
	3	dull	absent	dark grey	0.31
	10	dull	absent	pale violet	0.58
	14	dull	absent	violet	0.91
4. lime oil 15 days	2	greenish yellow	absent	violet blue	0.26
	3	dull	absent	darkgrey	0.31
	10	dull	absent	pale violet	0.60
	14	dull	absent	pale violet	0.92
5. lime oil 30 days	2	greenish yellow	absent	violet blue	0.26
	3	dull	absent	dark grey	0.31
	9	dull	absent	pale violet	0.60
	13	dull	absent	pale violet	0.92
6. lime oil 45 days	2	greenish yellow	violet	violet blue	0.26
	3	dull	absent	dark grey	0.32
	9	dull	absent	pale violet	0.60

Substance	Spot no.	Color under UV light at 254 nm	Color under UV light at 365 nm	Color after use spraying reagent	R_f
	13	dull	absent	pale violet	0.92
7. lime oil 60 days	2	greenish yellow	violet	violet blue	0.26
	3	dull	absent	dark grey	0.32
	9	dull	absent	pale violet	0.60
	13	dull	absent	pale violet	0.92
8. lime oil 90 days	2	greenish yellow	violet	violet blue	0.26
	3	dull	absent	dark grey	0.32
	9	dull	absent	pale violet	0.60
	13	dull	absent	pale violet	0.92
9. (+)-limonene	5	dull	absent	violet	0.59
9.β-myrcene	1	dull	absent	reddish violet	0.93

Table 4.5 Some interested points of R_f value of TLC chromatogram from Figure 4.4

Substance	Spot no.	Color under UV light at 254 nm	Color under UV light at 365 nm	Color after use spraying reagent	R_f
1. α-terpineol	1	greenish yellow	absent	reddish brown, dark blue rim	0.36
2. β-myrcene	1	dull	absent	reddish violet	0.93
3. lemon oil	3	greenish yellow	absent	violet	0.26
	4	greenish yellow	violet	pale grey	0.39
	5	greenish yellow	bright	bluish pale grey	0.44
	6	dull	absent	pale grey	0.52

Substance	Spot no.	Color under UV light at 254 nm	Color under UV light at 365 nm	Color after use spraying reagent	R_f
	7	dull	absent	dark red, violet blue rim	0.77
4. fresh peel lime oil	3	dull	absent	bluish violet	0.30
	4	greenish yellow	absent	light blue	0.32
	5	greenish yellow	bright blue	pale grey	0.35
	9	dull	absent	bluish violet	0.56
	10	dull	absent	violet light blue	0.61
	13	dull	absent	violet	0.93
5. pressed peel lime oil	3	greenish yellow	pale violet	light bluish grey	0.34
	9	dull	absent	light blue	0.68
	11	dull	absent	violet	0.93
6. 45 day fermented peel lime oil	3	dull	absent	violet	0.34
	8	dull	absent	light blue	0.68
	10	dull	absent	violet	0.93
7. (+)-limonene	1	greenish yellow	absent	light blue	0.33
	5	greenish yellow	absent	pale violet	0.67
8. (1S)-(-)-α-pinene	4	dull	absent	grey	0.36

4.2 Determination of Chemical Components of Distilled Lime Oil by GC Analysis

4.2.1 Optimum condition of GC

Capillary column	DB-1, 30m x 0.53mm ID x 1.5μm film thickness
Carrier gas	Nitrogen 40 mL/min
Pressure of carrier gas in column	50 kPa
Sample concentration	1% (v/v) in methanol
Volume of sample	1μL
Injection port	230°C
Detector	250°C
Column temperature	70°C (10 min)--2°C /min→80°C--7°C /min→220°C /min (5 min)
Stop time	40 min

Results were shown in Figures 4.4-4.8. GC chromatograms of standards, α-terpineol, β-myrcene, (+)-limonene and (1S)-(-)-α-pinene were shown in Figures 4.14-4.17 (Appendix B). It was clarified that some changes of chemical components could be started to observe after fermentation at 45 day. But this method needs to use standards to identify the kind of each component of essential oil. Because of insufficient standard it should be insisted the results by GC/MS analysis.

