CHAPTER 3

RESULTS

3.1 Isolation of Limonin

Limonin from lime seeds is crystallized powder with bitter taste and pale yellowish white color, which gave a yield around 755.03 $\pm$ 32.67 mg/kg, purity of 90.19 $\pm$ 2.14% (86-92%) compared to the standard limonin.

Table 4 Limonin recovery from lime seeds extraction

Tests	Used lime seeds (Kg)	Extracted limonin (g)	Limonin Recovery (mg/Kg)	Recovery percentage
1	1.20	0.86	716.67	0.07
2	1.20	0.89	741.67	0.07
3	1.10	0.81	736.36	0.07
4	1.00	0.79	790.00	0.08
5	1.10	0.82	745.45	0.07
6	1.20	0.96	800.00	0.08
Av	1.13	0.86	755.03	0.08
SD	0.08	0.06	32.67	0.00

3.2 Identifications of Limonin

The TLC chromatogram of purified limonin in eluents of benzene, ethanol, water and acetic acid (200:47:15:1 v/v/v/v, upper phase) was a single spot with relative *Rf* values of 0.43 (compared to standard limonin with relative *Rf* values of 0.43), and was in agreement with that suggested by Hsu,*et al* (1973). But the TLC chromatogram of purified limonin in the other eluents of benzene, ethanol, water, acetic acid, and isopropanol (185 : 47 : 30 : 1 : 15 v/v/v/v/v, upper phase) was single spot with relative *Rf* values of 0.55 (compared to standard limonin with relative *Rf* values of 0.56)

The ^{1}H-NMR spectral data of limonin in Table 5 were in agreement

with that suggested by Dreyer (1965) and Bennett and Hasegawa (1981). They started from the high field end of the spectrum:

Table 5 ^{1}H-NMR spectral data of limonin (500 MHz in $CDCl_3$)

Proton No.	Chemical Shift, δ (ppm)	
	Standard limonin	Purified limonin extracted from lime seeds
1	4.306(1H.s)	4.272(1H.s)
2	1.003-2.227(2H,m)	1.973-2.026(2H,m)
3		
4		
5	3.371(1H,d,j=5)	3.191(1H,s)
6	1.939(2H,d,j=5)	1.857-1.909(2H,m)
7		
8		
9	2.711-2.772(1H,m)	2.382-2.575(1H,m)
10		
11	2.850-2.991(2H,m)	2.712-2.850(2H,m)
12	2.251-2.405(2H,m)	2.041-2.066(2H,m)
13		
14		
15	4.662(1H,s)	4.083(1H,s)
16		
17	5.238(1H,s)	5.528(1H,s)
18	1.093(3H,s)	1.092(3H,s)
19	4.513(1H,d,j=13))	4.639(1H,dd,j=5, 12)
20		
21	6.504(1H,s)	6.506(1H,dd,j=2, 5)
22	7.377(1H,s)	7.567(1H,s)
23	7.552(1H,s)	7.629(1H,s)
24	1.078(3H,s)	1.124(3H,s)
25	1.325(3H,s)	1.204(3H,s)
26	1.332(3H,s)	1.231(3H,s)

The FT-IR spectral exhibited a single broadened, high intensity, carbonyl absorption band at 1756 (C=O stretching vibration for saturated ester, lactone) and 1709 cm^{-1} (C=O stretching vibration for saturated six membered ring ketone), and characteristic bands assigned to a furan ring at 1502 and 883 cm^{-1}(Dreyer, 1965 and 1966), 1273 and 1028 cm^{-1} (C-O stretching vibration for epoxy ring)

The optical rotation of limonin and standard limonin were identical in profile and exhibited a negative occurring at 130^{o} (Acetone; c 1.01).

The ES-MS gave a molecular ion peak at m/z 471 consistent with the molecular formula $C_{26}H_{30}O_8$, which implied five degrees of unsaturation.

The DSC spectrum showed that extracted limonin from lime seeds could be melted at a temperature of 298°C.

Limonin was insoluble in water, but was soluble in organic solvents, such as alcohol, chloroform, acetonitrile and acetone, and its aqueous solution has a pH of 5.8±0.75 (n=3) (Table 6).

Table 6 Approximate solubility of extracted limonin from lime seeds in pure solvents

Solvents	Approximate solubility (mg/ml)	pH (n = 3)
Water	<0.001	5.8±0.75
Ethyl alcohol	0.05	-
Acetone	1	-
Acetonitrile	1	-
Chloroform	0.5	-
Glycerin	<0.002	6.21±0.68

3.3 Quantitative Analysis of Limonin

The peak of HPLC chromatogram was performed at 9-10 minutes for each run.

The data of standard limonin concentration of 5 to 25 ppm and area under the curve of HPLC chromatogram was obtained. Then the calibration curve of standard limonin was constructed as shown in Figure 5 with the slope of the straight line of 15,681. The validation of HPLC-chromatogram had been done both intra-day and inter-day, as shown in Figure 6 and 7 respectively

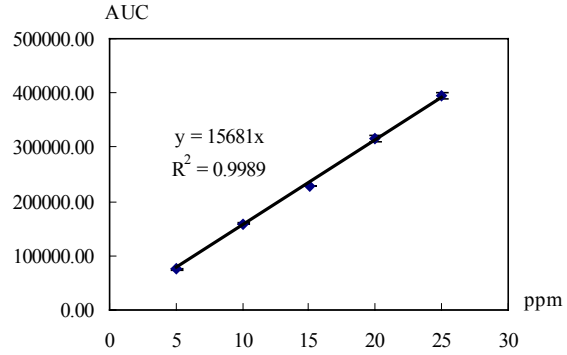

Figure 5 Standard curve of limonin (n = 4)

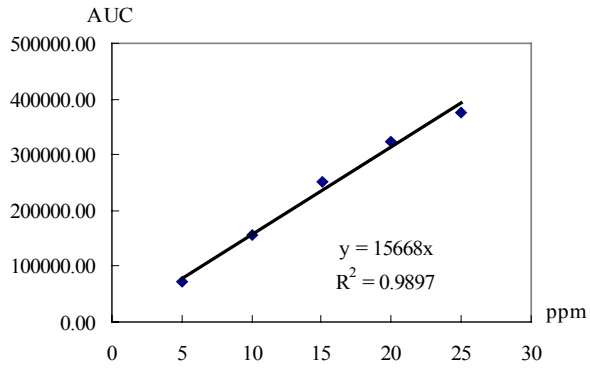

Figure 6 Intra-day validation curve of various concentrations of standard limonin (4 times in a day)

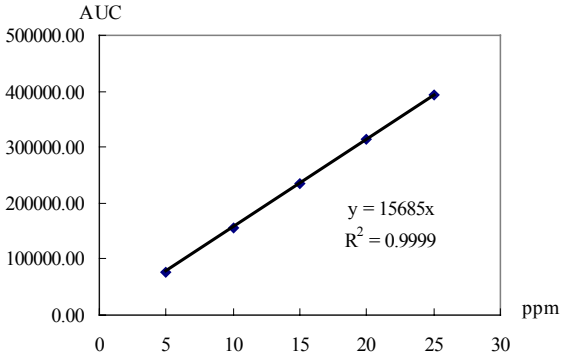

Figure 7 Inter-day validation curve of various concentrations of standard limonin (Once a day for 4 consecutive days)

3.4 Stability of Limonin from Lime Seeds in Aqueous Solution

Limonin was not stable in a basic solution of high pH. The remaining concentration of limonin could not be measured in buffered solution of pH 10, 11, and 12, but limonin still remained in buffered solution of pH 2-9. The percentage of the remaining concentration of limonin ($\%D/D_0$) was calculated (where D_0 was the remaining concentration of limonin or D at time zero). Then the graphs between the percentages of the remaining concentration of limonin ($\%D/D_0$) and times in various pH and temperatures were made. They were all curved lines as shown in Figures 8, 10, and 12. On the other hand the graphs were plotted between natural logarithm value of percentage of remaining concentration of limonin and time in various pH and temperatures; they were all straight lines as shown in Figure 9, 11, and 13. So it was assumed that degradation of limonin in various pH-buffered solutions was the apparent first order reaction. The slopes of all straight lines in various pH and temperatures from figures 9, 11, and 13 were collected in Table 7. These slopes were reaction rate constants in various pH and at different temperatures. From Table 7, the graph between the reaction rate constants and pH in various temperatures were plotted as shown in Figure 14. The obtained curves were called pH-rate profiles; they showed that limonin was most stable at pH 5-7, whose reaction rate constant was the lowest. Moreover the higher temperature of 80^{o}C more affected the stability of limonin rather than the lower temperatures of 70^{o}C and 45^{o}C respectively. When the data from Table 7 were plotted between the logarithmic form of reaction rate constant and the reciprocal of absolute temperature in various pH, the straight lines were obtained and called Arrhenius plots as shown in Figure 15. The slopes from Arrhenius plots then were calculated to activation energy as shown in Table 8.

The Arrhenius plot of log $k_{observed}$ against the reciprocal of the absolute temperature at various pH was made and the slopes of various pH were obtained. Then Arrhenius activation energies of various pH were calculated by equation Ea = slope x 2.303 x gas constant where gas constant =

1.987 cal/^{o}K-mole as shown in Table 8. In the study, the pH which was most changed by the loss of limonin was 9.

The analysis of variance indicated that limonin was significantly affected both by temperature and by pH (P<0.05), as well as by the time of storage in different environments. Moreover it was found that pH; temperature and time co-affected the remaining concentrations of limonin by all 2-ways ANOVA with 0.05 confidence levels (see Appendix 1, Table 1A).

The degradation of limonin at 45^{o}C in aqueous pH 5 buffered solutions took a period of 7200 minutes for 99.98% limonin to reduce to 22.16% limonin remaining concentration, whereas limonin in aqueous pH 7 and 6 buffered solutions took a period of 5760 minutes for 100% limonin to reduce to 23.77% and 22.01% limonin concentrations respectively. In addition the degradation of limonin in aqueous pH 4 buffered solutions took a period of 1440 minutes, whereas limonin in aqueous pH 3, 2, 8, and 9 buffered solutions took a period of 300 minutes or less for 100% limonin to reduce to 15-23% limonin concentrations.

The degradation of limonin at 70^{o}C and 80^{o}C in aqueous pH 5 buffered solution also took a period of 7200 minutes, but the limonin concentrations were reduced from 100.01% to 15.15% and 11.28% remaining concentration respectively, whereas limonin in aqueous pH 7 and 6 buffered solutions took a period of 5760 minutes for 100% limonin to reduce to 14.39% and 11.54% limonin concentration respectively at 70^{o}C, and reduced to 10.70% and 9.97% limonin concentration respectively at 80^{o}C. The degradation of limonin in aqueous pH 4 buffered solutions took a period of 1440 minutes, whereas limonin in aqueous pH 3, 2, 8, and 9 buffered solutions took a period of 300 minutes or less and the limonin concentrations were reduced from 100% to 7-13%.

