CHAPTER IV

RESULTS AND DISCUSSION

4.1 Optimization of Headspace-Solid Phase Microextraction (HS-SPME) Conditions

Some of the HS-SPME variables were considered. The major factor studied in this work included the type of fiber coating, the sample volume, the extraction temperature, the extraction time.

4.1.1 The Result of Type of the Fiber Coating

In this test, two different SPME fibers were tested: 75 µm CAR/PDMS and 50/30 µm DVB/CAR/PDMS. The results of the influence of the type of fiber coating showed in Figure 4.1.

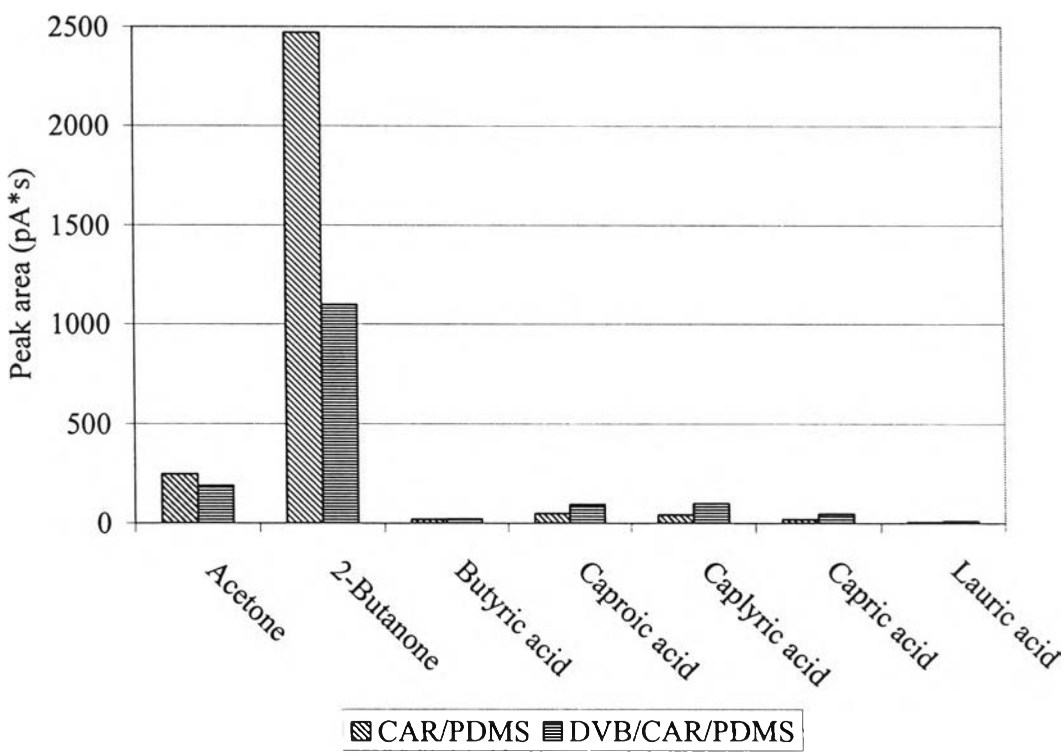

Figure 4.1 Extraction profile obtained with different fibers for 7 target analytes.

The highest response for acetone and 2-butanone were attained when using the CAR/PDMS fiber because the CAR/PDMS fiber offered more effective for extracting small molecules than the DVB/CAR/PDMS. While the DVB/CAR/PDMS gave the highest response for caproic acid, caprylic acid, capric acid, and lauric acid because the DVB phase in the DVB/CAR/PDMS fiber was suitable for high molecular weight substances. Both fibers showed no difference for responses of butyric acid and lauric acid because the butyric acid have low molecular weight, high polarity and the volatility are low compared to 2-butanone. It was equally extracted by both fibers. In Figure 4.1, as can be obviously observed, higher peak area of 2-butanone were obtained with the CAR/PDMS fiber, hence this fiber was selected for further studies.

