CHAPTER 4
RESULTS AND DISCUSSION

Part I Determination of vitamins in *M. citrifolia* and *P. emblica* fruits

Solid-phase extraction was used for the separation of vitamins B and C from *M. citrifolia* and *P. emblica*. Then the fraction of each sample was evaporated and dissolved in acidified water (pH 3.7). This solution was filtered through a 0.45 μm nylon membrane. Then the filtrate was subjected to HPLC determination. The experimental parameters, e.g. wavelength, mobile phase, flow rate etc. of the HPLC methods for the determination of *M. citrifolia* and *P. emblica* were also investigated.

4.1 Chromatographic Method

4.1.1 Separation of water-soluble vitamins

Water soluble vitamins in *M. citrifolia* and *P. emblica* fruits were determined by high performance liquid chromatography. The liquid chromatographic conditions used for the determination of ascorbic acid (vitamin C) are summarized in Table 4.1.

Table 4.1 HPLC conditions for Analysis of Ascorbic acid.

Operating parameters	Conditions
1. Stationary Phase	Hyperclone® C18, 5 µm (250 x 4.6 mm)
2. Mobile Phase	2% Acetic acid in water
3. Flow Rate	0.8 mL.min^{-1}
4. Wavelength	210 nm
5. Injection Volume	1 µL

A representative chromatogram of ascorbic acid is shown in Fig. 4.1

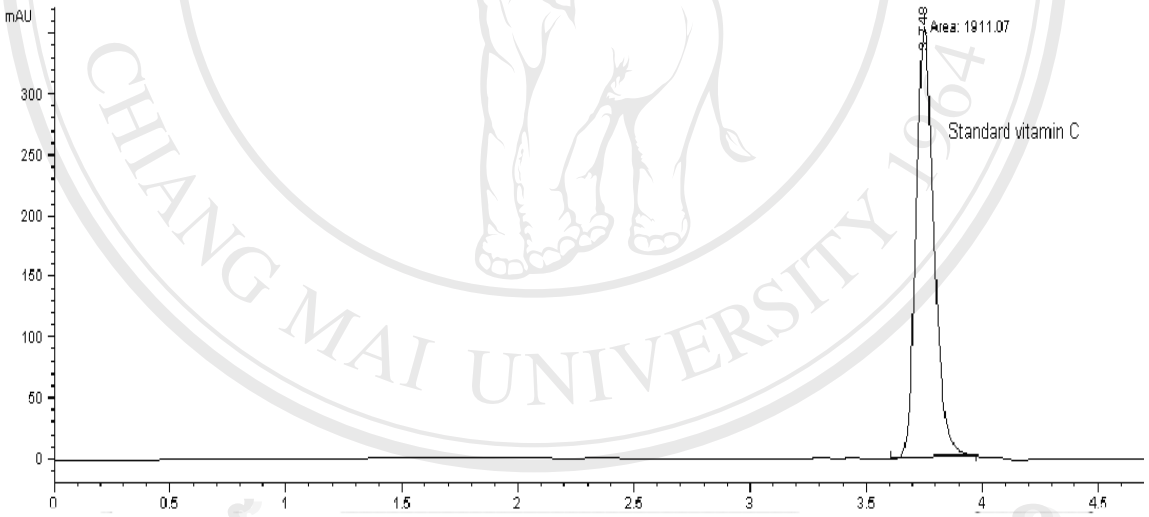

Figure 4.1 HPLC chromatogram of ascorbic acid (600.0 µg.mL^{-1}). Mobile phase: 2% acetic acid in water (pH 2.8), Flow rate 0.8 mL.min^{-1}, UV detector at 210 nm.

The chromatographic conditions used for the determination of thiamine hydrochloride (vitamin B_1), riboflavin (vitamin B_2), nicotinamide (vitamin B_3) and pyridoxine hydrochloride (vitamin B_6) are summarized in Table 4.2.

Table 4.2 HPLC conditions for Analysis of vitamin B_1, B_2, B_3 and B_6

Operating parameters	Conditions
1. Stationary Phase	Hyperclone® C18, 5 µm (250 x 4.6 mm)
2. Mobile Phase	5 mM Sodium-1-octanesulfonate, pH 2.5: Acetonitrile (75:25, v/v)
3. Flow Rate	1 $mL.min^{-1}$
4. Wavelength	280 nm
5. Injection Volume	2 µL

Well defined peaks of thiamine hydrochloride, riboflavin, nicotinamide and pyridoxine hydrochloride were obtained (Fig. 4.2 - 4.5) when using the HPLC conditions as presented in Table 4.2.

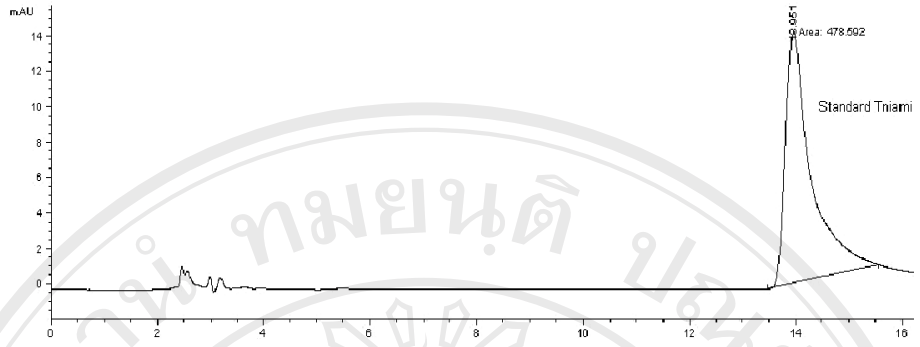

Figure 4.2 Typical chromatogram of pure standard thiamine HCl (500.0 µg.mL^{-1}), Mobile phase: 5 mM Sodium-1-octanesulfonate, pH 2.5:Acetonitrile (75:25, v/v), Flow rate 1.0 mL.min^{-1}, at 280 nm.

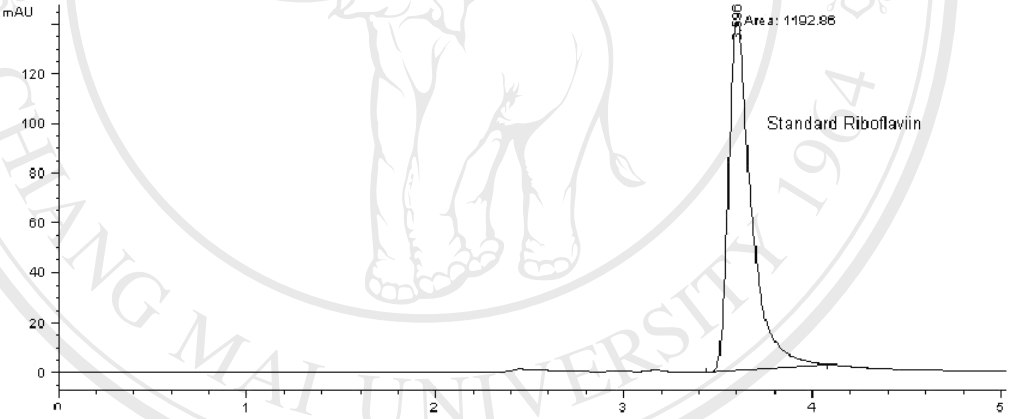

Figure 4.3 Typical chromatogram of pure standard riboflavin (500.0 µg.mL^{-1}), Mobile phase: 5 mM Sodium-1-octanesulfonate, pH 2.5: Acetonitrile (75:25, v/v), Flow rate 1.0 mL.min^{-1}, at 280 nm.

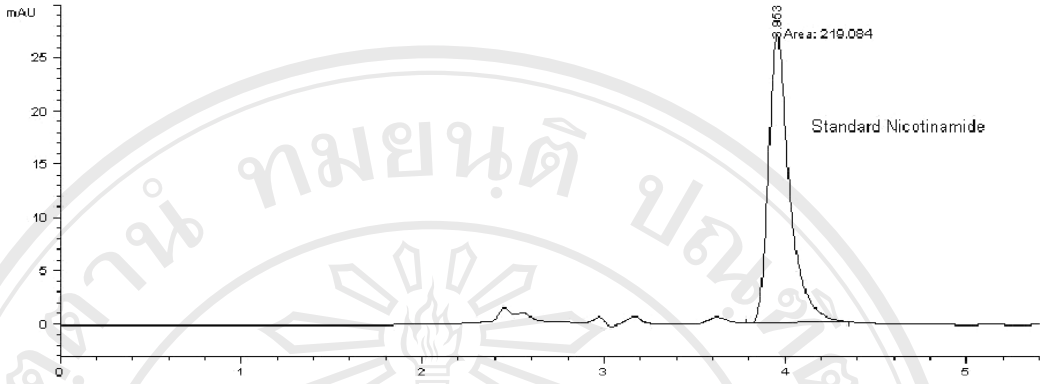

Figure 4.4 Typical chromatogram of pure standard nicotinamide (500.0 µg.mL^{-1}), Mobile phase: 5 mM Sodium-1-octanesulfonate, pH 2.5: Acetonitrile (75:25, v/v), Flow rate 1.0 mL.min^{-1}, at 280 nm.

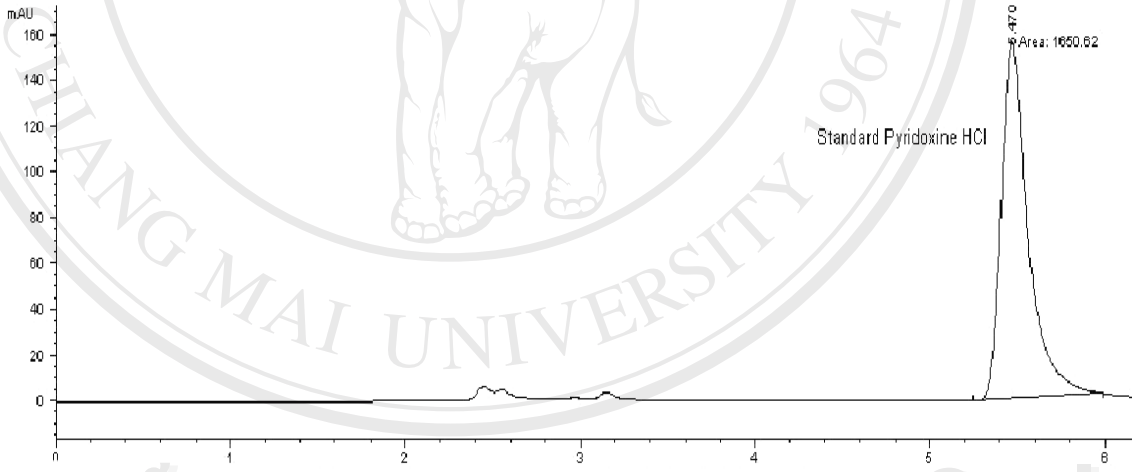

Figure 4.5 Typical chromatogram of pure standard pyridoxine HCl (500.0 µg.mL^{-1}), Mobile phase: 5 mM Sodium-1-octanesulfonate, pH 2.5:Acetonitrile (75:25, v/v), Flow rate 1.0 mL.min^{-1}, at 280 nm.

4.1.2 Separation of fat-soluble vitamins

The liquid chromatographic method used for the determination of α-tocopherol (vitamin E) and β-carotene is summarized in Table 4.3.

Table 4.3 HPLC conditions for Analysis of vitamin E and β-carotene

Operating parameters	Conditions
1. Stationary Phase	Inersil® ODS-3, 5 μm (250 x 4.6 mm)
2. Mobile Phase	Ethanol (solvent A): Methanol (solvent B)
3. Flow Rate	Gradient elution
4. Wavelength	295 and 450 nm
5. Injection Volume	2 μL

Gradient elution for the analysis fat-soluble vitamins

Time	A (%)	B (%)	Flow ($mL.min^{-1}$)
0	35	65	1
6	35	65	1
9	0	100	1.2
15	35	65	1

Note: A: Ethanol, B: Methanol

The chromatographic separation of α-tocopherol (vitamin E) and β-carotene using the gradient elution program is shown in Fig. 4.6. The elution order was: vitamin E and β-carotene. Vitamin were separated to the baseline and eluted as sharp peak within 15 min.

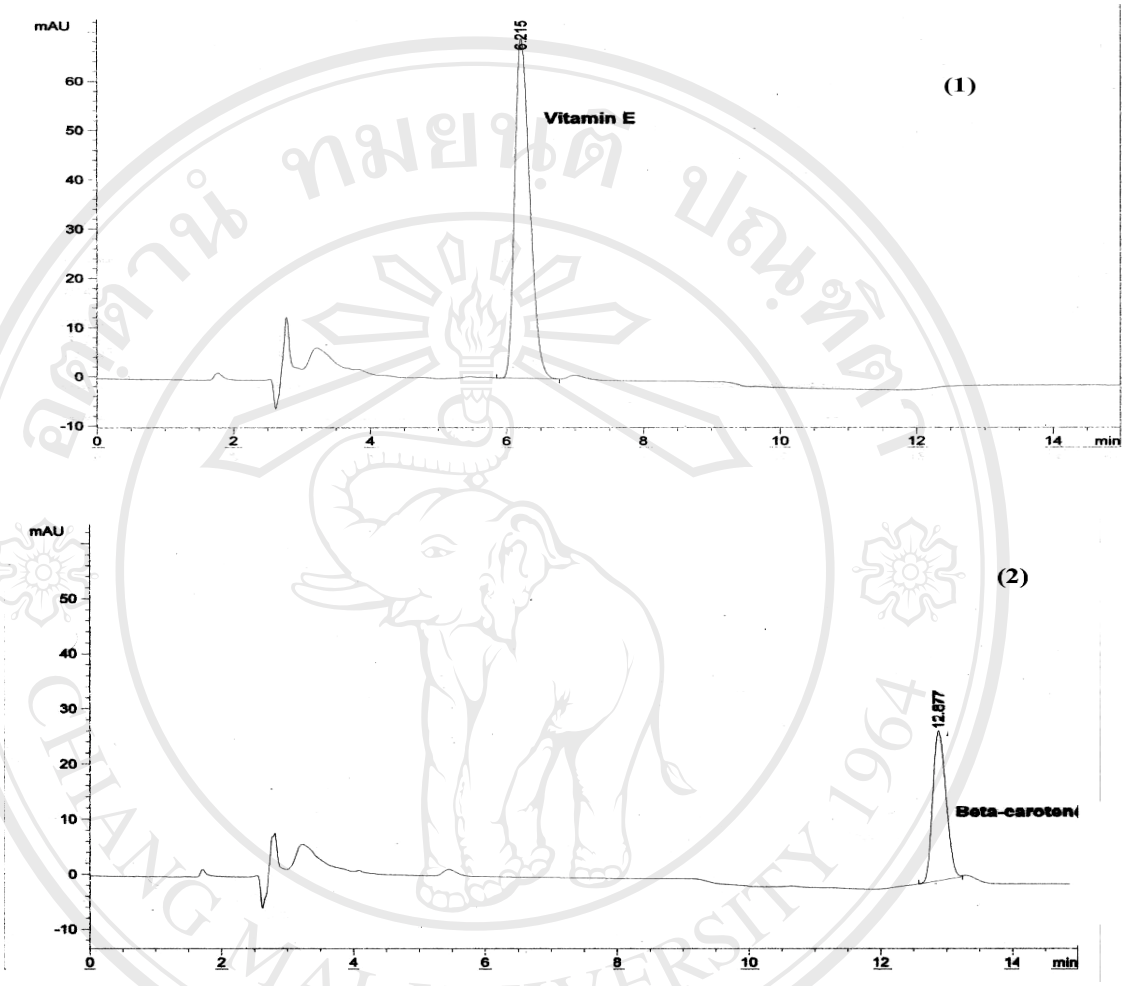

Figure 4.6 HPLC chromatogram of standard: (1) α-tocopherol (30.0 μg.mL^{-1}); (2) β-carotene (40.0 μg.mL^{-1}). The initial setting of UV detector was set at 295 nm and was then changed to 450 nm after 8 min.

4.1.3 Construction of calibration curves

4.1.3.1 Calibration Curve of Ascorbic acid

Stock standard solution containing 1000.0 $\mu g.mL^{-1}$ of ascorbic acid was prepared in water and stored in a glass-stoppered bottle at 4 ºC in the dark. Various concentrations of this solution containing 100.0, 300.0, 500.0, 700.0 and 1000.0 $\mu g.mL^{-1}$ were prepared by appropriate dilutions with the mobile phase solution. These solutions were analyzed by RP-HPLC using the conditions as listed in Table 4.1. Results are shown in Figs. 4.7 and 4.8.

Table 4.4 Relationship between L-ascorbic acid concentrations (100.0-1000.0 $\mu g.mL^{-1}$) and peak areas.

Concentration of L-ascorbic acid ($\mu g.mL^{-1}$)	**Peak area**
100.0	361.06
300.0	1045.99
500.0	1673.83
700.0	2317.99
1000.0	3263.22

Table 4.5 Relationship between L-ascorbic acid concentrations (5.0-800.0 µg.mL^{-1}) and peak areas.

Concentration of L-ascorbic acid (µg.mL^{-1})	**Peak area**
5.0	10.87
10.0	25.46
25.0	82.53
50.0	174.62
100.0	362.18
200.0	722.56
300.0	1045.20
400.0	1369.50
500.0	1673.58
600.0	2026.14
700.0	2317.76
800.0	2645.87

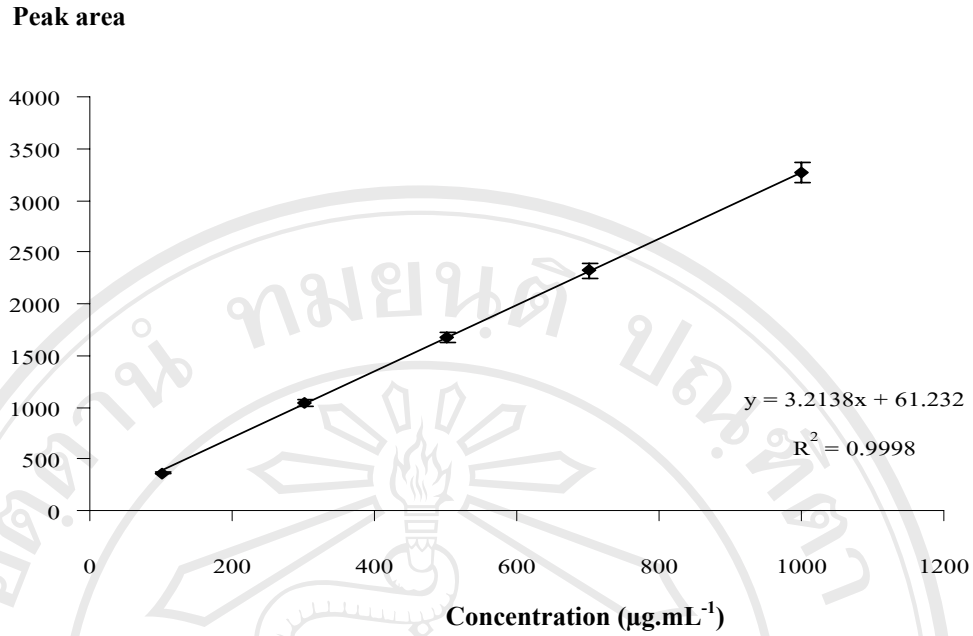

Figure 4.7 Calibration curve of standard ascorbic acid, concentrations ranging from 100.0 to 1000.0 µg.mL^{-1}.

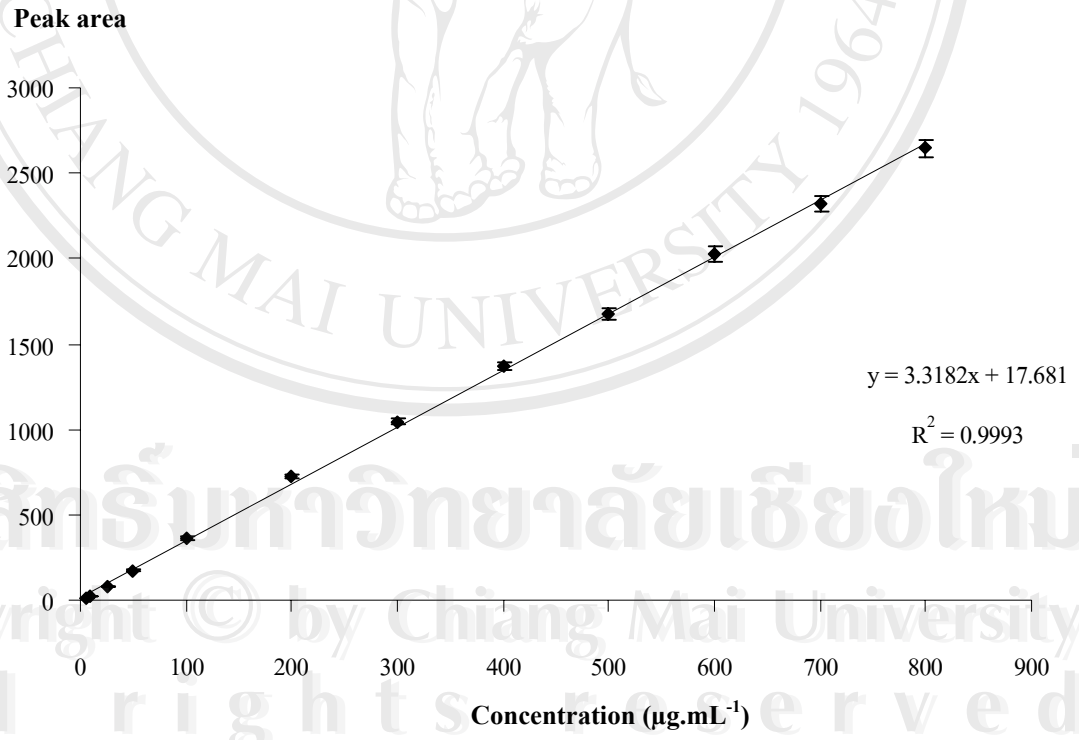

Figure 4.8 Calibration curve of standard ascorbic acid, concentrations ranging from 5.0 to 800.0 µg.mL^{-1}.