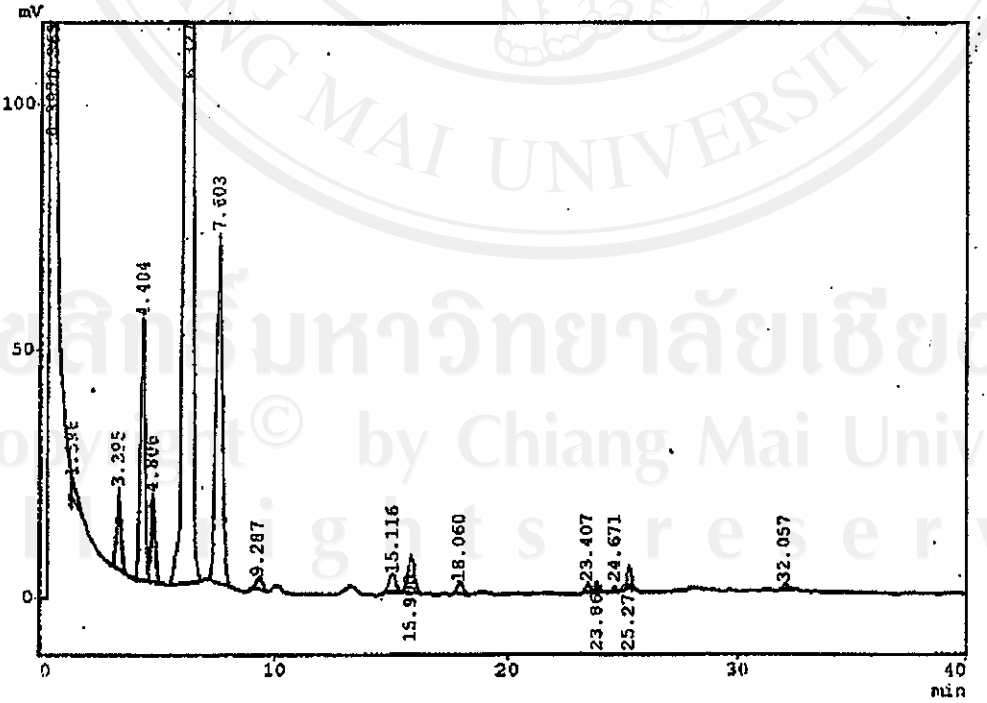


Figure 4.4 GC chromatogram of distilled lime oil from fresh peel

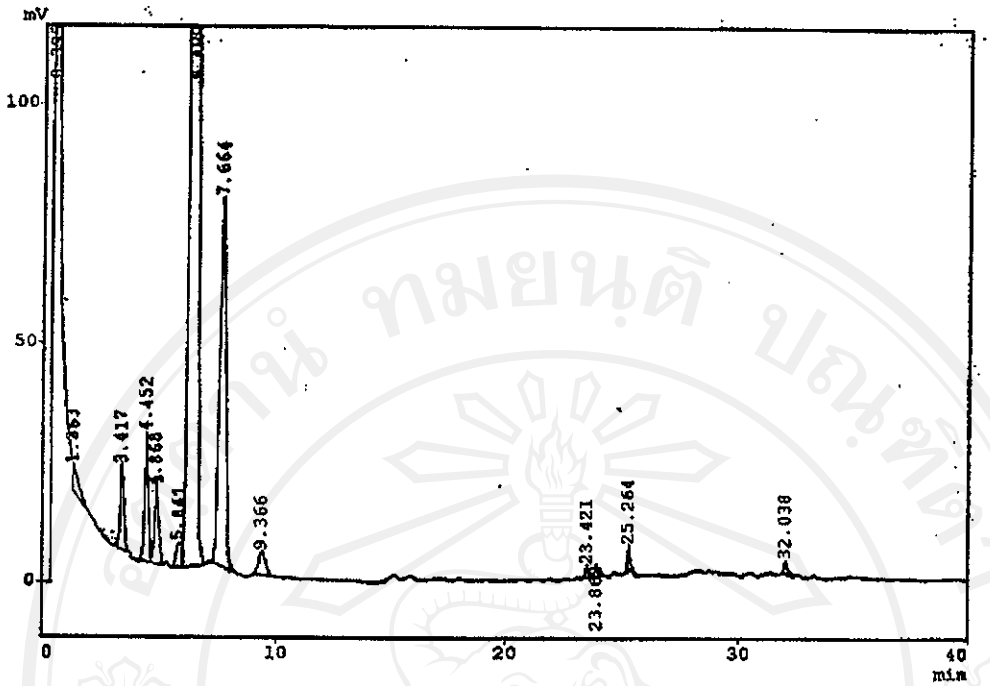


Figure 4.5 GC chromatogram of distilled lime oil from pressed peel

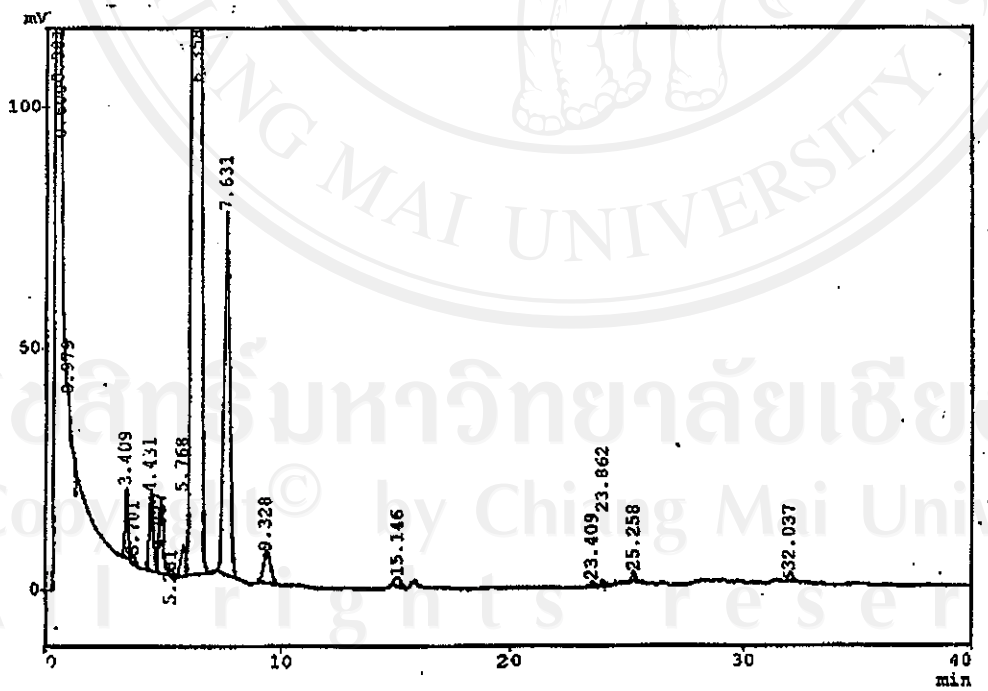


Figure 4.6 GC chromatogram of distilled lime oil from 15 day fermented peel

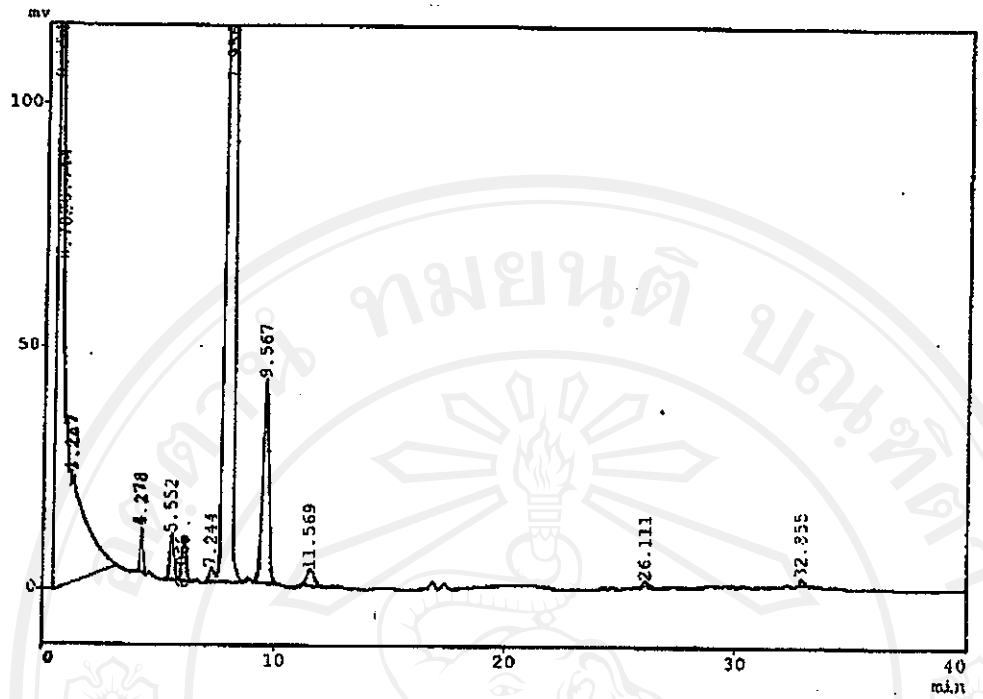


Figure 4.7 GC chromatogram of distilled lime oil from 30 day fermented peel

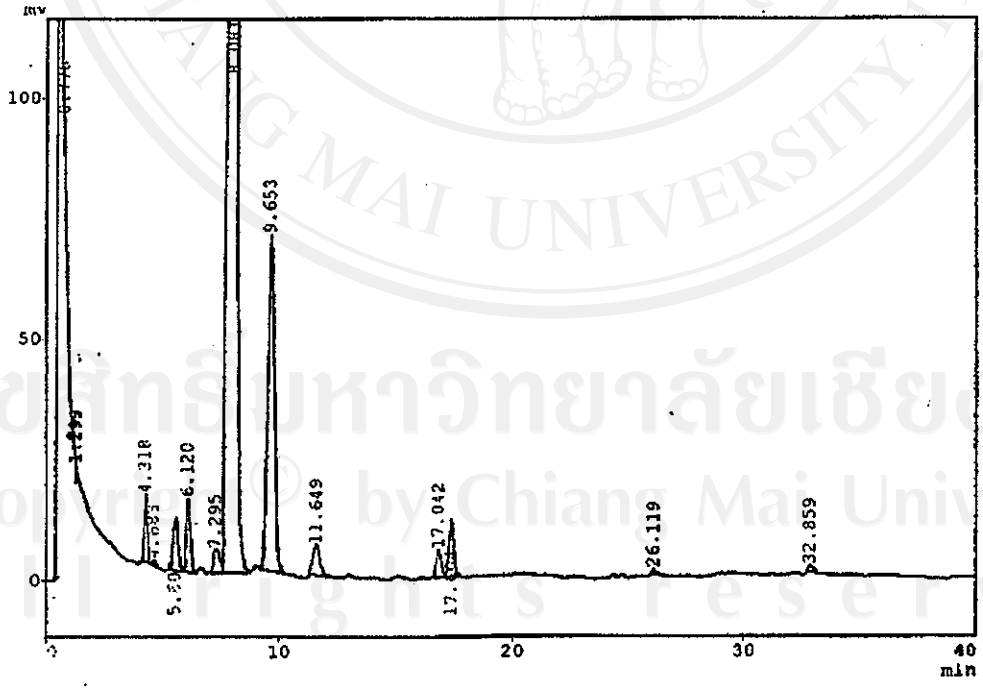


Figure 4.8 GC chromatogram of distilled lime oil from 45 day fermented peel

4.3 Determination of Chemical Components of Distilled Lime Oil by GC/MS Analysis