4.1.2 The Result of the Sample Volume

Phase ratio can affect extraction efficiency. In this test, two different sample volumes were inspected. Using 20 mL vials, 5 and 10 mL of sample volume (phase ratio = 2.0 and 1.0, respectively) were tested. The results were shown in Figure 4.2. Most of the target analytes showed a plateau from 5 to 10 mL onward, whereas lauric acid showed a peak using 10 mL of solution. Because of the volatility are lower compared to other compounds, resulting in lower sensitivity in the large headspace.

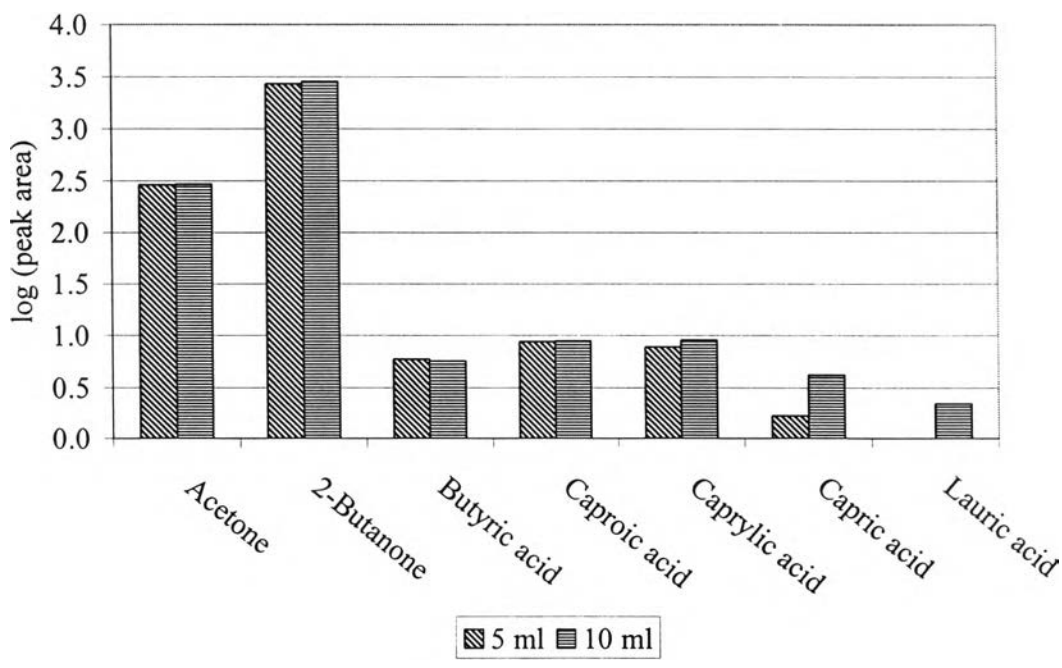

Figure 4.2 Influence of sample volume on the HS-SPME.

In HS-SPME, the small gas phase is respect to the sample. The sensitivity reduces when the headspace volume is large. Therefore a solution volume of 10 mL was selected for further experiments.

4.1.3 The result of the Extraction Temperature

The influence of the extraction temperature was investigated using the CAR/PDMS fiber. The fiber was exposed to the headspace of the sample vial for 20 min at different temperatures. The results of the comparison of different extraction temperatures are depicted in Figure 4.3 and Figure 4.4. Increasing temperatures from 30°C to 60°C indicated higher compounds retainment that resulted in higher enrichment of most volatile compounds on the fiber, (excepted acetone and butanone at 60°C). High temperatures are supposed to release more volatile compounds into the headspace; however, they can adversely affect the absorption of the volatile compounds on the fiber coating due to decreasing of partition coefficients. Therefore, the extraction of acetone and butanone tended to be reduced as the temperature increased to 60 °C. Moreover, at high temperature (60 °C), milk sample gave rise to heat flavor. From these reasons, the best extraction temperature was 45°C.

