CHAPTER 4

RESULTS AND DISCUSSION

4.1 Isolation of *Monascus purpureus*

The number of rice grains showing the growth of fungus after treatment by surface sterilization

After washing with 95% ethanol and 0.1-5.0% sodium hypochlorite solution, the number of rice grains with the growth of strain were obtained. The results showed different number of growth rice grains when washing with different concentration of sodium hypochlorite and with different time of washing as shown in Table 4.1.

Table 4.1 Number of rice grains with growth of strain

	Sodium hypochlorite solution															
	5.0 %v/v				2.5 %v/v				1.0 %v/v				0.1 %v/v			
	Time (minutes)															
	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
No. of grains	6	5	4	4	5	4	4	3	8	6	6	5	12	10	9	9

The number of rice grains with the growth of strain was highest when washed with 0.1% solution of sodium hypochlorite for 1 minute. The colonies of the strain are shown in Figure 4.1 (a) and (b).

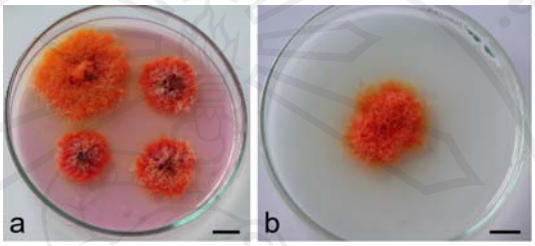


Figure 4.1 Isolation of *Monascus purpureus* from commercial Chinese red yeast rice available in local market. a. Cultivation of surface sterile red yeast rice on Rose Bengal agar, b. Isolated *M. purpureus* growing on PDA showing pure strain. Bars = 1 cm.

The best condition for isolation by surface sterile technique was to wash with 95% ethanol fallowed by 1 minute washing with 0.1% sodium hypochlorite solution for 30 seconds. The highest number of growth obtained is 12 rice grains.

The Rose Bengal agar used contains chloramphenicol which is antibacterial agent, therefore, the growth colonies are confirmed not to be bacteria (Jarvis, 1973**;** Ottow and Glathe, 1968; Gamble and Orcutt, 1951).

4.2 Identification of fungal strain

The colonies of *M. purpureus* strain CMU001 on PDA (Figure 4.2a) was initially white and became orange to red later. Ascomata 36–72 μm, non-ostiolate (cleistothecia), hyaline to reddish brown, superficial, globose, stalked, soon evanescent asci (Figure 4.2b,c). Ascospores 5–6.2 × 5 μm, hyaline or slightly orange, smooth-walled (Figure 4.2d, f). Anamorph present. Conidia 7.5–8.5 × 5–6.2 μm, hyaline, thick- and smooth-walled, subglobose with a very broadly truncate base (Figure 4.2e, f). The morphological characteristics of stalked cleistothecia and soon evanescent asci, which are no longer recognizable at maturity, are those of *Monascus*, as described by Tieghem (Tieghem, 1884). The characteristics of ascomata, ascospores and conidia of the strain CMU001 showed similarity to *M. purpureus* (Went, 1895).

In growth observation of the strain CMU001 on PDA, colonies also show the lava shaped, helix mycelium, red pigment production and starch hydrolysis activity (Figure 4.3). The growth is impossible in 6% NaCl and 30% ethanol. These confirmed characteristics of *M. purpureus* (Iizuka and Lin, 1981).

The strain isolated from red rice is studied taxonomically according to the procedure of Iizuka and Lin (1981). *M. purpureus* CMU001 is used in all experiments. The main morphological and cultural characteristics of the investigated microorganisms are shown in Table 4.2 comparing to *M. purpureus* Went.

Table 4.2 Morphological and cultural characterisation of *M. purpureus* Went and *M. purpureus* CMU001.

Cultural and morphological characters	7 days of cultivation on PDA at room temperature	
	M. purpureus Went[a]	*M. purpureus* CMU001
Colony shape	Lava	Lava
Mycelium shape	Helix	Helix
Production of red pigment	initially white and became orange to red later	initially white and became orange to red later
6% NaCl tolerance	Not be tolerant	Not be tolerant
30% ethanol tolerance	Not be tolerant	Not be tolerant
Starch hydrolysis activity	Found clear zone	Found clear zone

[a] Referent from Yongsmith (1999)

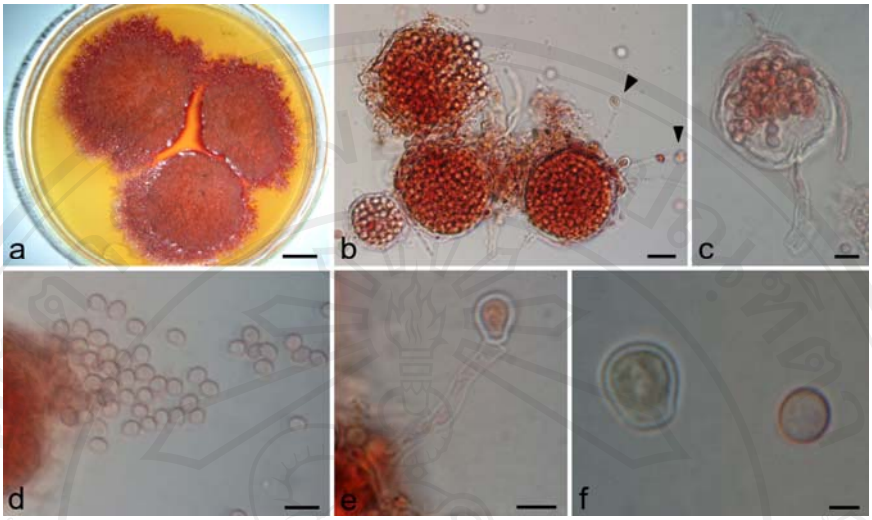


Figure 4.2 *Monascus purpureus* strain CMU001. a. Colonies in PDA, b. Various stages of ascomata development and conidial formation (arrowed), c. Several asci developed in stalked cleistothecium, d. Subglobose or ellisoidal ascospores, e. Conidiophore bearing conidium, f. Comparison of broadly truncate base conidium and globose ascospore. Bars: a. = 1 cm, b-d. = 10 µm, e. = 5 µm, f. = 2 µm.

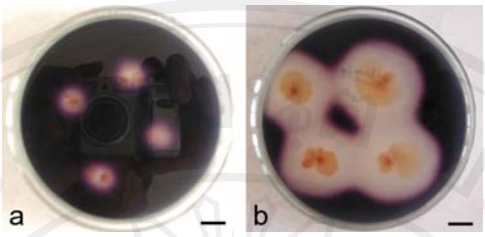


Figure 4.3 Starch hydrolysis activities of *M. purpureus* strain CMU001 after one (a) and two (b) weeks. Bars = 1 cm.

4.3 Preparation of red yeast rice

Preparation of red yeast rice, glutinous rice grains were immersed in water for 6 hours following by steaming for 20 minutes. After cooling, 50 grams of steam rice was put in 250 ml flask and sterile at 15 psi and 121 ^{o}C for 15 minutes. One week old precultured *M. purpureus* CMU001 was used as inoculum. The inoculated rice was incubated at 30 ^{o}C for 2 or 3 weeks. Humidity and pH were measured before and after inoculation. The end-product was dried in the oven at 65 ^{o}C for 6 hours to obtain dried red yeast rice.

Yield of red yeast rice

The yield of red yeast rice was evaluated as percentage yield. The percentage yields were obtained by using the following equation.

$$\text{Percentage yield} = \frac{\text{Weight of dried end product}}{\text{Weight of steamed rice used}} \times 100$$

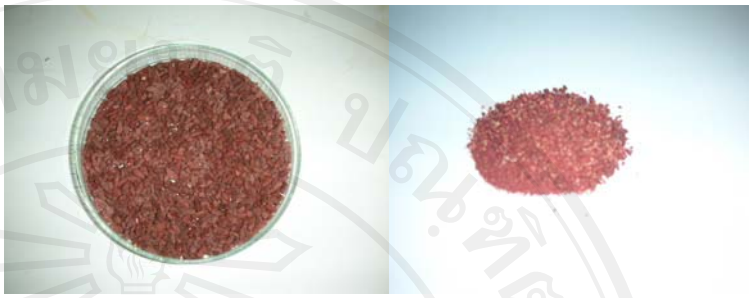
The percentage yield of prepared red yeast rice after 2 and 3 weeks cultivation using *Oryza sativa* L. cv. Mali105, *Oryza sativa* L. cv. Kam, *Oryza sativa* L. cv. RD6 and *Oryza sativa* L. cv. SPT1 rice are shown in Table 4.3.

All varieties gave red yeast rice production varying from pale red to darker red depending on the kind of rice used as shown in Figure 4.4.

Table 4.3 Percentage yield of red yeast rice

Fermented rice	Without soybean milk		With soybean milk	
	2 weeks	3 weeks	2 weeks	3 weeks
	% yield	% yield	% yield	% yield
Oryza sativa L. cv. Mali105	16.39	12.37	25.22	13.69
Oryza sativa L. cv. Kam	28.50	22.56	22.92	19.81
Oryza sativa L. cv. RD6	18.41	16.02	15.24	14.10
Oryza sativa L. cv. SPT1	17.03	15.28	15.93	13.40

Commercial red yeast rice



Oryza sativa L. cv.

Mali105 2 weeks

without soybean milk

Oryza sativa L.cv.