Thus it was assumed that the degradation of limonin in an aqueous solution followed the apparent first order reaction whether in any pH and at any temperature. It was noticed that degradation of limonin in various pH buffered solutions at any temperature could be divided into 3 groups, group I

was the degradation in pH 5, 7 and 6, of which reaction rate constants were less than $10x10^{-4}min^{-1}$. The degradation of limonin with a reaction rate constant more than $10x10^{-4}$ min^{-1} but less than $40x10^{-4}$ min^{-1} was found in group II, which was limonin in pH 4 buffered solutions. And group III was the degradation of limonin in aqueous buffered solutions of pH 3, 2, 8 and 9, which the reaction rate constants were more than $40x10^{-4}$ min^{-1}, and were determined as extreme pH effects.

In conclusion, limonin was completely degraded in pH 10 to 12 aqueous solutions. Its degradation follows the apparent first order reaction; it was most stable in pH 5 with the reaction rate constant of 2.0636×10^{-4} min^{-1}, at $45^{o}C$ with 70%RH, and activation energy of 2.45 Kcal/mole.

In a very high pH buffered solution (pH 10-12), limonin was destroyed, but in the acid range $k_{observed}$ (the observed rate constant) could be evaluated as $\log k_{observed} = \log k_1 + pH$, which k_1 or k_H could be obtained from the intercept value of the plot of $\log k_{observed}$ against pH, where k_0 was the average reaction-rate constant in the acid region. So does the alkaline region, $\log k_{observed}$ is equal to $\log k_2 + p(OH^-)$ or $\log k_{observed} = \log k_2 - 14 + pH$, which k_2 or k_{OH} could be obtained from the intercept of the plot of $\log k_{observed}$ against pH as well, where k_o was the average reaction-rate in the alkaline region. Therefore $k_{observed} = k_o + k_H[H]$ in acid region, and $k_{observed} = k_o + k_{OH}[OH]$ in the alkaline region are shown in Table 9, which could be used for calculating the reaction rate constant in any pH in various temperatures.

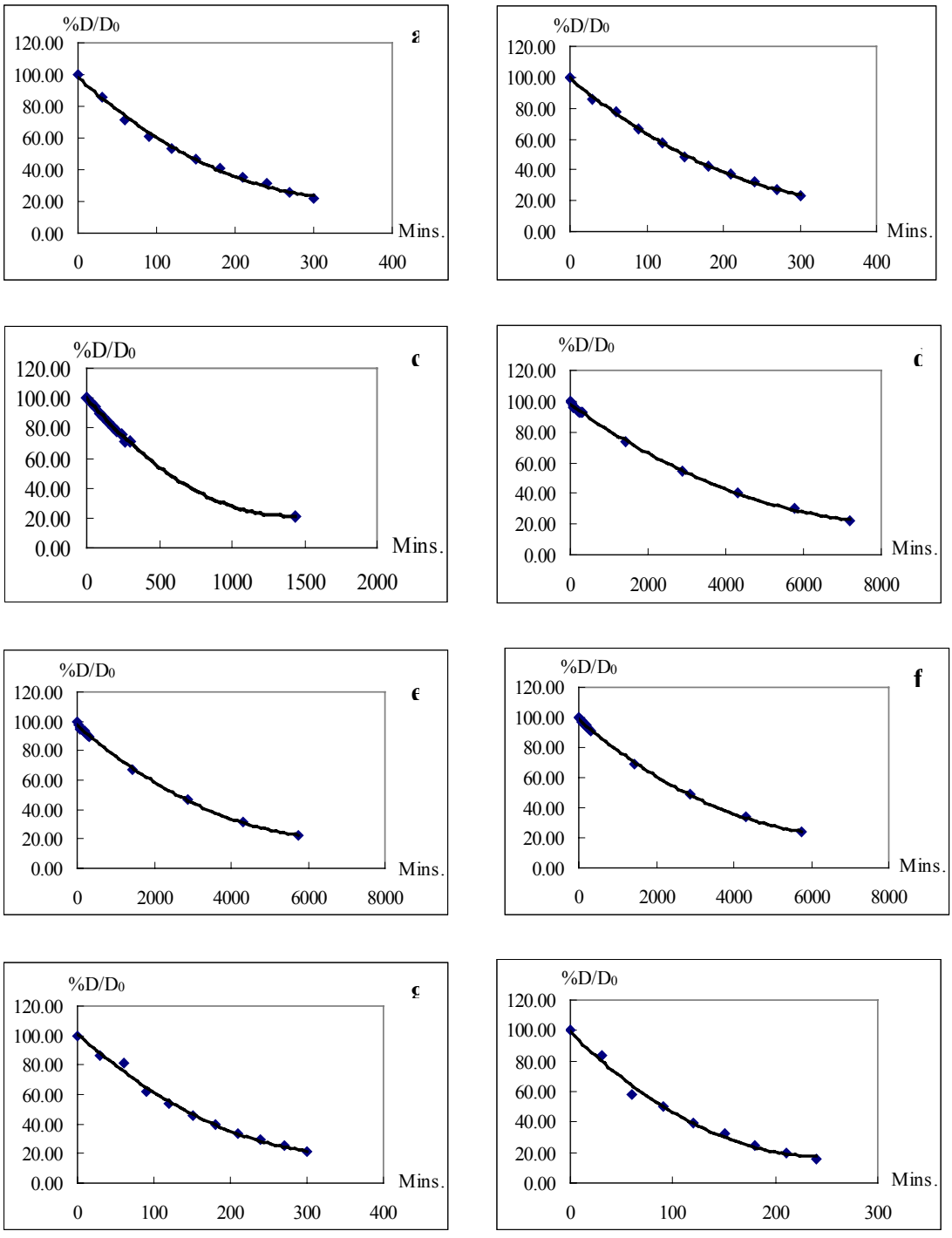

Figure 8 Degradation curve of limonin in buffered solution at 45°C, 70%RH, plotted between the percentage of the remaining concentration of limonin and time, (a) pH 2, (b) pH 3, (c) pH 4, (d) pH 5, (e) pH 6, (f) pH 7, (g) pH 8, and (h) pH 9 (n = 3)

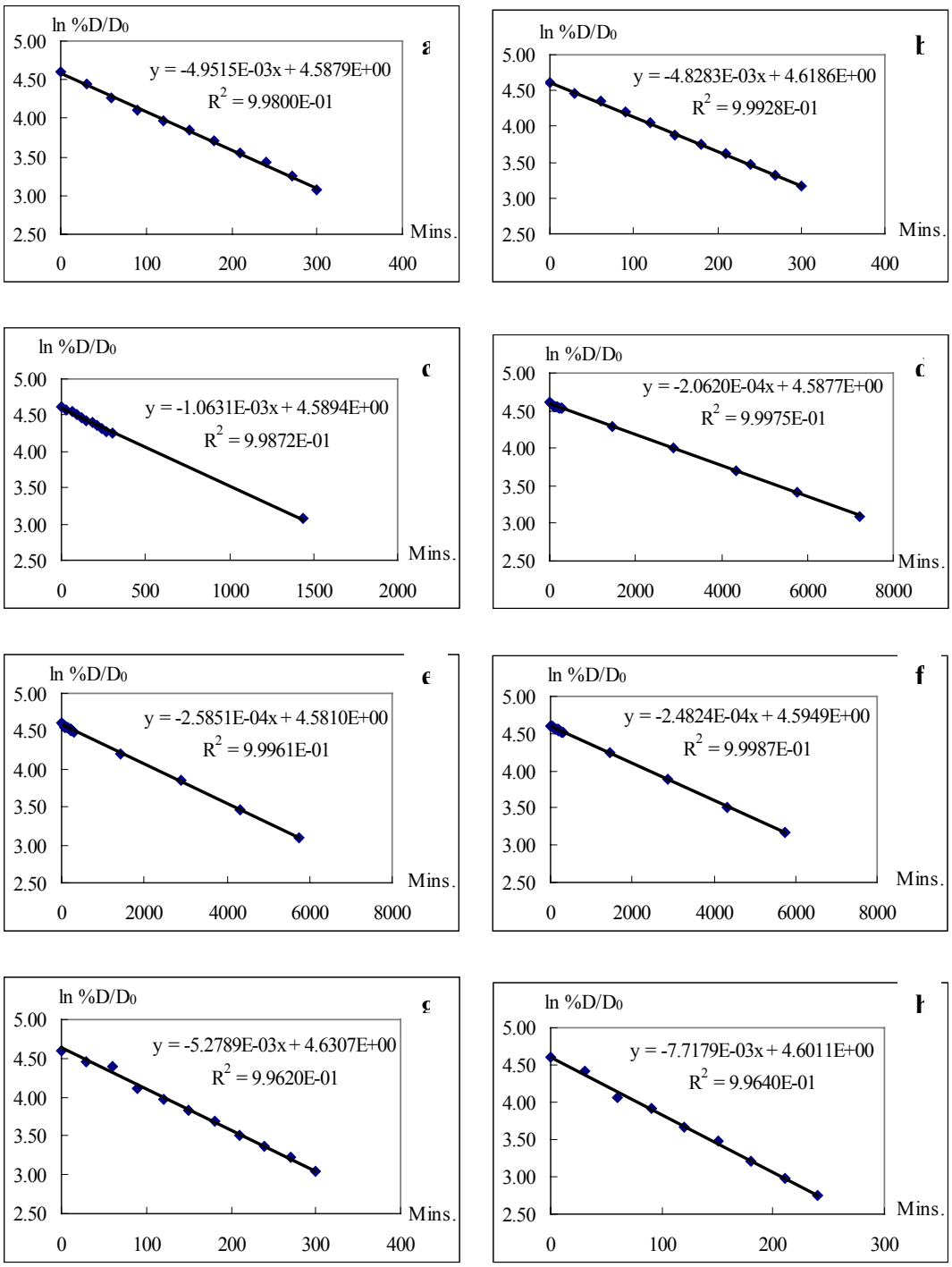

Figure 9 Degradation curve of limonin in buffered solution at 45°C, 70%RH, plotted between the natural logarithmic form of the percentage of the remaining concentration of limonin and time, (a) pH 2, (b) pH 3, (c) pH 4, (d) pH 5, (e) pH 6, (f) pH 7, (g) pH 8, and (h) pH 9 (n = 3)