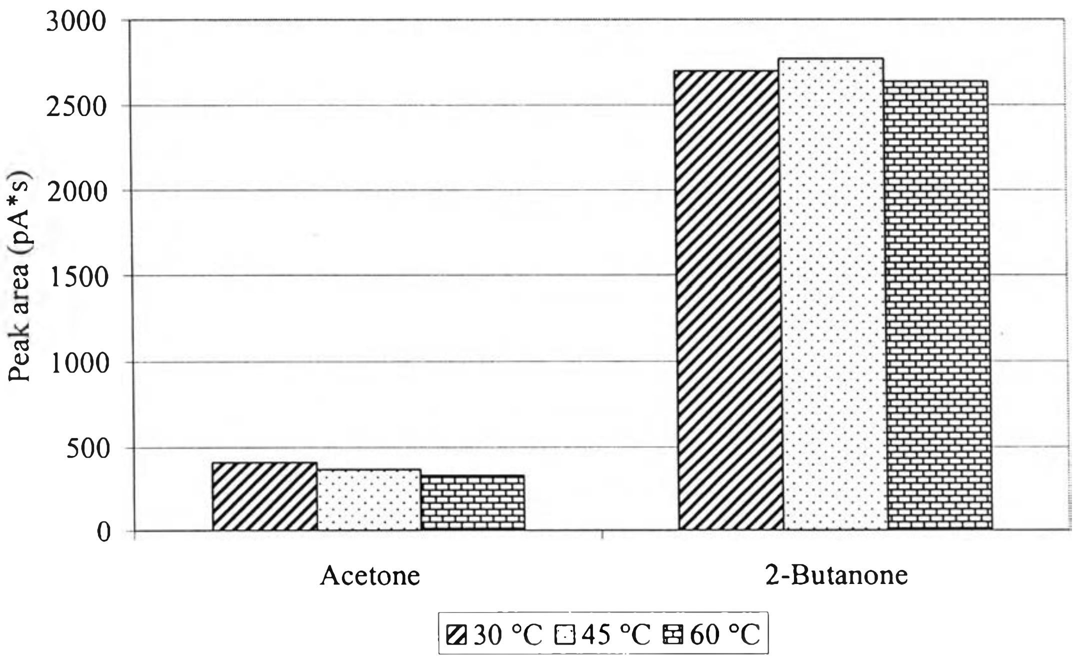

Figure 4.3 Effect of temperature on ketone compounds.

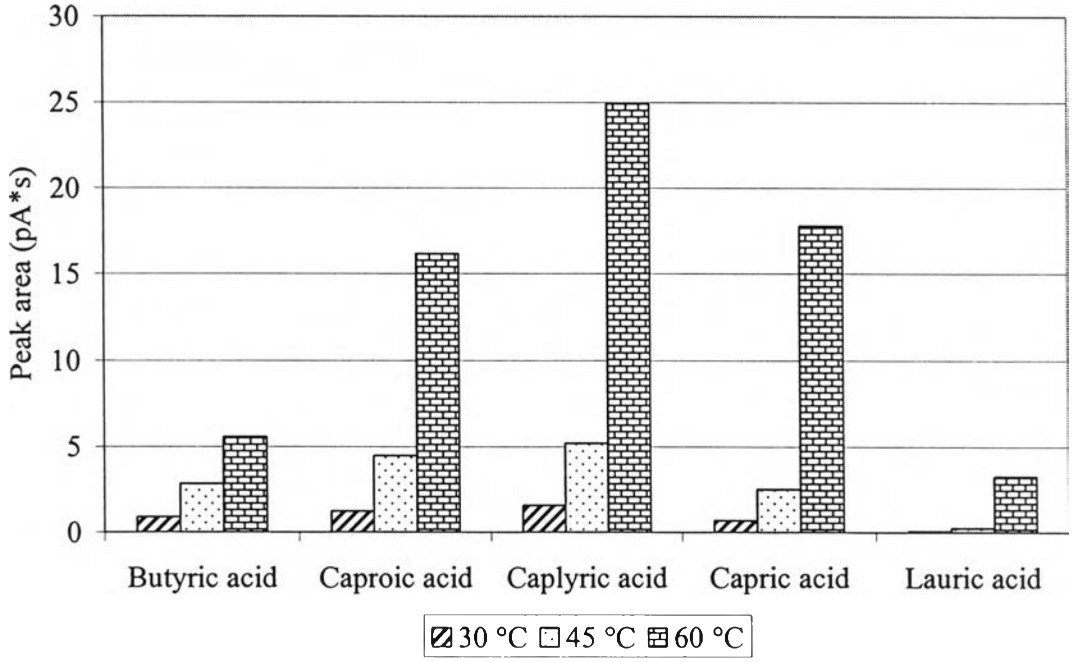

Figure 4.4 Effect of temperature on short-chain fatty acid compounds.