Mali105 3 weeks

without soybean milk

(a) (b)

Figure 4.4 Product of red yeast rice, (a) Red yeast rice (grains), (b) Red yeast rice (powder), (continued)

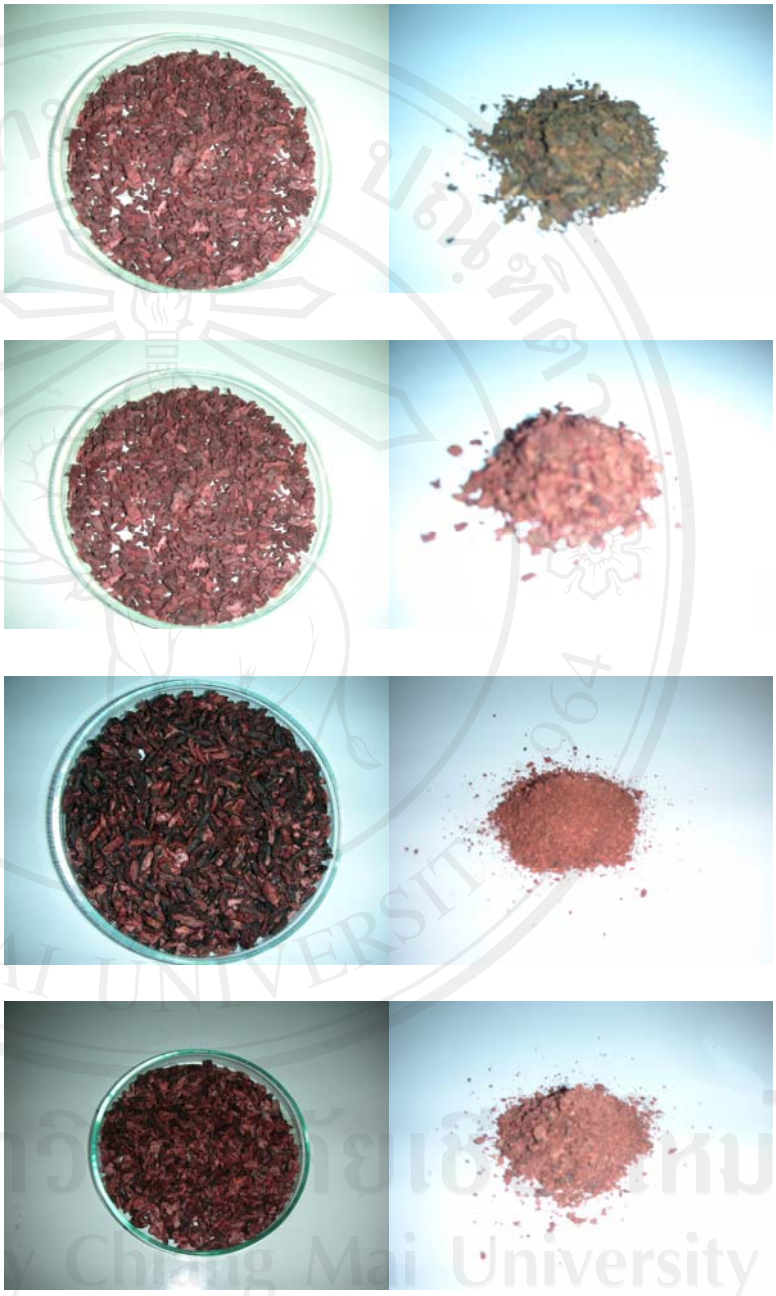
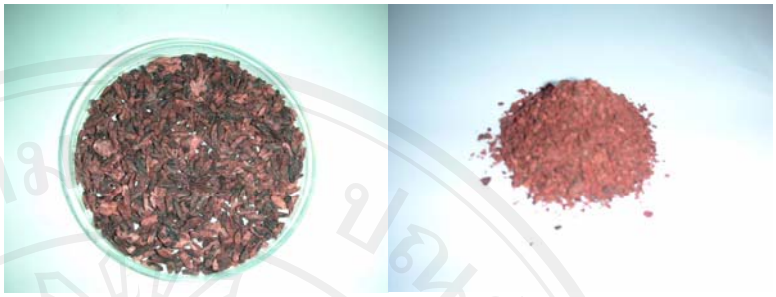


Figure 4.4 Product of red yeast rice, (a) Red yeast rice (grains), (b) Red yeast rice (powder), (continued)

Oryza sativa L. cv. Kam

2 weeks

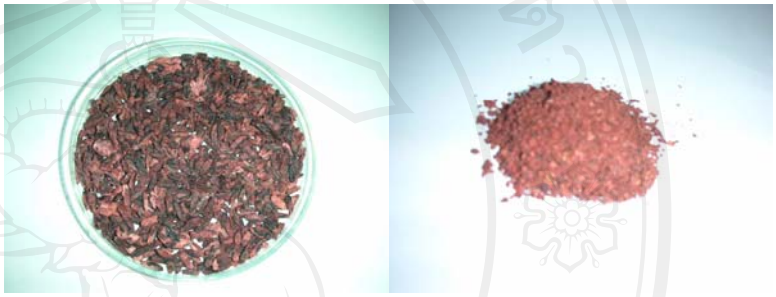
with soybean milk



Oryza sativa L. cv. Kam

3 weeks

with soybean milk



Oryza sativa L. cv. RD6

2 weeks

without soybean milk

Oryza sativa L. cv. RD6

3 weeks

without soybean milk

(a) (b)

Figure 4.4 Product of red yeast rice, (a) Red yeast rice (grains), (b) Red yeast rice (powder), (continued)

Oryza sativa L. cv. RD6

2 weeks

with soybean milk

Oryza sativa L. cv. RD6

3 weeks

with soybean milk

Oryza sativa L. cv. SPT1

2 weeks

without soybean milk

Oryza sativa L. cv. SPT1

3 weeks

without soybean milk

(a) (b)

Figure 4.4 Product of red yeast rice, (a) Red yeast rice (grains), (b) Red yeast rice (powder), (continued)

Oryza sativa L. cv. SPT1

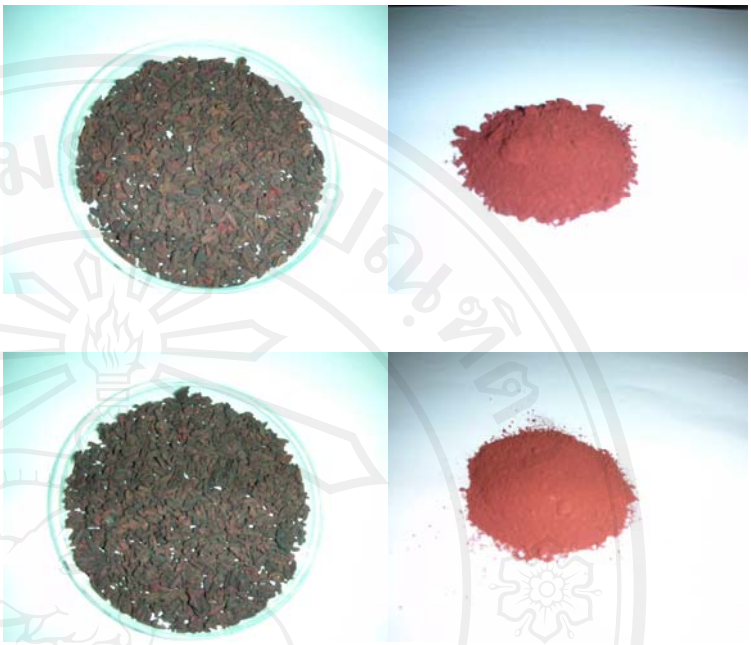
2 weeks

with soybean milk

Oryza sativa L. cv. SPT1

3 weeks

with soybean milk



(a) (b)

Figure 4.4 Product of red yeast rice, (a) Red yeast rice (grains), (b) Red yeast rice (powder).

Red yeast rice of *Oryza sativa* L. cv. Kam is not sticked together. The rice grains are not broken with red color. The color of red yeast rice of *Oryza sativa* L. cv. Mali105 is bright red and the grains are sticked together with pink-red color powder. The grains of red yeast rice of *Oryza sativa* L. cv. SPT1 and *Oryza sativa* L. cv. RD6 are almost the same with complete grains of darker color.

4.4 Measuring the content of red pigment

The content of red pigment in the 75% ethanol extract (Table 4.4) are expressed as absorbance unit per gram (AU/g) of red yeast rice powder. The absorbance was measured at 500 nm which is the maximum wave length for red color.

Table 4.4 Red pigment content from fermented 1 cultivar of normal rice and 3 cultivar of glutinous rice with *M. purpureus*

Time	AU(AU/g)							
	Oryza sativa L. cv. Mali105		*Oryza sativa* L. cv. Kam		*Oryza sativa* L. cv. RD6		*Oryza sativa* L. cv. SPT 1	
	Without soybean milk	With soybean milk	Without soybean milk	With soybean milk	Without soybean milk	With soybean milk	Without soybean milk	With soybean milk
2 weeks	4.51 ±0.00	3.60 ±0.01	3.63 ±0.01	6.99 ±0.01	30.11 ±0.00	45.04 ±0.01	29.63 ±0.02	36.09 ±0.01
3 weeks	1.42 ±0.00	0.91 ±0.00	6.06 ±0.02	5.06 ±0.01	39.90 ±0.02	42.16 ±0.00	34.13 ±0.00	42.70 ±0.01

After preparation of red yeast rice, the difference varieties gave red yeast rice products varying from pale red to darker red depending on the kind of rice used. The shape and the texture of each kinds of the product depend on the rice used.

Many reports indicated the absorbance of red pigment from *M. purpureus* at 400-500 nm (Babitha *et al*., 2007; Yongsmith *et al*., 2000 and Singhapol, 2005). The results show maximum absorbance around 500 nm that was used for measuring of red pigment. The red color is due to the presence of rubropunctamine and monascorubramine which are derived from orange colored compounds rubropunctatin and monascorubrin respectively (Yongsmith, 1999). Amine compounds seem to play an important role for derivatization of orange colored compounds by ring opening and shift rearrangement reaction. Consequently, from Table 4.3, most of the rice variety expressed more intense red color when adding soybean milk solution during red yeast rice preparation.

The red pigment produced from glutinous rice seems to be higher than normal rice, Mali105. The result is controversial to the one reported earlier by Pinthong *et al*. (2004). Controlling of humidity and low efficiency of starch degradation may be reasonable explanation.

In this report, it was found that, under control condition, glutinous rice gave more intense red color products. The result shows the most intense red color was observed from using RD6 with addition of soybean milk harvested with in 2 weeks. Among the glutinous rice used, the lowest quality product was obtained with Kam rice (purple rice).

4.5 Monacolins in red yeast rice

An earlier report (Li *et al*., 2004) shows the presence of 14 kinds of monacolins such as monacolin K (MK) or mevinolin, monacolin J (MJ), monacolin L (ML), monacolin M (MM), monacolin X (MX) and their hydroxyacid form, MK acid form (MKA), MJ acid form (MJA), ML acid form (MLA), MX acid form (MXA), MM acid form (MMA) as well as dehydromonacolin K (DMK), dihydromonacolin L (DML), compactin (P1), and 3α-hydroxy-3,5-dihydromonacolin L (HDML).

The results obtained by detecting at 237 nm shows about 12 interesting peaks. The chromatograms of a 3 weeks period of fermentation with and without the addition of soybean milk using non-glutinous rice, *Oryza sativa* L. cv. Mali105, glutinous rice; *Oryza sativa* L. cv. Kam, *Oryza sativa* L. cv. RD6 and *Oryza sativa* L. cv. SPT1 are shown in Figure 4.5.