4.1.3.2 Calibration Curve of vitamins B_1, B_2, B_3 and B_6

The standard stock solutions of thiamine hydrochloride (vitamin B_1), pyridoxine hydrochloride (vitamin B_6) and nicotinamide (vitamin B_3) were prepared by dissolving 10.0 mg of the commercial products, without prior purification, in 10 ml of water. Stock solution (1000.0 μg mL^{-1}) of riboflavin (vitamin $B_{2)}$ was prepared by dissolving 10.0 mg of the commercial product in 3 ml of 1 M phosphoric acid and diluted up to 10 ml with water and then sonicated for 2 min. Working standard solutions of thiamine hydrochloride with concentrations ranging from 10.0 to 100.0 μg mL^{-1}, riboflavin (10.0 to 100.0 μg mL^{-1}), nicotinamide (10.0 to 100.0 μg mL^{-1}) and pyridoxine hydrochloride (0.50 to 50.0 μg mL^{-1}) were prepared by making appropriate dilutions of their stock solutions. These solutions were analyzed by RP-HPLC using the conditions as described in Table 4.2. Results are shown in Fig. 4.9 (standard solution of vitamin B_1), Fig. 4.10 (standard solution of vitamin B_2), Fig. 4.11 (standard solution of vitamin B_3) and Fig. 4.12 (standard solution of vitamin B_6).

Table 4.6 Relationship between thiamine hydrochloride concentrations and peak areas.

Concentration of Thiamine hydrochloride (μg.mL^{-1})	**Peak area**
10.0	3.05
30.0	17.35
50.0	34.29
70.0	51.17
100.0	75.13

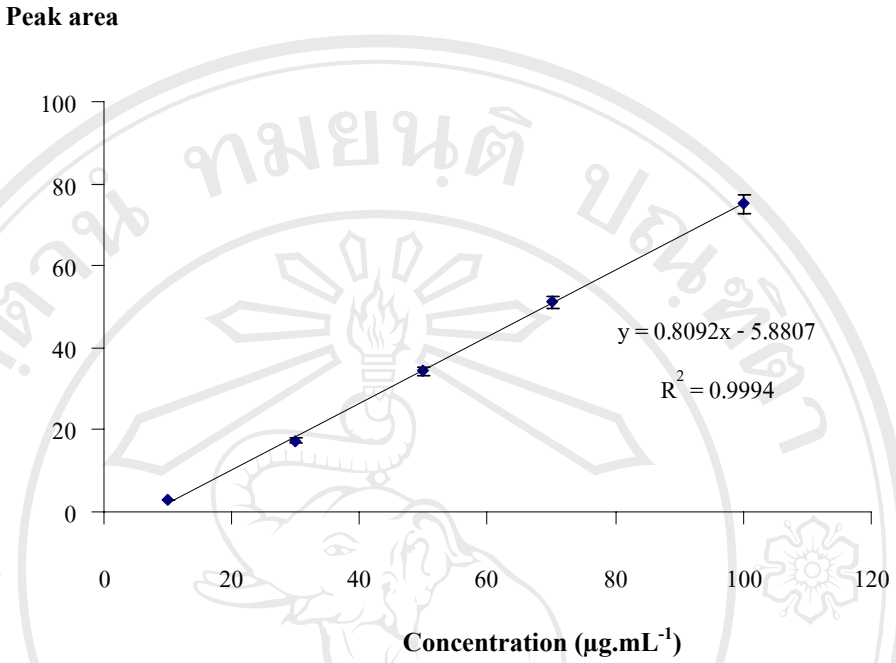

Figure 4.9 Calibration curve of standard thiamine hydrochloride, concentrations ranging from 10.0 to 100.0 µg.mL^{-1}.

Table 4.7 Relationship between riboflavin concentrations and peak areas.

Concentration of Riboflavin (µg.mL^{-1})	Peak area
10.0	11.11
30.0	32.09
50.0	54.62
70.0	80.06
100.0	112.05

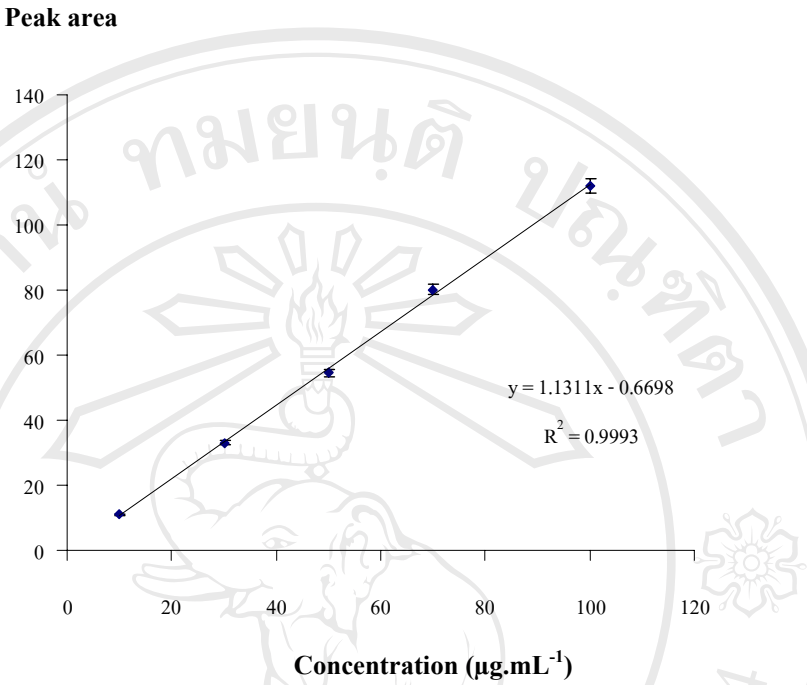

Figure 4.10 Calibration curve of standard riboflavin, concentrations ranging from 10.0 to 100.0 µg.mL^{-1}.

Table 4.8 Relationship between nicotinamide concentrations and peak areas.

Concentration of Nicotinamide (µg.mL^{-1})	**Peak area**
10.0	3.03
30.0	9.65
50.0	16.49
70.0	22.74
100.0	32.04

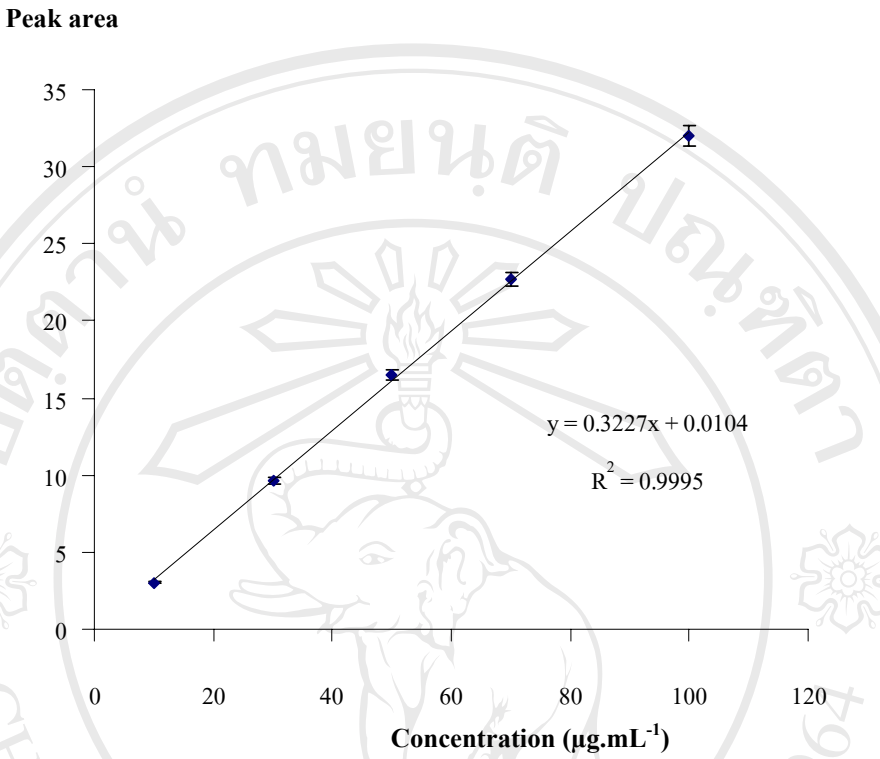

Figure 4.11 Calibration curve of standard nicotinamide, concentrations ranging from 10.0 to 100.0 µg.mL^{-1}.

Table 4.9 Relationship between pyridoxine hydrochloride concentrations and peak areas.

Concentration of nicotinamide (µg.mL^{-1})	Peak area
0.5	3.23
5.0	15.13
10.0	30.30
20.0	60.99
30.0	91.22
50.0	154.25

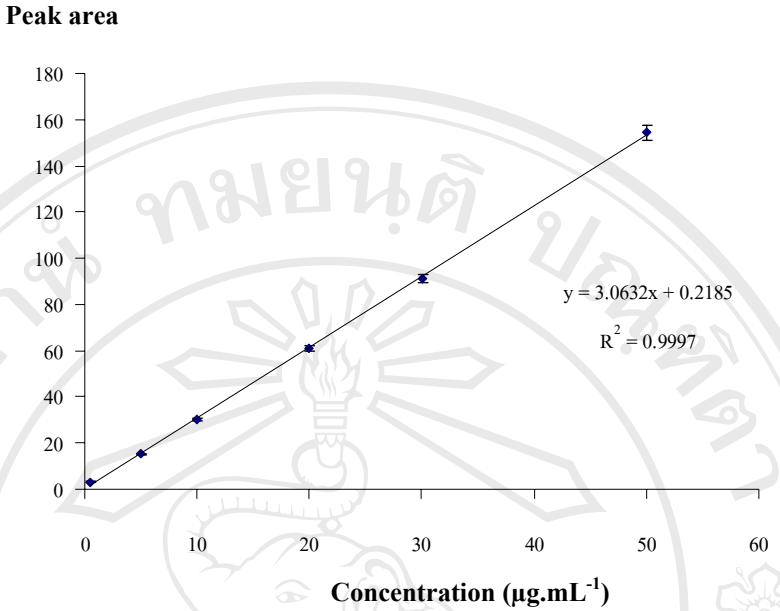

Figure 4.12 Calibration curve of standard pyridoxine hydrochloride, concentrations ranging from 0.5 to 50.0 µg.mL^{-1}.

4.1.3.3 Calibration Curves of α-tocopherol and β-carotene

The standard stock solutions of α-tocopherol and β-carotene were prepared by dissolving 1.0 mg of standard α-tocopherol and β-carotene in 10 ml of ethanol. Working standard solutions containing of α-tocopherol 1.0 to 10.0 µg mL^{-1}, and 1.0 to 10.0 µg mL^{-1} of β-carotene were prepared by dilution from standard stock solution. These solutions were analyzed by RP-HPLC using the condition as described in Table 4.3. Results are shown in Fig. 4.13 (standard solution of α-tocopherol) and Fig. 4.14 (standard solution of β-carotene).

Table 4.10 Relationship between α-tocopherol concentrations and peak areas.

Concentration of α-tocopherol ($\mu g.mL^{-1}$)	Peak area
1.0	24.37
3.0	93.71
5.0	162.93
7.0	232.12
10.0	323.51

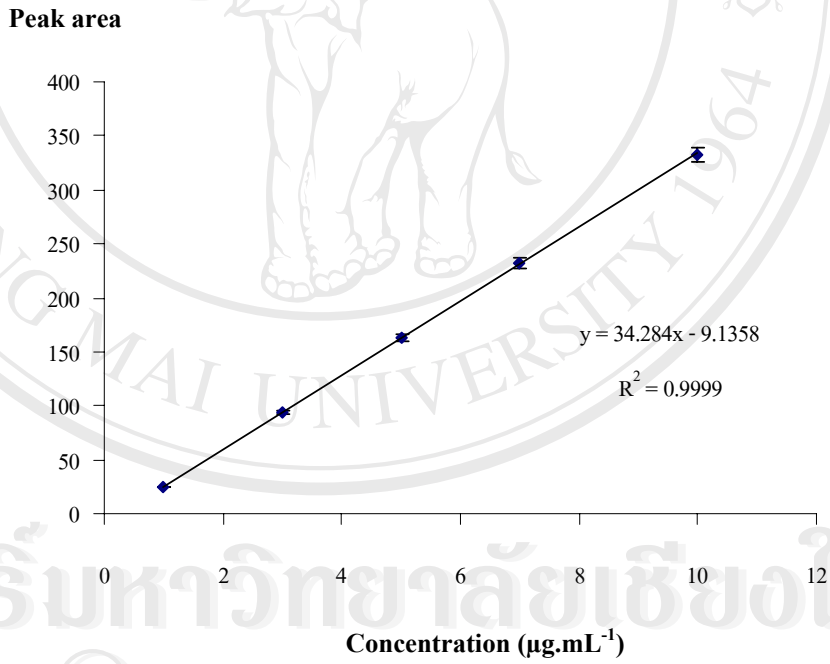

Figure 4.13 Calibration curve of standard α-tocopherol, concentrations ranging from 1.0 to 10.0 $\mu g.mL^{-1}$.

Table 4.11 Relationship between β-carotene concentrations and peak areas.

Concentration of β-carotene ($\mu g.mL^{-1}$)	Peak area
1.0	5.62
3.0	21.94
5.0	38.29
7.0	55.78
10.0	81.05

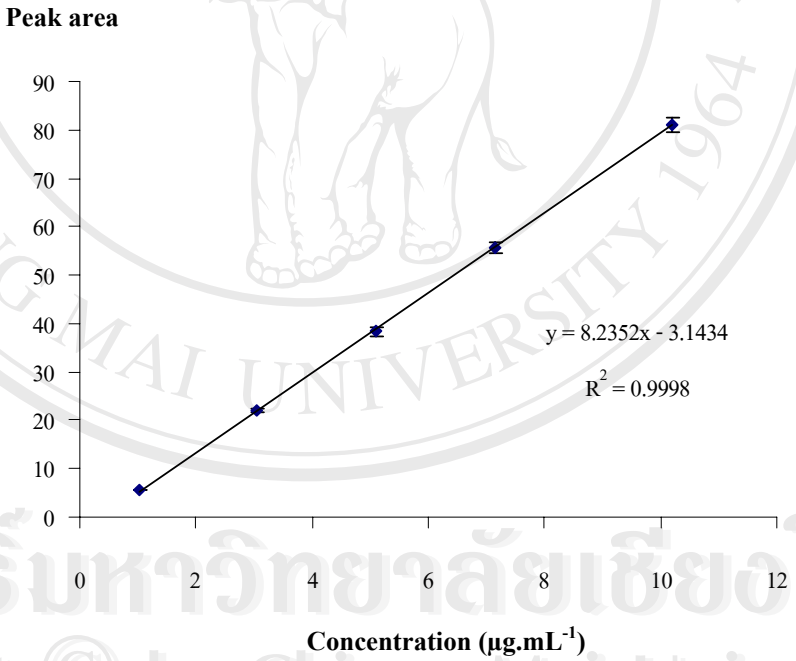

Figure 4.14 Calibration curve of standard β-carotene, concentrations ranging from 1.0 to 10.0 $\mu g.mL^{-1}$.

4.2 Validation of the Method

The HPLC method was validated by a standard procedure to evaluate if adequate accuracy, precision, selectivity and linearity had been achieved.

4.2.1 Limit of Detection

The limit of detection (LOD) was determined by decreasing the concentration of standard vitamin solutions (ascorbic acid, thiamine hydrochloride, riboflavin, nicotinamide, pyridoxine hydrochloride, α-tocopherol and β-carotene) and then measuring the signal to noise ratio from the result chromatograms. The limit of detection was obtained when the signal peak height was three times the noise or measurement of the signal-to-noise peak height ratio of 3:1. Table 4.12 shows the LOD values for the vitamins tested.

Table 4.12 Limits of detection of vitamins

Analyte	LOD ($\mu g.mL^{-1}$)
Ascorbic acid	0.50
Thiamine Hydrochloride	0.50
Riboflavin	0.10
Nicotinamide	2.00
Pyridoxine Hydrochloride	0.05
α-Tocopherol	0.01
β-Carotene	0.01

4.2.2 Limit of Quantitation

The limit of quantitation (LOQ) of standard vitamin solutions (ascorbic acid, thiamine hydrochloride, riboflavin, nicotinamide, pyridoxine hydrochloride, α-tocopherol and β-carotene) was achieved by measurement of the signal-to-noise peak height ratio of 10:1. The limit of quantitative was determined by testing diluents of the lowest concentration of standard solution (ascorbic acid, thiamin hydrochloride, riboflavin, nicotinamide, pyridoxine hydrochloride, α-tocopherol and β-carotene) used for linearity, and then measuring the signal-to-noise ratio from the result chromatograms. The limits of quantitation of each standards vitamin solution (ascorbic acid, thiamin hydrochloride, riboflavin, nicotinamide, pyridoxine hydrochloride, α-tocopherol and β-carotene) are shown in Table 4.13.

Table 4.13 Limits of quantitation of vitamins

Analyte	LOQ ($\mu g.mL^{-1}$)
Ascorbic acid	1.50
Thiamine Hydrochloride	1.50
Riboflavin	0.50
Nicotinamide	5.00
Pyridoxine Hydrochloride	0.50
α-Tocopherol	0.05
β-Carotene	0.03

4.2.3 Linearity studies

Linear range of each vitamin was determined. The calibration graph of each vitamin was constructed by plotting the peak areas of vitamin against concentration using linear regression analysis.

4.2.3.1 Linearity of Ascorbic acid

A series of ten solutions containing vitamin C in the concentrations ranging from 100.0 – 1000.0 $\mu g.mL^{-1}$ were prepared. Linearity of vitamin C was determined. Results are shown in Fig. 4.15.

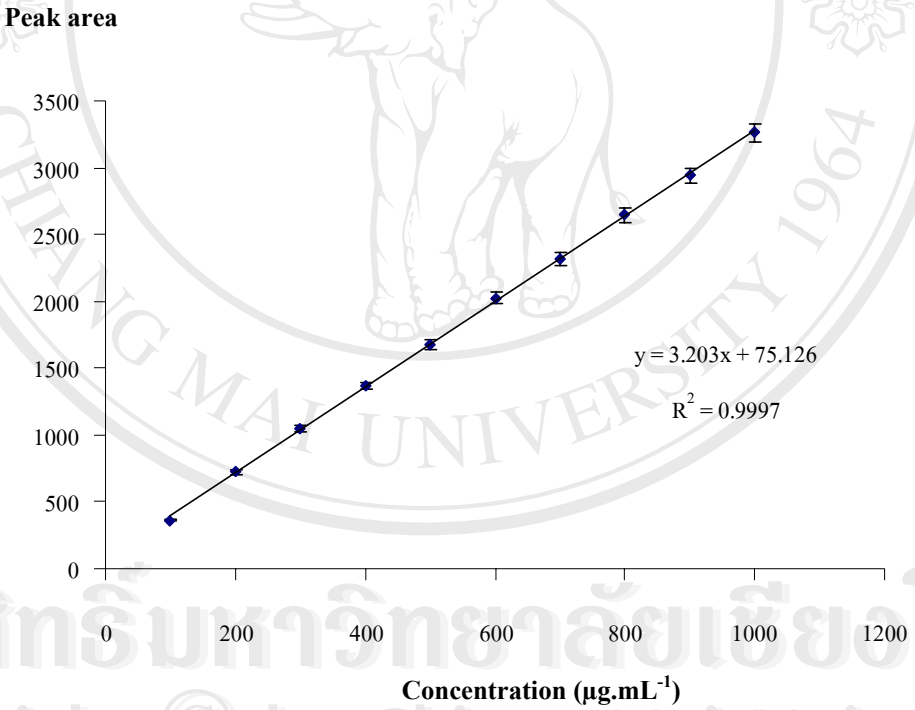

Figure 4.15 Linearity of standard ascorbic acid, concentrations ranging from 100.0 to 1000.0 $\mu g.mL^{-1}$.