4.1.4 The Result of the Extraction Time

Different times were examined in this extraction method. Since SPME is based on equilibrium between three phases in the sample, i.e., the liquid phase, the gas phase, and the fiber itself, the equilibrium time was to be determined. From the results were depict in Figure 4.5 and 4.6. The equilibration times for most analytes were 45 min. The shorter extraction times could be chosen for quantitation and application; however, care must be taken to control the exposure time and constant temperature in the system. Since there were a lot of samples per week and they could go bad. Therefore we wanted the shorter analysis time per sample. Extraction time of 20 min was chosen.

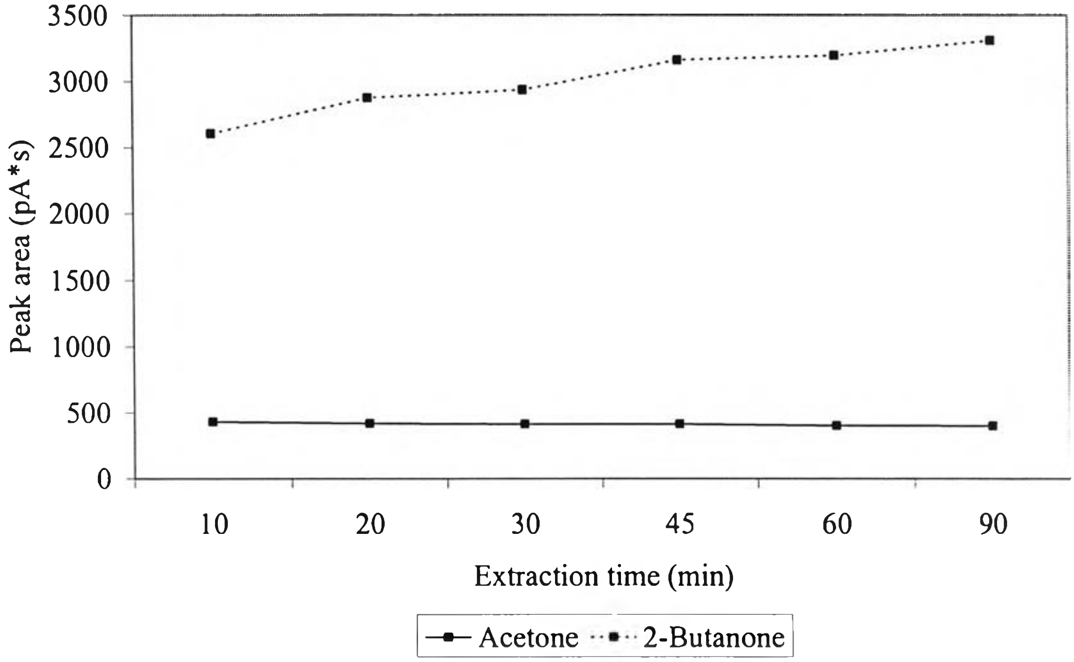

Figure 4.5 Extraction curves for the ketone compounds.