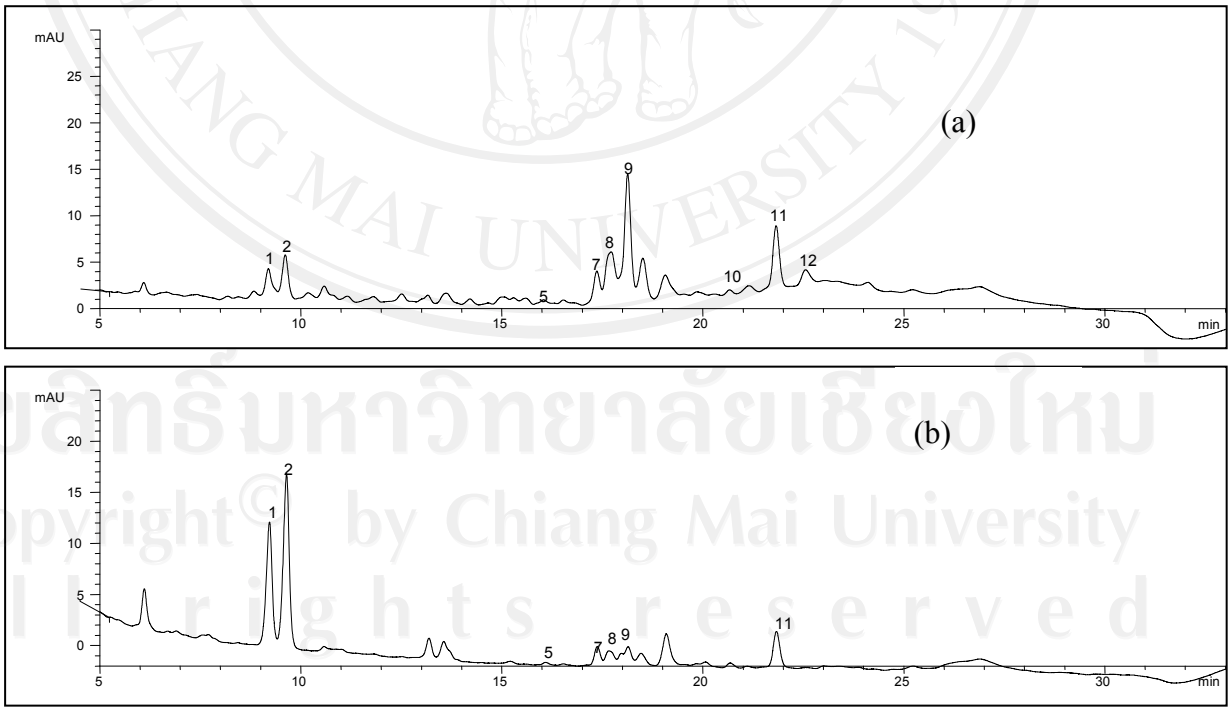


Figure 4.5 Chromatographic chemical profiling of monacolins in fermented red yeast rice from non-glutinous rice and glutinous rice, (continued)

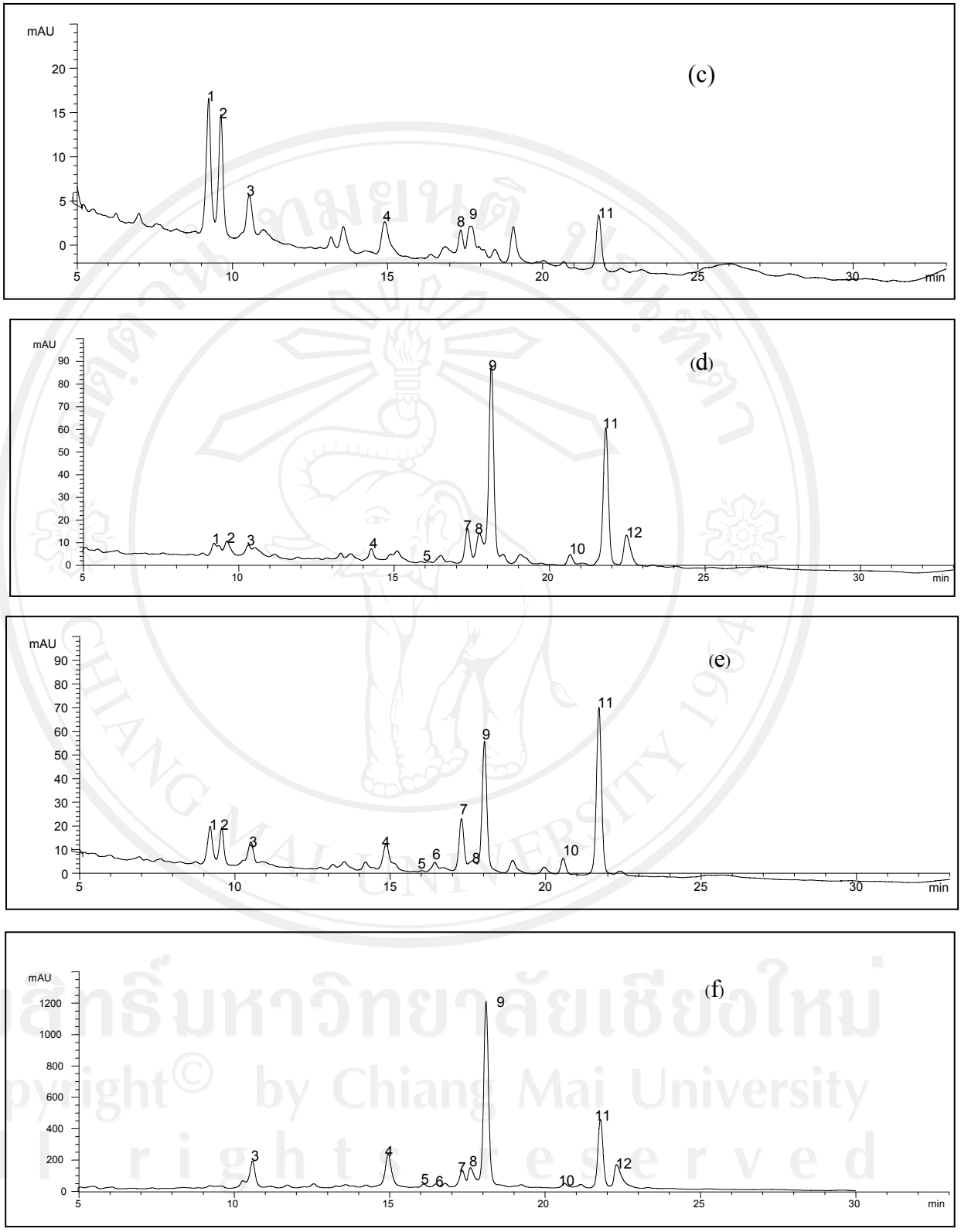


Figure 4.5 Chromatographic chemical profiling of monacolins in fermented red yeast rice from non-glutinous rice and glutinous rice, (continued)

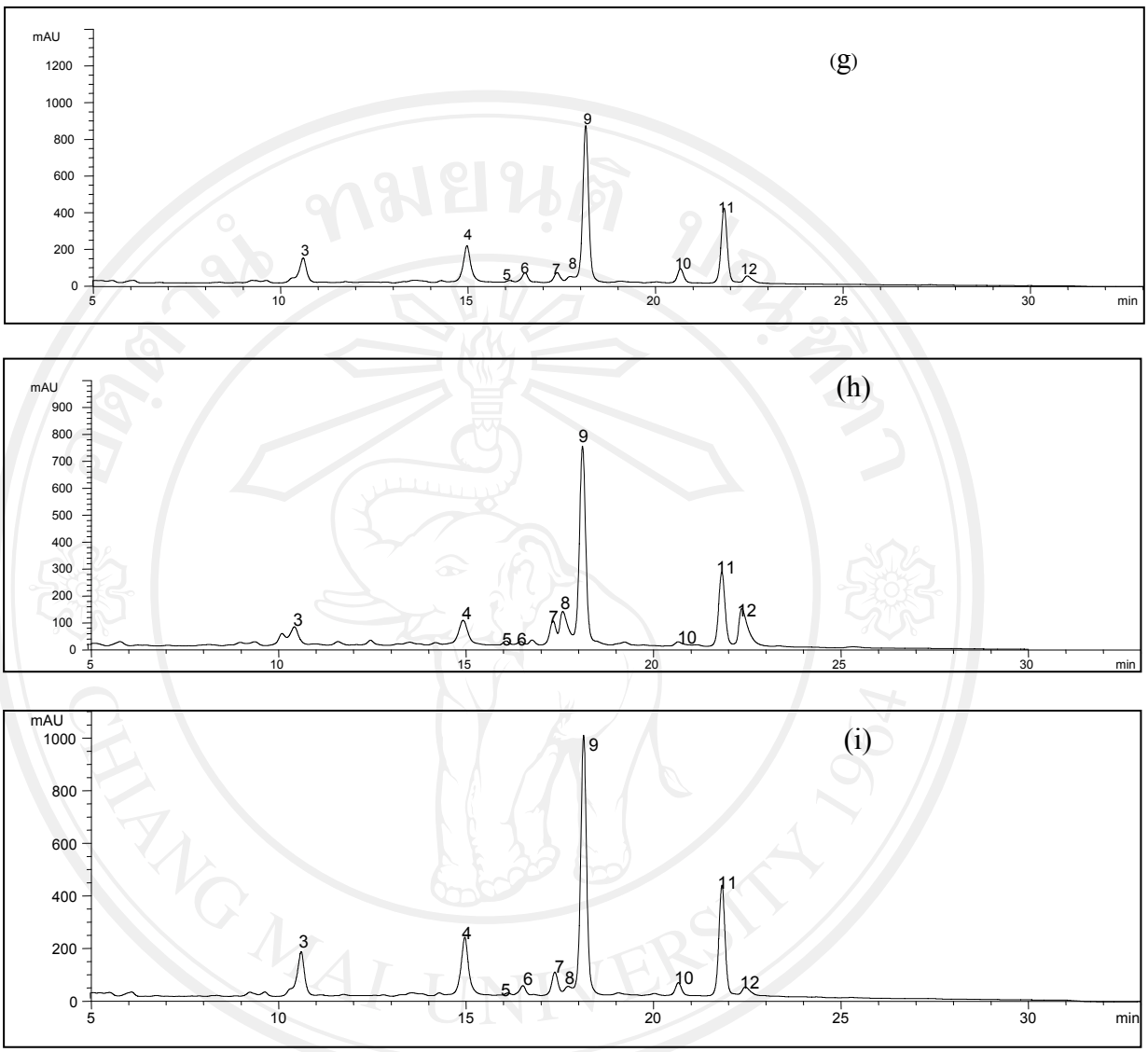


Figure 4.5 Chromatographic chemical profiling of monacolins in fermented red yeast rice from non-glutinous rice and glutinous rice, (a) commercial Chinese red yeast rice (Comryr), (b) and (c) *Oryza sativa* L. cv. Mali105 without and with soybean milk, (d) and (e) *Oryza sativa* L. cv. Kam without and with soybean milk, (f) and (g) *Oryza sativa* L. cv. RD6 without and with soybean milk, (h) and (i) *Oryza sativa* L. cv. SPT1 without and with soybean milk