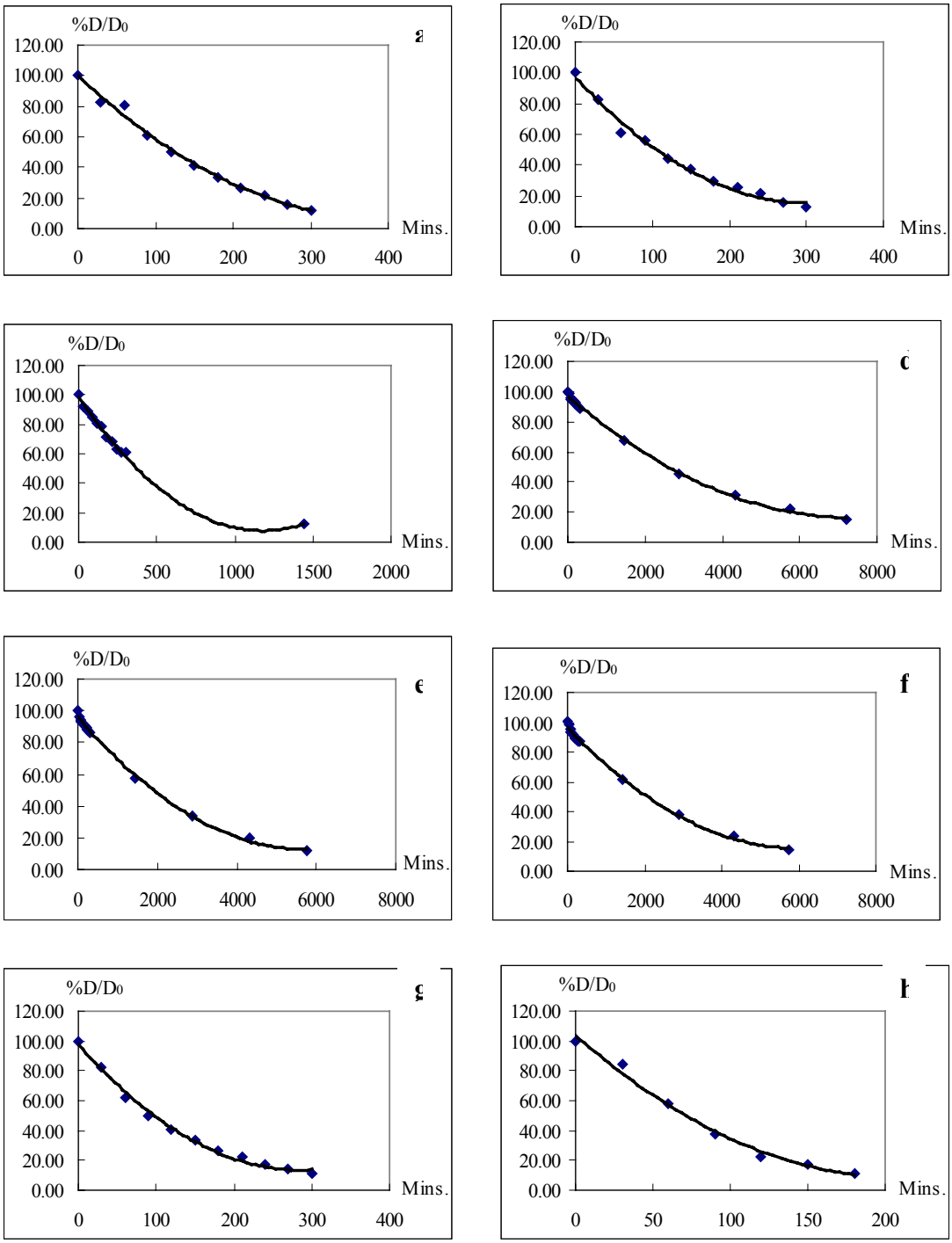

Figure 10 Degradation curve of limonin in buffered solution at 70°C, 70%RH, plotted between the percentage of the remaining concentration of limonin and time, (a) pH 2, (b) pH 3, (c) pH 4, (d) pH 5, (e) pH 6, (f) pH 7, (g) pH 8, and (h) pH 9 (n = 3)

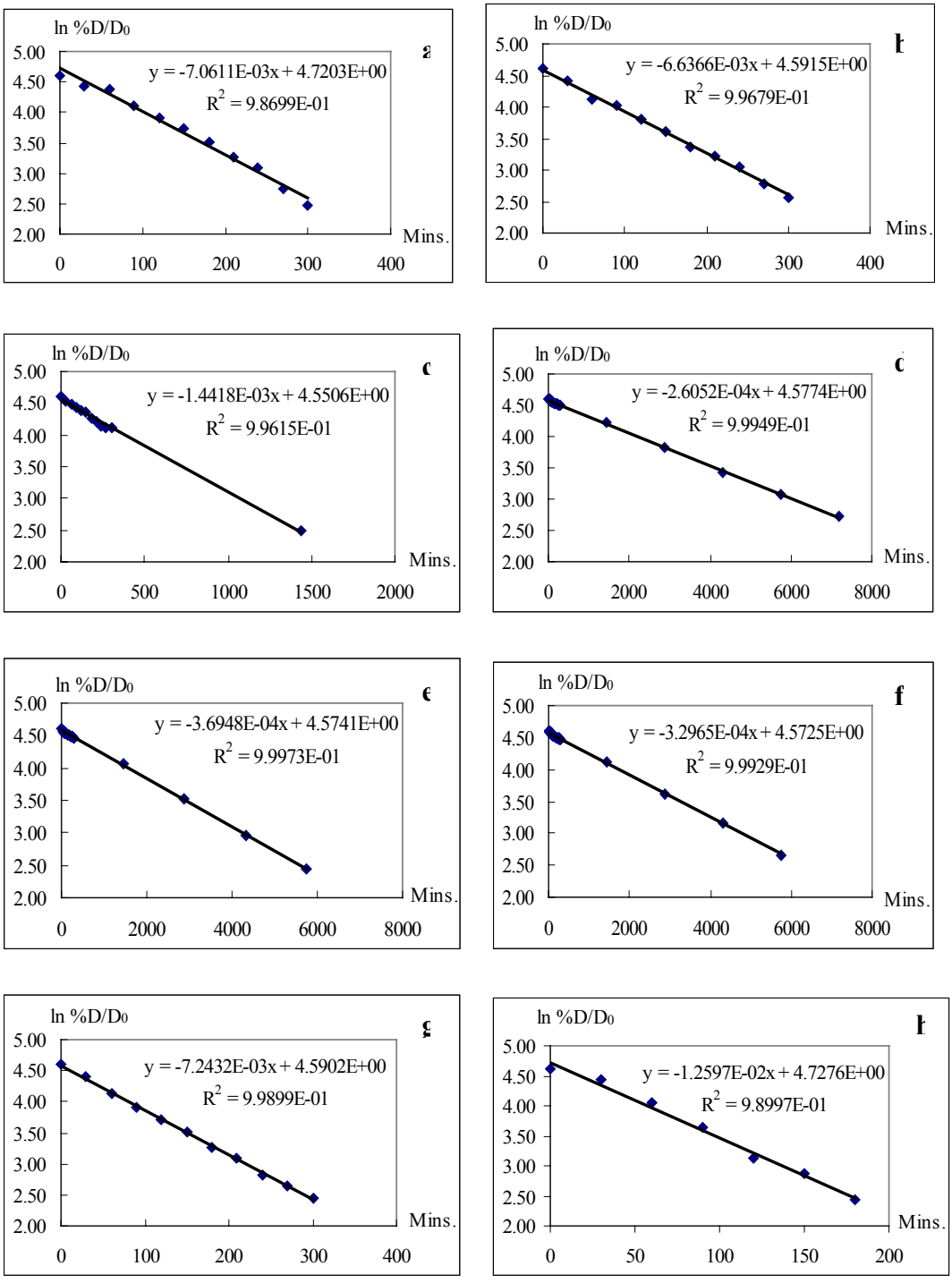

Figure 11 Degradation curve of limonin in buffered solution at 70°C, 70%RH, plotted between the natural logarithmic form of the percentage of the remaining concentration of limonin and time, (a) pH 2, (b) pH 3, (c) pH 4, (d) pH 5, (e) pH 6, (f) pH 7, (g) pH 8, and (h) pH 9 (n = 3)

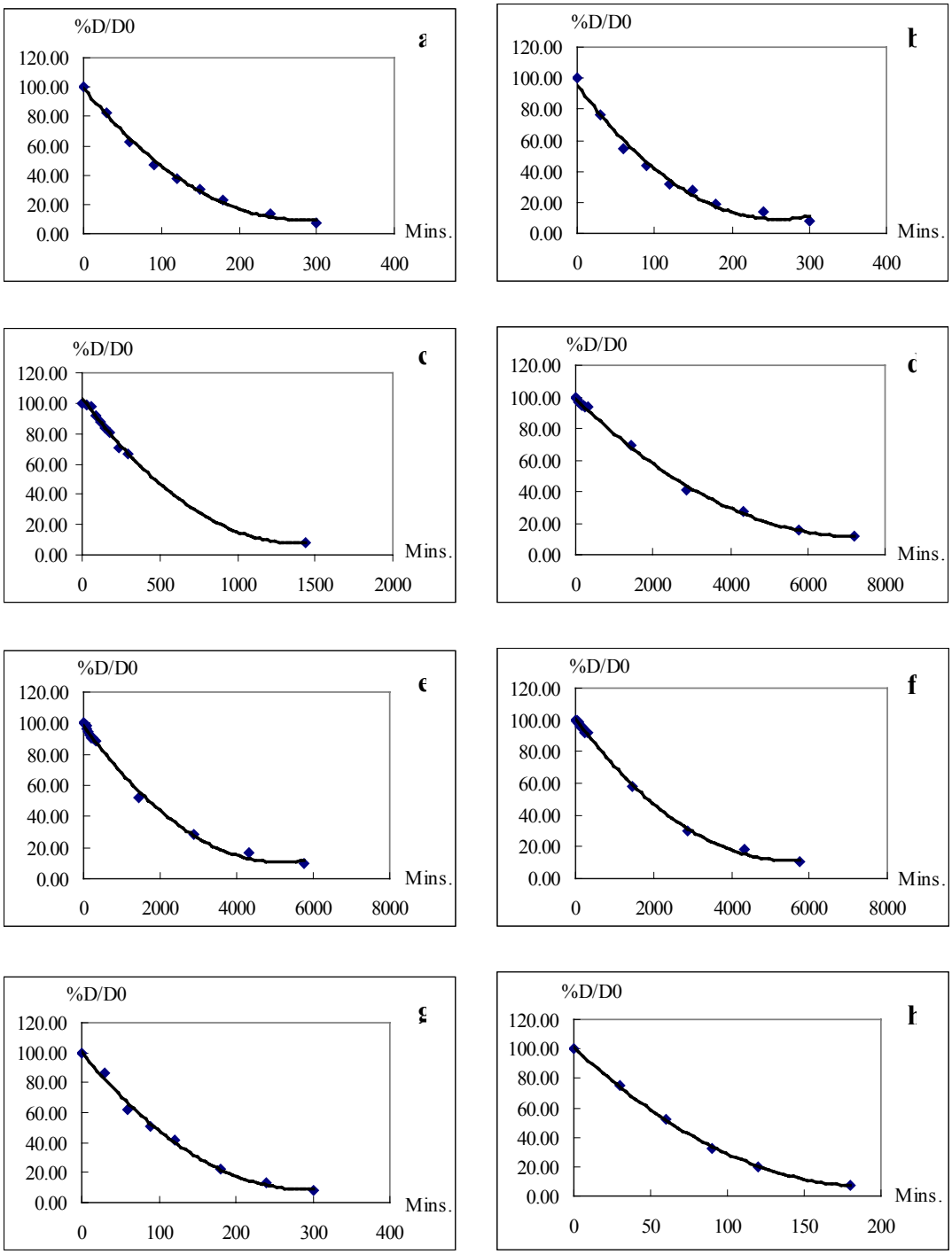

Figure 12 Degradation curve of limonin in buffered solution at 80°C, 70%RH, plotted between the percentage of the remaining concentration of limonin and time, (a) pH 2, (b) pH 3, (c) pH 4, (d) pH 5, (e) pH 6, (f) pH 7, (g) pH 8, and (h) pH 9 (n = 3)