4.3.1 Optimum condition of GC/MS

Capillary column	Alltech 15897 AT-1MS (100% Dimethylpolysiloxane), 30m x 0.25mm ID x 0.25μm film thickness
Carrier gas	Helium 1.0 mL/min
Sample concentration	1% (v/v) in methanol
Volume of sample	1μL
Inlet	Split 200: 1, 230°C
Column temperature	70°C (10 min)--2°C /min→80°C--7°C /min→220°C /min (5 min)
Stop time	40 min
MS quadrupole	150°C
MS source	230°C

Results of determination of chemical components of distilled lime oil by GC/MS analysis using search libraries: WILEY275.L and NIST98.L were shown in Figures 4.9-4.10 and Tables 4.6-4.7. It was found that after fermentation components of distilled lime oil were changed. There were 27 chemical components that could observe clearly in unfermented pressed peel lime oil and 16 chemical components in 45 day fermented lime oil. The major components identified of unfermented pressed peel lime oil were limonene (68.78%), *p*-cymene (8.51%), γ-terpinene (7.82%), α-terpineol (3.44%), terpinen-4-ol (2.18%), β-pinene (1.51%), β-myrcene (1.40%), α-terpinolene (1.03%) and β-bisabolene (1.01%). The 45 day fermented lime oil contained limonene (74.17%), γ-terpinene (11.65%), *p*-cymene (3.77%), α-terpineol (2.68%), β-myrcene (1.59%), α-terpinolene (1.31%), terpinen-4-ol (1.30%), α-pinene (1.13%) and β-pinene (1.04%) as the major components.