Table 4.5 Data of chemical profiling of monacolins in fermented red yeast rice (peak area) from non-glutinous rice and glutinous rice without soybean milk for 3 weeks

Peak No	**MW[a]**	**Ref.[b]**	**Compound**	**Comryr[c]**	***Oryza sativa* L. cv. Mali105**	***Oryza sativa* L. cv. Kam**	***Oryza sativa* L. cv. RD6**	***Oryza sativa* L. cv. SPT1**
1	-	-	Unidentified	50.19 ±0.01	128.94 ±0.12	66.72 ±0.00	ND	ND
2	-	-	Unidentified	62.14 ±0.00	182.08 ±0.07	112.61 ±0.04	ND	ND
3	-	-	Unidentified	ND	ND	84.48 ±0.04	2528.99 ±0.13	1289.37 ±0.08
4	422	Calaf *et al*., 1997; Stubbs *et al*., 1986.	Monacolin K Acid form (MKA)	ND	ND	81.61 ±0.02	4397.43 ±0.04	2457.56 ±0.29
5	390	Brown *et al*., 1976; Endo, 1985.	Compactin (P1)	39.49 ±0.03	3.02 ±0.08	4.90 ±0.03	536.09 ±0.01	376.07 ±0.11
6	-	-	Unidentified	ND	ND	ND	536.79 ±0.01	331.89 ±0.03
7	424	Li *et al*., 2004	Monacolin M acid form (MMA)	73.92 ±0.06	21.62 ±0.51	186.41 ±0.06	1792.46 ±0.01	1307.55 ±0.15
8	404	Endo, 1979; Endo, 1980.	Monacolin K (MK)	126.49 ±0.32	22.72 ±0.69	198.29 ±0.01	2138.68 ±0.01	2122.45 ±0.12
9	358	-	Unkwown	211.72 ±0.11	22.95 ±0.95	1030.99 ±0.12	14742.30 ±0.00	9654.09 ±0.02
10	406	Li *et al*., 2004.	Monacolin M (MM)	68.36 ±0.39	ND	64.59 ±0.05	875.56 ±0.01	593.00 ±0.17
11	386	Ma *et al*., 2000; Li *et al*., 2005.	Dehydromonacolin K (DMK)	233.89 ±0.55	45.54 ±0.03	773.51 ±0.09	5733.99 ±0.01	3650.18 ±0.03
12	-	-	Unidentified	209.32 ±0.73	ND	244.95 ±0.02	3465.02 ±0.02	2890.51 ±0.22

Table 4.6 Data of chemical profiling of monacolins in fermented red yeast rice (peak area) from non-glutinous rice and glutinous rice with soybean milk for 3 weeks

Peak No	**MW[a]**	**Ref.[b]**	**Compound**	***Oryza sativa* L. cv. Mali105**	***Oryza sativa* L. cv. Kam**	***Oryza sativa* L. cv. RD6**	***Oryza sativa* L. cv. SPT1**
1	-	-	Unidentified	174.56 ±0.01	191.00 ±0.05	ND	ND
2	-	-	Unidentified	148.35 ±0.00	163.41 ±0.03	ND	ND
3	-	-	Unidentified	82.57 ±0.02	163.93 ±0.01	2374.28 ±0.08	2837.37 ±0.03
4	422	Calaf *et al*., 1997; Stubbs *et al*., 1986.	Monacolin K acid form (MKA)	75.92 ±0.00	212.76 ±0.04	3579.93 ±0.02	3947.22 ±0.04
5	390	Brown *et al*., 1976; Endo, 1985.	Compactin (P1)	ND	5.68 ±0.01	325.70 ±0.00	403.80 ±60.27
6	-	-	Unidentified	ND	55.68 ±0.01	940.37 ±0.01	783.07 ±0.01
7	424	Li *et al*., 2004.	Monacolin M Acid form (MMA)	45.55 ±0.00	276.07 ±0.00	1014.3 ±0.01	1489.96 ±0.01
8	404	Endo, 1979; Endo, 1980.	Monacolin K (MK)	70.69 ±0.00	80.71 ±0.03	595.71 ±0.00	667.89 ±0.00
9	358	-	Unkwown	ND	660.56 ±0.08	10336.9 ±0.00	11952.90 ±0.00
10	406	Li *et al*., 2004.	Monacolin M (MM)	ND	85.13 ±0.12	1602.02 ±0.01	1355.48 ±153.45
11	386	Ma *et al*., 2000; Li *et al*., 2005.	Dehy-dromonacolin K (DMK)	79.96 ±0.00	863.15 ±0.22	5369.92 ±0.01	5761.05 ±0.01
12	-	-	Unidentified	ND	ND	1266.94 ±0.01	1424.89 ±0.01

ND = Not detectable

[a] Molecular weight

[b] Reference

[c] commercial Chinese red yeast rice

Table 4.7 Compactin, Monacolin K content in 3 weeks old red yeast rice

Fermented rice	**Compactin[a] (mg/g)**	**Monacolin[a] K (mg/g)**	**Compactin[b] (mg/g)**	**Monacolin[b] K (mg/g)**
Comryr[c]	1.62 ± 0.00	2.00 ± 0.01	Not available	
Oryza sativa L. cv. Mali105	0.12 ± 0.00	0.36 ± 0.01	Not detectable	1.12 ± 0.00
Oryza sativa L. cv. Kam	0.20 ± 0.00	3.13 ± 0.00	0.23 ± 0.00	1.28 ± 0.00
Oryza sativa L. cv. RD6	21.98 ± 0.00	33.79 ± 0.00	13.35 ± 0.00	9.41 ± 0.00
Oryza sativa L. cv. SPT1	15.42 ± 0.01	33.54 ± 0.00	16.56 ± 2.47	10.55 ± 0.00

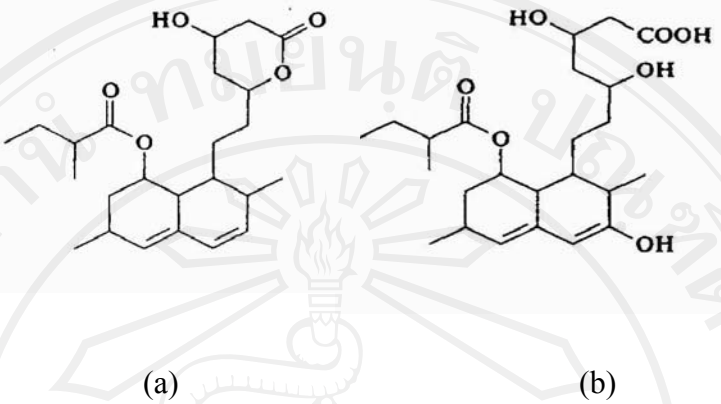
[a] fermented rice without soybean milk

[b] fermented rice with soybean milk

[c] commercial Chinese red yeast rice

The chromatographic profile of commercial Chinese red yeast rice and Thai non-glutinous rice (*Oryza sativa* L. cv. Mali105) had common similarities while Thai glutinous rice varieties had almost the same profile. Thai glutinous rice seems to give more intense monacolins peaks than non-glutinous rice and commercial Chinese red yeast rice. The relative retention time, enrichment techniques and LC-MS were used to confirm the position of monacolins peaks. The peaks identified were based on the results obtained as shown in Table 4.5-4.7 with their areas.

The amount of compactin and monacolin K was determined using standard compounds as shown in Table 4.7. It was found that *Oryza sativa* L. cv. RD6 fermented without the addition of soybean milk yielded the highest amounts of monacolin K and compactin, 33.79 mg/g dry weight and 21.98 mg/g dry weight, respectively. As compared to the other types of rice tested. Thai glutinous rice seems to give a more satisfactory amount of monacolin K and compactin than both Thai non-glutinous rice and commercial Chinese red yeast rice. A report by Chen & Hu (2005) indicated that the highest amount of monacolin K was 2.52 mg/g in red yeast rice. The reported amount is less than in Thai glutinous red yeast rice (*Oryza sativa* L. cv. RD6). The study on red yeast rice made from Thai glutinous rice would be beneficial to improve its quality. Monacolin K and compactin could be obtained without adding soybean milk. The result does not correspond to the increase of the red color as when soybean milk is added. The addition of soybean milk darkened the color rather than increased monacolin K. Perhaps there is some nitrogen source involved in the red color change instead of monacolins synthesis (Pinthong *et al.*, 2004).



(a) (b)

Figure 4.6 (a) Monacolin K in lactone form, (b) Monacolin K in acid form

Another interesting compound is the peak number 9 where the molecular weight was found to be 358 by LC-MS. The structure of the compound may have something to do with monacolin K but with the loss of H_2O (dehydration) and two methyl groups. The compound seems to be related to monacolin K but with the loss of H_2O or β-hydroxyl group. Dehydromonacolin K is also relevant. Monacolin K is not active in its natural form but will be active by hydrolysis of the lactone moiety to form β-hydroxy acid (Fig. 4.6). It is the hydroxy acid opened lactone structure which is active for inhibition of HMG CoA reductase (Alberts *et al.*, 1980; Trenin *et al.*, 1997). The β-hydroxy acid contributed to a competitive inhibition of HMG-CoA reductase and the production of cholesterol. The loss of β-hydroxyl group in the case of compound number 9 and dehydromonacolin K may decrease the inhibition efficiency. The study on the relevancy of these compounds, which seem to be more abundant, in the presence of monacolin K or compactin is of interest for further study.