4.2.3.2 Linearity of Thiamine hydrochloride, Riboflavin, Nicotinamide and Pyridoxine hydrochloride

Linearity of thiamine hydrochloride with concentrations 10.0 - 500.0 μg.mL^{-1}, riboflavin (10.0 - 200.0 μg mL^{-1}), nicotinamide (10.0 - 900.0 μg mL^{-1}) and pyridoxine hydrochloride (0.50 - 50.0 μg mL^{-1}) were studied. Results are shown in Fig. 4.16 (standard solution of vitamin B_1), Fig. 4.17 (standard solution of vitamin B_2), Fig. 4.18 (standard solution of vitamin B_3) and Fig. 4.19 (standard solution of vitamin B_6).

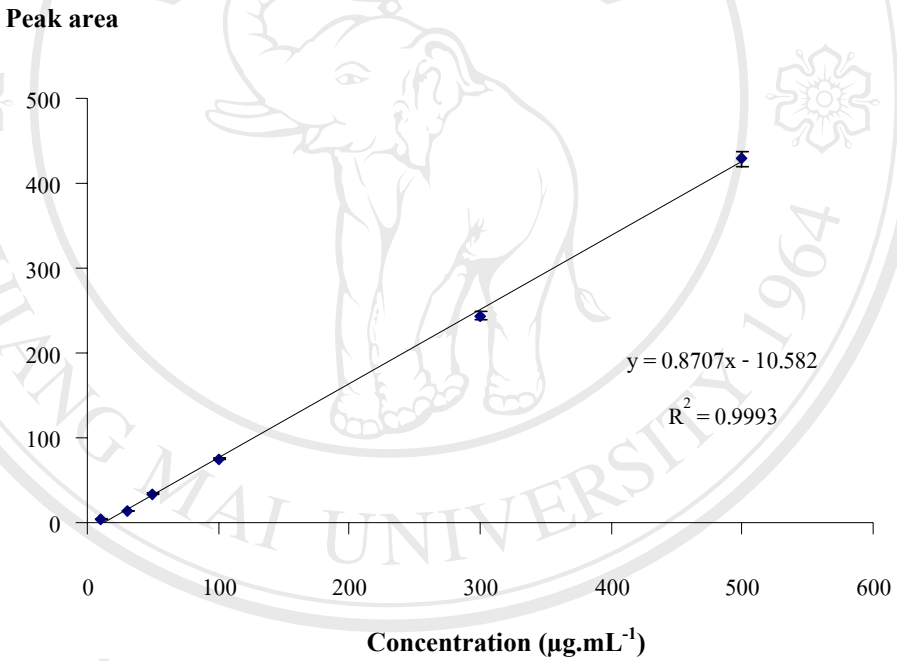

Figure 4.16 Linearity of standard thiamine hydrochloride, concentrations ranging from 10.0 to 500.0 μg.mL^{-1}.

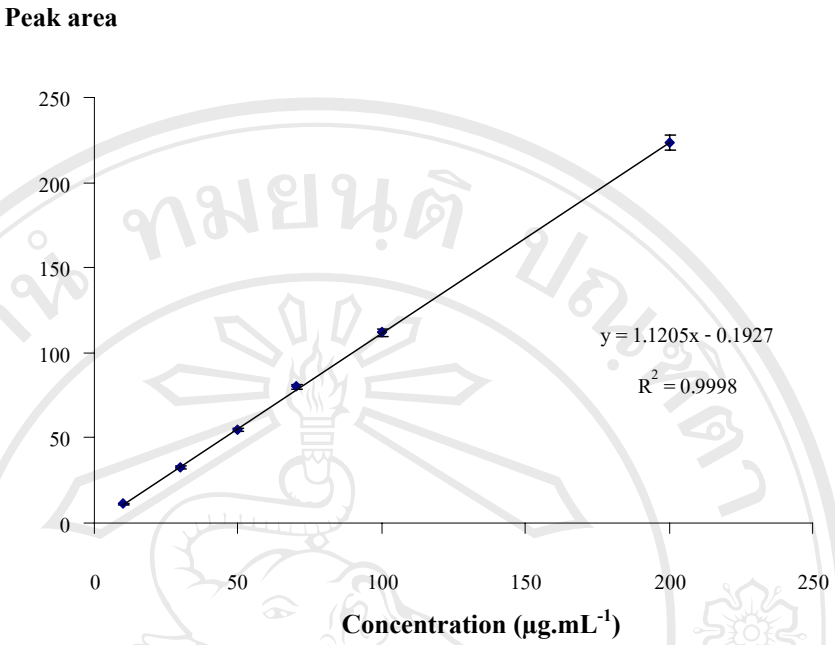

Figure 4.17 Linearity of standard riboflavin, concentrations ranging from 10.0 to 200.0 µg.mL^{-1}.

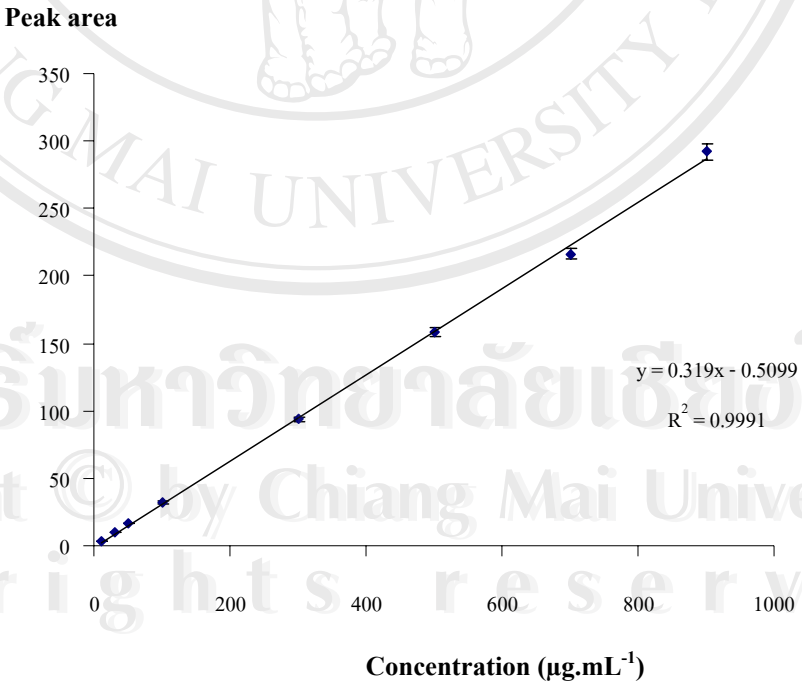

Figure 4.18 Linearity of standard nicotinamide, concentrations ranging from 10.0 to 900.0 µg.mL^{-1}.

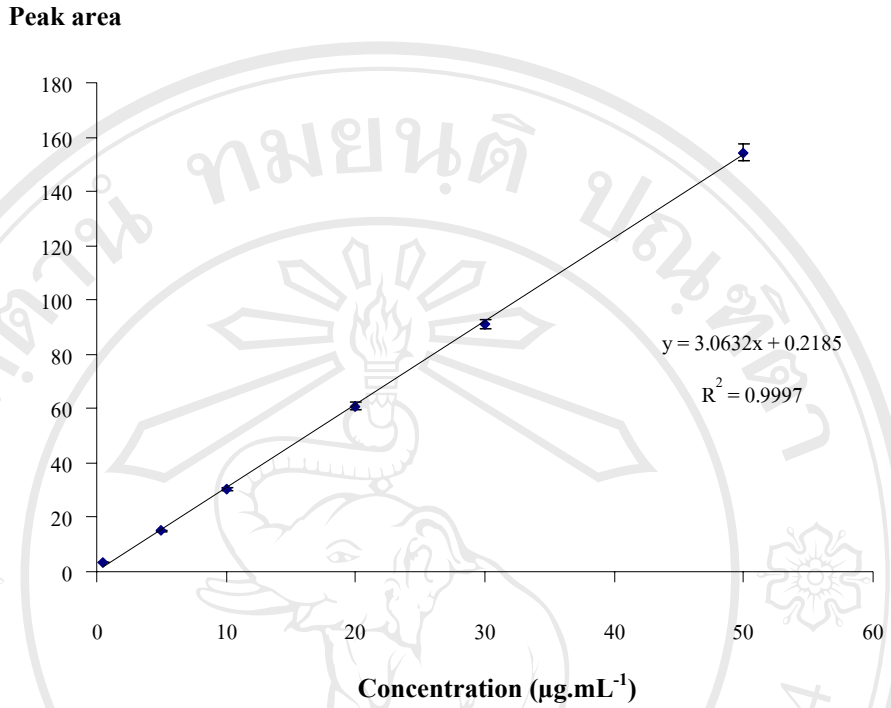

Figure 4.19 Linearity of standard pyridoxine hydrochloride, concentrations ranging from 0.5 to 50.0 µg.mL^{-1}.

4.2.3.2 Linearity of α-Tocopherol and β-Carotene

Linearity was checked for each vitamin using varies standard solutions with concentrations ranging from 0.5 - 100.0 μg mL^{-1} of α-tocopherol (Fig. 4.20) and 1.0 - 100.0 μg $mL^{-1}$of β-carotene (Fig. 4.21). Linearity regression was used to determine the slope and intercept.

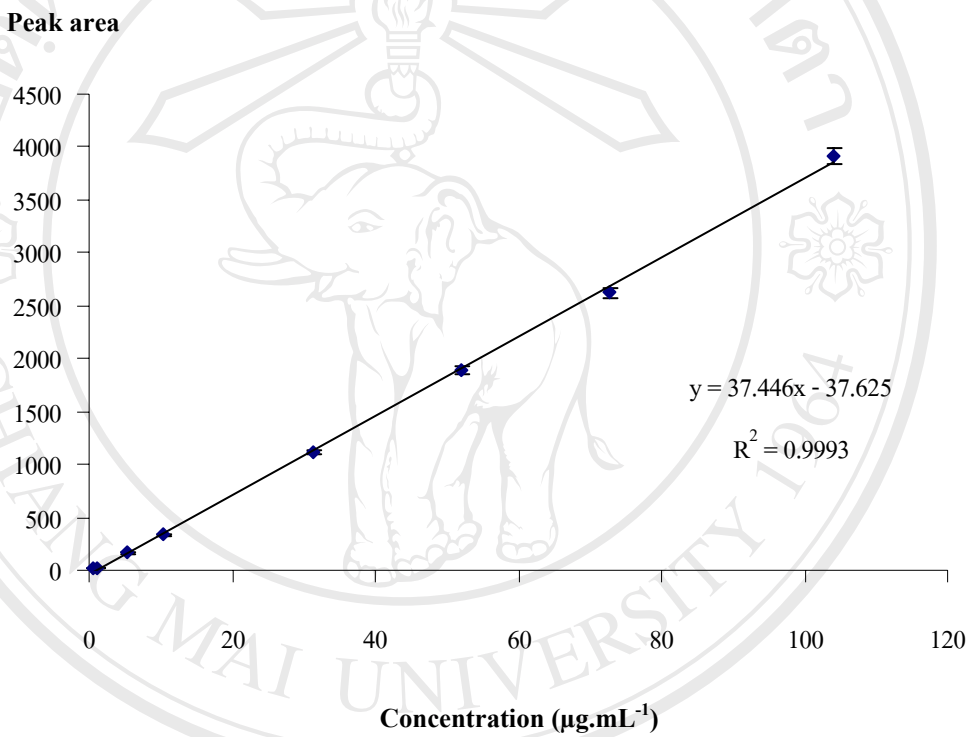

Figure 4.20 Linearity of standard α-tocopherol, concentrations ranging from 0.5 to 100.0 μg.mL^{-1}.

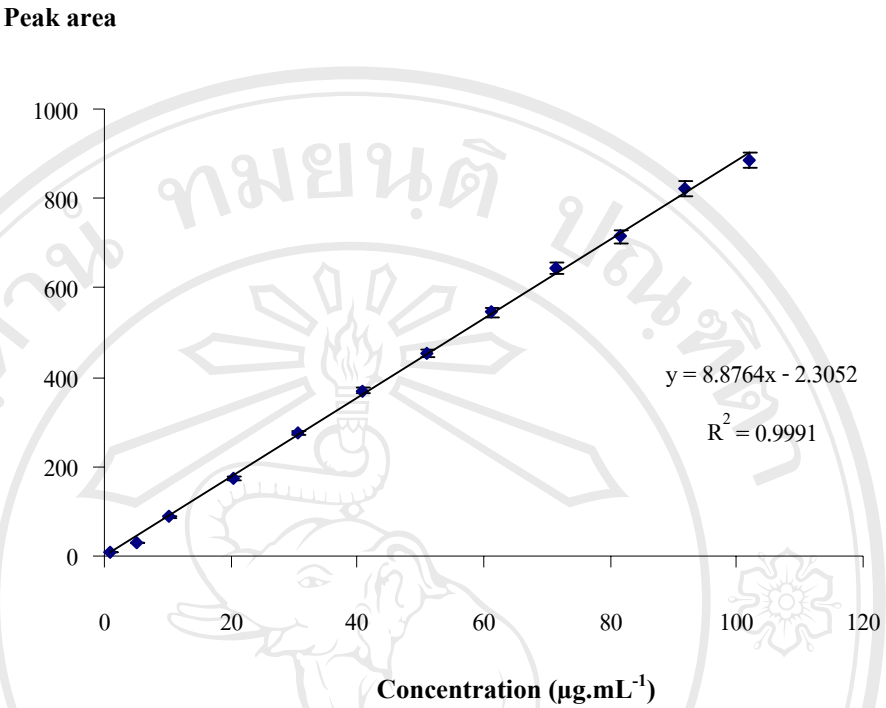

Figure 4.21 Linearity of standard β-carotene, concentrations ranging from 0.5 to 100.0 µg.mL^{-1}.

4.2.4 Accuracy Assay

The accuracy of the method was determined by investigating the recovery of samples of spiking standard vitamin C, thiamine HCl, riboflavin, nicotinamide, pyridoxine HCl, α-tocopherol and β-carotene into sample extracts and comparing the measured value to the true value.

4.2.4.1 Accuracy of vitamin C in fruits of *M. citrifolia* and *P. emblica*

The accuracy of vitamin C in *M. citrifolia* and *P. emblica* fruits were determined the mean recoveries were presented in Table 4.14 and Table 4.15.

Table 4.14 Recovery assay of L-ascorbic acid by RP-HPLC (n=5) in *M. citrifolia* fruits.

Concentration of L-ascorbic acid added ($\mu g.mL^{-1}$)	Concentration found (Mean ± S.D.) ($\mu g.mL^{-1}$)	Recovery (%)	Relative error (%)
400.0	402.82 ± 0.76	100.71 ± 0.24	2.82
500.0	501.43 ± 1.15	100.29 ± 0.36	1.43
600.0	600.57 ± 0.93	100.10 ± 0.93	0.57

Table 4.15 Recovery assay of L-ascorbic acid by RP-HPLC (n=5) in *P. emblica* fruits.

Concentration of L-ascorbic acid added ($\mu g.mL^{-1}$)	Concentration found (Mean ± S.D.) ($\mu g.mL^{-1}$)	Recovery (%)	Relative error (%)
400.0	396.69 ± 0.86	99.17 ± 0.27	-3.31
500.0	496.16 ± 0.67	99.23 ± 0.21	-3.84
600.0	597.31 ± 0.79	99.55 ± 0.25	-2.69

4.2.4.2 Accuracy of vitamins B group in *M. citrifolia* and *P. emblica* fruits.

The accuracies of thiamine hydrochloride *M. citrifolia* and *P. emblica* fruits were determined the mean recoveries were shown in Table 4.16 to Table 4.23.

Table 4.16 Recovery assay of thiamine hydrochloride by RP-HPLC (n=5) in *M. citrifolia* fruits.

Concentration of thiamine HCl added ($\mu g.mL^{-1}$)	Concentration found (Mean ± S.D.) ($\mu g.mL^{-1}$)	Recovery (%)	Relative error (%)
10.0	9.98 ± 0.63	99.80 ± 0.16	-0.02
20.0	20.12 ± 0.74	100.60 ± 0.42	0.12
30.0	29.95 ± 0.82	99.83 ± 0.24	-0.05

Table 4.17 Recovery assay of riboflavin by RP-HPLC (n=5) in *M. citrifolia* fruits.

Concentration of riboflavin added ($\mu g.mL^{-1}$)	Concentration found (Mean ± S.D.) ($\mu g.mL^{-1}$)	Recovery (%)	Relative error (%)
10.0	10.16 ± 0.86	101.60 ± 0.51	0.16
20.0	19.87 ± 0.67	99.35 ± 0.46	-0.13
30.0	29.78 ± 0.79	99.26 ± 0.27	-0.22

Table 4.18 Recovery assay of nicotinamide by RP-HPLC (n=5) in *M.citrifolia* fruits.

Concentration of nicotinamide added ($\mu g.mL^{-1}$)	Concentration found (Mean ± S.D.) ($\mu g.mL^{-1}$)	Recovery (%)	Relative error (%)
10.0	9.94 ± 0.48	99.40 ± 0.18	-0.06
20.0	20.05 ± 0.56	100.25 ± 0.26	0.05
30.0	29.84 ± 0.79	99.47 ± 0.37	-0.16

Table 4.19 Recovery assay of pyridoxine hydrochloride by RP-HPLC (n=5) in *M. citrifolia* fruits.

Concentration of pyridoxine HCl added ($\mu g.mL^{-1}$)	Concentration found (Mean ± S.D.) ($\mu g.mL^{-1}$)	Recovery (%)	Relative error (%)
10.0	9.92 ± 0.48	99.20 ± 0.18	-0.08
20.0	20.12 ± 0.08	100.60 ± 0.11	0.12
30.0	29.94 ± 0.24	99.80 ± 0.25	-0.06

Table 4.20 Recovery assay of thiamine hydrochloride by RP-HPLC (n=5) in *P. emblica* fruits.

Concentration of thiamine HCl added ($\mu g.mL^{-1}$)	Concentration found (Mean ± S.D.) ($\mu g.mL^{-1}$)	Recovery (%)	Relative error (%)
10.0	10.15 ± 1.25	99.17 ± 0.16	0.15
20.0	19.97 ± 0.87	99.85 ± 0.07	-0.03
30.0	30.07 ± 0.64	100.23 ± 0.28	0.07

Table 4.21 Recovery assay of riboflavin by RP-HPLC (n=5) in *P. emblica* fruits.

Concentration of riboflavin added ($\mu g.mL^{-1}$)	Concentration found (Mean ± S.D.) ($\mu g.mL^{-1}$)	Recovery (%)	Relative error (%)
10.0	9.92 ± 1.25	99.20 ± 0.83	-0.08
20.0	20.36 ± 2.37	101.80 ± 0.67	0.36
30.0	29.73 ± 1.65	99.10 ± 0.42	-0.27

Table 4.22 Recovery assay of nicotinamide by RP-HPLC (n=5) in *P. emblica* fruits.

Concentration of nicotinamide added ($\mu g.mL^{-1}$)	Concentration found (Mean ± S.D.) ($\mu g.mL^{-1}$)	Recovery (%)	Relative error (%)
10.0	10.17 ± 0.23	101.70 ± 1.56	0.17
20.0	19.92 ± 1.52	99.60 ± 0.38	-0.08
30.0	30.16 ± 0.51	100.53 ± 0.85	0.16

Table 4.23 Recovery assay of pyridoxine hydrochloride by RP-HPLC (n=5) in *P. emblica* fruits.

Concentration of pyridoxine HCl added ($\mu g.mL^{-1}$)	Concentration found (Mean ± S.D.) ($\mu g.mL^{-1}$)	Recovery (%)	Relative error (%)
10.0	9.91 ± 1.28	99.10 ± 1.53	-0.09
20.0	19.85 ± 2.17	99.25 ± 0.97	-0.15
30.0	29.76 ± 1.85	99.20 ± 1.74	-0.24

4.2.4.3 Accuracy of α-tocopherol and β-carotene in *M. citrifolia* and *P. emblica* fruits.

The accuracies of α-tocopherol and β-carotene *M. citrifolia* and *P. emblica* fruits were determined the mean recoveries were shown in Table 4.24 to Table 4.27.

Table 4.24 Recovery assay of α-tocopherol by RP-HPLC (n=5) in *M. citrifolia* fruits.

Concentration of α-tocopherol added ($\mu g.mL^{-1}$)	**Concentration found (Mean ± S.D.) ($\mu g.mL^{-1}$)**	**Recovery (%)**	**Relative error (%)**
5.0	4.98 ± 0.21	99.60 ± 2.66	-0.02
10.0	10.08 ± 0.08	100.80 ± 0.50	0.08

Table 4.25 Recovery assay of β-carotene by RP-HPLC (n=5) in *M. citrifolia* fruits.

Concentration of β-carotene added ($\mu g.mL^{-1}$)	**Concentration found (Mean ± S.D.) ($\mu g.mL^{-1}$)**	**Recovery (%)**	**Relative error (%)**
5.0	4.99 ± 0.03	99.80 ± 1.40	-0.01
10.0	10.15 ± 0.15	101.50 ± 0.34	0.15

Table 4.26 Recovery assay of α-tocopherol by RP-HPLC (n=5) in *P. emblica* fruits.