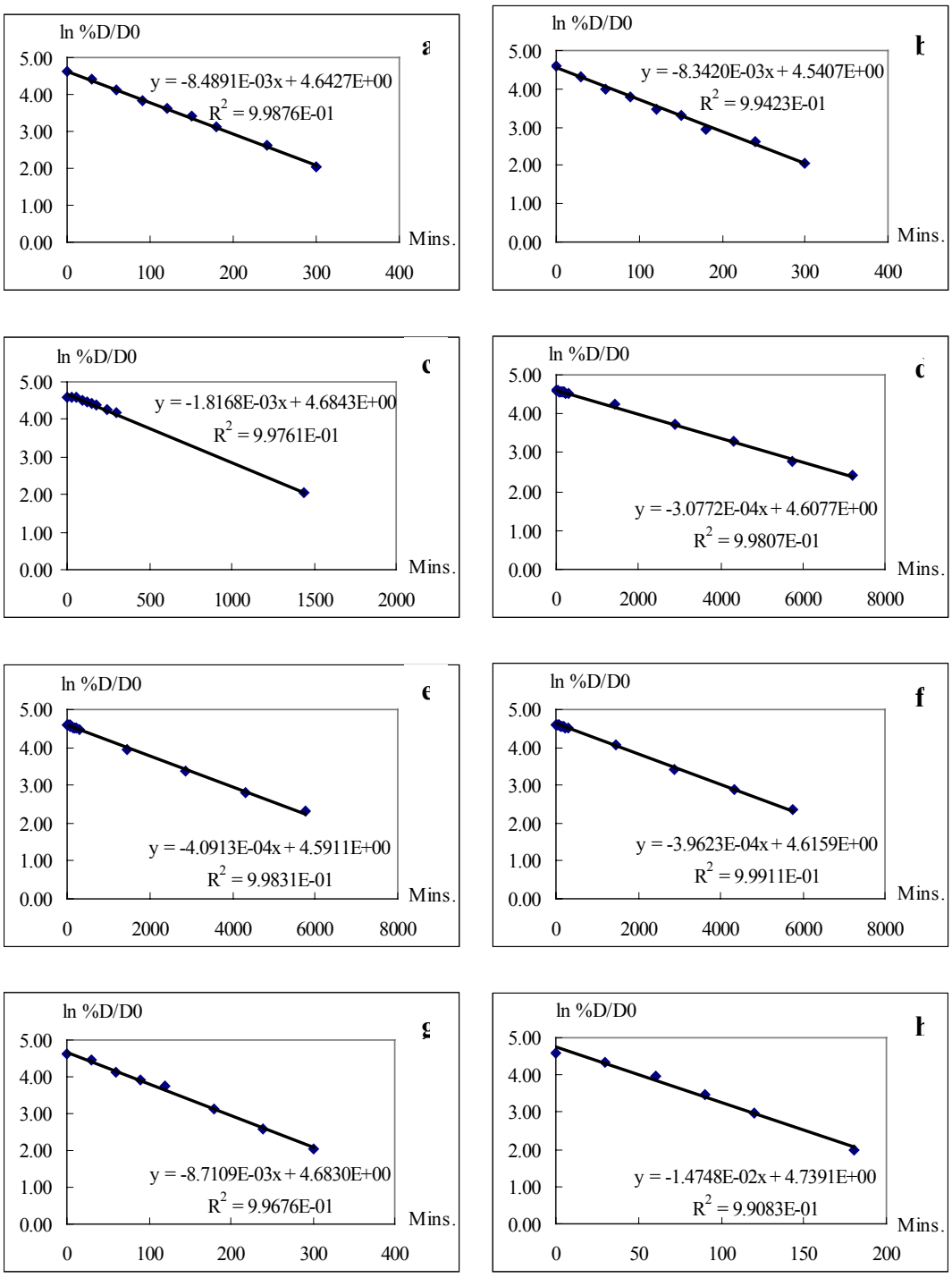

Figure 13 Degradation curve of limonin in buffered solution at 80°C, 70%RH, plotted between the natural logarithmic form of the percentage of the remaining concentration of limonin and time, (a) pH 2, (b) pH 3, (c) pH 4, (d) pH 5, (e) pH 6, (f) pH 7, (g) pH 8, and (h) pH 9 (n = 3)

Table 7 Reaction rate constant (min^{-1}) of limonin decomposition in various pH and temperatures, 70%RH

pH	45°C	70°C	80°C
2	4.9515×10^{-3}	7.0611×10^{-3}	8.4891×10^{-3}
3	4.8283×10^{-3}	6.6366×10^{-3}	8.3420×10^{-3}
4	1.0631×10^{-3}	1.4418×10^{-3}	1.8168×10^{-3}
5	2.0636×10^{-4}	2.6052×10^{-4}	3.0772×10^{-4}
6	2.5851×10^{-4}	3.6948×10^{-4}	4.0913×10^{-4}
7	2.4824×10^{-4}	3.2965×10^{-4}	3.9623×10^{-4}
8	5.2789×10^{-3}	7.2432×10^{-3}	8.7109×10^{-3}
9	7.7179×10^{-3}	1.2597×10^{-2}	1.4748×10^{-2}

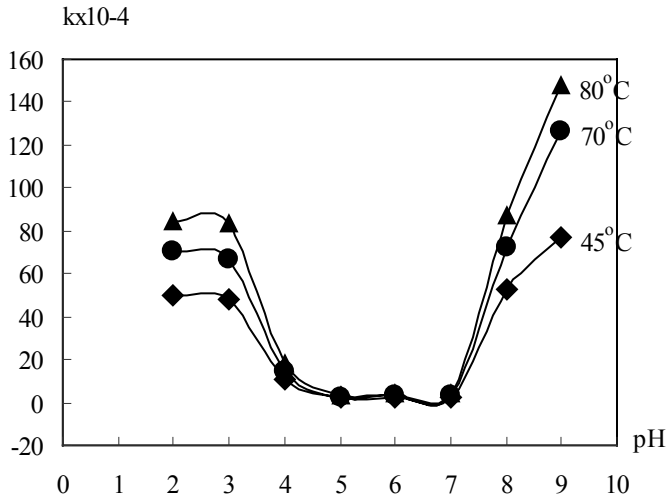

Figure 14 pH-rate profile of limonin decomposition at 45°C (▯), 70°C (▯), and 80°C (▯)

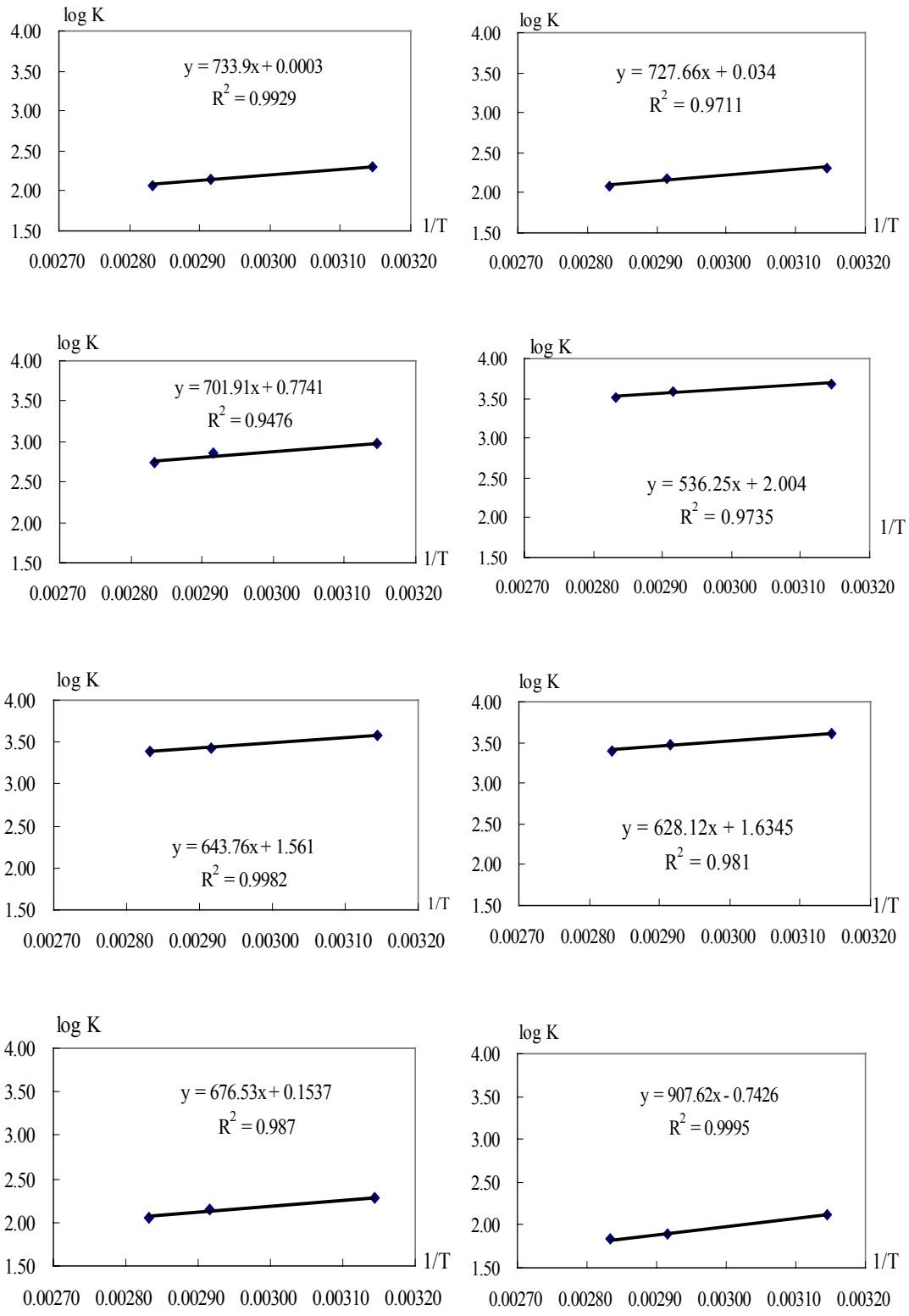

Figure 15 Arrhenius plot of limonin at various pH (a) pH 2, (b) pH 3, (c) pH 4, (d) pH 5, (e) pH 6, (f) pH 7, (g) pH 8, and (h) pH 9

Table 8 Arrhenius activation energy of limonin decomposition at various pH (calculated from equation Ea = slope x 2.303 x 1.987/1,000)

pH	slope	Ea (Kcal/mol)
2	733.90	3.36
3	727.66	3.33
4	701.99	3.21
5	536.25	2.45
6	643.76	2.95
7	628.12	2.87
8	676.53	3.10
9	907.62	4.15

Table 9 $k_{observed}$ (k_{obs}) of reaction affected by extreme pH buffered solution (acid pH range 2, 3, basic pH range 8, 9).