If the increase of the compounds number 9 and dehydromonacolin K cause the decrease of Monacolin K and compactin, the optimum condition for the production of high concentration of monacolin K and compactin may be obtained by blocking the production of the compound number 9 and dehydromonacolin K. However, many kinds of fungi also produce mycotoxin (Pattanagul *et al.*, 2008; Lin *et al.,* 2008). Therefore, the determination of some mycotoxin such as citrinin should be carried out accurately. The amount of citrinin is not allowed more than 200 ng/g in Japan. In China and the European Economic Community, the similar citrinin level is still under debate (Mandt, 1998).

4.6 Antioxidant activity in red yeast rice

4.6.1 Total antioxidant capacity

In order to compare between each red yeast rice sample prepared using different condition, the total antioxidant capacity is the selected method. The kinds of antioxidants in red yeast rice have rarely been reported therefore the specific method for the specific group of compounds cannot be used. Total antioxidant capacity may cover the activity of most of the antioxidants possibly present in red yeast rice such as flavonoids, polyphenols, carotenoids, alkaloids, vitamins, etc.

The total antioxidant capacity results of red yeast rice were determined by the same method as described previously. The total antioxidant capacity in red yeast rice reported in Table 4.8 showed higher amounts of antioxidant capacity when glutinous rice was used except purple glutinous rice (*Oryza sativa* L. cv. Kam) which gave the lower amount in spite of being glutinous rice. The addition of soybean milk increased the antioxidant activity. For comparison of the cultivation time in Table 4.8, 3 weeks

of cultivation increased the total antioxidant capacity for glutinous rice while the decrease was found in the case of non-glutinous and purple glutinous rice.

Regarding the increase of the total antioxidant capacity, the period of growth can be used for explanation. In the first balanced phase or trophophase the rate of uptake and utilization of nutrients is maximal. Secondary metabolites or some antioxidants are rarely produced in this phase. When the nutrients are depleted and become limited, the growth rate slows and the storage phase begins. It is in this phase that the synthesis of secondary metabolites begins (Garrway and Evans, 1984). In the case of glutinous rice (*Oryza sativa* L. cv. RD6 and *Oryza sativa* L. cv. SPT1) and the addition of soybean milk, the growth may reach the second storage phase faster. When the cultivation time is longer the production of secondary metabolite or antioxidants may accumulate more. The decrease in the case of *Oryza sativa* L. cv. Mali105 and *Oryza sativa* L. cv. Kam when cultivation time was 3 weeks, may have been due to the degradation of the metabolites.

Another parameter that should be considered is the presence of some antioxidants in soybean milk used. The higher activities obtained in case of adding soybean milk may be due to the presence of isoflavones which are polyphenol compounds and closely related to antioxidant flavonoids (*http://www.Soybean - Wikipedia, the free encyclopedia.htm).* The activity of isoflavones however may be terminated after fermentation process. For further work, it is possible to determine the isoflavones content before and after fermentation.

Table 4.8 Total antioxidant capacity in red yeast rice (GAE (mg/ml))

Fermented Rice	mg gallic acid equivalent (GAE)/ ml extract		
	Without soybean milk	With soybean milk	
	2 weeks	2weeks	3 weeks
Comryr*	0.09±0.00		
Mali105	0.17±0.00	0.26±0.01	0.15±0.00
Kam	0.15±0.01	0.22±0.01	0.19±0.00
RD6	0.35±0.01	0.50±0.01	0.53±0.01
SPT1	0.36±0.01	0.48±0.00	0.53±0.01

Comryr* Commercial Chinese red yeast rice

GAE= Gallic acid equivalent

4.6.2 β-carotene bleaching method (BCB)

The production of free radicals in human caused by lipid peroxidation reaction is very interesting. The antioxidants that can reduce such free radicals can be determined by β-carotene bleaching method. In this method, unsaturated fatty acid is used to give lipid peroxidased free radicals. The radicals can react and decolorize β-carotene. In the presence of antioxidants β-carotene bleaching will not occur. By the way, anti lipid peroxidation free radical can be monitored by measuring the degree of decolorization.

Table 4.9 The half-inhibition concentration (IC_{50}) values of the antioxidant activity measured using β–carotene bleaching method of standard BHT (μg/ml) and red yeast rice samples

Fermented Rice	**IC_{50} (mg/ml)**		
	Without soybean milk	**With soybean milk**	
	2 weeks	**2weeks**	**3 weeks**
BHT	0.54±0.01		
Comryr*	Not determined		
Oryza sativa L. cv. Mali105	0.23±0.01	0.24±0.01	0.18±0.01
Oryza sativa L. cv. Kam	0.17±0.01	0.10±0.00	0.11±0.01
Oryza sativa L. cv. RD6	0.18±0.00	0.09±0.01	0.09±0.00
Oryza sativa L. cv. SPT1	0.12±0.00	0.09±0.03	0.09±0.00

Comryr* Commercial Chinese red yeast rice

The results in Table 4.9 show that, in order to obtain IC_{50}, red yeast rice was prepared from glutinous rice, in which *Oryza sativa* L. cv. RD6 and *Oryza sativa* L. cv. SPT1 were more effective. The IC_{50} of *Oryza sativa* L. cv. RD6 and *Oryza sativa* L. cv. SPT1 extract was lower indicating that they gave higher potency levels. The addition of soybean milk during cultivation shows improved activity for glutinous rice. The result is concurrent to the antioxidant capacity in Table 4.8.

The rapid use of starch in reaching the second phase of growth in the case of glutinous rice seems to enhance the production of fungal secondary metabolites. The production of darker red pigment was concurrent to the increase of antioxidant potency. Many secondary metabolites are produced by fungi, among these are compounds derived from polyketides. The possible compounds are pigments and phenolic compounds most of which have antioxidant activity. Aniya *et al*. (2000) reported one of the secondary metabolites as an antioxidant of the mold, *Monascus anka* is a form of dimerumic acid. Dimerumic acid inhibited NADPH-and iron (II)-dependent lipid peroxidation of rat liver microsomes at 20 and 200 μM, respectively (Tiara *et al*., 2002).

To support the results obtained, the determination of the content of antioxidants such as carotenoids, phenolic compounds, flavonoids, alkaloids and polyketide derivatives should be done.

In conclusion, the highest antioxidant activity was obtained in red yeast rice of 3 weeks old from SPT1 and RD6 with the addition of soybean milk. This result is concurrent to the production of red pigments.

4.7 Volatile aroma compounds of red yeast rice prepared from Thai glutinous rice

In order to test for the sensory characteristic of red yeast rice products using different kinds of rice grains and different fermentation techniques, the products were simply sniffed without statistical method and odor description was compiled in Table 4.10 and Table 4.11

Table 4.10 Sensory characteristic of red yeast rice prepared from Thai glutinous rice without an addition of soybean milk (2weeks solid fermentation).

Fermented rice	Odor description
Oryza sativa L. cv. Mali105	Sweet odor with a little smell of fermented products such as alcohols and acids.
Oryza sativa L. cv. Kam	Odor of starch with a little smell of starch off-flavor
Oryza sativa L. cv. RD6	Mild odor or undetectable odor
Oryza sativa L. cv. SPT1	Mild fermented product odor

Table 4.11 Sensory characteristic of red yeast rice prepared from Thai glutinous rice with an addition of soybean milk (2weeks solid fermentation).

Fermented rice	Odor description
Oryza sativa L. cv. Mali105	Strong smell of fermented product especially alcoholic smell
Oryza sativa L. cv. Kam	Mild odor of alcohol or undetectable odor
Oryza sativa L. cv. RD6	Mild odor of alcohol or undetectable odor
Oryza sativa L. cv. SPT1	Mild odor of starch or starch off-flavor

Study on the aroma compounds present in red yeast rice is important to understand the contribution of these compounds to red yeast rice aroma. The solid phase micro-extraction method followed by the GC-MS analysis is one of the effective techniques for this purpose. The identification of volatile compounds was done by means of searching and matching the mass spectra of the compounds with the known reference compounds in the database library of volatile compounds. Chromatograms of volatile compounds in red yeast rice fermented without an addition of soybean milk were obtained as shown in Figure 4.7

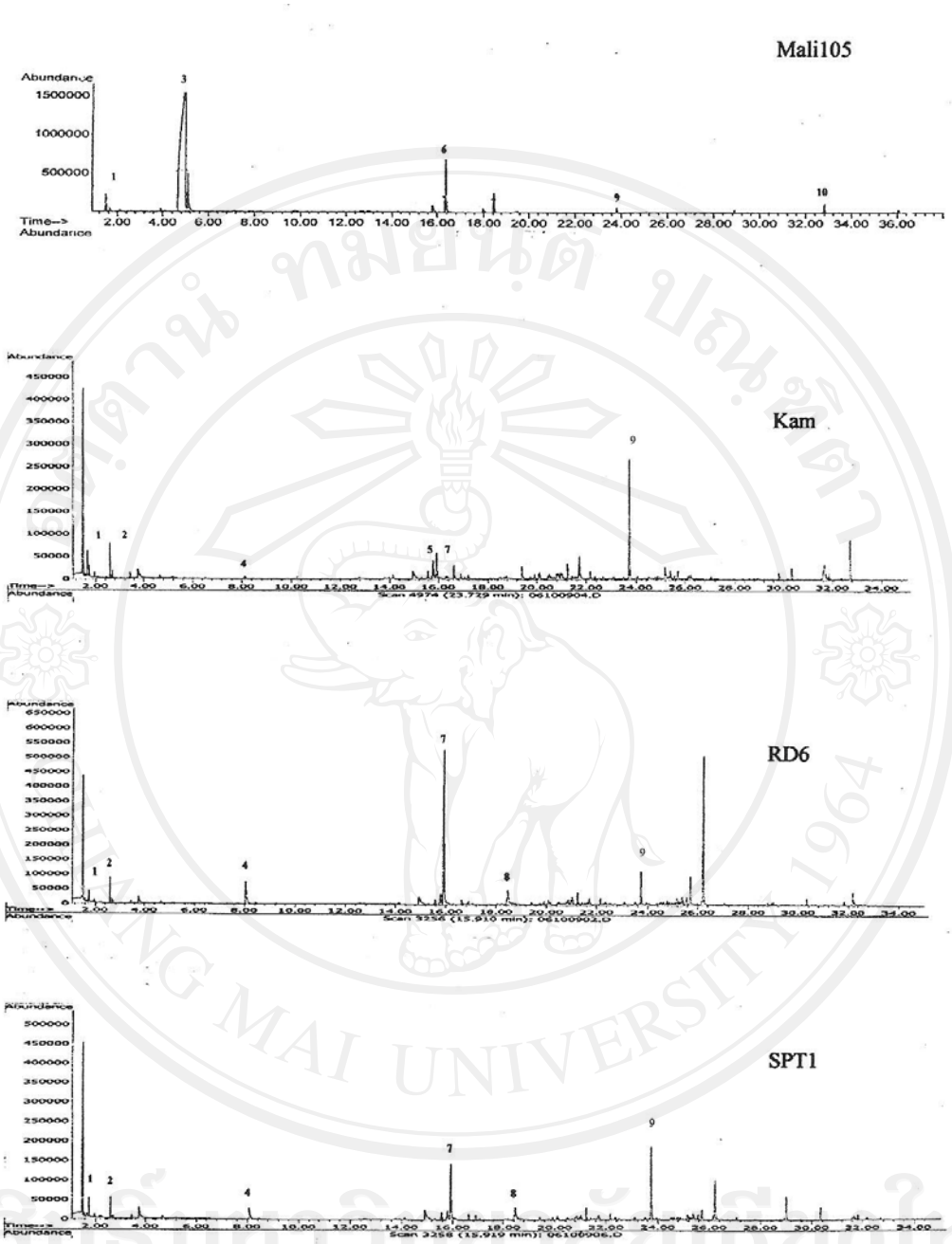


Figure 4.7 Chromatogram of volatile aroma compounds of red yeast rice fermented for 2 weeks without an addition of soybean milk.