Concentration of α-tocopherol added ($\mu g.mL^{-1}$)	Concentration found (Mean ± S.D.) ($\mu g.mL^{-1}$)	Recovery (%)	Relative error (%)
5.0	5.03 ± 0.32	100.60 ± 0.47	0.03
10.0	9.97 ± 0.16	99.70 ± 2.15	-0.03

Table 4.27 Recovery assay of β-carotene by RP-HPLC (n=5) in *P. emblica* fruits.

Concentration of β-carotene added ($\mu g.mL^{-1}$)	Concentration found (Mean ± S.D.) ($\mu g.mL^{-1}$)	Recovery (%)	Relative error (%)
5.0	4.96 ± 0.01	99.20 ± 0.07	-0.04
10.0	10.03 ± 0.07	100.30 ± 1.16	0.03

4.2.5 Precision

The precision of the LC method for determining vitamins was confirmed by analyzing each sample (n=5), using the proposed HPLC method. All the relative standard deviation for vitamin content in both samples was less than 2%.

4.2.5.1 Precision of vitamin C in *M. citrifolia* and *P. emblica* fruits.

The results for within-day and between-day precision are presented in Tables 4.28 and 4.29.

Table 4.28 Precision of vitamin C in *M. citrifolia* fruits.

Concentration of vitamin C added ($\mu g.mL^{-1}$)	Within-day variability (n=5)		Between-day variability (n=5)	
	Concentration found (Mean ± S.D.) ($\mu g.mL^{-1}$)	R.S.D.	Concentration found (Mean ± S.D.) ($\mu g.mL^{-1}$)	R.S.D.
400.0	398.16 ± 0.08	0.02	400.03 ± 0.04	0.01
500.0	500.02 ± 0.08	0.01	498.57 ± 0.01	0.00
600.0	597.02 ± 0.12	0.02	600.10 ± 0.26	0.04

Table 4.29 Precision of vitamin C in *P. emblica* fruit.

Concentration of vitamin C added ($\mu g.mL^{-1}$)	Within-day variability (n=5)		Between-day variability (n=5)	
	Concentration found (Mean ± S.D.) ($\mu g.mL^{-1}$)	R.S.D.	Concentration found (Mean ± S.D.) ($\mu g.mL^{-1}$)	R.S.D.
400.0	400.03 ± 0.15	0.04	400.03 ± 0.09	0.02
500.0	498.73 ± 0.25	0.05	499.84 ± 0.16	0.03
600.0	600.08 ± 0.31	0.05	598.89 ± 0.28	0.05

4.2.5.2 Precision of vitamin B in *M. citrifolia* and *P. emblica* fruits.

The results for within-day and between-day precision are presented in Table 4.30 to Table 4.37.

Table 4.30 Precision of vitamin B_1 in *M. citrifolia* fruits.

Concentration of vitamin B_1 added ($\mu g.mL^{-1}$)	Within-day variability (n=5)		Between-day variability (n=5)	
	Concentration found (Mean ± S.D.) ($\mu g.mL^{-1}$)	R.S.D.	Concentration found (Mean ± S.D.) ($\mu g.mL^{-1}$)	R.S.D.
10.0	9.98 ± 0.03	0.30	10.01 ± 0.03	0.30
20.0	20.01 ± 0.11	0.55	19.87 ± 0.12	0.60
30.0	30.02 ± 0.03	0.10	29.98 ± 0.03	0.10

Table 4.31 Precision of vitamin B_2 in *M. citrifolia* fruits.

Concentration of vitamin B_2 added ($\mu g.mL^{-1}$)	Within-day variability (n=5)		Between-day variability (n=5)	
	Concentration found (Mean ± S.D.) ($\mu g.mL^{-1}$)	R.S.D.	Concentration found (Mean ± S.D.) ($\mu g.mL^{-1}$)	R.S.D.
10.0	10.04 ± 0.15	1.49	10.02 ± 0.17	1.70
20.0	19.86 ± 0.21	1.06	20.05 ± 0.24	1.20
30.0	30.01 ± 0.06	0.20	29.99 ± 0.05	0.17

Table 4.32 Precision of vitamin B_3 in *M. citrifolia* fruits.

Concentration of vitamin B_3 added ($\mu g.mL^{-1}$)	Within-day variability (n=5)		Between-day variability (n=5)	
	Concentration found (Mean ± S.D.) ($\mu g.mL^{-1}$)	R.S.D.	Concentration found (Mean ± S.D.) ($\mu g.mL^{-1}$)	R.S.D.
10.0	9.97 ± 0.13	1.30	10.03 ± 0.09	0.90
20.0	20.08 ± 0.05	0.25	19.84 ± 0.05	0.25
30.0	29.79 ± 0.03	0.10	29.89 ± 0.01	0.03

Table 4.33 Precision of vitamin B_6 in *M. citrifolia* fruits.

Concentration of vitamin B_6 added ($\mu g.mL^{-1}$)	Within-day variability (n=5)		Between-day variability (n=5)	
	Concentration found (Mean ± S.D.) ($\mu g.mL^{-1}$)	R.S.D.	Concentration found (Mean ± S.D.) ($\mu g.mL^{-1}$)	R.S.D.
10.0	9.86 ± 0.02	0.20	9.97 ± 0.02	0.20
20.0	20.07 ± 0.16	0.80	19.91 ± 0.21	1.05
30.0	30.03 ± 0.03	0.10	29.93 ± 0.04	0.13

Table 4.34 Precision of vitamin B_1 in *P. emblica* fruits.

Concentration of vitamin B_1 added ($\mu g.mL^{-1}$)	Within-day variability (n=5)		Between-day variability (n=5)	
	Concentration found (Mean ± S.D.) ($\mu g.mL^{-1}$)	R.S.D.	Concentration found (Mean ± S.D.) ($\mu g.mL^{-1}$)	R.S.D.
10.0	10.01 ± 0.01	0.10	9.99 ± 0.01	0.10
20.0	19.88 ± 0.26	1.31	19.88 ± 0.26	1.31
30.0	30.02 ± 0.03	0.10	30.06 ± 0.02	0.07

Table 4.35 Precision of vitamin B_2 in *P. emblica* fruits.

Concentration of vitamin B_2 added ($\mu g.mL^{-1}$)	Within-day variability (n=5)		Between-day variability (n=5)	
	Concentration found (Mean ± S.D.) ($\mu g.mL^{-1}$)	R.S.D.	Concentration found (Mean ± S.D.) ($\mu g.mL^{-1}$)	R.S.D.
10.0	9.99 ± 0.02	0.20	10.01 ± 0.02	0.20
20.0	19.96 ± 0.16	0.80	19.93 ± 0.11	0.55
30.0	30.01 ± 0.01	0.03	29.84 ± 0.01	0.03

Table 4.36 Precision of vitamin B_3 in *P. emblica* fruits.

Concentration of vitamin B_3 added ($\mu g.mL^{-1}$)	Within-day variability (n=5)		Between-day variability (n=5)	
	Concentration found (Mean ± S.D.) ($\mu g.mL^{-1}$)	R.S.D.	Concentration found (Mean ± S.D.) ($\mu g.mL^{-1}$)	R.S.D.
10.0	10.02 ± 0.07	0.70	10.07 ± 0.11	1.09
20.0	20.08 ± 0.24	1.19	19.98 ± 0.21	1.05
30.0	29.99 ± 0.12	0.40	30.01 ± 0.09	0.30

Table 4.37 Precision of vitamin B_6 in *P. emblica* fruits.

Concentration of vitamin B_6 added ($\mu g.mL^{-1}$)	Within-day variability (n=5)		Between-day variability (n=5)	
	Concentration found (Mean ± S.D.) ($\mu g.mL^{-1}$)	R.S.D.	Concentration found (Mean ± S.D.) ($\mu g.mL^{-1}$)	R.S.D.
10.0	9.98 ± 0.01	0.10	9.96 ± 0.01	0.10
20.0	20.01 ± 0.12	0.60	20.06 ± 0.08	0.40
30.0	30.05 ± 0.01	0.03	29.94 ± 0.01	0.03

4.2.5.3 Precision of α-tocopherol and β-carotene in *M. citrifolia* and *P. emblica* fruits.

The results for within-day and between-day precision are presented in Table 4.38 to Table 4.41.

Table 4.38 Precision of α-tocopherol in *M. citrifolia* fruits.

Concentration of α-tocopherol added ($\mu g.mL^{-1}$)	Within-day variability (n=5)		Between-day variability (n=5)	
	Concentration found (Mean ± S.D.) ($\mu g.mL^{-1}$)	R.S.D.	Concentration found (Mean ± S.D.) ($\mu g.mL^{-1}$)	R.S.D.
5.0	5.00 ± 0.02	0.40	5.00 ± 0.02	0.40
10.0	10.09 ± 0.08	0.79	10.05 ± 0.11	1.09

Table 4.39 Precision of α-tocopherol in *P. emblica* fruits.

Concentration of α-tocopherol added ($\mu g.mL^{-1}$)	Within-day variability (n=5)		Between-day variability (n=5)	
	Concentration found (Mean ± S.D.) ($\mu g.mL^{-1}$)	R.S.D.	Concentration found (Mean ± S.D.) ($\mu g.mL^{-1}$)	R.S.D.
5.0	4.96 ± 0.05	1.01	5.01 ± 0.04	0.80
10.0	9.87 ± 0.02	0.20	10.01 ± 0.07	0.70

Table 4.40 Precision of β-carotene in *M. citrifolia* fruits.

Concentration of β-carotene added ($\mu g.mL^{-1}$)	Within-day variability (n=5)		Between-day variability (n=5)	
	Concentration found (Mean ± S.D.) ($\mu g.mL^{-1}$)	R.S.D.	Concentration found (Mean ± S.D.) ($\mu g.mL^{-1}$)	R.S.D.
5.0	4.97 ± 0.08	1.61	5.00 ± 0.02	0.40
10.0	10.08 ± 0.11	1.09	10.04 ± 0.07	0.70

Table 4.41 Precision of β-carotene in *P. emblica* fruits.

Concentration of β-carotene added ($\mu g.mL^{-1}$)	Within-day variability (n=5)		Between-day variability (n=5)	
	Concentration found (Mean ± S.D.) ($\mu g.mL^{-1}$)	R.S.D.	Concentration found (Mean ± S.D.) ($\mu g.mL^{-1}$)	R.S.D.
5.0	4.95 ± 0.03	0.61	4.97 ± 0.03	0.60
10.0	10.02 ± 0.01	0.10	9.94 ± 0.01	0.10

4.3 Determination of vitamin C contents in *M. citrifolia* and *P. emblica* fruits

The chromatographic system used for the determination of vitamin C consisted of a liquid chromatograph, tertiary pump, variable wavelength detector, auto-sample, auto injector and auto degasser, using the chromatographic conditions as presented in Table 4.1.

The quantification was focused on vitamin C in *M. citrifolia* and *P. emblica* fruits. It was quantified by means of an external calibration curve in the concentration range from 100.0 to 1000.0 $\mu g.mL^{-1}$ of vitamin C. The calibration equation for vitamin C was constructed by plotting the UV responses against the vitamin C concentrations at four concentration levels (analysed in triplicate). Linear calibration curve was obtained over the range 100.0 to 1000.00 $\mu g.mL^{-1}$ of vitamin C with r^2 = 0.9998. The limit of detection (LOD), defined as the minimum concentration capable of giving a chromatographic signal three times higher than background noise, was estimated at 0.50 $\mu g.mL^{-1}$. The limit of quantification (LOQ) was 1.50 $\mu g.mL^{-1}$. The recoveries of vitamin C in *M. citrifolia* fruits were found to be 100.10 ± 0.93 to 100.71 ± 0.24 % (Table 4.1) and recoveries of vitamin C in *P. emblica* were found to be 99.17 ± 0.27 to 99.55 ± 0.25% (Table 4.15) indicating an agreement between the true value and the value found. The precision of the method based on within-day repeatability was assessed, by replicate injections (n = 5) of two or three standard solutions covering different concentrations levels: low, medium, and high, where peak areas were measured, in comparison to the peak area of the external standard. The precisions of vitamin C in *M. citrifolia* fruits varied from 0.01 to 0.04% and in *P. emblica* varied from 0.02 to 0.05% as shown in Table 4.28 and Table 4.29.

The chromatographic peak of vitamin C was observed retention time at 3.7 min. Figs. 4.22 and 4.23 show the chromatograms of vitamin C in *M. citrifolia* and *P. emblica* respectively. Table 4.42 shows vitamins C content in *M. citrifolia* and *P. emblica* fruits.

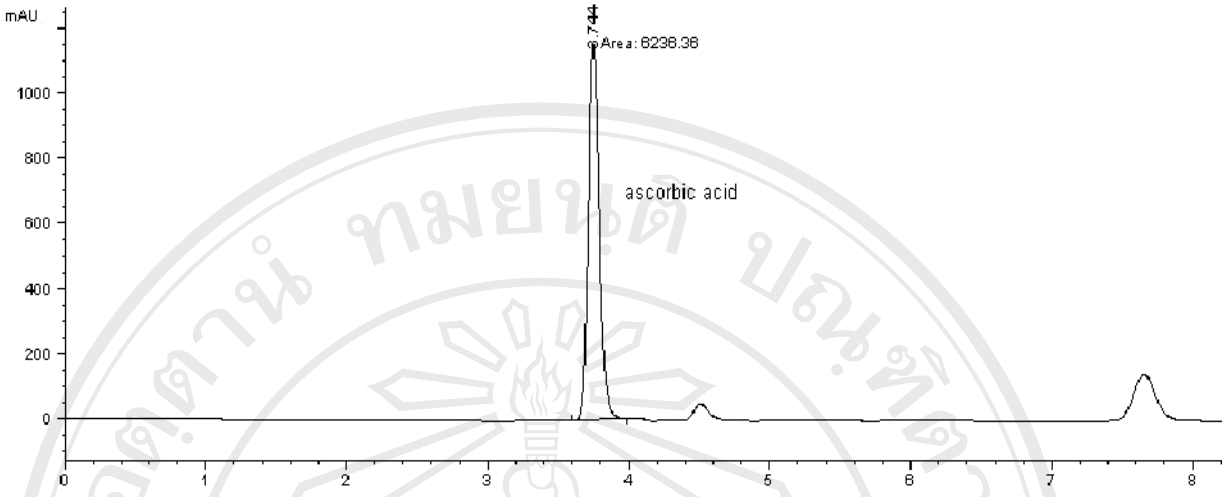

Figure 4.22 Chromatogram for aqueous extract of *M. citrifolia* at 210 nm.

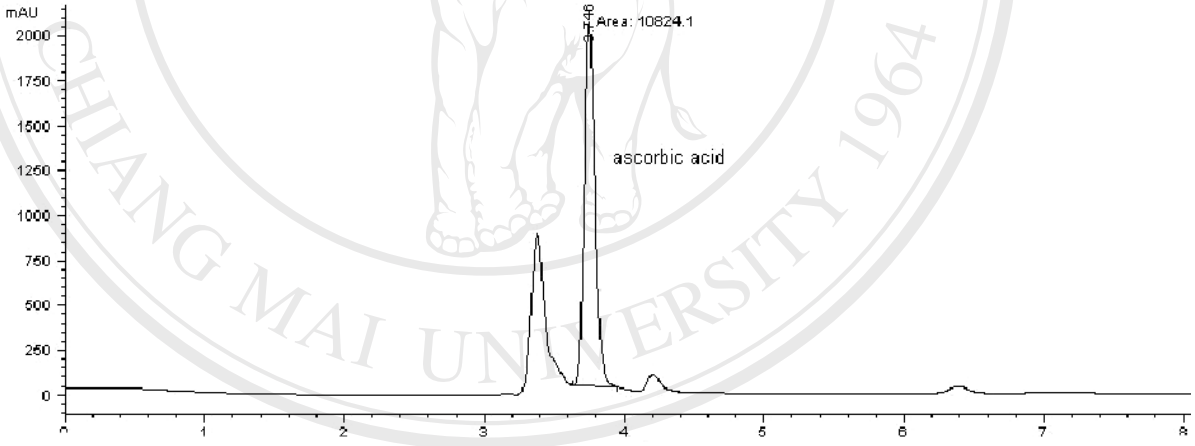

Figure 4.23 Chromatogram for aqueous extract of *P. emblica* at 210 nm.

Table 4.42 Quantitative HPLC determination of vitamin C in aqueous solution of *M. citrifolia* and *P. emblica* (n = 3)

Sample name	Average area under curve (mAU)	Concentration ($\mu g.mL^{-1}$)	Sample weight (g)	vitamin C found ($mg.g^{-1}$)
M. citrifolia	185.35	59.09	10.8608	0.08
P. emblica	167.18	53.52	10.9495	0.21

4.4 Determination of vitamins B contents in *M. citrifolia* and *P. emblica* fruits

The retention time of each vitamin was investigated. Vitamin standards were chromatographed separately in order to determine the retention times. In the HPLC determination of thiamine HCl, riboflavin, nicotinamide and pyridoxine HCl, the presence of an acidic ion-pair reagent is important. For fruit samples, the presence of octanesulphonate resulted in the best separation of interfering substances. Linear calibration curves were obtained over the concentration ranges 10.0-100.0 $\mu g.mL^{-1}$ of thiamine HCl, 10.0-100.0 $\mu g.mL^{-1}$ of riboflavin, 10.0-100.0 $\mu g.mL^{-1}$ of nicotinamide and 0.5-50.0 $\mu g.mL^{-1}$ of pyridoxine HCl respectively with the correlation coefficient of 0.9999. The limit of detection (LOD), defined as the minimum concentration capable of giving a chromatographic signal three times higher than background noise, was estimated at 0.50 $\mu g.mL^{-1}$ of thiamine HCl (vitamin B_1), 0.10 $\mu g.mL^{-1}$ of riboflavin (vitamin B_2), 2.00 $\mu g.mL^{-1}$ of nicotinamide (vitamin B_3) and 0.05 $\mu g.mL^{-1}$ of pyridoxine HCl (vitamin B_6). The limit of quantification (LOQ) was 1.50 $\mu g.mL^{-1}$ of thiamine HCl (vitamin B_1), 0.50 $\mu g.mL^{-1}$ of riboflavin (vitamin B_2), 5.00 $\mu g.mL^{-1}$ of nicotinamide (vitamin B_3) and 0.50 $\mu g.mL^{-1}$ of pyridoxine HCl (vitamin B_6). The

recoveries of vitamin B_1, B_2, B_3 and B_6 in *M. citrifolia* were found to be 99.83 ± 0.24 to 100.60 ± 0.42 % of vitamin B_1 (Table 4.16), 99.26 ± 0.27 to 101.60 ± 0.51 % of vitamin B_2 (Table 4.17), 99.40 ± 0.18 to 100.25 ± 0.26 % of vitamin B_3 (Table 4.18) and 99.20 ± 0.18 to 100.60 ± 0.11 % of vitamn B_6 (Table 4.19) respectively and the recoveries of vitamin B_1, B_2, B_3 and B_6 in *P. emblica* were found to be 99.17 ± 0.16 to 100.23 ± 0.28 % of vitamin B_1 (Table 4.20), 99.10 ± 0.42 to 101.80 ± 0.67 % of vitamin B_2 (Table 4.21), 99.60 ± 0.38 to 101.70 ± 1.56 % of vitamin B_3 (Table 4.22) and 99.10 ± 1.53 to 99.25 ± 0.97 % of vitamin B_6 (Table 4.23) respectively. The precisions of vitamin B_1, B_2, B_3 and B_6 in *M. citrifolia* are varied from 0.10 to 0.60 % of vitamin B_1, 0.20 to 1.70 % of vitamin B_2, 0.03 to 1.30 % of vitamin B_3, 0.20 to 1.05 % of vitamin B_6 respectively and in *P. emblica* varied from 0.07 to 1.31 % of vitamin B_1, 0.03 to 0.80 % of vitamin B_2, 0.30 to 1.19 % of vitamin B_3 and 0.03 to 0.6 % of vitamin B_6 respectively as shown in Table 4.30 to Table 4.37.

Well defined separation peaks of water-soluble vitamins B_2, B_3 and B_6 are observed retention time at 3.5 min, 3.9 min and 5.5 min respectively. Figs. 4.24 and 4.25 show the content of vitamin B in *M. citrifolia* and *P. emblica*.