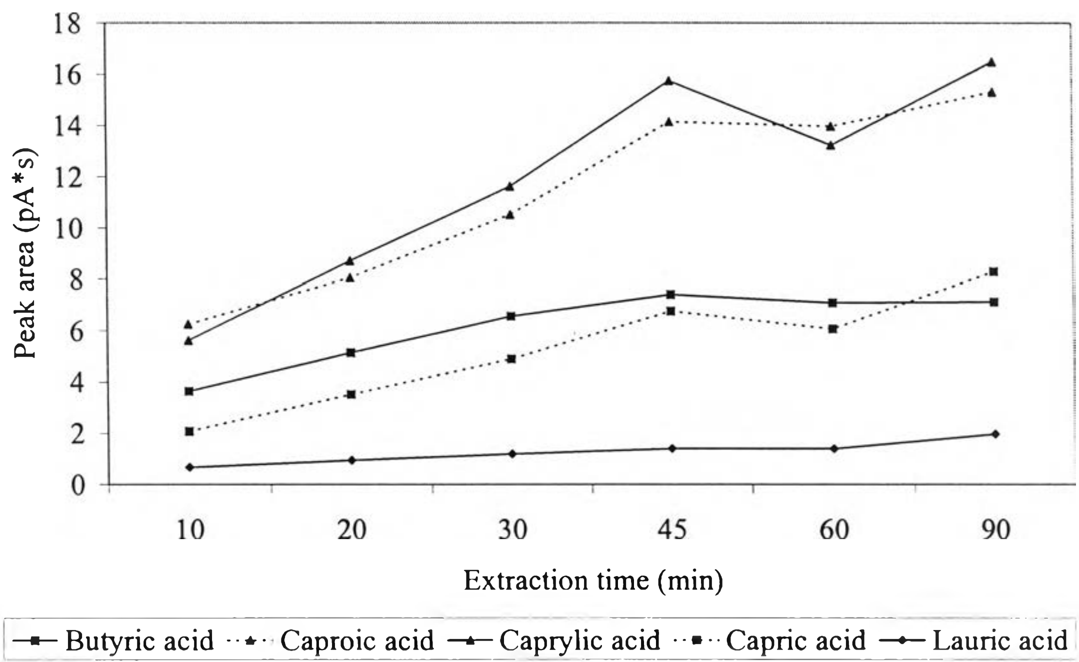

Figure 4.6 Extraction curves for the short-chain fatty acid compounds.

In summary, the optimized condition of SPME for best extraction of the seven volatile compounds considered were using the CAR/PDMS fiber, 45°C for extraction temperature, 20 min for sampling time, and 10 mL for sample volume.

4.2 The Result of Response Factors

In the present study, response factors for each volatile compound were calculated by equation in section 3.5 were summarized in Table 4.1.

Table 4.1 Response factors of individual volatile compounds.

Volatile compound	Response factor
Acetone	0.134
2-butanone	0.023
Butyric acid	28.022
Caproic acid	7.309
Caprylic acid	3.296
Capric acid	2.934
Lauric acid	7.657

The validity of response factors depends on the assumption that there were different affinities of each compound to the SPME fiber and to the GC detector. For example, the highest response factor for butyric acid was calculated, because butyric acid has low affinity to SPME fiber and low sensitivity to the GC detector. In this study, we used response factors for quantitation analysis of volatile compound.

4.3 The Result of Calibration Curves

The mixed standard of FAMEs C_4-C_{24} solutions covering the concentration range of each fatty acid (Table 3.3) were plotted by peak area versus concentrations of FAME. The 5 points calibration curve was shown in Appendix A. The results of linear equations and correlation coefficients were shown in Table 4.2.

Table 4.2 The linear equations and correlation coefficients of calibration curve.

FAMEs	Linear equation	Correlation coefficient (r^2)
C14:0	y = 116392x - 566896	0.9990
C14:1	y = 104176x - 572932	0.9964
C16:0	y = 132464x - 1000000	0.9992
C16:1	y = 121562x - 791181	0.9950
C18:0	y = 150749x - 1000000	0.9990
C18:1	y = 254700x - 2000000	0.9991
C18:2	y = 108634x - 821755	0.9917
C18:3	y = 83377x - 746351	0.9829

From Table 4.2, the data of the 5 points calibration curve are acceptable for quantitation purpose because the correlative coefficients (r^2) ranged from 0.9829 for C18:3 to 0.9992 for C16:0. The linear equations are used for quantitative analyses of corresponded fatty acids.

4.4 The Result of Organoleptic Test

The results of organoleptic test were showed in Table 4.6. The color of milk samples was observed to change from white to yellowish-white in the first to the fifth lactation and in early lactation to late lactation. Because the higher milk fat related to higher lactation and higher lactation stage. The odor of milk samples was found no differences which may be due to our lack of well trained sniffer.