The composition of volatile aroma compounds of red yeast rice using *Oryza sativa* L. cv. Mali105 which is a non-glutinous rice is significantly different from all glutinous rice. The chromatogram of *Oryza sativa* L. cv. Mali105 shows 20 peaks. Most identified compounds of *Oryza sativa* L. cv. Mali105 are alcohols. Glutinous rice; *Oryza sativa* L. cv. Kam, *Oryza sativa* L. cv. RD6 and *Oryza sativa* L. cv. SPT1 contain various groups of volatile compounds such as alcohols, aldehydes and methyl ketones. The compounds identified in both non-glutinous and glutinous red yeast rice are show in Table 4.12 with relative percentage of each compound.

Table 4.12 Volatile compounds of red yeast rice without an addition of soybean milk.

Peak number	Compounds	Relative percentage (%)			
		Oryza sativa L. cv. Mali105	*Oryza sativa* L. cv. Kam	*Oryza sativa* L. cv. RD6	*Oryza sativa* L. cv. SPT1
1	Ethanol	0.42	3.44	1.42	3.13
2	Isopentanal	ND	4.50	3.64	3.28
3	2,3-Butanediol	71.40	ND	ND	ND
4	2-Heptanone	ND	ND	7.54	3.69
5	4-Ethylresorcinol	ND	4.58	ND	ND
6	Phenethyl alcohol	5.84	ND	ND	ND
7	2-Nonanone	ND	5.58	34.51	13.79
8	Octanoic acid	ND	ND	6.12	4.72
9	Isoamyl benzoate	0.48	23.87	4.22	16.07
10	Ethyl palmitate	0.93	ND	ND	ND

ND = not detectable

The addition of soybean milk for red yeast rice production gave different results concerning to the volatile aroma compounds composition as shown in Figure 4.8.

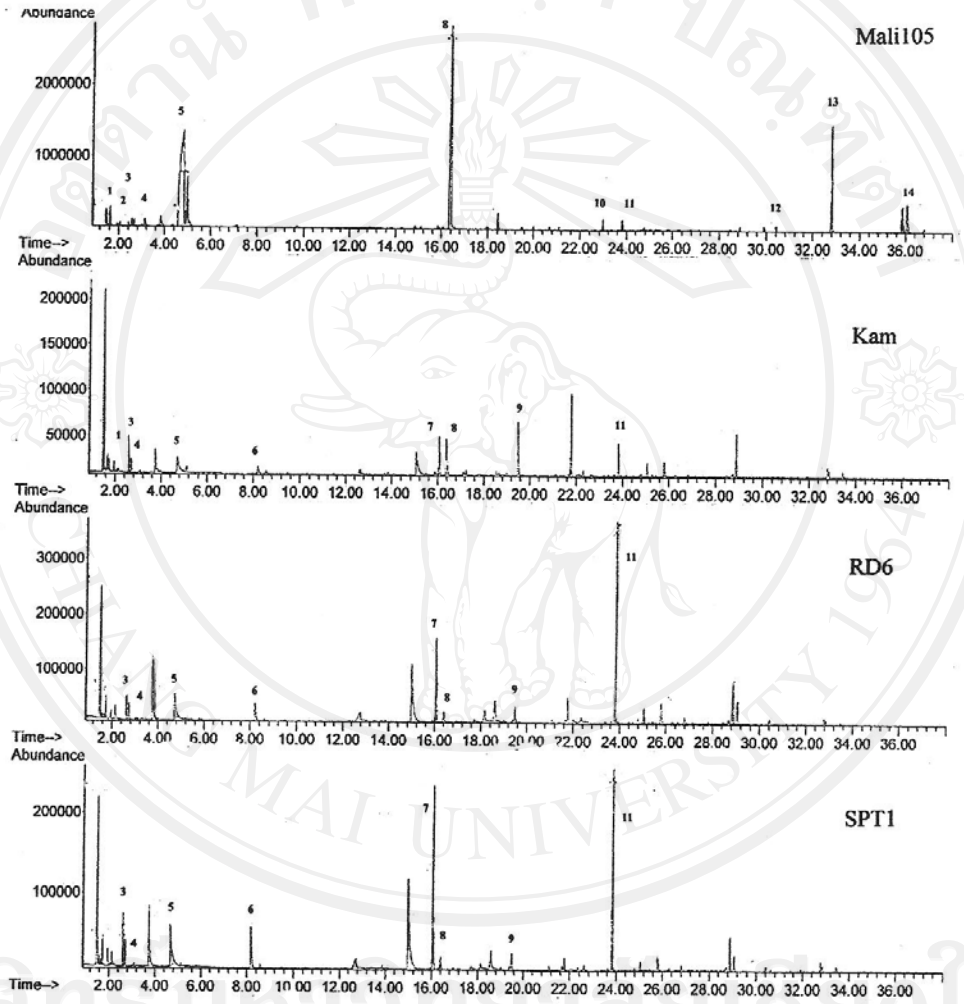


Figure 4.8 Chromatogram of volatile aroma compounds of red yeast rice fermented for 2 weeks with an addition of soybean milk

Thirteen compounds were identified and one compound was tentatively identified in all red yeast rice. These compounds and their relative percentage are shown in Table 4.13

Table 4.13 Volatile compounds of red yeast rice with an addition of soybean milk

Peak number	Compounds	Relative percentage (%)			
		Oryza sativa L. cv. Mali105	*Oryza sativa* L. cv. Kam	*Oryza sativa* L. cv. RD6	*Oryza sativa* L. cv. SPT1
1	Ethanol	1.90	2.07	ND	ND
2	Isobutanol	0.34	ND	ND	ND
3	Isopentanal	0.84	4.71	1.78	3.35
4	2-Methylbutanal	0.60	2.72	1.35	1.90
5	2,3-Butanediol	39.0	ND	5.96	7.55
6	2-Heptanone	ND	ND	2.75	5.19
7	2-Nonanone	ND	7.14	9.42	17.66
8	Phenethyl alcohol	28.30	8.19	1.60	ND
9	5-Butyldihydro-4-methyl-2 (3H)-furanone	ND	9.44	2.05	1.82
10	Phenethyl hexanonate	0.77	ND	ND	ND
11	Isoamyl benzoate (tentative)	0.82	5.38	18.97	15.98
12	Isopropyl myristate	0.36	ND	ND	ND
13	Ethyl palmitate	8.31	ND	ND	ND
14	Ethyl oleate	4.40	ND	ND	ND

ND = not detectable

The comparison of chromatographic profiles and the composition of volatile aroma compounds between different red yeast rice products shows the different results of glutinous rice to non-glutinous rice. Mali 105 has more alcohols and esters while Kam rice, RD6 rice and SPT1 rice shows the presence of other fermentation products such as methyl ketones. Among these compounds, 2, 3-butanediol is dominant in Mali105. 2, 3-Butanediol as a normal constituent of wine is the second most abundant, thus representing an important potential source of aroma. Due to its very high threshold value about 150 mg/l (Shinohara, 1984; Dubois, 1994), 2, 3-butanediol is generally not expected to affect the sensory qualities of alcoholic beverages appreciably. Another important consideration is that the high and low levels of production of acetoin (a pleasant buttery odor compound) and 2, 3-butanediol is exhibited constantly by each species with an inverse pattern; production of these compounds could, therefore, be used as a discriminating characteristic among the different species of yeast. Yeasts that are high producers of 2, 3-butanediol are always low producers of acetoin, and vice versa. Thus, *S. cerevisiae* is generally characterized by high production of 2, 3-butanediol and low production of acetoin, whereas *K. apiculata* and *Sc. ludwigii* are always low butanediol and high acetoin-producers. Only a few strains of *S. cerevisiae* were found to be able to accumulate abnormally high quantities of acetoin (Romano and Suzzi, 1993) and low amounts of 2, 3-butanediol (Romano *et al.*, 1996b). The structure of 2, 3-butanediol and acetoin are shown in Figure 4.9. In contrast, aroma constituents of normal rice without fermentation are shown to contain fewer alcohols as reported by Bullard and Holguin (1977) in Table 4.14.

Table 4.14 Compounds associated with volatile aroma of unprocessed (raw) rice[a]

Acetaldehyde	*o*-xylene	2-nonanone
Acetone	2-acetylfuran	nonanal
2-metrypropanal	a-pinene	2,4-octadienal
1-propanal	*n*-propylbenzene	1,2-dimetryl-3-ethylbenzene
2-buanone	1-ethyl-4-methylbenzene	1,2,4,5-tatramethylbenzene
butanal	benzaldehyde	2-nonenal
3-methylbutanal	1,3,5-trimethylbenzene	1,2,3,4-tetramethylbenzene
2-methylbutanal	6-methyl-5-hepten-2-one	decanone
Benzene	2-octanone	naphthalene
3-penten-2-one	2-*n*-penthylfuran	decanal
2-pentanone	octanal	2,4-nonadienal
pentanal	1,2,4-trimethylbenzene	2-decenal
2,5-dimethylfuran	2,4-heptadienal	2-undecanone
2-methylpentanal	δ-carene	2-methylnaphthalene
Toluene	1,2,3-trimethylbenzene	2,4-decadienal
2-hexanone	*p*-diethylbenzene	2-dodecanone
ethylbenzene	2-octenal	2-ethylnaphthalene
p-xylene	*m*-diethylbenzene	2-dodecenal
m-xylene	1,3-dimethyl-5-ethylbenzene	18 unidentified ketone
2-heptanone	*o*-diethylbenzene	8 unidentified alkylbenzenes
Heptanal	*p*-methylbenzaldehyde	3 unidentified aldehydes
2-butylfuran	1,3-dimethyl-4-ethylbenzene	2 unidentified furans
2,4-hexadienal	1,4-dimethyl-2-ethylbenzene	

[a] Bullard and Holguin (1997).