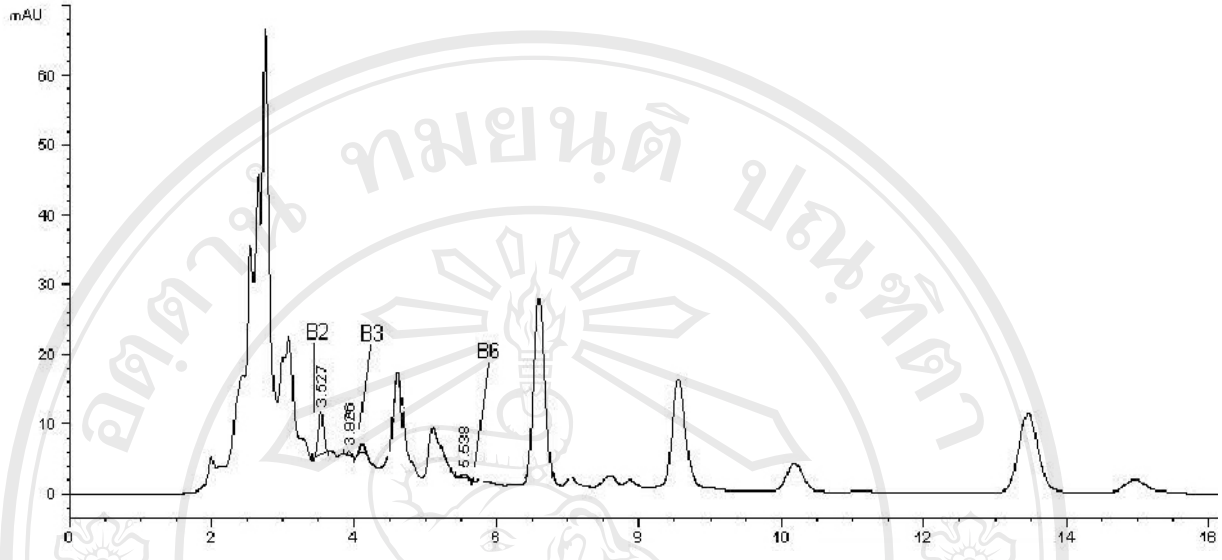

Figure 4.24 Chromatogram for aqueous extract *M. citrifolia* at 280 nm.

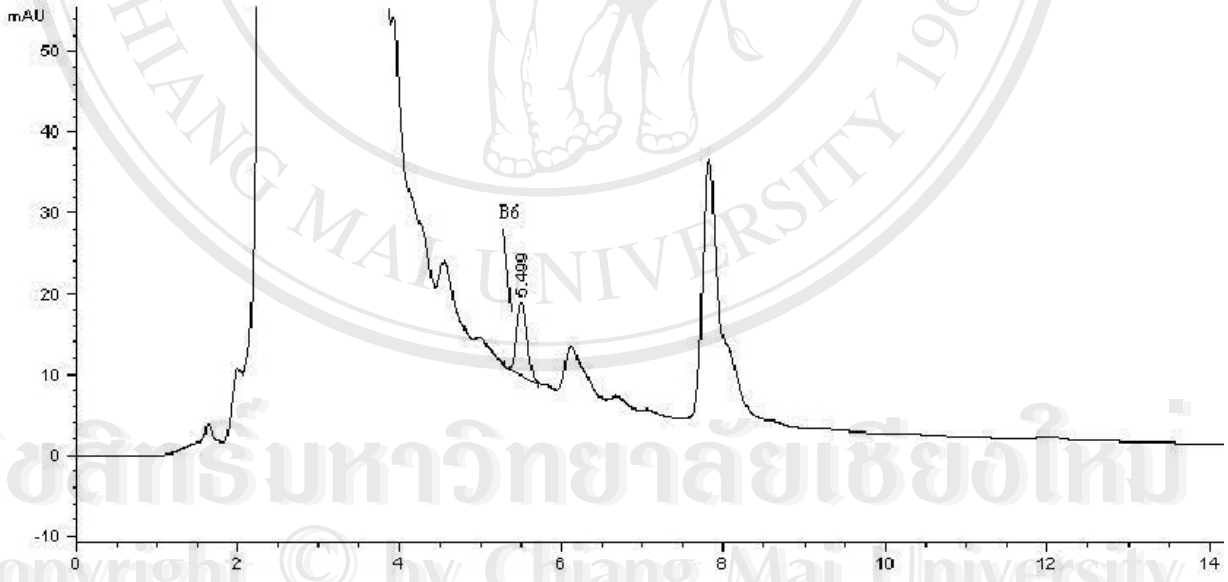

Figure 4.25 Chromatogram for aqueous extract of *P. emblica* at 280 nm.

Table 4.43 Quantitative HPLC determination of vitamins B in aqueous solution of *M. citrifolia* and *P. emblica* (n = 3)

Sample name	Sample weight (g)	Average area under curve (mAU)		Concentration ($\mu g.mL^{-1}$)	Vitamin found ($mg.g^{-1}$)
M. citrifolia	10.01	B_1	ND	ND	ND
		B_2	31.75	28.66	0.18
		B_3	17.02	52.71	0.99
		B_6	4.47	1.22	0.23
P. emblica	10.05	B_1	ND	ND	ND
		B_2	ND	ND	ND
		B_3	ND	ND	ND
		B_6	44.72	14.61	0.07

ND = not detected

4.5 Determination of α-tocopherol and β-carotene contents in *M. citrifolia* and *P. emblica* fruits

In this investigation, α-tocopherol and β-carotene in *M. citrifolia* and *P. emblica* were quantified by reverse-phase HPLC with UV detection using ethanol-methanol as mobile phase. This mobile phase was too non-polar for the rapid elution of α-tocopherol and β- carotene. In reverse-phase separations of fat-soluble vitamins, the retention times of measured vitamins varied between 6.2 min for α-tocopherol and 12.0 min for β-carotene (Fig. 4.6). Detection limits defined as a signal three times the height of the noise level were 0.01 $\mu g.mL^{-1}$ of α-tocopherol and 0.01 $\mu g.mL^{-1}$ of β-carotene (Table 4.12). The limit of quantification (LOQ) was 0.05 $\mu g.mL^{-1}$ of α-tocopherol and 0.03 of β- carotene (Table 4.13). Linear calibration curves were obtained over the concentration ranges 1.0-10.0 $\mu g.mL^{-1}$ of α-tocopherol ($r^2 = 0.9999$) (Table 4.10) and 1.0-10.0 $\mu g.mL^{-1}$ of β- carotene ($r^2 = 0.9998$) (Table 4.11). The recoveries of α-tocopherol and β- carotene in *M. citrifolia* were found to be 99.60 ±

2.66 to 100.80 ± 0.50 % (Table 4.24) and 99.80 ± 1.40 to 101.50 ± 0.34 % (Table 4.25) respectively and the recoveries of α-tocopherol and β- carotene in *P. emblica* were found to be 99.70 ± 2.15 to 100.60 ± 0.47 % (Table 4.26) and 99.20 ± 0.07 to 100.30 ± 1.16 % (Table 4.27) respectively. The precisions of α-tocopherol and β-carotene in *M.citrifolia* are varied from 0.40 to 1.09 % of α-tocopherol and 0.40 to 1.61 % of β- carotene respectively and in *P. emblica* varied from 0.20 to 1.01 % of α-tocopherol and 0.10 to 0.61 % of β- carotene respectively as shown in Table 4.38 to Table 4.41. The chromatographic separation of α-tocopherol and β-carotene in *M. citrifolia* and *P. emblica* were performed. Well defined peaks were observed as shown in Fig. 4.26 and Fig. 4.27. The contents of α-tocopherol and β-carotene found in *M. citrifolia* were 0.31 and 0.01 $mg.g^{-1}$ respectively and those found in *P. emblica* were 0.04 and 0.03 $mg.g^{-1}$. Table 4.44 shows α-tocopherol and β-carotene content in *M. citrifolia* and *P. emblica*

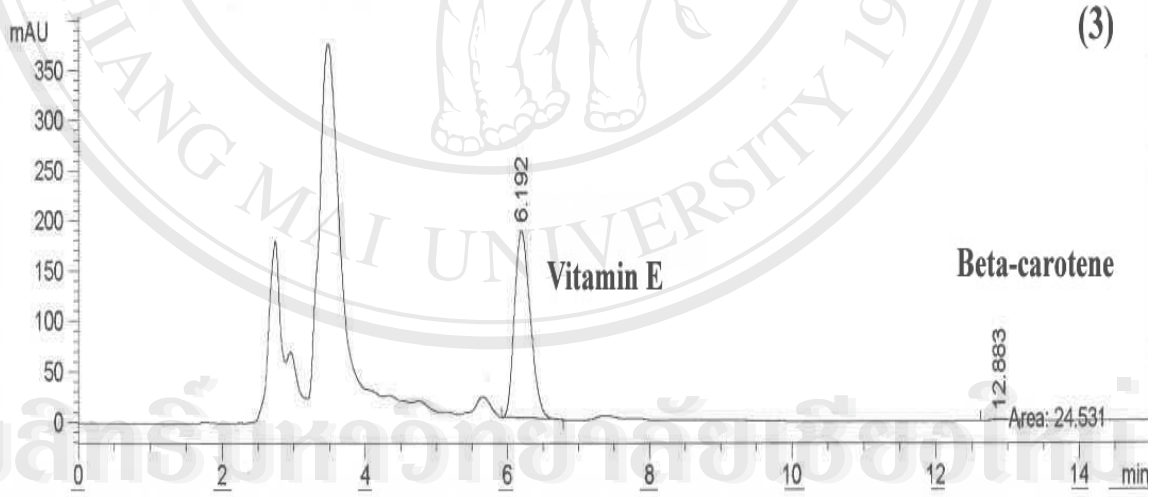

Figure 4.26 HPLC chromatogram of α-tocopherol and β- carotene in organic extract of *M. citrifolia*

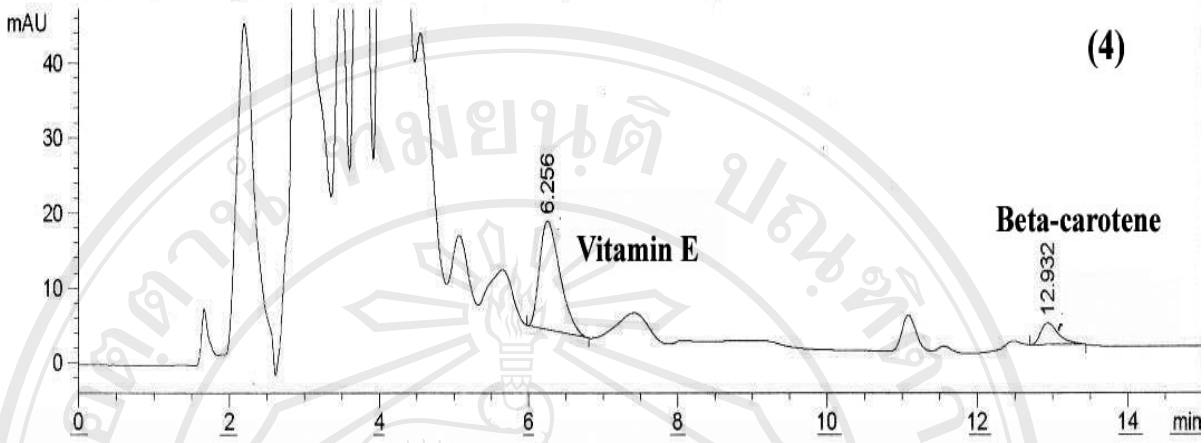

Figure 4.27 HPLC chromatogram of α-tocopherol and β- carotene in organic extract of *P. emblica*

Table 4.44 Quantitative HPLC determination of α-tocopherol and β- carotene in organic extracts of *M. citrifolia* and *P. emblica*

Sample name	Sample weight (g)	Average area under curve (mAU)		Concentration ($\mu g.mL^{-1}$)	Vitamin found ($mg.g^{-1}$)
M. citrifolia	50.7496	α-tocopherol	2872.31	75.55	0.31
		β- carotene	24.24	3.34	0.01
P. emblica	50.5634	α-tocopherol	282.70	8.39	0.04
		β- carotene	49.57	6.25	0.03

Part II Determination of vitamins in fermented juices containing *M. citrifolia* and *P. emblica*

The proposed HPLC method described for fruit extracts was applied to the determination of vitamins in fermented juices containing *M. citrifolia* and *P. emblica*. Seven formulations fermented fruit juices of each plant were prepared to obtain the desired formulates.

The pH of each fermented juice products containing *M. citrifolia* and *P. emblica* was measured by means of pH meter. It was found that the pH are in the ranges of 3.25 to 4.45 and 2.91 to 3.94 for *M. citrifolia* and *P. emblica* as shown in Table 4.45 and Table 4.46, respectively.

Table 4.45 pH of fermented juices containing *M. citrifolia* from various product processes at different fermentation period.

	pH						
Formulas	**D0**	**D7**	**D15**	**D30**	**D45**	**D60**	**D90**
1	4.15	4.09	3.51	3.51	3.68	3.43	3.35
2	3.69	3.75	3.38	3.31	3.42	3.28	3.25
3	4.25	4.31	4.00	3.79	3.76	3.56	3.49
4	3.86	3.98	3.30	3.34	3.44	3.27	3.22
5	0.00	0.00	4.45	4.32	4.19	4.24	4.20
6	4.22	4.26	3.93	3.86	4.00	3.58	3.43
7	4.08	4.13	4.07	4.27	4.18	3.85	3.73

Note: D0 – D90 represent 0^{th}, 7^{th}, 15^{th}, 30^{th}, 45^{th}, 60^{th} and 90^{th} days of fermentation.

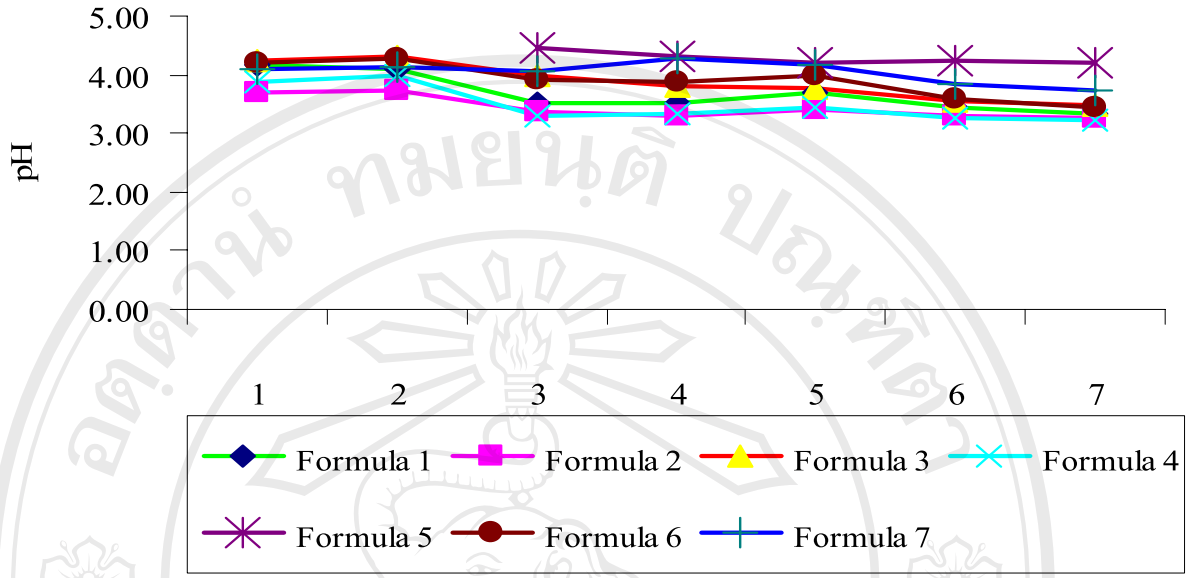

Figure 4.28 The pH of fermented juices containing *M. citrifolia* from various production processes at different fermentation period.

Table 4.46 pH of fermented juices containing *P. emblica* from various production processes at different fermentation period.

	pH						
Formulas	**D0**	**D7**	**D15**	**D30**	**D45**	**D60**	**D90**
1	3.94	3.42	3.38	3.27	3.39	3.31	2.95
2	3.37	2.99	3.03	2.92	3.05	3.07	3.12
3	3.72	3.18	3.17	3.29	3.41	3.35	3.25
4	3.20	2.91	2.97	2.91	3.01	3.08	3.15
5	0.00	0.00	3.54	3.30	3.40	3.33	3.24
6	3.84	3.35	3.31	3.25	3.34	3.27	3.29
7	3.77	3.28	3.28	3.17	3.25	3.29	3.36

Note: D0 – D90 represent 0^{th}, 7^{th}, 15^{th}, 30^{th}, 45^{th}, 60^{th} and 90^{th} days of fermentation.

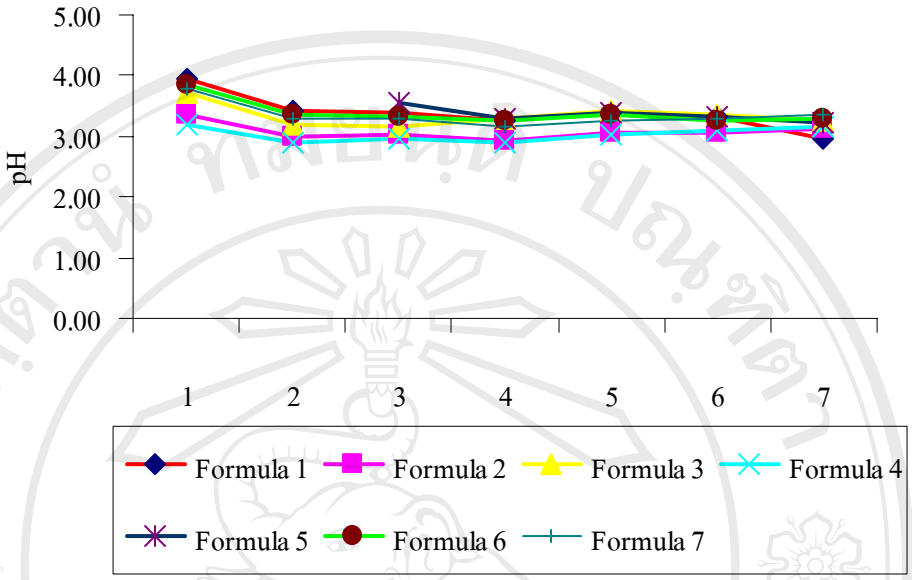

Figure 4.29 The pH of fermented juices containing *P. emblica* from various production processes at different fermentation period.

For contemplation, measurement acid-base of fermentation fruits containing the *M. citrifolia* was prepared. It was found that the pH of formula 2 and 4 are lower than those of the other formula because the Royal jelly's of formula are more acid than that of sugar. So the time taken for fermentation is due 90th days, pH of the above 2 formulae cited are lower than that of formula because it produces lactic acid by adding germ into the ferment at media to obtain lactic acid.

Upon measurement of pH values of liquid fermentation media of *P. emblica,* it was found that the average pH were about 3.22-4.45. At the first interval of fermentation the pH is higher than those obtained by intervals.

4.6 Determination of vitamin C contents in fermented juices containing *M. citrifolia* and *P. emblica.*

Aliquots of 7 fermented juice products of *M. citrifolia* and *P. emblica* were taken for HPLC analysis, after the 0^{th}, 7^{th}, 15^{th}, 30^{th}, 45^{th}, 60^{th} and 90^{th} days of fermentation.

The contents of vitamin C in fermented juices of *M. citrifolia* are reported in Table 4.47 (Fig 4.30) and Table 4.48 (Fig. 4.31), respectively.

Table 4.47 Vitamin C contents in fermented juices containing *M. citrifolia* of various production processes at different fermentation period.

Formulas	Day after fermentation						
	Vitamin C (μg.mL^{-1})						
	D0	D7	D15	D30	D45	D60	D90
1	257.22	84.21	36.79	19.49	12.11	ND	ND
2	412.83	283.67	182.61	39.94	15.10	ND	ND
3	202.97	113.47	49.64	27.86	8.36	ND	ND
4	457.10	284.98	154.95	42.49	18.33	3.26	ND
5	0.00	0.00	117.37	85.07	29.76	5.04	ND
6	279.52	187.81	33.98	5.98	ND	ND	ND
7	219.70	124.36	59.78	29.39	6.32	ND	ND

ND = not detect

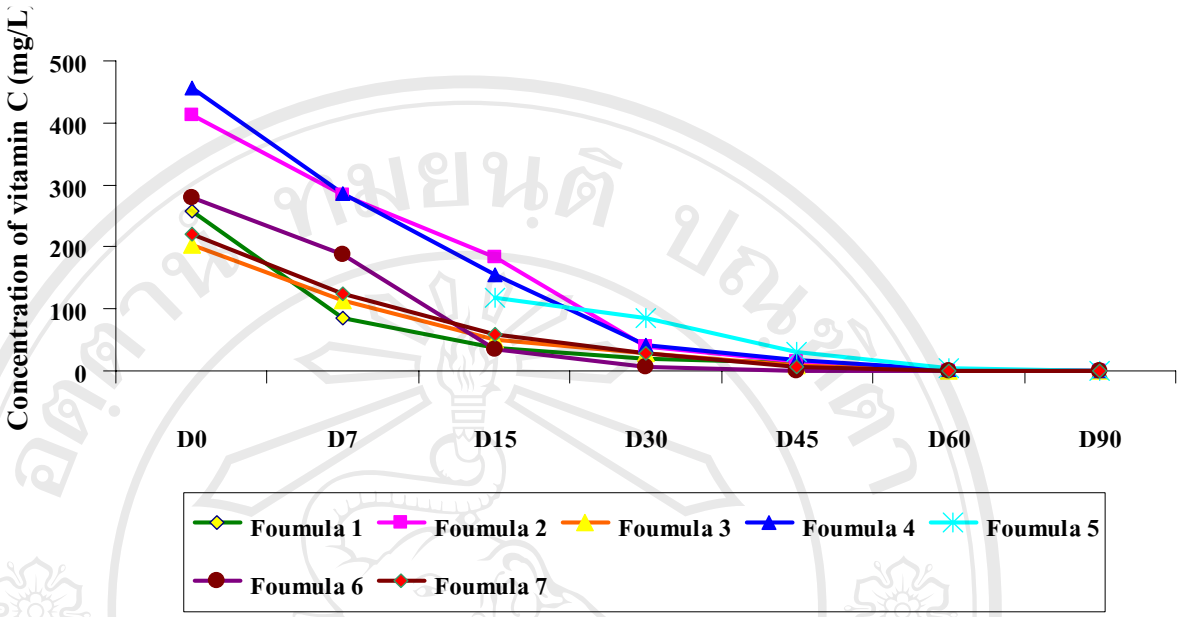

Figure 4.30 Vitamin C contents in fermented juices containing *M. citrifolia*.