Region	Temp(^{o}C)	$k_H(min^{-1})$	$k_o x10^{-4}(min^{-1})$	$k_{obs} = k_0 + k_H[H]$
Acid	45	0.0521	48.90	$k_{obs}= 48.9x10^{-4}+0.0521[H]$
	70	0.1047	68.49	$k_{obs}= 68.49x10^{-4}+0.1047[H]$
	80	0.0879	84.16	$k_{obs}= 84.16x10^{-4}+0.0879[H]$
Region	Temp(^{o}C)	$k_{OH}(min^{-1})$	$k_o x10^{-4}(min^{-1})$	$k_{obs} = k_0 + k_{OH}[OH]$
Alkaline	45	$2.10x10^{-9}$	64.99	$k_{obs} = 64.99x10^{-4}+2.10x10^{-9}[OH]$
	70	$7.19x10^{-10}$	99.20	$k_{obs}= 99.20x10^{-4}+7.19x10^{-10}[OH]$
	80	$1.07x10^{-9}$	117.30	$k_{obs}=117.30x10^{-4}+1.07x10^{-9}[OH]$

3.5 Stability of Limonin from Lime Seeds in Solid State

The graphs between the percentage of the remaining concentrations of limonin and time in various temperatures were made. They were all curved lines as shown in Figure 16. In addition, the graphs plotted between the natural logarithmic value of the percentage of the remaining concentration of limonin and time in various temperatures, they were all straight lines as shown in Figure 17. So it was assumed that degradation of limonin was the

first order reaction. The slopes of all straight lines from Figure 17 are in Table 10, these slopes were the reaction rate constant at various temperatures. The Arrhenius plot of solid limonin between logarithm values of rate constant versus the reciprocal of absolute temperature was a straight line as shown in Figure 18, with a slope of 473.95 day^{-1}, so activation energy could be calculated as 3,123.11 kcal/mole, which was many times more than apparent first order reaction rate constant of limonin in every tested pH-buffered solution (pH 2-9). It follows that limonin in solid state took a longer period of degradation around 360 days at every studied temperature (45°C, 70°C, and 80°C). Solid limonin in 45°C, 70%RH environment was reduced to 58.13 ± 4.06 % limonin concentration within a year, whereas limonin in 70°C and 80°C, with 70%RH environment, were reduced to 48.27 ± 3.88 % and 42.83 ± 3.76% limonin concentration respectively. So the reaction rate constants at 45°C, 70°C, and 80°C were $1.58x10^{-3}$, $2.03x10^{-3}$, and $2.22x10^{-3}$ day^{-1} respectively. Therefore it could be concluded that limonin in a solid state was more stable than limonin in an aqueous buffered solution. In addition, limonin in 45°C, 70%RH environment was more stable than in 70°C, and 80°C, with 70%RH environment consecutively.

The analysis of variance showed that limonin preserved in a solid state was significantly affected by temperature and by the time of storage (P <0.05). Moreover the statistical analysis of variance showed that temperature and time co-affected the remaining concentration of limonin by UNIANOVA analysis with 0.05 confidence level (Appendic 1, Table 4A).

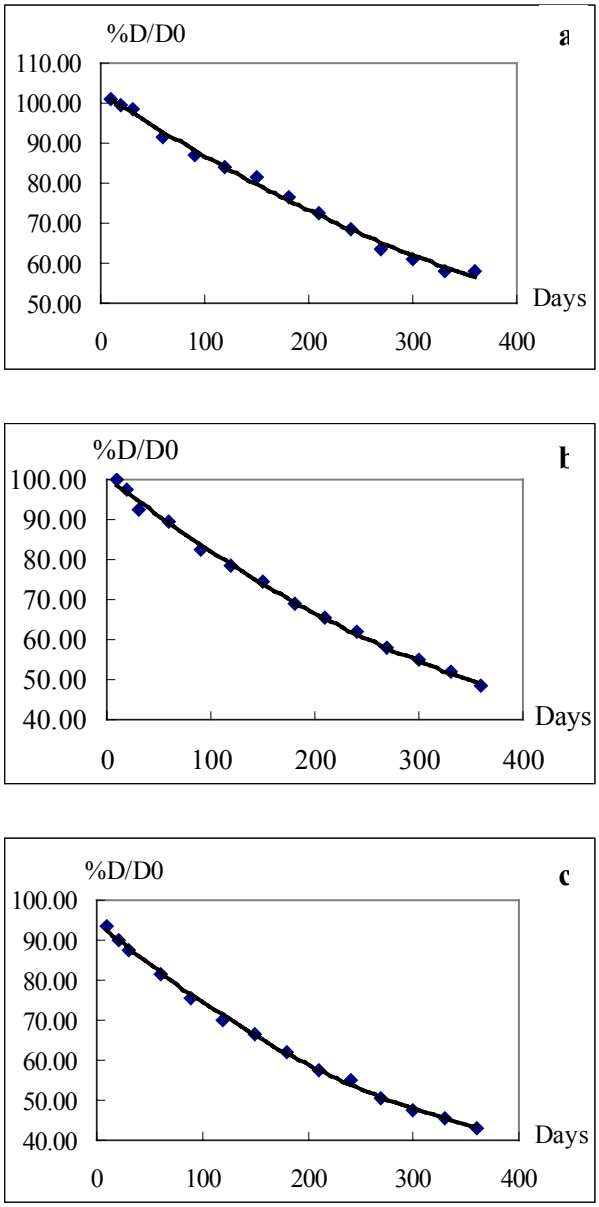

Figure 16 Degradation curve of limonin in a solid state at various temperature, 70%RH, plotted between the percentage of the remaining concentration of limonin and time (a) 80^{o}C, (b) 80^{o}C, and (c) 80^{o}C

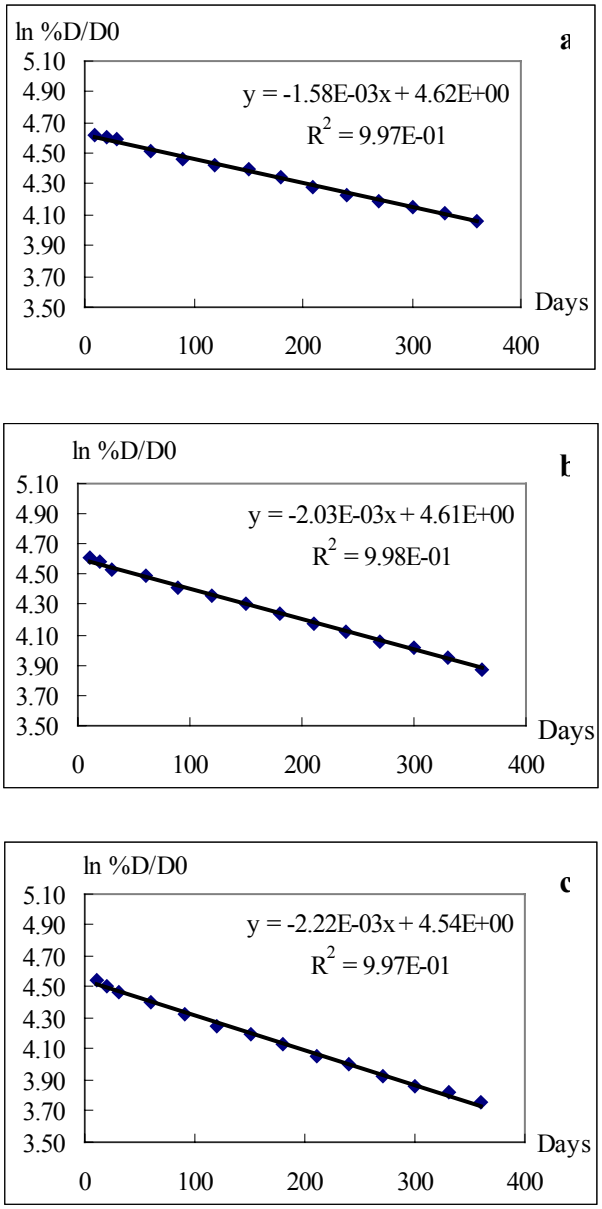

Figure 17 Degradation curve of limonin in a solid state at various temperatures, 70%RH, plotted between the natural logarithmic form of the percentage of the remaining concentration of limonin and time (a) 45°C, (b) 70°C, and (c) 80°C

Table 10 Reaction rate constant (day^{-1}) of solid limonin in various temperatures 70%RH

°C	1/T ($kelvin^{-1}$)	k $x10^{-3}$ (day^{-1})	log k
45	0.0031	1.58	2.80
70	0.0029	2.03	2.69
80	0.0028	2.22	2.65

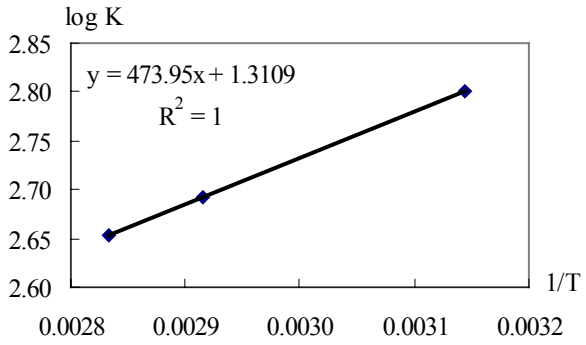

Figure 18 Arrhenius plot of solid limonin

3.6 The Study of Macrophage-Stimulation Activity of Limonin

The macrophage-stimulating activity of limonin was evaluated as the number of exudated cells in the peritoneal cavity (peritoneal exudated cells: PEC number), the percentage of phagocytic (PP), and phagocytic index (PI) after limonin administration for several days.

The PEC number of different groups of mice fed with different concentrations of limonin is shown in Figure 19. It showed that PEC numbers in mice fed with limonin increased and dependent on the limonin concentration. In addition PEC numbers increased when the days of limonin administrations were increased.

The statistic multivariate analysis of variance by Scheffe's post hoc test indicated that PEC numbers in mice treated with 0.5 ml/day/mouse of 20 ppm of limonin or more (50, 100, and 200 ppm) were significantly different

from untreated and PBS-treated mice at $p<0.05$. But PEC numbers in mice treated with 0.5 ml/day/mouse of 10 ppm of limonin or less (5 ppm) were not significantly difference from untreated and PBS-treated mice ($p<0.05$), and PEC numbers in mice treated with 0.5 ml/day/mouse of 20 ppm of limonin were not significantly different from mice treated with 0.5 ml/day/mouse of 10 ppm of limonin at $p<0.05$ (Appendix 1, Table 7A). Additionally, it was indicated that PEC in mice treated with limonin for 2 days was significantly different from mice treated with limonin for 4 days and 6 days by statistic multivariate analysis of variance by Scheffe's post hoc test (Appendix 1, Table 10A).

In conclusion, the dose of 0.5 ml/day/mouse of limonin of 20 ppm or less did not significantly change the PEC number, where as the dose of 0.5 ml/day/mouse of limonin of 50 ppm or more significantly increased the PEC number. The more limonin concentration administered to the mice, the greater the increase in the PEC number. In addition, the greater the number of days of limonin administration, the greater increase in the PEC number in every group of limonin treated mice with confident level of 95%.

However the statistic multivariated tests of between subjects affected by Scheffe's multiple contrast indicated that PEC number was significantly affected by the concentration of limonin, days after the limonin administration, and the coincidence of concentration and days after limonin administration at $p<0.05$ (Appendix 1, Table 6A).

The percentages of phagocytic cell (PP), and the phagocytic index (PI), are shown in Figures 20 and 21. These show that the phagocytic uptake of fluorescent particles by the PEC in mice fed with limonin has increased. The PP and PI in mice treated with limonin were more than the untreated and PBS-treated mice, and the more limonin concentration administered to the mice, the greater increase in the PP and PI.