(a) (b)

Figure 4.9 The structure of (a) 2, 3-butanediol and (b) acetoin

The methyl ketones appear in red yeast rice from glutinous rice. These compounds may be formed as intermediates of fatty acids pathway. *Monascus purpureus* converts short-chain fatty acids to methyl ketones. The regulation of the metabolic pathway is similar to that found in *Penicillium roquefortii*. There are differences in the actual amount of precursors metabolized. The fermentation of fatty acid mixtures led to methyl ketone mixtures. The metabolism of each fatty acid was dependent on the precursor composition (Kranz *et al.*, 2004).

In case of addition of soybean milk, there are more complexities of aroma composition. phenethyl alcohol and isoamyl benzoate become dominant in Mali 105 and glutinous rice respectively. The formation of aromatic alcohol and aromatic acid may be initiated from an amino acid, phenylalanine. Stark *et al*. (2002) described the extractive bioconversion of 2-phenylethanol from l-phenylalanine. l-phenylalanine was converted to 2-phenylethanol by *Saccharomyces cerevisiae* in fed-batch culture. The step of benzoic acid formation from phenylalanine was proposed by Bergstrom *et al*. (1995). Other acid moieties of esters formed may be derived from fatty acids. Theses compounds, all together, can contribute to the fermented product and alcoholic aroma.

4.8 Mycotoxin (citrinin) in red yeast rice

The results obtained by detecting at 345 nm shows . The chromatograms of 3 weeks period of fermentation with and without the addition of soybean milk using non-glutinous rice, *Oryza sativa* L. cv. Mali105, glutinous rice; *Oryza sativa* L. cv. Kam, *Oryza sativa* L. cv. RD6 and *Oryza sativa* L. cv. SPT1 are shown in Figure 4.10.

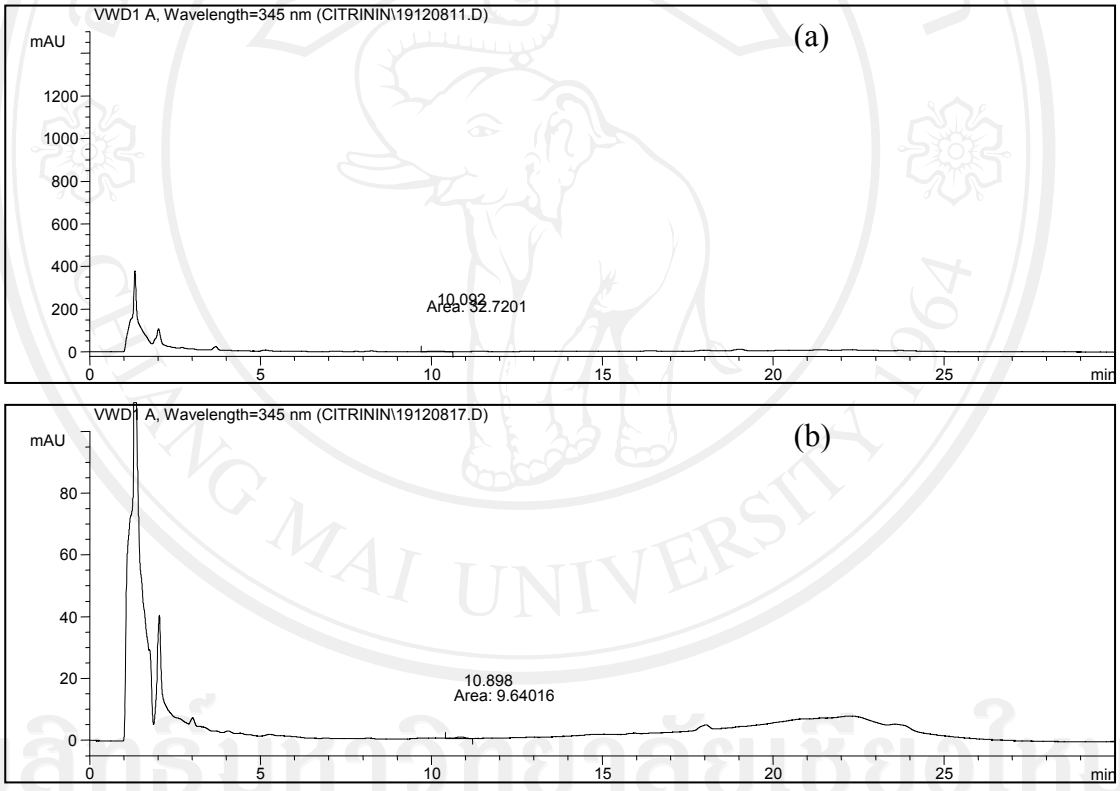


Figure 4.10 Chromatographic chemical profiling of citrinin in fermented red yeast rice from non-glutinous rice and glutinous rice, (continued)

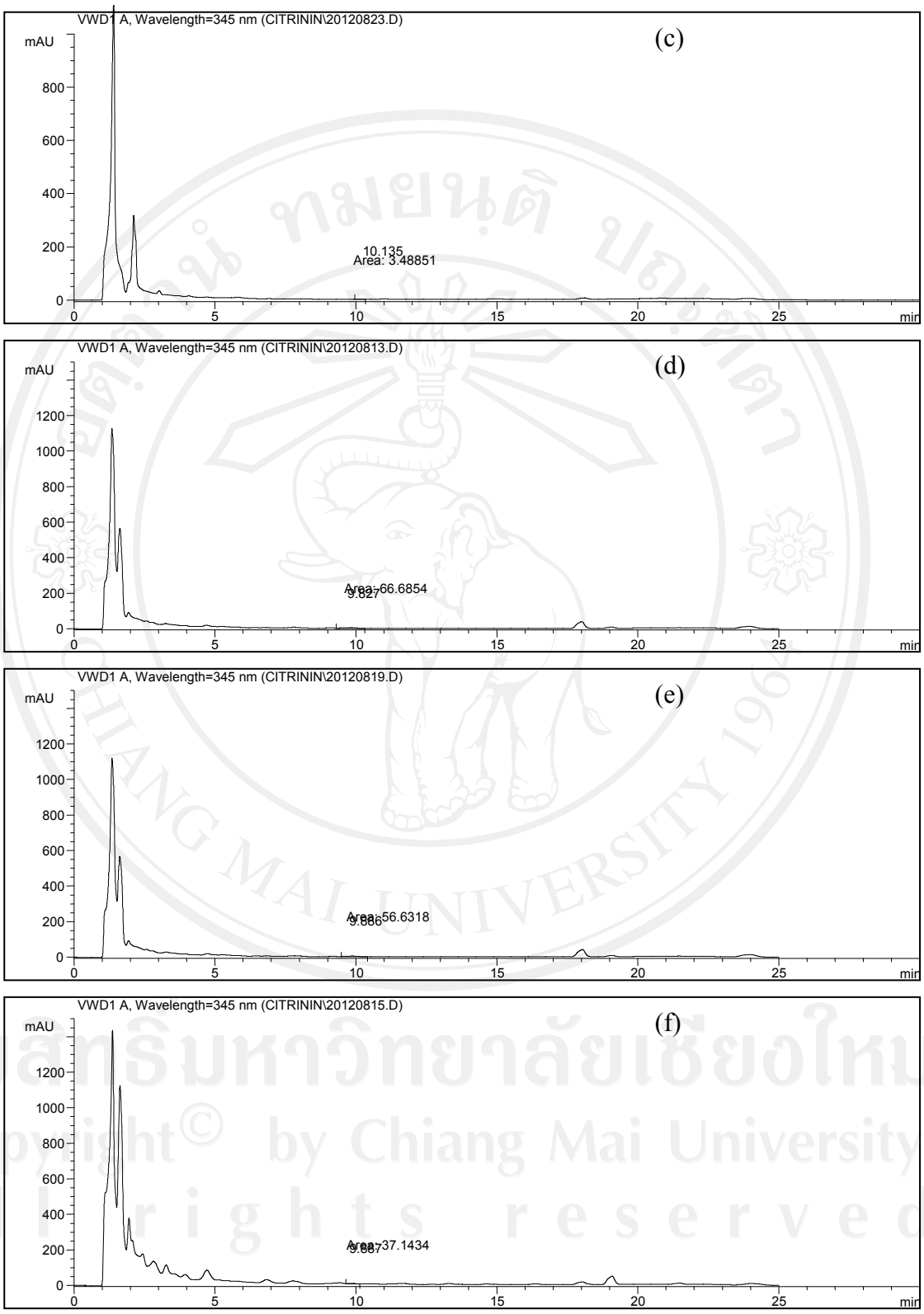


Figure 4.10 Chromatographic chemical profiling of citrinin in fermented red yeast rice from non-glutinous rice and glutinous rice, (continued)