The color of milk samples were found differences between milk from dairy cow fed with different feedstuff (grass or hay and corn silage). All milk samples from dairy cow fed grass or hay had white color, while milk samples from cow fed corn silage had white to yellowish-white color. The odor of milk samples from dairy cow fed corn silage have buttery odor more than milk samples from dairy cow fed grass and hay. It might be a result of that the grass and hay in Thailand is low quality feedstuff. We were found that the dairy cow fed grass and hay as roughage, the dairy cow produce low milk fat. In addition, the quantity of carotene in corn silage is more than the quantity of carotene in grass and hay therefore milk samples from dairy cow fed corn silage have yellowish-white color. The odor of milk samples was sniffed to difference between milk from dairy cow fed with different feedstuff.

Table 4.3 Results of Organoleptic test for milk.

Factor	Color	Odor
Lactation Number		
1→5	White → Yellowish-white	No difference
Lactation Stage		
Early→Late	White → Yellowish-white	No difference
Feedstuff		
Grass and hay	White	Mild butter
Corn silage	Yellowish-white	Butter
Environmental		
Cement House (Farm B)	White → Yellowish-white	Butter
Terrain House (Farm C)	White → Yellowish-white	Butter

The last factor, the milk samples were collected from dairy cows raised in cement house and terrain house. Both farms gave corn silage as roughage, so the color and odor of milk sample were observed to resembling in the different farm environment. The milk samples have yellowish-white color and buttery odor.

4.5 The Result and Discussion of Determination of the Volatile Component in Milk Produced in Thailand

The amounts of volatile components in a sample were estimated by the response factors. The volatile components of various cow milks from different lactation numbers, different stage of lactation, different feedstuff, and different dairy farms were quantified using the optimized SPME method.

Table 4.4 to 4.7 showed the concentration of the volatile components in milk samples, which were below the flavor threshold level. Except, the concentration of butyric acid in the study of lactation number, the concentration of butyric acid, caproic acid, and lauric acid in late lactation in the study of lactation stage, and the concentration of butyric acid in the milk from cow fed corn silage as roughage in the study of feedstuff were above the flavor threshold level. Because of the differences of individual cows and other several factors. All data were analysed using one-way analysis of variance (one-way ANOVA). The variances are lactation number, stage of lactation, feedstuff, and environmental factor. The significant differences were considered by P-values. If the P-values is less than 0.05, the data are significantly different.

4.5.1 Influence of Lactation Number on Volatile Component in Cow's Milk

Milk sample was collected from dairy cows. Different lactation numbers from the first lactation to the fifth lactation were studied. The results were showed in Appendix B, Table 4.4 and Figure 4.7.

Table 4.4 P-values and the concentrations (ppm) of volatile components in cow's milk from various lactation numbers.

Volatile	Lactation Number					*P-Value*
Component	1	2	3	4	5	
Acetone	17.717	19.279	17.012	18.282	26.355	*0.7049*
Butanone	2.896	3.904	2.553	1.831	3.990	*0.8606*
Butyric acid	17.825	19.694	23.770	31.019	35.129	*0.8697*
Caproic acid	4.374	5.813	6.884	8.702	9.393	*0.8818*
Caprylic acid	2.896	3.040	3.450	4.822	4.582	*0.9418*
Capric acid	4.065	5.908	5.332	7.894	6.272	*0.9303*
Lauric acid	6.809	8.510	7.297	8.450	10.324	*0.9695*