It was shown that amounts of vitamin C decreased with increasing fermentation time. Vitamin C was no found from the 90th day of fermentation. (Fig. 4.30)

Table 4.48 Vitamin C contents in fermented juices containing *P. emblica* of various production processes at different fermentation period.

Formulas	Day after fermentation						
	Vitamin C (μg.mL^{-1})						
	D0	D7	D15	D30	D45	D60	D90
1	558.51	358.16	231.29	44.11	21.12	ND	ND
2	725.03	548.93	280.57	123.57	34.33	10.14	ND
3	793.54	740.49	391.11	197.34	97.12	18.75	ND
4	824.77	566.57	279.14	137.62	31.39	11.49	ND
5	0.00	0.00	634.85	414.07	173.64	25.73	ND
6	800.96	549.47	260.96	79.9	21.53	ND	ND
7	715.22	518.05	357.54	165.71	22.08	ND	ND

ND = not detect

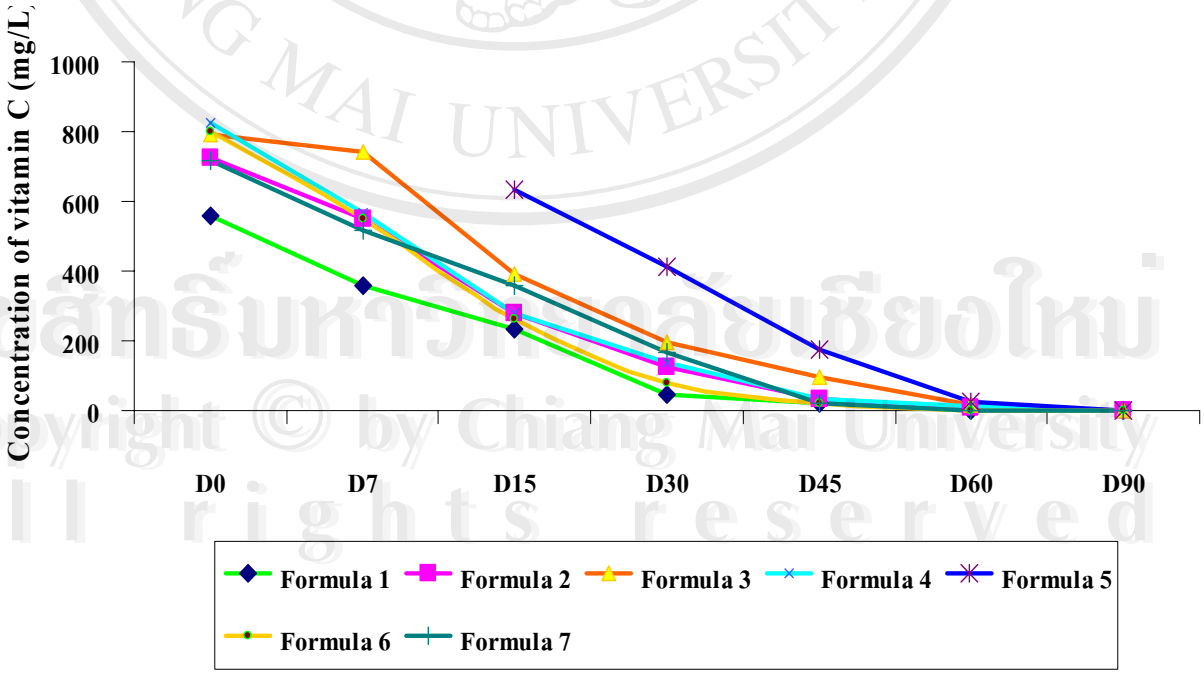

Figure 4.31 Vitamin C contents in fermented juices containing *P. emblica*.

Vitamin C observed in fermented juices containing *M. citrifolia* and *P. emblica* were ranges 3.26 - 457.10 $\mu g.mL^{-1}$ and 10.14 – 824.77 $\mu g.mL^{-1}$, respectively.

For product 5, during the first period raw cane-sugar as solid was added into *M. citrifolia* and *P. emblica* followed by addition of water on the 15th day of fermentation period. Therefore the evaluation of vitamin content of product 5 was started on the 15th day of fermentation period. Similarly, the amount of vitamin C decreased with increasing time. There is more vitamin C present in the formula containing honey than that containing raw cane-sugar (Fig. 4.30 and Fig. 4.31). Because vitamin C is easily destroyed during processing and storage through the action of metals such as copper and iron. Exposure to oxygen and prolonged heating in the presence of oxygen could destroy ascorbic acid. Thus, the stability of vitamin C in fortified foods depends on the characteristics of the product, processing method, and type of packaging used [179].

4.7 Determination of vitamins B contents in fermented juices containing *M. citrifolia* and *P. emblica*

The proposed method was again applied to the determination of vitamin B group in fermented juices. The samples were analysed after the 0th, 7th, 15th, 30th, 45th, 60th and 90th day of fermentation and triplicate analyses were performed for each sample (Table 4.49 to Table 4.50).

There is no vitamin B present in product 5 containing *P. emblica*, but there are vitamins B_2, B_3 and B_6 present in product 5 containing *M. citrifolia* at the 15th and 30th day of fermentation, only vitamin B_3 was observed at the 45th day of fermentation. Vitamins B group in all fermented juice products are decreased with increasing time (Fig. 4.32 to Fig. 4.44). During fermentation, some by-products were present (e.g. ethyl alcohol, methyl alcohol, acetaldehyde and iso-propanol). Therefore, produced organic solvents effected the stability of vitamin C, vitamin B_1, B_2, B_3 and B_6 in product. Especially thiamine (vitamin B_1), one of the most unstable B vitamins, degraded easiest. Baking, pasteurization, or boiling of foods fortified with thiamine can reduce its content by up to 50 percent. The stability of thiamine during storage depends greatly on the moisture content of the food. Thiamine is

highly sensitive to heat and alkalis. Riboflavin (vitamin B_2) is very stable during thermal processing, storage and food preparation. Riboflavin, however, is susceptible to degradation on exposure to light. The use of light-proof packaging material prevents its deterioration. Nicotinamide (vitamin B_3) is one of the most stable vitamins and the main loss occurs from leaching into cooking water. Pyridoxine (vitamin B_6) losses depend on the type of thermal processing. For example, high losses of B_6 occur during sterilization of liquid infant formula; in contrast, B_6 in enriched flour and corn meal is resistant to baking temperatures. B_6 is susceptible to light induced degradation and exposure to water can cause leaching and consequent losses. Pyridoxine is sensitive to light, heat and alkalis [179, 180].

Table 4.49 Vitamins B contents in fermented juices containing *M. citrifolia* of various production processes at different fermentation period.

		Concentration (μg.mL^{-1})						
	Vitamins	**D0**	**D7**	**D15**	**D30**	**D45**	**D60**	**D90**
Formula 1	**B1**	168.84	24.78	ND	ND	ND	ND	ND
	B2	69.22	56.09	30.33	10.39	ND	ND	ND
	B3	187.90	153.60	62.27	32.73	23.04	ND	ND
	B6	4.30	4.02	2.80	ND	ND	ND	ND
Formula 2	**B1**	216.36	109.12	34.49	24.49	ND	ND	ND
	B2	11.02	ND	ND	ND	ND	ND	ND
	B3	215.74	183.17	126.08	46.36	ND	ND	ND
	B6	2.81	2.02	ND	ND	ND	ND	ND
Formula 3	**B1**	159.00	24.61	ND	ND	ND	ND	ND
	B2	55.67	46.12	32.70	ND	ND	ND	ND
	B3	121.69	116.24	86.65	40.41	ND	ND	ND
	B6	13.14	5.30	ND	ND	ND	ND	ND
Formula 4	**B1**	235.22	101.5	32.93	24.03	18.55	ND	ND
	B2	12.52	ND	ND	ND	ND	ND	ND
	B3	207.62	168.02	114.86	47.59	ND	ND	ND
	B6	3.53	2.42	ND	ND	ND	ND	ND
Formula 5	**B1**	ND	ND	ND	ND	ND	ND	ND
	B2	ND	ND	51.19	30.82	ND	ND	ND
	B3	ND	ND	104.29	67.77	44.48	ND	ND
	B6	ND	ND	3.69	2.68	ND	ND	ND
Formula 6	**B1**	139.91	21.51	ND	ND	ND	ND	ND
	B2	49.55	41.45	28.14	ND	ND	ND	ND
	B3	180.09	133.07	114.20	51.16	18.62	ND	ND
	B6	3.86	ND	ND	ND	ND	ND	ND
Formula 7	**B1**	270.52	220.01	171.05	98.25	ND	ND	ND
	B2	39.76	30.44	ND	ND	ND	ND	ND
	B3	107.43	54.64	29.97	ND	ND	ND	ND
	B6	3.25	2.63	1.77	ND	ND	ND	ND

Table 4.50 Vitamins B contents in fermented juices containing *P. emblica* of various production processes at different fermentation period.

		Concentration (μg.mL^{-1})						
	Vitamins	**D0**	**D7**	**D15**	**D30**	**D45**	**D60**	**D90**
Formula1	**B1**	ND	ND	ND	ND	ND	ND	ND
	B2	29.17	17.70	ND	ND	ND	ND	ND
	B3	214.95	120.88	85.49	70.25	ND	ND	ND
	B6	3.39	2.29	ND	ND	ND	ND	ND
Formula2	**B1**	ND	ND	ND	ND	ND	ND	ND
	B2	85.77	32.48	29.08	ND	ND	ND	ND
	B3	585.92	414.23	241.54	ND	ND	ND	ND
	B6	4.22	ND	ND	ND	ND	ND	ND
Formula3	**B1**	ND	ND	ND	ND	ND	ND	ND
	B2	72.00	47.59	33.09	ND	ND	ND	ND
	B3	217.08	175.39	102.82	77.21	ND	ND	ND
	B6	4.47	3.65	2.09	ND	ND	ND	ND
Formula4	**B1**	ND	ND	ND	ND	ND	ND	ND
	B2	40.06	32.40	20.38	ND	ND	ND	ND
	B3	454.39	295.30	134.23	114.70	ND	ND	ND
	B6	5.22	4.89	3.16	ND	ND	ND	ND
Formula5	**B1**	ND	ND	ND	ND	ND	ND	ND
	B2	ND	ND	ND	ND	ND	ND	ND
	B3	ND	ND	ND	ND	ND	ND	ND
	B6	ND	ND	ND	ND	ND	ND	ND
Formula6	**B1**	ND	ND	ND	ND	ND	ND	ND
	B2	121.45	67.31	55.29	ND	ND	ND	ND
	B3	732.82	67.34	ND	ND	ND	ND	ND
	B6	2.12	ND	ND	ND	ND	ND	ND
Formula7	**B1**	ND	ND	ND	ND	ND	ND	ND
	B2	71.84	34.38	13.76	ND	ND	ND	ND
	B3	897.02	584.61	234.86	ND	ND	ND	ND
	B6	3.61	2.38	ND	ND	ND	ND	ND

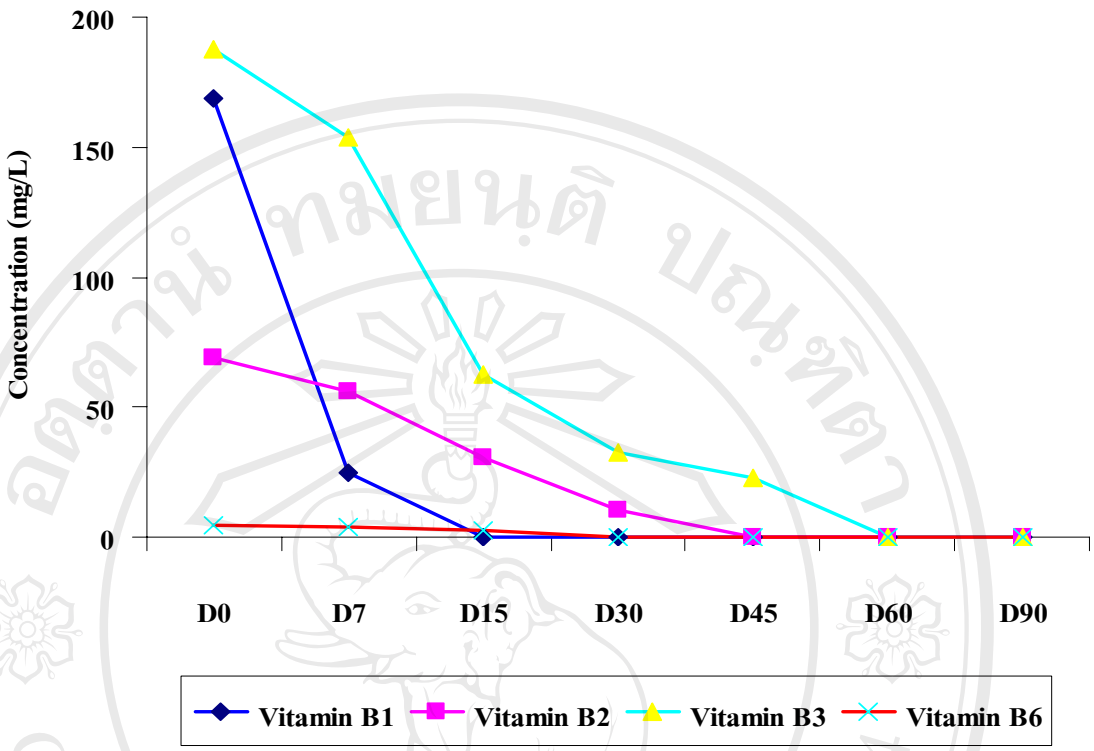

Figure 4.32 Vitamins B contents in fermented juices containing *M. citrifolia* in formula 1.

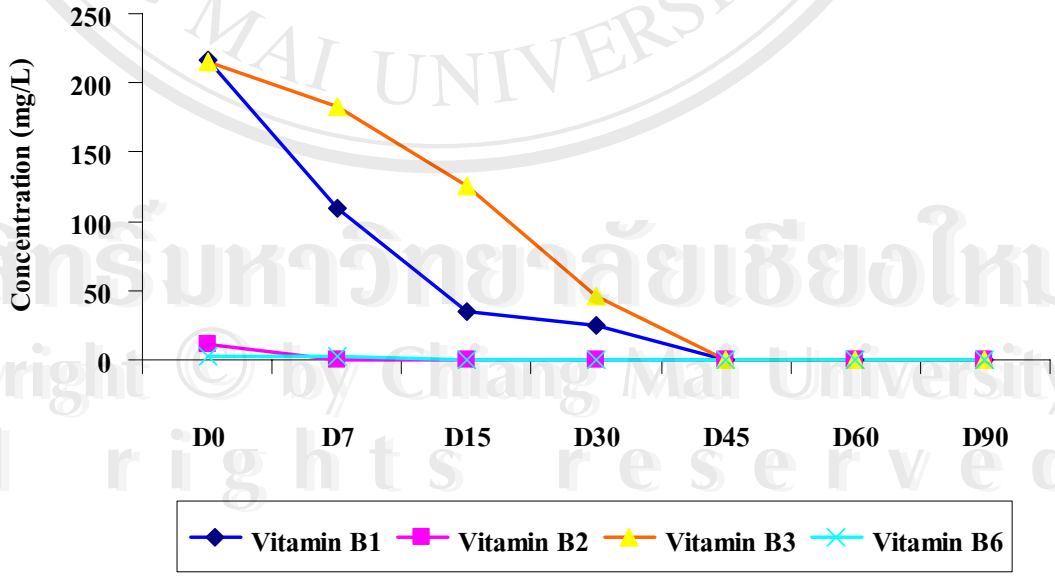

Figure 4.33 Vitamins B contents in fermented juices containing *M. citrifolia* in formula 2.

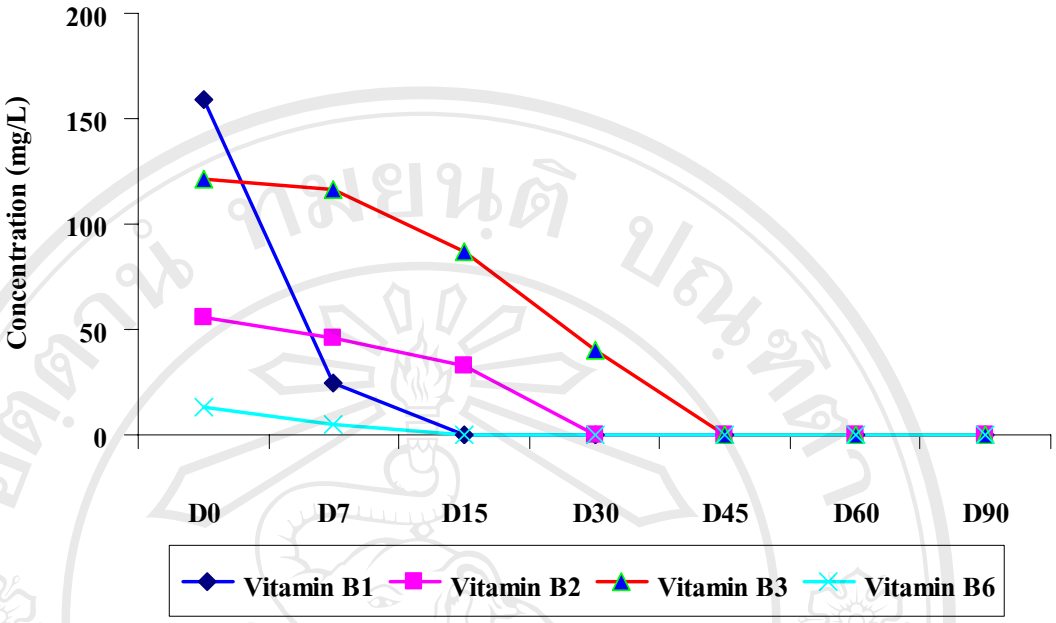

Figure 4.34 Vitamins B contents in fermented juices containing *M. citrifolia* in formula 3.

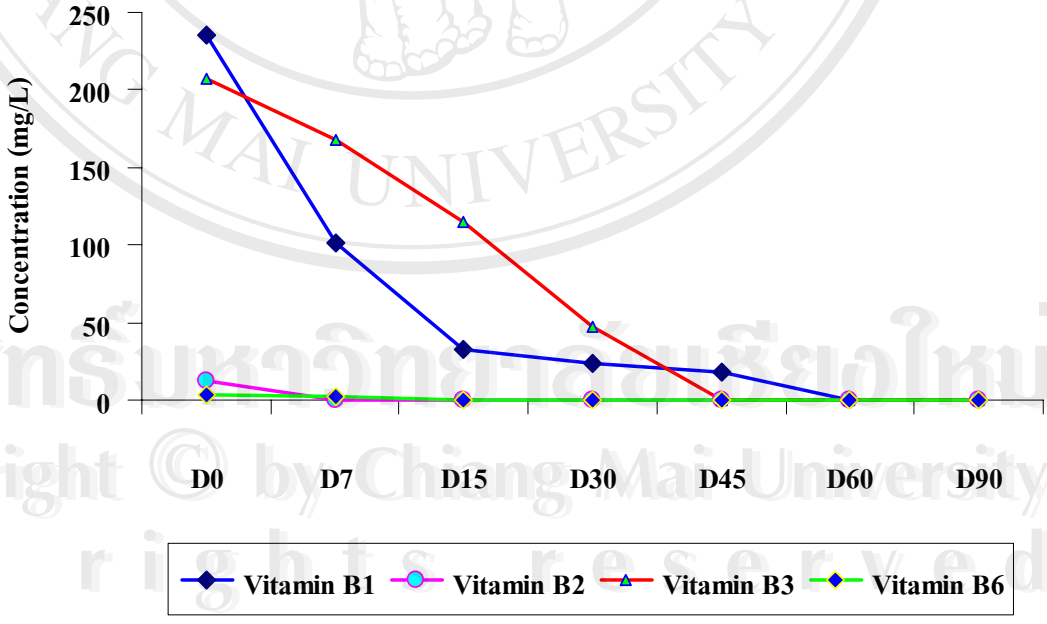

Figure 4.35 Vitamins B contents in fermented juices containing *M. citrifolia* in formula 4.