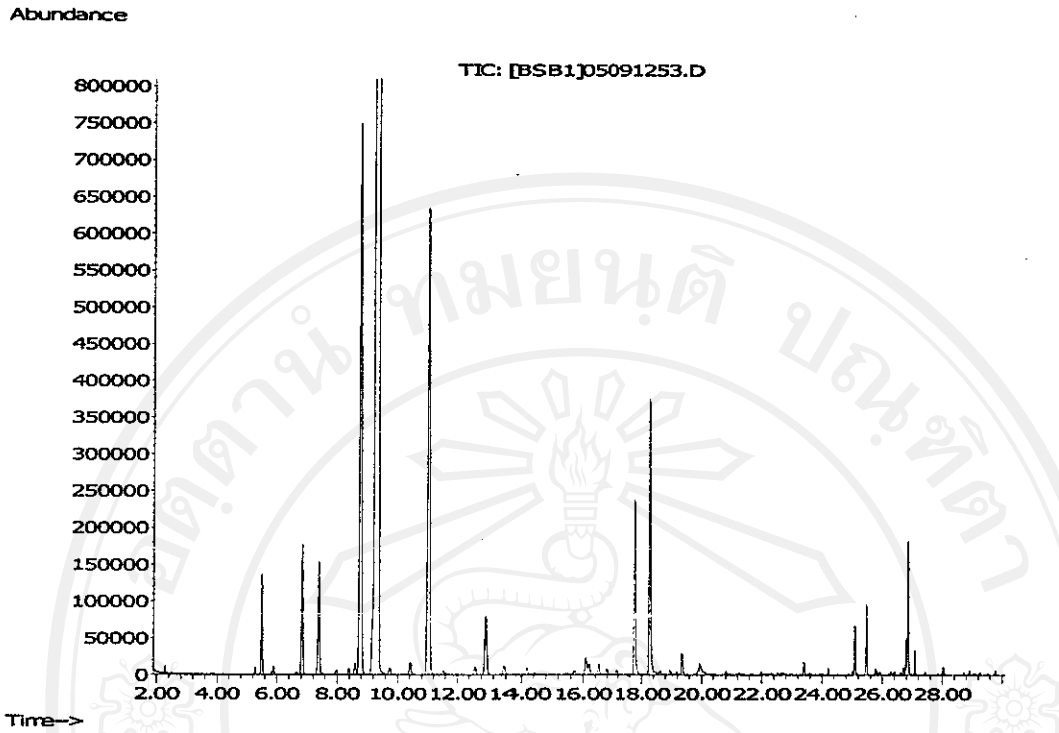


Figure 4.9 GC/MS chromatogram of distilled lime oil from pressed peel

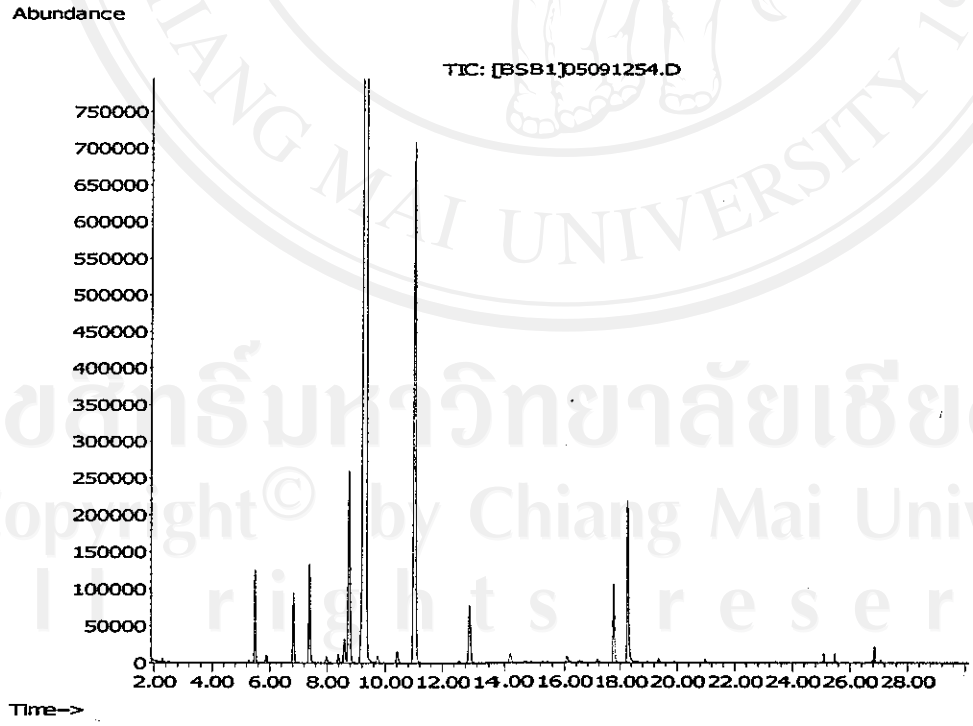


Figure 4.10 GC/MS chromatogram distilled lime oil from 45 day fermented peel

Table 4.6 Relative amounts of chemical components of distilled lime oil from pressed peel

Peak no.	R.T., min	Chemical component	% Relative amount
1	5.49	α-pinene	0.96
2	5.89	camphene	0.08
3	6.83	β-pinene	1.51
4	7.38	β-myrcene	1.40
5	8.60	α-terpinene	0.16
6	8.76	*p*-cymene	8.51
7	9.36	limonene	68.78
8	10.43	*trans*-β-ocimene	0.19
9	11.03	γ-terpinene	7.82
10	12.59	*p*-cymenyl	0.13
11	12.95	α-terpinolene	1.03
12	13.94	linalool	0.15
13	16.08	β-terpineol	0.27
14	16.19	unknown	0.18
15	16.52	prenitene	0.15
16	17.77	terpinen-4-ol	2.18
17	18.24	α-terpineol	3.44
18	19.35	ionene	0.26
19	19.94	citronellol	0.20
20	23.40	citronellyl acetate	0.11
21	25.09	*trans*-caryophyllene	0.37
22	25.48	α-bergamotene	0.56
23	26.70	α-bisabolene	0.07
24	26.78	farnesene	0.23
25	26.85	β-bisabolene	1.01