The statistic multivariate analysis of variance by Scheffe's post hoc test indicated that PP in mice treated with 0.5 ml/day/mouse of PBS and limonin of every concentration was significantly different from untreated

mice (P<0.05). Additionally, PP in mice treated with 0.5 ml/day/mouse of 20 ppm of limonin or more (50, 100, and 200 ppm) were significantly different from untreated, PBS-treated, but PP in mice treated with 0.5 ml/day/mouse of 10 ppm of limonin or less (5 ppm) were not significantly difference from PBS-treated mice (p<0.05) (Appendix 1, Table 8A). It was also indicated PP in mice treated with limonin for 2 days was significantly different from mice treated with limonin for 4 days and 6 days by statistic univariate analysis of variance by Scheffe's post hoc test (Appendix 1, Table 11A).

PI was affected by limonin administration the same as PP. The statistical analysis of variance by Scheffe's post hoc test indicated that PI in mice treated with 0.5 ml/day/mouse of 10 ppm of limonin or more (20, 50, 100, and 200 ppm) was significantly different from untreated and PBS-treated mice at p<0.05. But PI in mice treated with 0.5 ml/day/mouse of 10 ppm of limonin were not significantly different from 5 ppm limonin-treated mice, nor was PI in mice treated with 0.5 ml/day/mouse of 5 ppm of limonin (p<0.05) (Appendix 1, Table 9A). It also indicated that PI in mice treated with limonin for 2 days were significantly different from mice treated with limonin for 4 days and 6 days by statistic univariate analysis of variance by Scheffe's post hoc test (Appendix 1, Table 11A).

The statistic multivariated tests between subjects effect by Scheffe's multiple contrast indicated that PEC number, PP and PI in limonin-treated mice were significantly affected by the concentration of limonin, days between limonin administration, and coincidence of concentration and days between limonin administration at p<0.05 (Appendix 1, Table 6A).

Over all, it was concluded that the immunological effect of limonin on macrophage stimulation was obtained at the dose of 0.5 ml/day/mouse of limonin of 50 ppm or more which would significantly change the PEC number, PP, and PI, and the stronger limonin concentration administered to the mice, the more increase in the PEC number, PP and PI.

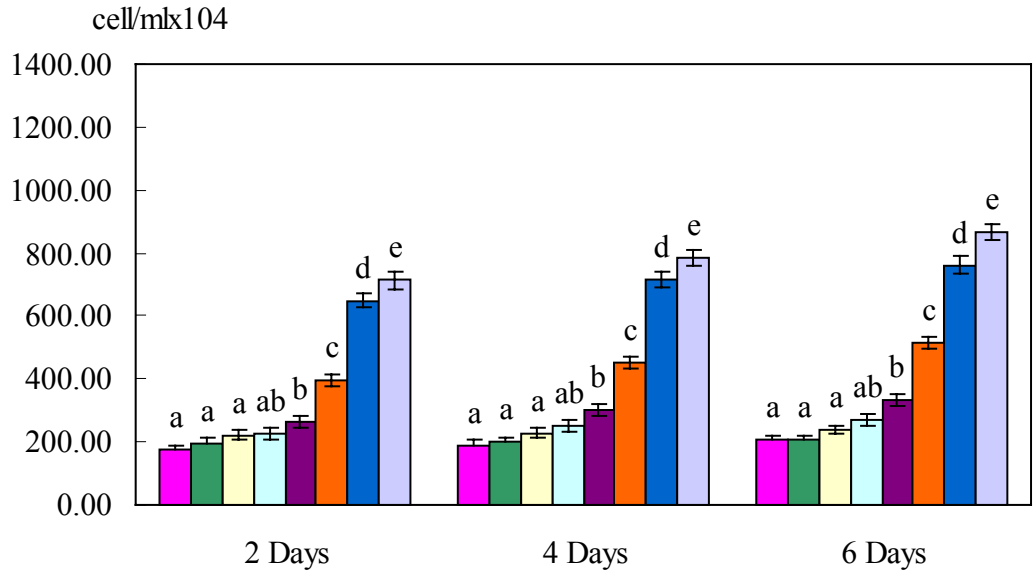

Figure 19 PEC numbers of different groups of mice on different days after limonin administration (untreated, PBS-treated, limonin 5 ppm, limonin 10 ppm, limonin 20 ppm, limonin 50 ppm, limonin 100 ppm, limonin 200 ppm (n = 4) Values with different letter are significantly different. $P<0.05$.

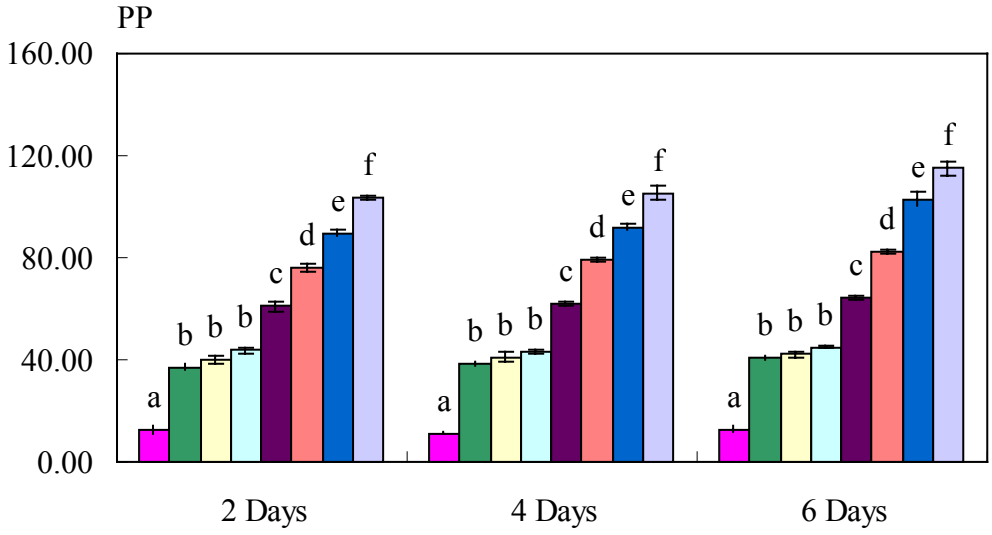

Figure 20 PP of different groups of mice on different days after 0.5 ml/day/mouse of limonin administration (untreated, PBS-treated, 5 ppm limonin, 10 ppm limonin, 20 ppm limonin, 50 ppm limonin, 100 ppm

limonin, 200 ppm limonin (n = 4) Values with different letter are significantly different. $P<0.05$.

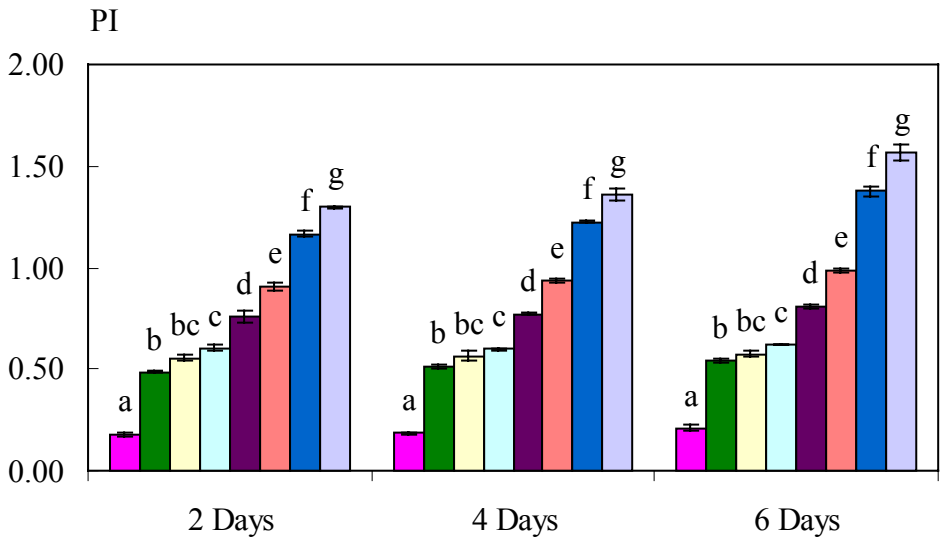

Figure 21 PI of different groups of mice on different days after 0.5 ml/day/mouse of limonin administration (untreated, PBS-treated, 5 ppm limonin, 10 ppm limonin, 20 ppm limonin, 50 ppm limonin, 100 ppm limonin, 200 ppm limonin (n = 4) Values with different letter are significantly different. $P<0.05$.

3.7 Effect of Limonin on the Hematological Parameters

Administration of limonin increased the total WBC count in mice as shown in Figure 22. The maximum WBC count was obtained on the 12th day in the mice treated with .0.5 ml/day/mouse of 200 ppm of limonin around 470.00 ± 13.04 x 10^4 cell/ml. The maximum WBC count was 320.83 ± 15.30 x 10^4 cell/ml on the 18th day in untreated mice, 370.00 ± 13.78 x 10^4 cell/ml on the 15th day in PBS-treated mice, 373.33 + 8.16 x 10^4 cell/ml and 373.33 ± 10.80 x 10^4 cell/ml on the 15th day in mice treated with .0.5 ml/day/mouse of 5 ppm and 10 ppm respectively, 382.50 ± 8.22 x 10^4 cell/ml on the 12th day in mice treated with .0.5 ml/day/mouse of 20 ppm, 397.50 ± 16.36 x 10^4 cell/ml on the 30th day, 430.00 ± 10.49 x 10^4 cell/ml on the 27th day in mice treated with .0.5 ml/day/mouse of 50 ppm, and 100 ppm of limonin respectively (Appendix 3, Table 7C)

The statistic univariate tests between subjects effect by Scheffe's multiple contrast and Post Hoc test at $P<0.05$ indicated that the total WBC count was significantly affected by the concentration of limonin, and days between limonin administration (Appendix 1, Table 13A). And there was a significant difference in total WBC count between untreated mice and mice treated with 0.5 ml/day/mouse of every studied concentration of limonin and at any interval of days of limonin administration ($P<0.05$). There was a significant difference in total WBC count between mice treated with 0.5 ml/day/mouse of 50 ppm or more (100 and 200 ppm) of limonin and mice treated with 0.5 ml/day/mouse of 20 ppm or less (5 and 10 ppm) of limonin, PBS-treated, and untreated mice. But the total WBC count in mice treated with 0.5 ml/day/mouse of 5 and 10 ppm of limonin was not significantly different from PBS-treated mice at $P<0.05$. Likewise the total WBC count in mice fed with 0.5 ml/day/mouse of 10 ppm of limonin did not significantly differ from mice fed of 20 ppm of limonin. On the contrary, the total WBC count in mice treated with 0.5 ml/day/mouse of 50 ppm was significantly different from mice treated with 100 and 200 ppm of limonin (Appendix 1, Table 14A).

In conclusion, the higher dose of limonin 0.5 ml/day/mouse of 200 ppm of limonin) increased the total WBC count more than the lower dose of 0.5 ml/day/mouse of 100 ppm of limonin significantly on the 15^{th}, 27^{th}, and 30^{th} day. The dose of 0.5 ml/day/mouse of 100 ppm of limonin increased total WBC count more than the dose of 0.5 ml/day/mouse of 50 ppm of limonin significantly on the 3^{rd}, 12^{th},15^{th}, 21^{th}, 24^{th}, 27^{th}, and 30^{th} day (multivariate analysis of variance by Scheffe's post hoc test at $P<0.05$) (Appendix 1, Table 15A). On the other hand, the total WBC count in mice fed with 0.5 ml/day/mouse of 5, 10, and 20 ppm of limonin was not different from the PBS-treated mice. Thus limonin significantly affected the total WBC count only at a concentration of 50 ppm or more with 95% confidence level.