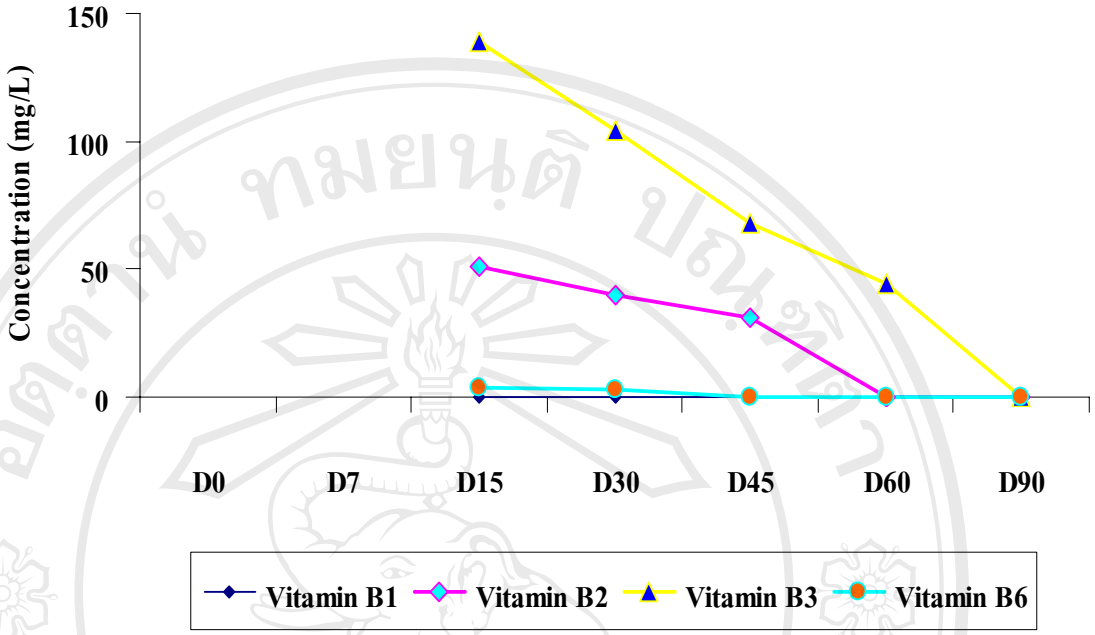

Figure 4.36 Vitamins B contents in fermented juices containing *M. citrifolia* in formula 5.

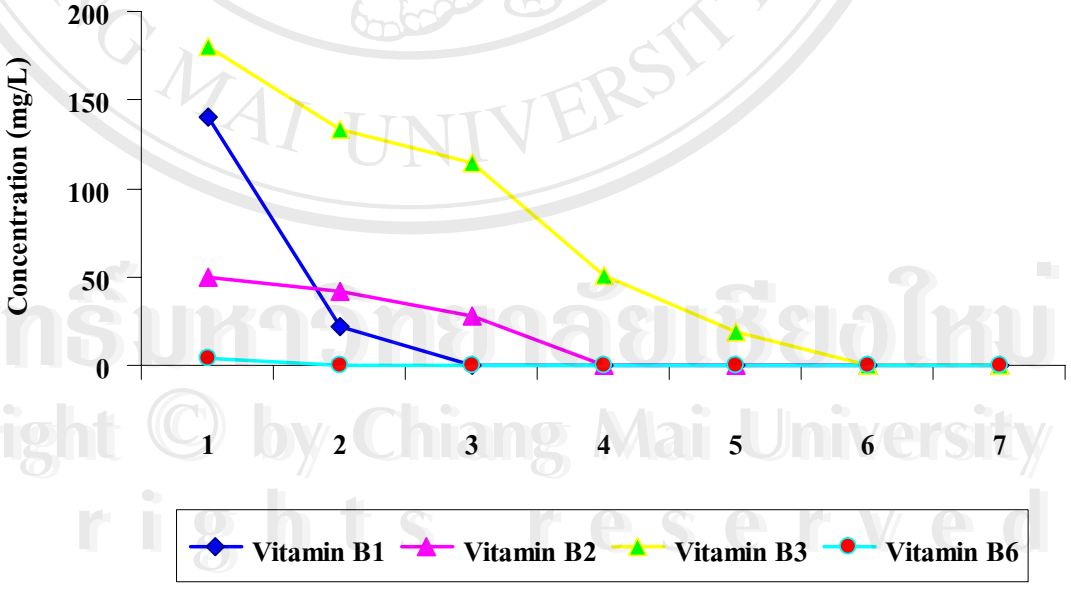

Figure 4.37 Vitamins B contents in fermented juices containing *M. citrifolia* in formula 6.

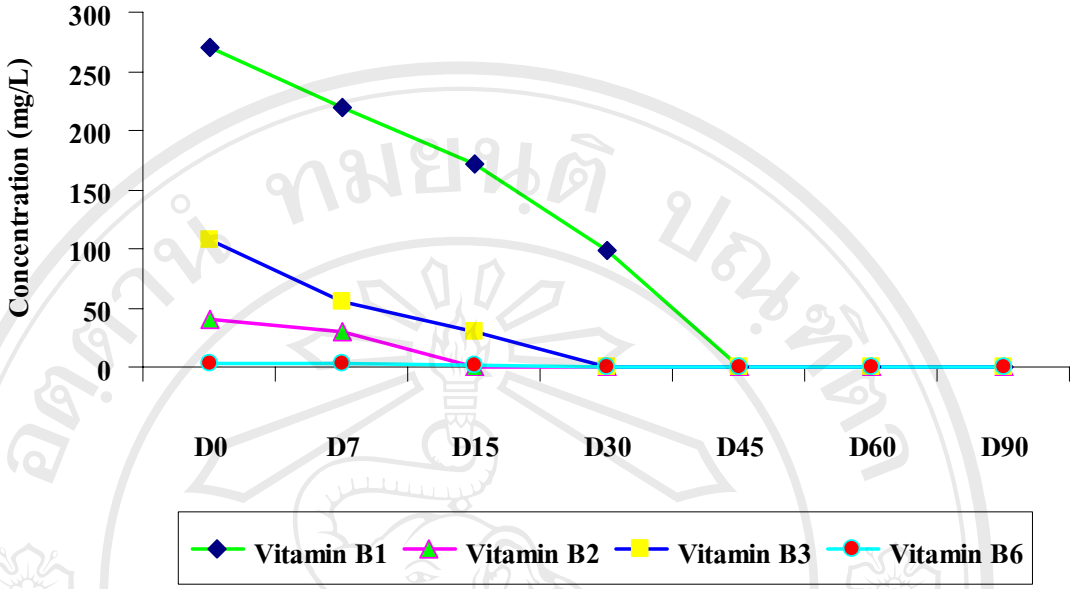

Figure 4.38 Vitamins B contents in fermented juices containing *M. citrifolia* in formula 7.

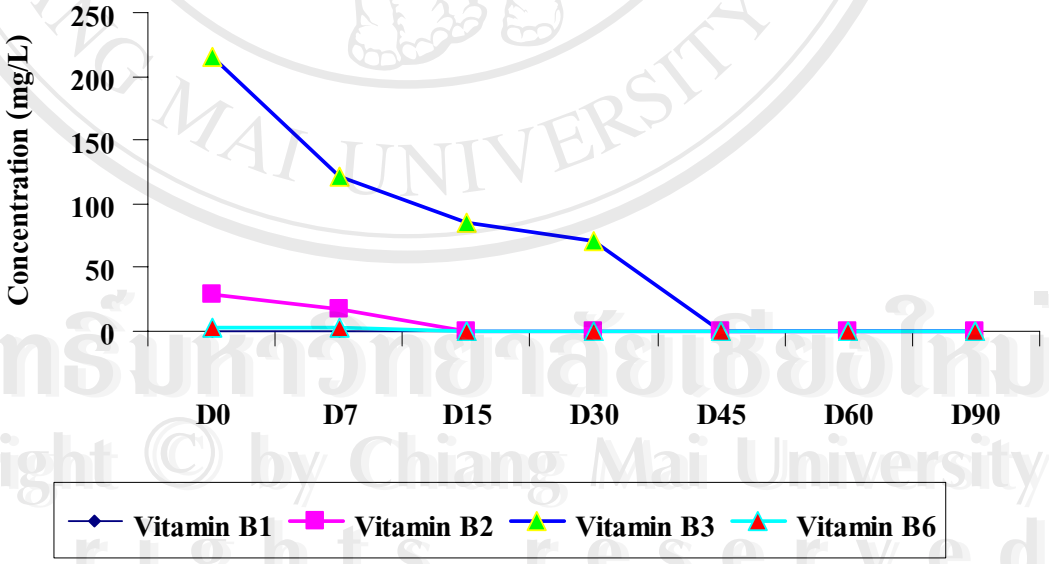

Figure 4.39 Vitamins B contents in fermented juices containing *P. emblica* in formula 1.

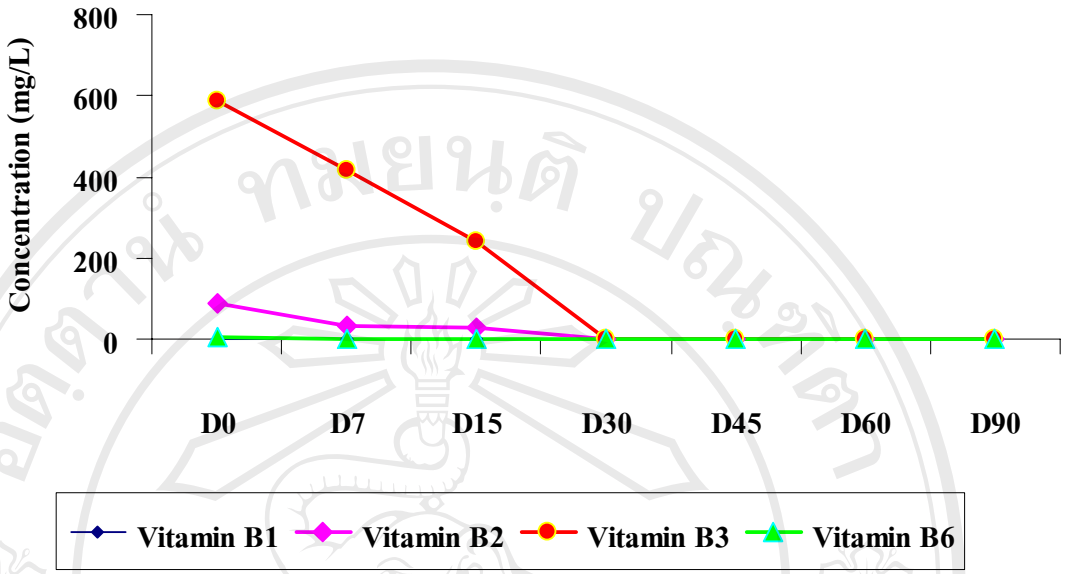

Figure 4.40 Vitamins B contents in fermented juices containing *P. emblica* in formula 2.

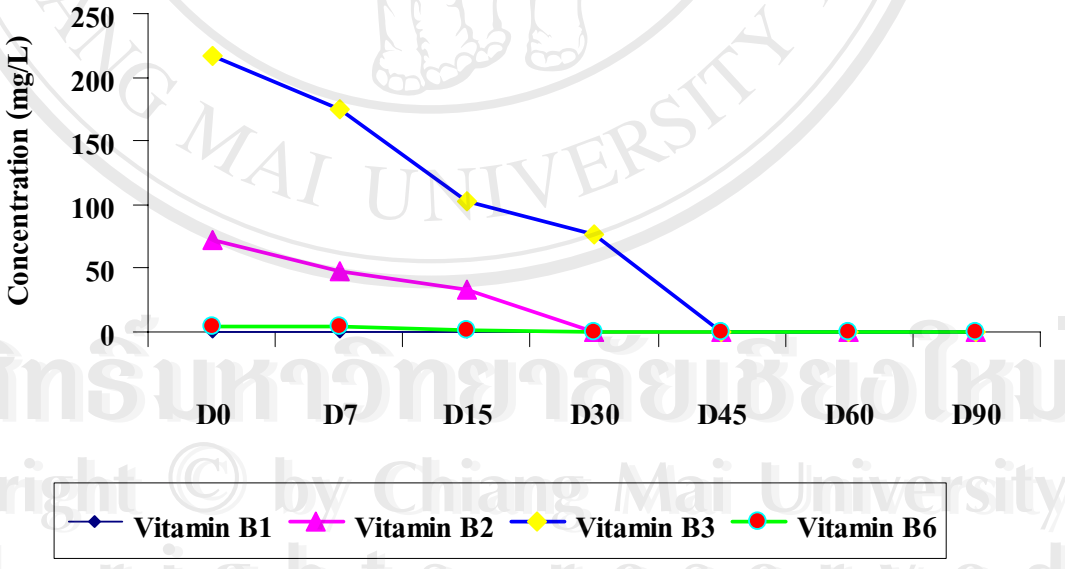

Figure 4.41 Vitamins B contents in fermented juices containing *P. emblica* in formula 3.

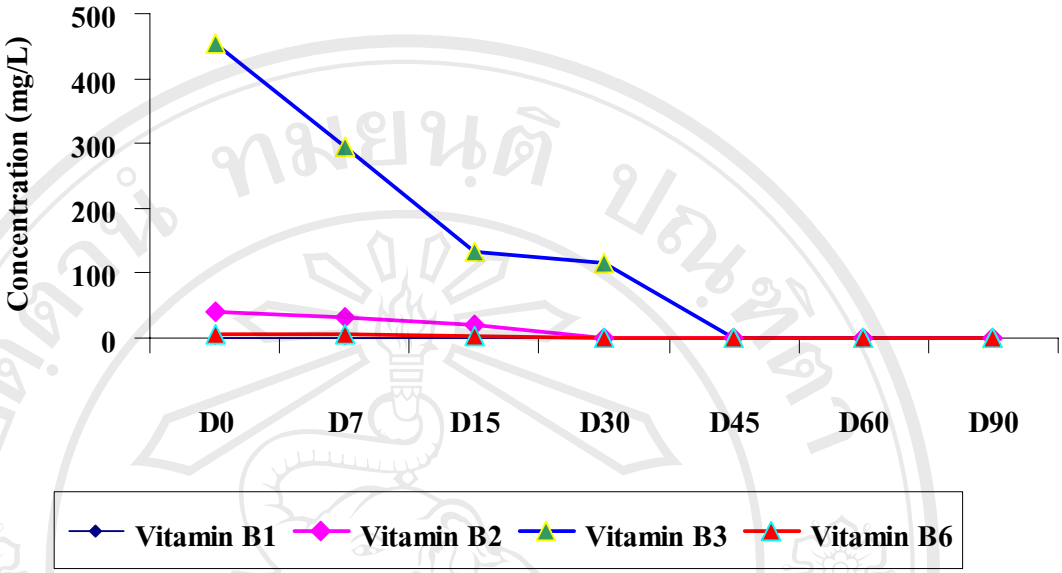

Figure 4.42 Vitamins B contents in fermented juices containing *P. emblica* in formula 4.

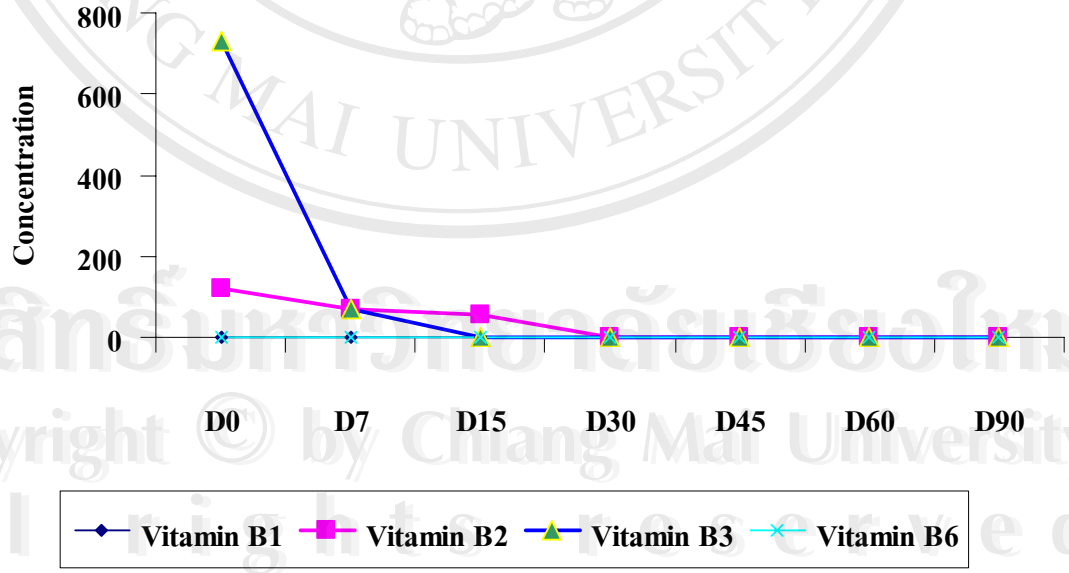

Figure 4.43 Vitamins B contents in fermented juices containing *P. emblica* in formula 6.

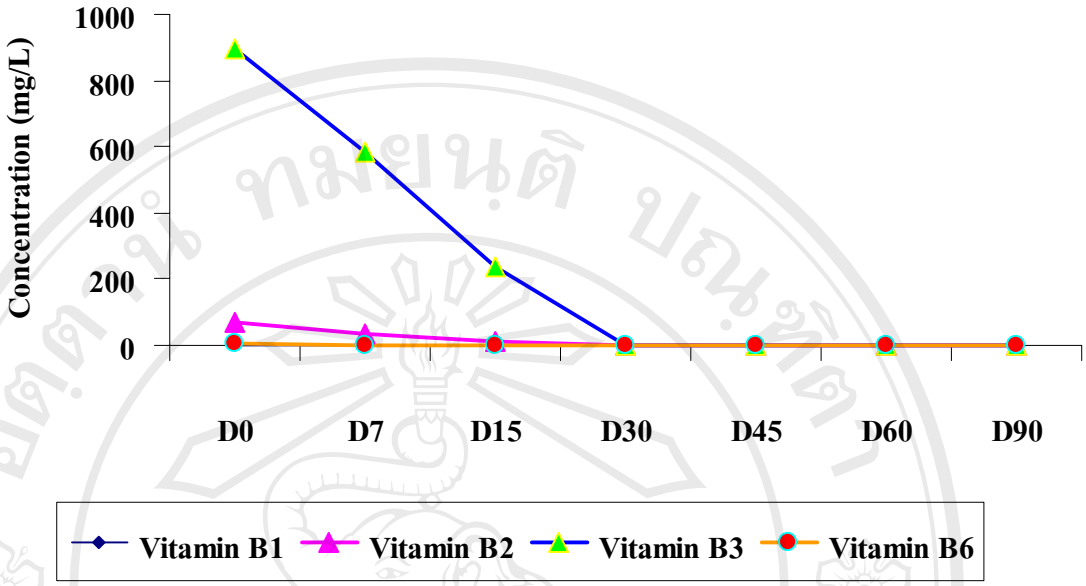

Figure 4.44 Vitamins B contents in fermented juices containing *P. emblica* in formula 7.

4.8 Stability of vitamins C and B in fermented juices containing *M. citrifolia* and *P. emblica*

4.8.1 Stability of vitamins C and B in fermented juices containing *M. citrifolia* and *P. emblica*

Table 4.51 The study on stability of vitamin C in fermented juices containing *M. citrifolia* and *P. emblica*

Formulae	Concentration ($mg.mL^{-1}$)						
	Days						
	D0	D7	D15	D30	D45	D60	D90
1	453.48	251.81	28.55	13.35	1.08	ND	ND
2	478.24	235.49	31.32	9.49	0.39	ND	ND
3	465.04	311.01	199.50	34.99	14.78	ND	ND
4	452.93	242.82	31.32	17.00	0.08	ND	ND

Formula 1: fermentation of *M. citrifolia* and raw cane-sugar with *L. casei*.

Formula 2: fermentation of *M. citrifolia* and honey with *L. casei*

Formula 3: fermentation of *P. emblica* and raw cane-sugar with *L. casei*.