Peak no.	R.T., min	Chemical component	% Relative amount
26	27.08	δ-cardinene	0.18
27	28.04	(-)-caryophyllene oxide	0.07

Table 4.7 Relative amounts of main components of distilled lime oil from 45 day fermented peel

Peak no.	R.T., min	Chemical component	% Relative amount
1	5.49	α-pinene	1.13
2	6.83	β-pinene	1.04
3	7.38	β-myrcene	1.59
4	8.38	isocineole	0.14
5	8.60	α-terpinene	0.45
6	8.76	*p*-cymene	3.77
7	9.34	limonene	74.17
8	10.42	*trans*-β-ocimene	0.24
9	11.03	γ-terpinene	11.65
10	12.95	α-terpinolene	1.31
11	14.18	fenchol	0.19
12	17.77	terpinen-4-ol	1.30
13	18.27	α-terpineol	2.68
14	25.09	*trans*-caryophyllene	0.09
15	25.48	α-bergamotene	0.09
16	26.85	β- bisabolene	0.15

After fermentation the kind of observed chemical components was less than before fermentation and two new chemical components, isocineole and fenchol, were observed. When compare with differential value of the relative amounts (with total peak area) of the same chemical components of distilled lime oil before and after

fermentation by GC/MS analysis in Table 4.8, it was shown that there were 14 same chemical components. The most increased components were monoterpenes, e.g. α-pinene, β-myrcene, α-terpinene, limonene, trans-β-ocimene, γ-terpinene and α-terpinolene. The most decreased components were oxygenated monoterpenes and sesquiterpenes e.g. β-pinene, *p*-cymene, terpinen-4-ol, α-terpineol, *trans*-caryophyllene, α-bergamotene and β-bisabolene.

From Table 4.8, the increased and decreased were 6 components from 14 same components which shown in Table 4.9 and Figure 4.11 (Figures 4.18-4.29 see in Appendix C). The increased components were limonene (+ 4.18%) and γ-terpinene (+ 3.70%). The decreased components were *p*-cymene (- 4.91%), α-terpineol (- 0.93%), β-bisabolene (- 0.88 %) and terpinen-4-ol (- 0.83%).

Table 4.8 Comparative relative amounts of the same chemical components of distilled lime oil before and after fermentation by GC/MS analysis

Peak no. Chemical components	Before fermentation % Relative amount	After fermentation % Relative amount	Differential value, %
1. α-pinene	0.98	1.14	+0.16
2. β-pinene	1.54	1.05	-0.49
3. β-myrcene	1.43	1.60	+0.17
4. α-terpinene	0.16	0.45	+0.29
5. *p*-cymene	8.69	3.78	-4.91
6. limonene	70.24	74.42	+4.18
7. *trans*-β-ocimene	0.19	0.25	+0.06
8. γ-terpinene	7.99	11.69	+3.70
9. α-terpinolene	1.06	1.31	+0.25
10. terpinen-4-ol	2.23	1.30	-0.93
11. α-terpineol	3.52	2.69	-0.83
12. *trans*-caryophyllene	0.39	0.09	-0.30

Peak no. Chemical components	Before fermentation % Relative amount	After fermentation % Relative amount	Differential value, %
13. α-bergamotene	0.57	0.09	- 0.48
14. β-bisabolene	1.03	0.15	- 0.88

Table 4.9 Increased and decreased relative amounts of major components of distilled lime oil after fermentation

Chemical component	Before fermentation % Relative amount	After fermentation % Relative amount	Differential value, %
1. *p*-cymene	8.69	3.78	- 4.91
2. limonene	70.24	74.42	+ 4.18
3. γ-terpinene	7.99	11.69	+ 3.70
4. terpinen-4-ol	2.23	1.30	- 0.93
5. α-terpineol	3.52	2.69	- 0.83
6. β-bisabolene	1.03	0.15	- 0.88