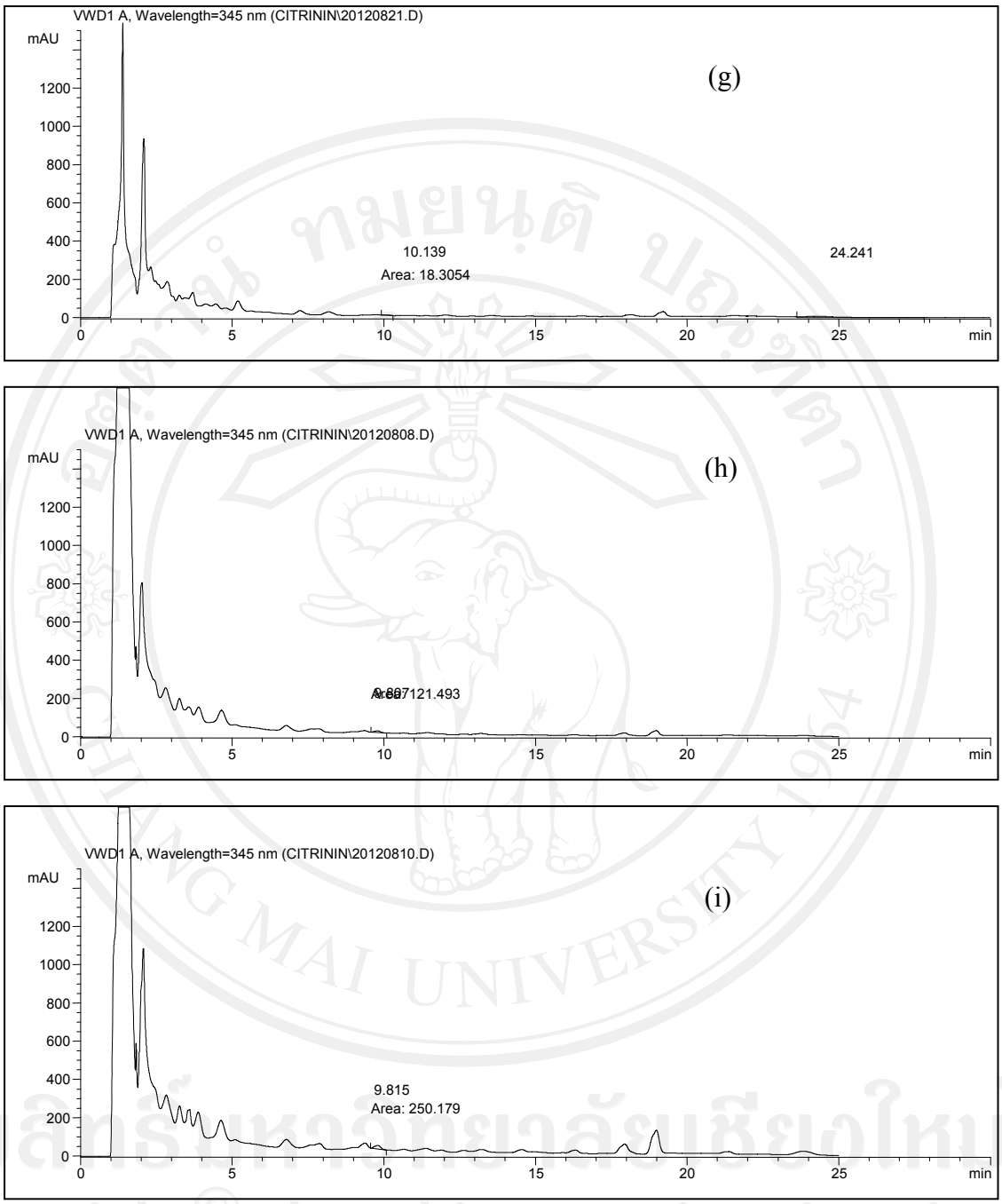


Figure 4.10 Chromatographic chemical profiling of citrinin in fermented red yeast rice from non-glutinous rice and glutinous rice, (a) commercial Chinese red yeast rice (Comryr), (b) and (c) *Oryza sativa* L. cv. Mali105 without and with soybean milk, (d) and (e) *Oryza sativa* L. cv. Kam without and with soybean milk, (f) and (g) *Oryza sativa* L. cv. RD6 without and with soybean milk, (h) and (i) *Oryza sativa* L. cv. SPT1 without and with soybean milk

Table 4.15 Citrinin contents in red yeast rice fermented without soybean milk

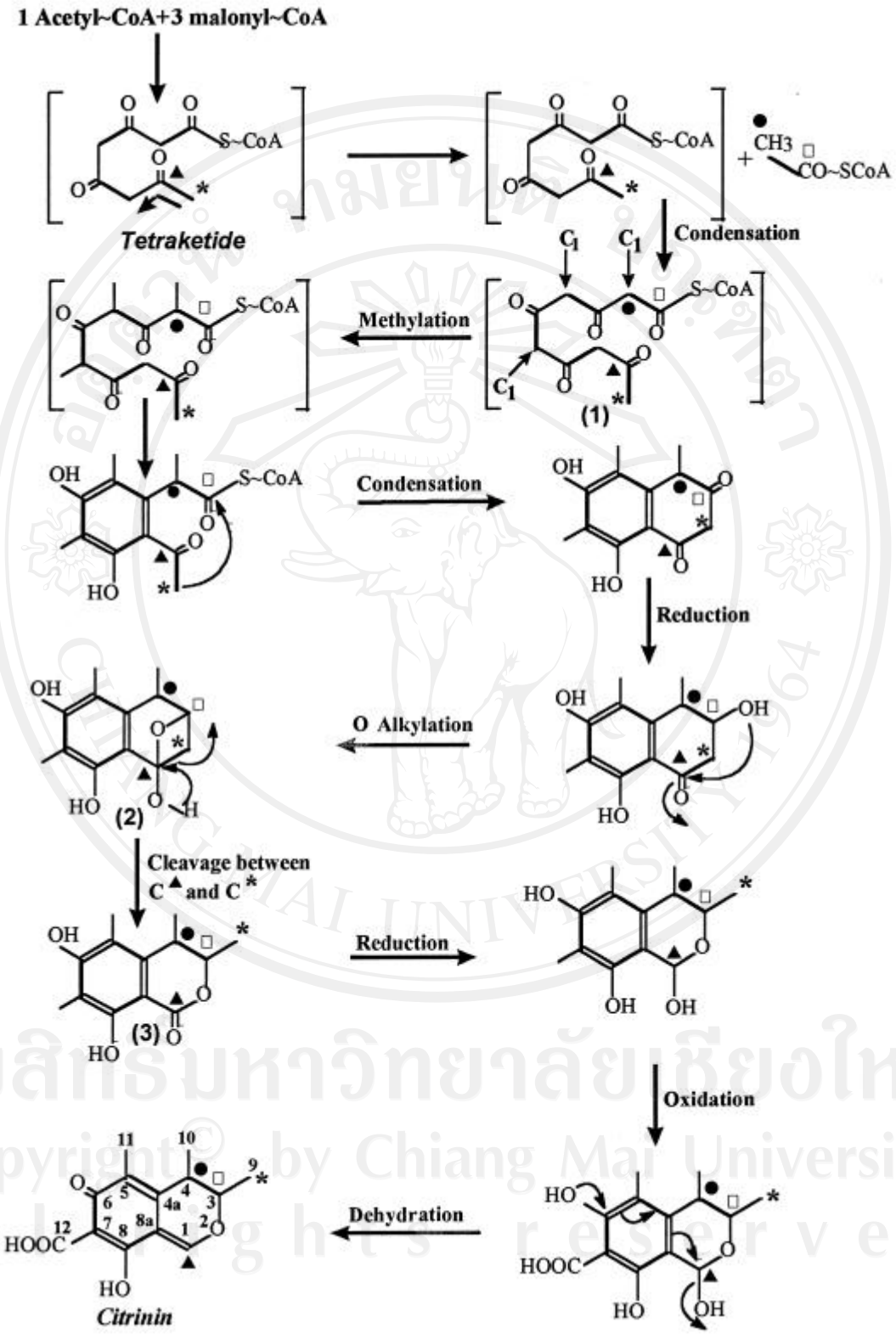
Fermented rice sample	2 weeks			3 weeks		
	Peak Area	ppb	mg/g of sample	Peak area	ppb	mg/g of sample
Comryr*	-	-	-	21.71	2524.42	0.002
Oryza sativa L. cv. Mali105	5.23	608.14	0.001	9.84	1136.25	0.001
Oryza sativa L. cv. Kam	78.13	9084.88	0.009	82.74	9620.93	0.009
Oryza sativa L. cv. RD6	115.37	13415.12	0.013	67.62	7862.62	0.007
Oryza sativa L. cv. SPT1	338.09	39312.20	0.039	123.16	14221.71	0.014

Comryr* Commercial Chinese red yeast rice

Table 4.16 Citrinin contents in red yeast rice fermented with soybean milk

Fermented rice sample	2 weeks			3 weeks		
	Peak area	ppb	mg/g of sample	Peak area	ppb	mg/g of sample
Oryza sativa L. cv. Mali105	6.00	697.67	0.0007	3.49	405.81	0.0004
Oryza sativa L. cv. Kam	55.30	6430.23	0.006	48.86	5681.39	0.005
Oryza sativa L. cv. RD6	89.05	10282.91	0.010	37.60	4372.09	0.004
Oryza sativa L. cv. SPT1	428.72	49851.16	0.049	251.29	29220.81	0.029

The results in Table 4.15, 4.16 shows that, among the glutinous rice the citrinin content of RD6 at 3 weeks with an addition of soybean milk was the lowest (0.004 mg/g). The addition of soybean milk during cultivation in most of the rice shows decreaseing of citrinin content for glutinous rice. The normal rice, Mali 105 with an addition of soybean milk at 3 weeks shows lower citrinin concentration of 0.0004 mg/g. Citrinin is also the secondary metabolites which are formed after maximum use of nutrients in normal growth. The rice that reaches the stage faster may start to produce citrinin earlier. The polyketide pathway is the major route for the formation of secondary metabolites (Chandler *et al*., 1992) including various mycotoxins (Simpson, 1986) in most of the filamentous fungi. Citrinin is a typical toxin also isolated in *M. ruber* (Blanc *et al,* 1995a). It is known that in *Aspergillus*, citrinin is formed by the condensation of one acetyl coenzyme A (acetyl-CoA) molecule with four malonyl-CoA molecules, followed by the addition of three methyl units as shown below.



Source : Hajjaj *et al*. (1999)

The possibility to decrease the amount of citrinin is to use peroxidase enzyme in the presence of lipids, especially, fatty acids. The amount of citrinin can be blocked by using peroxidase enzyme (Blanc *et al*., 1998). This result agree with the work of Hajjaj *et al*. (2000a) who studied the effect of 6-8 carbons fatty acids to the formation of citrinin in *Monascus*. The studied was carried out by liquid fermentation with 5.20 g/l of glucose, 5 g/l glutamate adding with octanoic acid which was the precursor of red pigment synthesis via polyketide synthesis pathway. The fatty acid increased the red pigment for 30-40 % while the amount of citrinin was decreased. The degradation of the newly synthesized citrinin (or an intermediate in the citrinin pathway) is done by hydrogen peroxide resulting from peroxisome proliferation induced by medium-chain fatty acids or methylketones. The results in this work showed the tentative decrease of citrinin when adding soybean milk. This may be due to the presence of lipids in soybean milk as well as the peroxidase activity.