The differential WBC count on the 12^{th} day is shown in Table 11, in which lymphocyte and monocyte increased, but neutrophil decreased, when

limonin was administrated to the mice. The analysis of variance by MANOVA indicated that groups of mice and days of limonin feeding both affected the neutrophil, lymphocyte, and monocyte significantly with 95% confident level (Appendix 1, Table 16A). In all tests, neutrophil and lymphocyte in mice fed with 200 ppm of limonin were significantly different from mice fed with 100 ppm of limonin, but monocyte was not significantly different (Appendix 1, Table 17A). Statistic multiple comparison by Scheffe's multiple contrast post hoc test indicated that there was not a significant difference on neutrophil, lymphocyte, and monocyte with 9, 12, and 15 days of limonin feeding (P <0.05) (Appendix 1, Table 18A). Thus, limonin of 200 ppm affected the count of neutrophil, lymphocyte, and monocyte more significantly than 100 ppm limonin. The days of limonin feeding affected the WBC-differentiated count, but not significantly. (P<0.05)

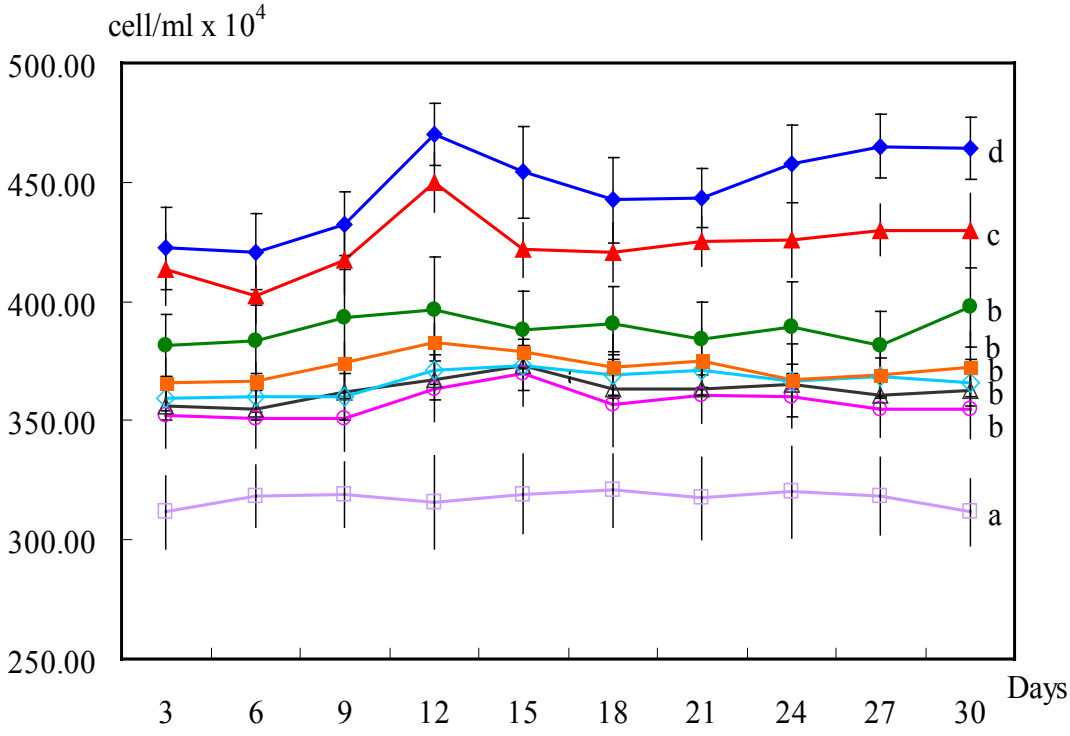

Figure 22 Total WBC count of different groups of mice on different days after limonin administration □ untreated, O PBS-treated,Δ limonin 5 ppm, limonin 10 ppm, limonin 20 ppm, limonin 50

ppm, ✠ limonin 100 ppm ♈ limonin 200 ppm (n = 6) Values with different letter are significantly different. $P<0.05$.

Table 11 The differential WBC count (in 25 large squares)on the 12th day after administration of untreated, PBS-treated, limonin 100 ppm treated, and 200 ppm treated mice

Cell types		Untreated	PBS	100ppm	200ppm
Neutrophil	Cell No.	194	210	257	249
	SD	11.58	7.07	4.08	7.36
	Percentage	61.48	57.80	57.04	53.01
Lymphocyte	Cell No.	114	144	180	204
	SD	9.70	5.85	7.75	6.65
	Percentage	36.15	39.68	40.00	43.44
Monocyte	Cell No.	8	9	13	17
	SD	2.74	2.04	2.58	4.08
	Percentage	2.37	2.52	2.96	3.55
Total	Cell No.	316	363	450	470

3.8 Effect of Limonin on Production of a Specific Antibody

The specific antibody titers of different groups of mice treated with different concentrations of limonin were shown in Table 12. The maximum antibody titer of 1024 was observed in the dose of 0.5 ml/day/mouse of 200 ppm of limonin treated mice on the 12th day after 20% SRBC immunization. The mice treated with 0.5 ml/day/mouse of 100 ppm of limonin gave the maximum antibody titer of 512 on the 15th day after 20% SRBC immunization. The mice treated with 0.5 ml/day/mouse of 50 ppm of limonin gave the maximum antibody titer of 128 on the 15th day after 20% SRBC immunization. The mice treated with 0.5 ml/day/mouse of 5 ppm, 10 ppm, and 20 ppm of limonin gave the maximum antibody titer of 64 on the 15th day, 15th day and 12th day after 20% SRBC immunization respectively, the same as mice treated with 0.5 ml/day/mouse of PBS gave the maximum antibody titer of 64 on the 15th day after 20% SRBC immunization. But untreated mice with 20% SRBC immunization gave the maximum antibody

titer of 32 on the 9th to 15th day after 20% SRBC immunization, whereas untreated and un-immunized mice gave the maximum antibody titer only 2. So there was a significant increase in the production of specific antibody to sheep red blood cell in mice treated with limonin. The antibody titer in mice treated with 0.5 ml/day/mouse of 50 ppm to 200 ppm of limonin remained the highest titer till the 18th day after 20% SRBC immunization, whereas antibody titer in mice treated with 0.5 ml/day/mouse of 5 to 20 ppm of limonin remained the highest titer till the 21st to 24th day after 20% SRBC immunization.

There was a significant difference in mice given 0.5 ml/day/mouse of every concentration of limonin from limonin-untreated mice without SRBC immunization. And the titer in mice fed with 0.5 ml/day/mouse of 5 of limonin were not significantly different from limonin untreated mice with SRBC immunization and PBS-treated mice. The titer in mice fed with 0.5 ml/day/mouse of 10 and 20 ppm of limonin were not significantly different from 5 ppm limonin-treated mice, as well as titer in mice fed with 0.5 ml/day/mouse of 50 ppm of limonin were not significantly different from mice fed with 10 and 20 ppm of limonin. But the titer in mice fed with 0.5 ml/day/mouse of 100 and 200 ppm of limonin were significantly different from every group of mice fed with limonin under 100 ppm concentration with 95% confident level (Appendix 1, Table 20A).

The dose-dependence of this parameter was subsequently examined by statistic analysis of variance by UNIANOVA with Scheffe's multiple contrast post hoc test, it is found that antibody titers of mice given 0.5 ml/day/mouse of 100 and 200 ppm of limonin were significantly different from every other group of mice ($P<0.05$) (Appendix 1, Table 20A). However mice given 0.5 ml/day/mouse of 200 ppm of limonin showed a higher antibody titer than mice given 0.5 ml/day/mouse of 100 ppm of limonin only on the 12th, 15th, 21st, and 30th day (Appendix 1, Table 21A)

In conclusion, the dose of 0.5 ml/day/mouse of 100 ppm or more of limonin affected the production of specific antibody in mice, and a higher concentration of limonin stimulated a higher antibody titer.

Table 12 The antigen-antibody titers of various groups of mice fed with different concentrations of limonin on different days after 5 day-20%SRBC immunization (n = 6)

Group	3	6	9	12	15	18	21	24	27	30
Untreated and Unimmunized	0	0	0	2	2	2	2	2	2	2
Untreated	0	16	32	32	32	32	32	32	32	16
PBS	0	16	32	32	64	64	32	32	32	16
Limonin5ppm	0	16	32	32	64	64	64	32	32	16
Limonin10ppm	0	16	32	32	64	64	64	64	32	16
Limonin20ppm	0	16	32	64	64	64	64	64	32	16
Limonin50ppm	0	16	32	64	128	128	64	64	32	16
Limonin100ppm	0	32	128	256	512	512	128	128	64	32
Limonin200ppm	0	128	256	1024	1024	1024	512	256	128	128

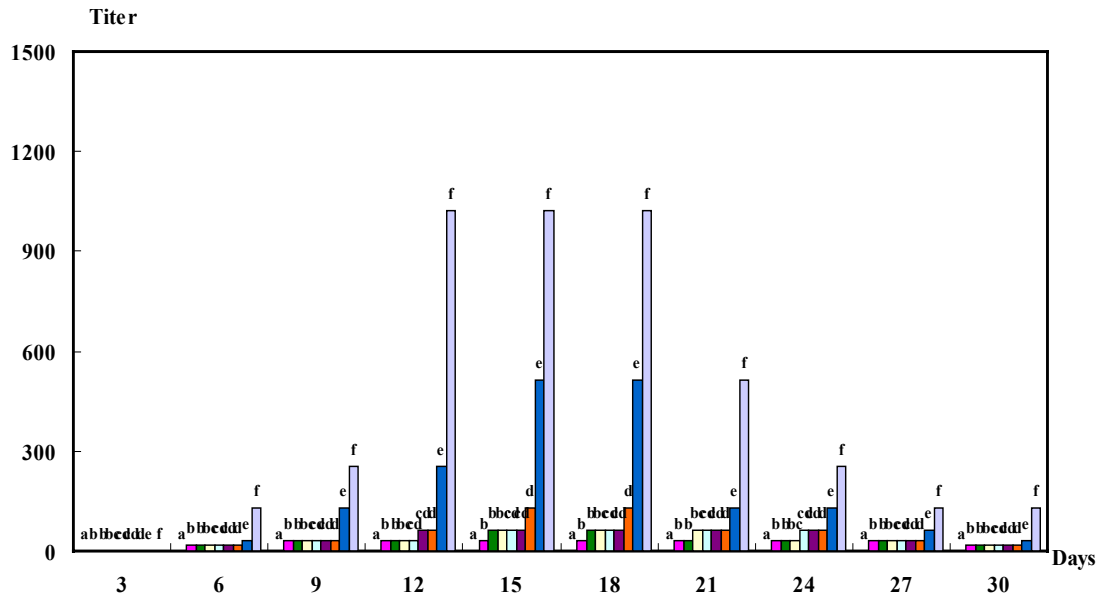

Figure 23 Antigen-antibody titers of different groups of mice fed with different concentration of limonin on different days after 5 days-20% SRBC-immunization. (untreated unimmunized , untreated immunized, PBS-treated, limonin 5 ppm, limonin 10 ppm, limonin 20 ppm, limonin 50 ppm, limonin 100 ppm, limonin 200 ppm) (n = 6) Values with different letters are significantly different. $P<0.05$.