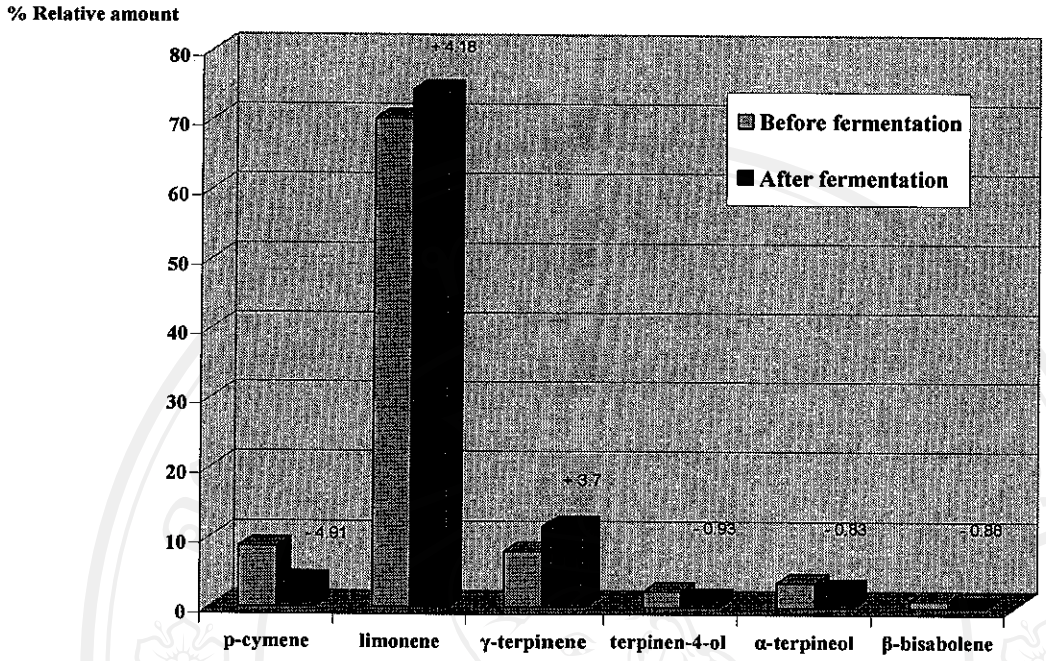


Figure 4.11 Increased and decreased relative amounts of major components of distilled lime oil after fermentation

There were studied about changes in essential oil during anaerobic fermentation (Ensiling) of odorous plant using enzymes and microorganisms. It was presented that the major components of essential oil changed both increased and decreased during fermentation. There was effect of the enzymes and microorganisms on essential oil content. The acidic condition of fermentation process provided a self-condensation between the double bond and the aldehyde groups of an open chain monoterpene to cyclic monoterpenes [54].

Due to hydro distillation lime oil was directly high heated and contacted acidic solution for long time so some components were changed and yield of aldehyde was too low [55]. So aldehyde from this research was not observed. Most of reactions were hydrolysis which acid was catalyst and rearrangement of terpenes, main components in lime oil. In additionally, bicyclic terpenes presented in distilled lime oil had chance to be hydrolysis and rearrangement reactions during distillation e.g. α-pinene, β- pinene, sabinene and thujene were changed to alcohol terpenes (α-

terpineol, terpinen-4-ol and borneol) and hydrocarbon terpenes (terpinolene, limonene, camphene and γ-terpinene [15].

So it may be assume that limonene and γ-terpinene were apart from reactions during distillation and these may due to many reactions occurred between fermentation too and the walls of oil cells may be broken down between acidic solution contacts for long time during fermentation. The decreased components may be converted to some increased components or be consumed by microorganisms during fermentation.

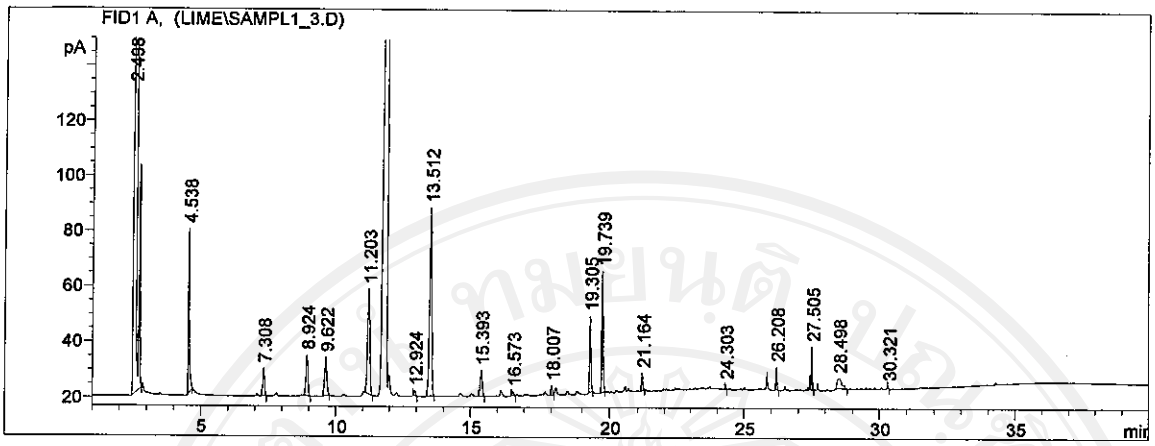
Aroma/flavor quality of lime oil is associated with an increase in oxygenated constitutes. Citral and some of the aliphatic alcohols (C8 to C10), linalool, geraniol, terpineol, borneol and their ester suupply the Characteristic notes. However, the freshness is undoubtedly due partly to the terpenes, among which limonene and natural diterpene actually have pleasant odors. If the oil is not fresh and if the terpenes are perceptibly decomposseed or oxidized, the odor is worthless no matter how good the oxygenated components are [53]. Some of the oxygenated compounds are produced during the distillation when the lime oil exposed to high temperatures in an acid environment [15]. Oxygenated derivatives of monoterpenes and sesquiterpenes those are more important than the terpene hydrocarbons as aroma chemical. The characteristic odor of many essential oils is representative of the combined odors of the oxygenated compounds [18]. So the odors of distilled lime oil after fermentation were changed because of the increased components, limonene and γ-terpinene, and the decreased components, oxygenated monoterpenes and sesquiterpenes. These opposite results caused the new odor occur different from general distilled lime oil. The note of new odor was becoming more pickle lime-like and citrus-like. However, the distilled lime oil before and after fermentation contained four major chemical components, limonene, γ-terpinene, *p*-cymene and α-terpineol, in various amounts which led to different odor too.