Formula 4: fermentation of *P. emblica* and honey with *L. casei*

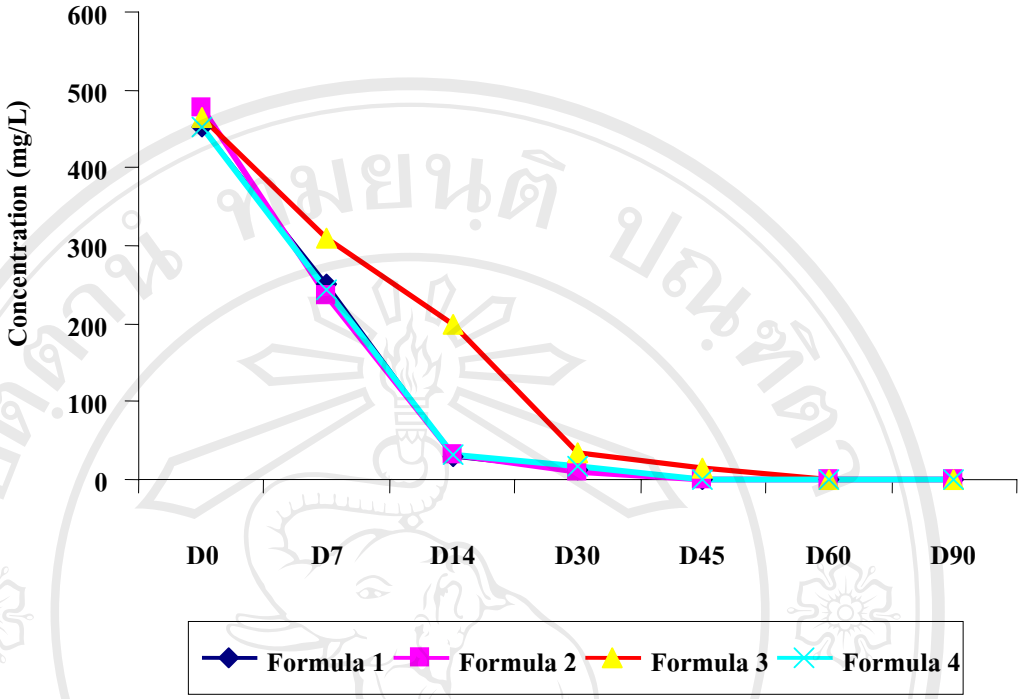

Figure 4.45 Degradation with day of vitamin C containing *M. citrifolia* and *P. emblica*

The stability of vitamin C contents in fermented juices containing *M. citrifolia* and *P. emblica* were investigated. It was found that the amounts of vitamin C decreased with increasing time. Vitamin C was observed on the 60th day of fermentation, because vitamin C decomposed by light and oxygen. Results are presented in Fig. 4.45.

Table 4.52 The study on stability of vitamins B contents in fermented juices containing *M. citrifolia* and *P. emblica*.

Formulas	Concentration ($mg.mL^{-1}$)						
	Days						
	Vitamins	**D0**	**D7**	**D15**	**D30**	**D45**	**D60**	**D90**
1	B1	98.53	37.05	0	0	0	0	0
	B2	99.40	73.84	55.05	29.81	13.43	0	0
	B3	98.15	79.06	31.00	0	0	0	0
	B6	100.89	81.90	36.78	26.17	14.23	5.78	0
2	B1	96.74	50.18	30.89	0	0	0	0
	B2	99.40	55.75	28.21	12.10	0	0	0
	B3	96.42	36.75	0	0	0	0	0
	B6	99.39	57.05	27.81	5.70	0	0	0
3	B1	93.51	47.55	28.21	0	0	0	0
	B2	98.94	63.73	33.93	13.71	0	0	0
	B3	98.02	83.94	40.34	0	0	0	0
	B6	100.90	79.48	36.29	25.09	12.27	5.62	0
4	B1	94.43	49.25	29.36	0	0	0	0
	B2	97.32	53.64	26.84	11.65	0	0	0
	B3	97.06	36.33	0	0	0	0	0
	B6	100.02	51.27	26.17	13.21	5.63	0	0

Formula 1: fermentation of *M. citrifolia* and raw cane-sugar with *L. casei*.

Formula 2: fermentation of *M. citrifolia* and honey with *L. casei*

Formula 3: fermentation of *P. emblica* and raw cane-sugar with *L. casei*.

Formula 4: fermentation of *P. emblica* and honey with *L. casei*

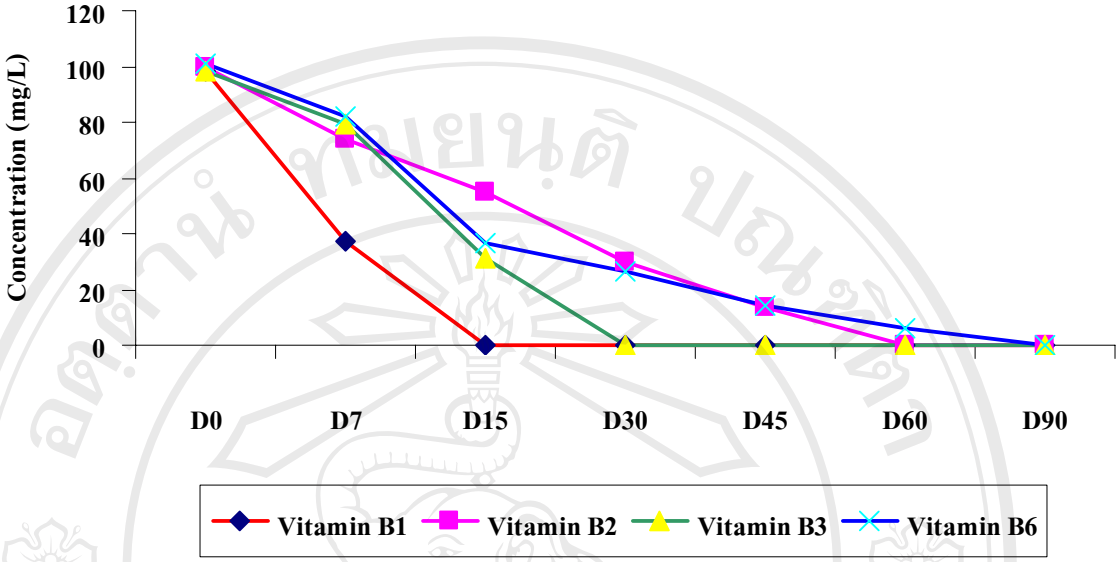

Figure 4.46 Degradation with day of vitamins B in *M. citrifolia* of formula1.

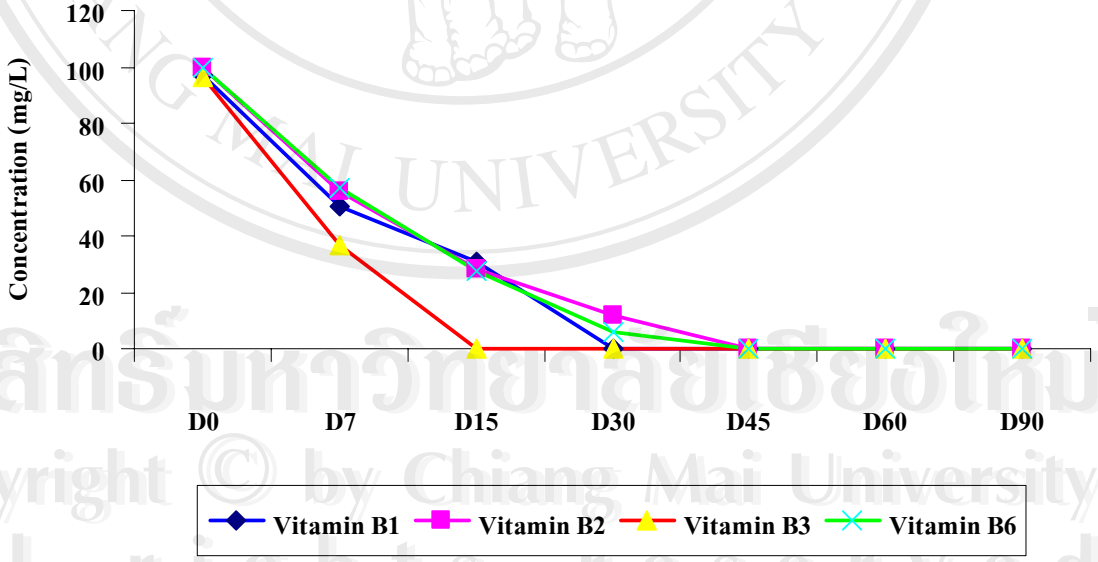

Figure 4.47 Degradation with day of vitamins B in *M. citrifolia* of formula 2.

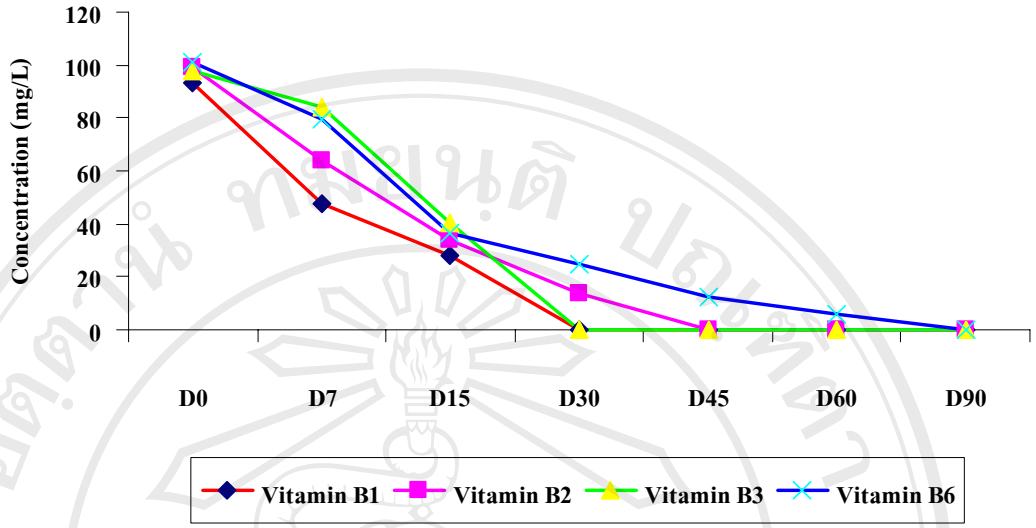

Figure 4.48 Degradation with day of vitamins B in *M. citrifolia* of formula 3.

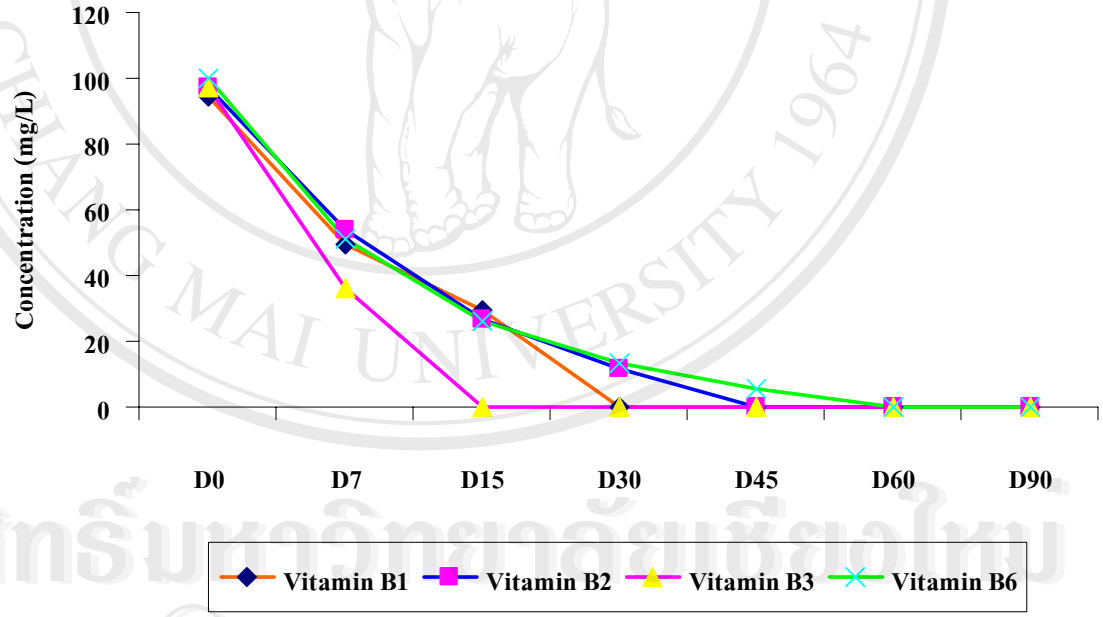

Figure 4.49 Degradation with day of vitamins B in *M. citrifolia* L. of formula 4.

Fig. 4.46 to Fig. 4.49 showed the relationship between the concentrations of vitamin B_1, B_2, B_3 and B_6 in the fermented juices product No. 1, 2, 3 and 4, when kept in the dark at 30 ^oC on the 0^{th}, 7^{th}, 15^{th}, 30^{th}, 45^{th}, 60^{th} and 90^{th} days after fermentation. The concentration of vitamin B_1, B_2, B_3 and B_6 decreased with increasing time. Vitamin B_1 is one of the most unstable B vitamins. Baking, pasteurization, or boiling of foods fortified with thiamine can reduce its content by up to 50 percent. The stability of thiamine during storage depends greatly on the moisture content of the food. Thiamine is highly sensitive to heat and alkalis. Vitamin B_2 is very stable during thermal processing, storage and food preparation. Riboflavin, however, is susceptible to degradation on exposure to light. Riboflavin also degraded in this experiment. The use of light-proof packaging material may prevent its deterioration. Vitamin B_3 is one of the most stable vitamins among B-vitamin and the main loss occurs from leaching into cooking water. However, from our experiment, vitamin B_3 is the most unstable B-vitamin in fermented juices. Vitamin B_6 losses depend on the type of thermal processing. For example, high losses of B_6 occur during sterilization of liquid infant formula; in contrast, B_6 in enriched flour and corn meal is resistant to baking temperatures. B_6 is susceptible to light induced degradation and exposure to water can cause leaching and consequent losses. From our experiment, vitamin B_6 is the most stable vitamin in fermented juices [179, 180].

4.9 The statistical treatment of analytical data of vitamins in seven fermented juices sample by ANOVA

4.9.1 Statistical analysis of Vitamins C and B in Fermented juice Products Containing *M. citrifolia* and *P. emblica*

In this investigation, seven formulae of fermented juices containing *M. citrifolia* and *P. emblica* were prepared separately. The amounts of vitamin C and vitamins B in each formula were quantified by mean of RP-HPLC.

4.9.2 Statistical analysis of vitamin C

The LC analyses of vitamin C in seven fermented juices containing *M. citrifolia* were carried out. The results were analyzed by using ANOVA. It indicated that the amounts of vitamin C found in all formulas (1-7) on the 0^{th}, 7^{th}, 15^{th}, 30^{th}, 45^{th}, 60^{th} and 90^{th} days after fermentation were statistically significance difference at the level of 0.05, but there is no significant difference at the 0.05 level for the amounts of vitamin C found in formulae 1, 2, 3, 6 and 7 on the 60^{th} day after fermentation.

The amounts of vitamin C in seven formulae containing *P. emblica* were also analyzed by RP-HPLC. Results were also evaluated by means of ANOVA. It showed that the amounts of vitamin C found in all formulae (1-7) on the 0^{th}, 7^{th}, 15^{th}, 30^{th}, 45^{th}, 60^{th} and 90^{th} days after fermentation were statistically significant difference at the level of 0.05, but there is no significant difference at the level of 0.05 for the amounts of vitamin C found in formulae 1, 6 and 7 on the 60^{th} days after fermentation. Vitamin C contents in all formulae decreased with increasing time.

4.9.3 Statistical analysis of vitamins B_1, B_2, B_3 and B_6

The amounts of vitamin B_1, B_2, B_3 and B_6 in seven formulae of fermented juices containing *M. citrifolia* and *P. emblica* were determined by HPLC. The results of vitamin B_1, B_2, B_3 and B_6 in formulas 1-7 were analyzed by means of ANOVA (Tables 4.53 and 4.54). Significant difference at the level of 0.05 means vitamins B in these mention formulae are different with the 95% confidence interval.

Table 4.53 Analysis of Vitamins B_1, B_2, B_3 and B_6 in Formulae 1-7 containing *M. citrifolia* by ANOVA.

Day after fermentation	Vitamin B	Formula	Significant difference at the level of 0.05	No significant difference at the level of 0.05
0	B_1	1 to 7	/	
	B_2	2 and 4		/
	B_3	1 and 6		/
		2 and 4		/
	B_6	4 and 7		/
		4 and 6		/
7	B_1	1, 3 and 6		/
	B_2	2, 4 and 5		/
	B_3	1 to 7	/	
	B_6	5 and 6		/
		4 and 7		/
15	B_1	1, 3, 5 and 6		/
		2 and 4		/
	B_2	2, 4 and 7		/
		1 and 6		/
	B_3	2, 4 and 6		/
	B_6	2, 3, 4 and 6		/

Table 4.53 (continued)

Day after fermentation	Vitamin B	Formula	Significant difference at the level of 0.05	No significant difference at the level of 0.05
30	B_1	1, 3,5 and 6		/
		2 and 4		/
	B_2	2, 3, 4, 6 and 7		/
	B_3	2, 4 and 6		/
	B_6	1, 2, 3, 4, 6 and 7		/
45	B_1	1, 3, 5, 6 and7		/
		2 and 4		/
	B_2	1, 2, 3, 4, 6 and 7		/
		5 and 7		/
	B_3	2, 3, 4 and 7		/
		1 and 6		/
	B_6	1 to 7	/	
60	B_1	1, 3, 4, 5, 6 and7		/
	B_2	1 to 7	/	
	B_3	1, 2, 3, 4, 6 and 7		/
	B_6	1 to 7	/	
90	B_1,B_2,B_3,B_6	1 to 7	/	

Table 4.54 Analysis of Vitamins B_1, B_2, B_3 and B_6 in Formulae 1-7 containing *P. emblica* by ANOVA.

Day after fermentation	Vitamin B	Formula	Significant difference at the level of 0.05	No significant difference at the level of 0.05
0	B_1	1 to 7	/	
	B_2	4 and 5		/
		3 and 7		/
	B_3	1 and 3		/
		6 and 7		/
	B_6	4 and 5		/
		1 and 7		/
		2 and 3		/
7	B_1	1 and 7	/	
	B_2	2 and 7		/
	B_3	1 to 7	/	
	B_6	2, 4 and 6		/
		1 and 7		/
15	B_1	1 to 7	/	
	B_2	1 and 4		/
		2 and 5		/
		2 and 3		/
	B_3	4, 5 and 6		/
		1 and 3		/
		2 and 7		/
		2, 4 and 6		/
	B_6	1, 2, 4, 6 and 7		/

Table 4.54 (continued)

Day after fermentation	Vitamin B	Formula	Significant difference at the level of 0.05	No significant difference at the level of 0.05
30	B_1, B_2,B_3 B_6	1 to 7 4, 5, 6 and 7 1 and 3	/	/ /
45	B_1, B_2, B_3 and B_6	1 to 7	/	
60	B_1, B_2, B_3 and B_6	1 to 7	/	
90	B_1, B_2, B_3 and B_6	1 to 7	/	

4.10 Discussion

The acidity of formulae containing honey with *L. casei* and without *L. casei* are lower than that of the formulae containing raw cane-sugar with *L. casei*. The amount of vitamins present in each formula is pH dependent. The degradation rate of vitamins in formulae containing honey with *L. casei* and without *L. casei* are less than those obtained from formulae containing raw cane-sugar with *L. casei* and without *L. casei*. There are no significant differences of vitamins between formulae containing honey with *L. casei* and without *L. casei*.

The treatment using potassium metabisulfite (KMS) as a bactericidal have no significant difference of vitamins between heating process treatment.

Ascorbic acid (vitamin C) are easily destroyed during processing and storage through the action of metals such as copper and iron. Exposure to oxygen and prolonged heating in the presence of oxygen and alkalis destroyed ascorbic acid. Thiamine (vitamin B_1) is one of the most unstable B vitamins. Baking, pasteurization,

or boiling of foods fortified with thiamine can reduce its content by up to 50 percent. The stability of thiamine during storage depends greatly on the moisture content of the food. Thiamine is highly sensitive to heat and alkalis. Riboflavin (vitamin B_2) is very stable during thermal processing, storage and food preparation. Riboflavin, however, is susceptible to degradation on exposure to light. The use of light-proof packaging material prevents its deterioration. Nicotinamide (vitamin B_3) is one of the most stable vitamins and the main loss occurs from leaching into cooking water. Pyridoxine (vitamin B_6) losses depend on the type of thermal processing. For example, high losses of B_6 occur during sterilization of liquid infant formula; in contrast, B_6 in enriched flour and corn meal is resistant to baking temperatures. B_6 is susceptible to light induced degradation and exposure to water can cause leaching and consequent losses. Pyridoxine is sensitive to light, heat and alkalis [179, 180].

The stability of vitamins in fermented products was also studied. Formula with lower pH, degradation of vitamins less occurred than do the formula with higher pH. During fermentation, some by-products were present (e.g. ethyl alcohol, methyl alcohol, acetaldehyde and iso-propanol). Therefore, produced organic solvents effected the stability of vitamin C, vitamin B_1, B_2, B_3 and B_6 in product. Light and oxygen may also contribute to the stability of such vitamins.

Vitamins degradation might be due to three factors, as following:

1. The production process, in which *M. citrifolia* and *P. emblica* fruits was crushed by the crusher, has too many surfaces contacted with oxygen in the air. For this reason, vitamins in *M. citrifolia* and *P. emblica* fruits were oxidized and destroyed by oxygen.

2. Heating process (treatment 7) destroyed vitamins in *M. citrifolia* and *P. emblica* fruits.

3. Production process, such as preparing and mixing, cause vitamins to be reduced. Each step of production process, vitamins in raw material were prone to be destroyed by light, oxygen and heating.

From the above reasons, vitamins in fermented juices were decreased along with the fermentation in our experiment.