3.9 The Effect of Processing Technique on Limonin Content in Lime Juices

The data on Table 13 showed that limonin in lime juice was around 10-17 ppm. Lime juice expressed by machine gave a higher yield of limonin than that expressed by hand. Heating extensively lowered the limonin content in lime juice both expressed by machine and by hand, whereas freezing had little effect on the limonin content in lime juice. Comparatively, limonin content in untreated lime juice expressed by machine was more than untreated lime juice expressed by hand, more than frozen lime juice expressed by machine, more than heated lime juice expressed by machine, more than frozen lime juice expressed by hand, and more than heated lime juice expressed by hand, consecutively. The statistic analysis of variance of processing effect on limonin content by one way ANOVA tests of between subjects effects with Scheffe's multiple contrasts indicated that processing of lime juices significantly affected limonin content with 95% confident level.

Table 13 Limonin content (ppm) in various processed lime juice. Values with different letters are significantly different. $P<0.05$

Processes	Test 1	Test 2	Test 3	Test 4	Average.	SD
Hand	16.99	16.92	16.91	16.89	16.93a	0.04
Machine	17.54	17.53	17.48	17.43	17.49b	0.05
Hand + Heat	10.34	10.06	10.00	9.97	10.09c	0.17
Machine + Heat	10.50	10.49	10.48	10.46	10.48d	0.02
Hand + Freeze	15.44	15.43	15.47	15.37	15.43e	0.04

Machine + Freeze	16.25	16.24	16.19	16.16	16.21f	0.04

3.10 The Effect of Processing Techniques on Immunological Effects

Immunological effects of lime juice were tested by total WBC count and specific antibody titer comparison in mice fed with different processed lime juices. The total WBC counts of mice fed with lime juice with various processing techniques are shown in Figure 24. It was found that total WBC count in mice fed with untreated lime juice expressed by machine was highest on the day 12^{th} at 568.33 $x10^4$ ± 15.38 $x10^4$ cell/ml(Appendix 3, Table 8C). The data showed that mice fed with untreated lime juice gave a higher total WBC count than processed juices, mice fed with lime juice expressed by machine gave a higher total WBC count than that expressed by hand both heated and frozen juices. Also mice fed with frozen lime juice both expressed by machine and hand gave a higher total WBC than that heated ones.

The statistical analysis of variance by one way ANOVA indicated that processing of lime juice affected the total WBC counts in mice significantly with 95% confidence level. But multiple comparison by Sheffe's multiple contrast post hoc test indicated that the total WBC count in mice fed with untreated lime juice expressed by hand was not significantly different from that in mice fed with untreated lime juice expressed by machine, and the total WBC count in mice fed with heated lime juice expressed by machine was significantly different from that in mice fed with frozen lime juice expressed by machine (P<0.05). Also the total WBC count in mice fed with heated lime juice expressed by hand was not significantly different from that in mice fed with frozen lime juice expressed by hand, and the total WBC count in mice fed with heated lime juice expressed by hand was not significantly different from that in mice fed with heated lime juice expressed by machine (P<0.05). Lastly, the WBC count in mice fed with frozen lime juice expressed by hand was not significantly different from that in mice fed with frozen lime juice expressed by machine. But the total WBC count in mice fed with either processed or unprocessed lime juice was significantly

different from that in mice untreated with lime juice, and the total WBC count in mice fed with unprocessed lime juice expressed either by hand or by machine was significantly different from that in mice fed with processed lime juice (P<0.05) (Appendix 1, Table 24A).

. The specific antibody titers of mice fed with lime juice with various processing techniques is shown in Table 14. The data showed that mice fed with untreated lime juice expressed by machine gave a maximum antibody titer of 1024 on the 9^{th} to 15^{th} day after 5 days 20% SRBC immunization, mice fed with untreated lime juice expressed by hand gave a maximum antibody titer of 512 on the 9^{th} to 12^{th} day after 5 days 20% SRBC immunization, mice fed with frozen lime juice expressed by machine gave a maximum antibody titer of 512 on the 12^{th} to 15^{th} day after 5 days 20% SRBC immunization, mice fed with frozen lime juice expressed by hand gave a maximum antibody titer of 256 on the 9^{th} to 18^{th} day after 5 days 20% SRBC immunization, mice fed with heated lime juice expressed by machine gave a maximum antibody titer of 256 on the 12^{th} to 18^{th} day after 5 days 20% SRBC immunization, mice fed with heated lime juice expressed by hand gave a maximum antibody titer of 128 on the 12^{th} to 18^{th} day after 5 days 20% SRBC immunization.

The statistical analysis of variance by one way ANOVA indicated that processing of lime juice affected specific antibody titer in mice significantly with 95% confident level. But multiple comparison by Sheffe's multiple contrast Post Hoc Test indicated that specific antibody titer in mice fed with untreated lime juice expressed by hand was not significantly different from that in mice fed with untreated lime juice expressed by machine, specific antibody titer in mice fed with heated lime juice expressed either by hand or by machine was not significantly different from that in mice fed with frozen lime juice expressed either by hand or by machine, and specific antibody titer in mice fed with either heated or frozen lime juice expressed by hand was not significantly different from that in mice fed with either heated or frozen lime juice expressed by machine (P<0.05).

Nevertheless specific antibody titer in mice fed with processed lime juice either heated or frozen and expressed either by hand or by machine was significantly different from that in untreated but immunized mice (Appendix 1, Table 26A).

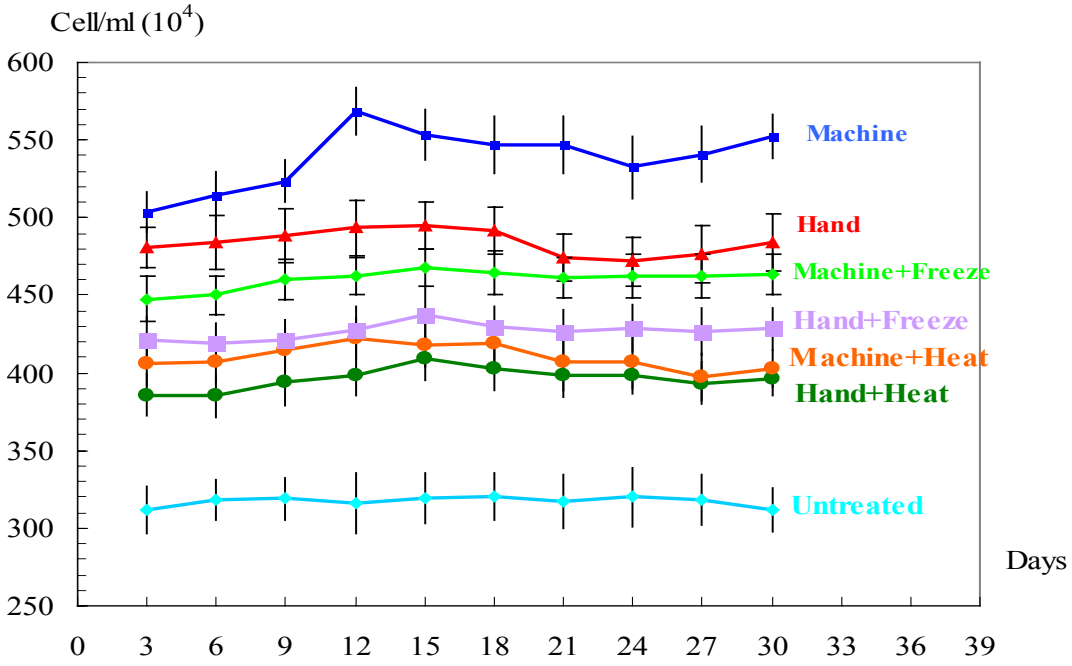

Figure 24 Total WBC count of different groups of mice on different days after treatment with differently processed lime juices. (expressed by machine, expressed by hand, expressed by machine and frozen, expressed by hand and frozen, expressed by machine and heated, expressed by hand and heated, untreated mice) (n = 6)

Table 14 The antigen-antibody titers of various groups of mice fed with differently processed lime juices on different days after 5 day-20%SRBC immunization (n = 6)

Group	3	6	9	12	15	18	21	24	27	30
Untreated Unimmunized	0	0	2	2	2	2	2	2	2	2

ControlUntreated	0	16	16	32	32	32	32	32	32	16
Hand	16	128	512	512	256	256	256	128	128	128
Machine	16	256	1024	1024	1024	512	512	256	128	128
Hand + Heat	0	64	64	128	128	128	64	32	32	32
Machine + Heat	2	64	64	256	256	256	128	128	64	32
Hand + Freeze	4	64	256	256	256	256	128	128	128	64
Machine + Freeze	8	128	256	512	512	256	256	128	128	64

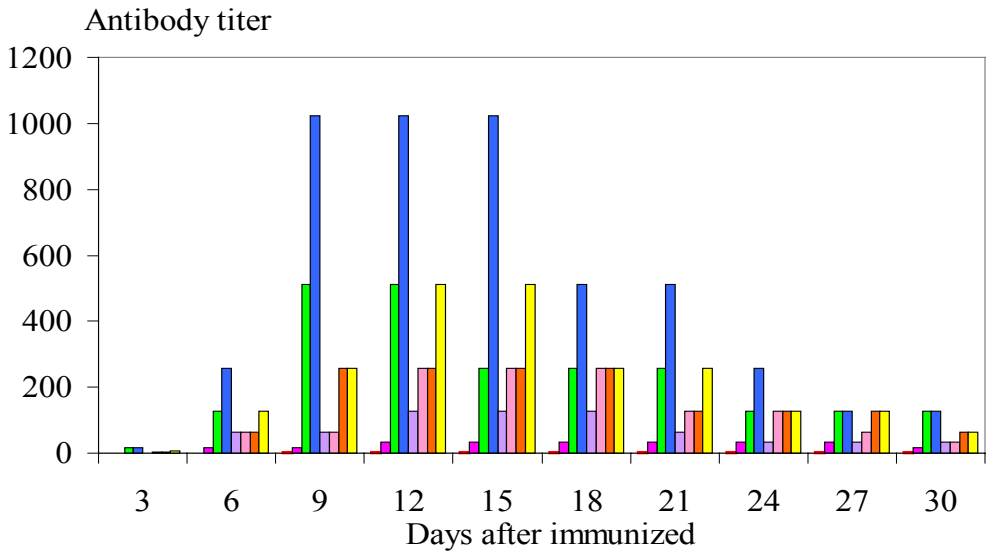

Figure 25 Antigen-antibody titers of different groups of mice fed with differently processed lime juices on different days after 5 days-20% SRBC-immunization. (untreated unimmunized, untreated immunized, lime juice expressed by hand, lime juice expressed by machine, lime juice expressed by hand and frozen, lime juice expressed by machine and frozen, lime juice expressed by hand and heated, lime juice expressed by machine and heated) (n = 6)