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

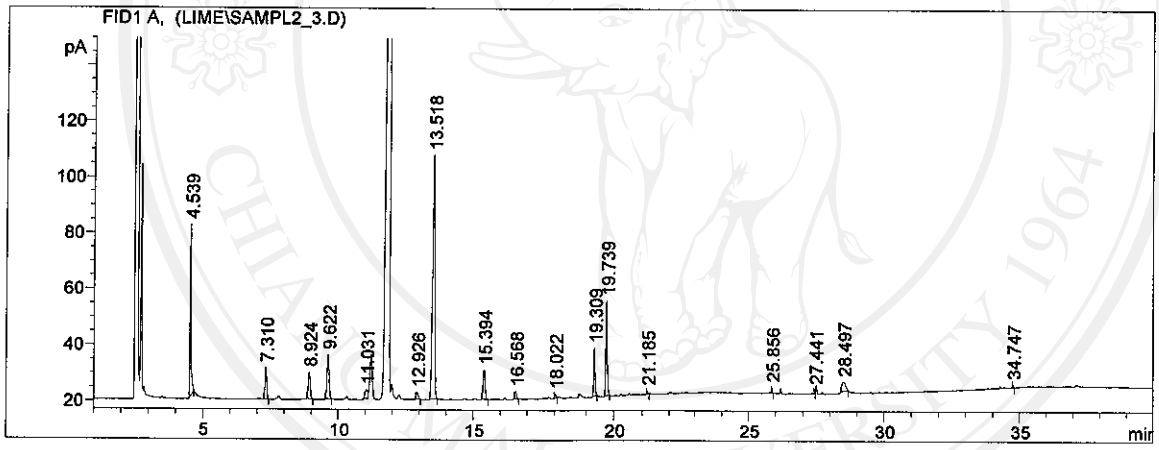
4.2.1 Optimum condition of GC

Capillary column	Alltech 15897 AT-1MS (100% Dimethylpolysiloxane), 30m x 0.25mm ID x 0.25μm film thickness
Carrier gas	Helium 1.0 mL/min
Sample concentration	0.01 g/mL methanol
Internal standard concentration	URETANE (Ethyl carbamate) 0.5 % w/v
Volume of sample	1μL
Inlet	Split 20: 1, 230°C
Detector	250°C
Column temperature	70°C (10 min)--2°C /min→80°C--7°C /min→220°C /min (5 min)
Stop time	40 min

Results were shown in Figures 4.12-4.13 and Table 4.10. It was found that amounts of α-terpineol were decreased from 28.57 ± 0.12 to 24.03 ± 0.06 mg/g after fermentation. Amounts of α-terpineol in lime oil were important role in perfumery industry and may be effects on the central nervous system (CNS) but no researches were confirmed the kind of component that was the best to stimulate CNS. The further study on stimulative effect of major component of distilled lime oil should be done.



Figures 4.12 GC chromatogram of lime oil before fermentation (Internal Standardization)



Figures 4.13 GC chromatogram of lime oil after fermentation (Internal Standardization)

Table 4.10 Amounts of α-terpineol of distilled lime oil

Substance	Amount of α-terpineol (mg/g)
distilled lime oil before fermentation	28.57 ± 0.12
distilled lime oil after fermentation	24.03 ± 0.06