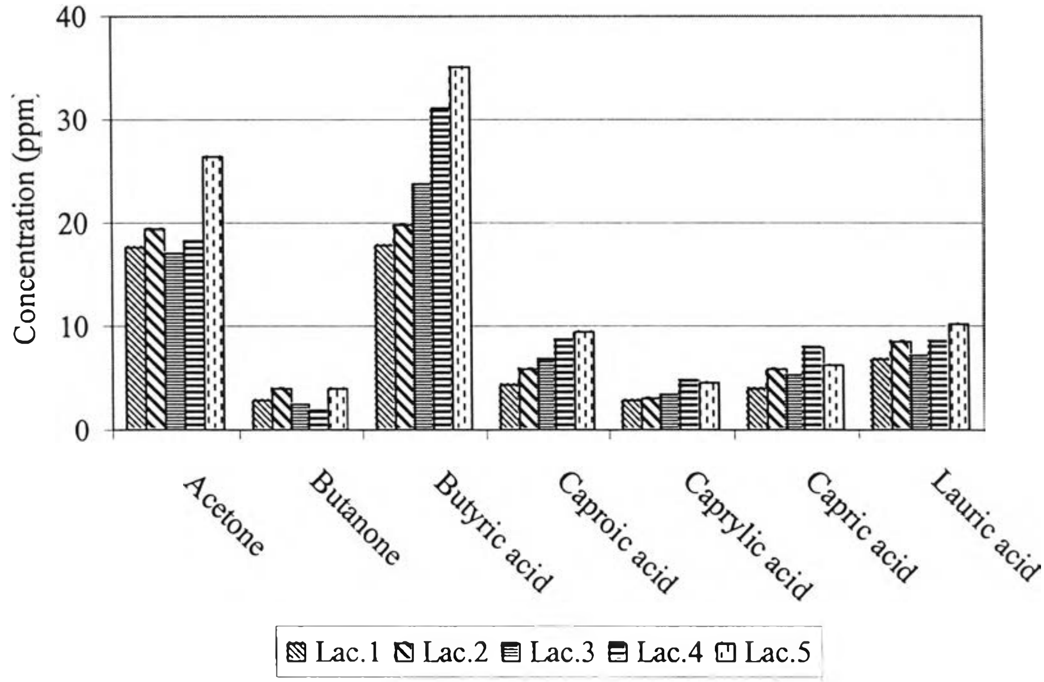

Figure 4.7 Influence of lactation number on volatile components in cow's milk.

The amount of acetone and short-chain fatty acids increased on average from the first lactation to the fifth lactation (17.012-26.355 ppm for acetone, 1.813-3.990 ppm for butanone, 17.825-35.129 ppm for butyric acid, 4.374-9.393 ppm for caproic acid, 2.896-4.822 ppm for caprylic acid, 4.065-7.894 ppm for capric acid, and 6.809-10.324 ppm for lauric acid). Moreover, in the fourth lactation and the fifth lactation, the amount of butyric acid exceeds the flavor threshold level. And in the fifth lactation, the amount of lauric acid exceeds the flavor threshold level. Therefore, milk samples produced by dairy cows on the fourth and the fifth lactation, tended to have off-flavor. When lactation number increased, the amount of milk fat increased (Table 4.8). Therefore, free fatty acid increased too.

4.5.2 Influence of Stage of Lactation on Volatile Component in Cow's Milk

The lactation stage is the main variable. At different lactation stage milk composition varies differently. Appendix B and Table 4.5 showed the result of stage of lactation contributing to concentration of volatile components in cow's milk.

Table 4.5 P-values and the concentrations (ppm) of volatile components in cow's milk from various lactation stages.

Volatile Component	Stage of Lactation			*P-Value*
	Early	Mid	Late	
Acetone	14.731	11.463	9.444	*0.111*
Butanone	0.850	0.404	0.276	*0.001*
Butyric acid	20.398	10.439	72.028	*0.001*
Caproic acid	5.631	2.554	19.518	*0.008*
Caprylic acid	2.743	0.999	9.587	*0.004*
Capric acid	5.153	2.304	12.751	*0.000*
Lauric acid	7.690	2.859	26.497	*0.000*

Variations of volatile components were shown in Figure 4.9. The concentrations of acetone and 2-butanone decreased from early lactation to late lactation (14.731 ppm to 9.444 ppm, 0.850 ppm to 0.276 ppm for acetone and 2-butanone, respectively). There was a highly significant ($P<0.05$) effect of lactation stage on 2- butanone in milk, while, no significant ($P>0.05$) was observed on of lactation stage on acetone. Because of 2-butanone is the main characteristic of associated feed. In late lactation, the farmers decreased amount of feed for dairy cows and leave the udder dry until the next calving. Consequently, concentration of 2-butanone was decreased. While 2-butanone is the main characteristic of associated feed, acetone is characteristic of the associated individual cow and feed, leading to no significant effect of lactation stage on acetone in milk.

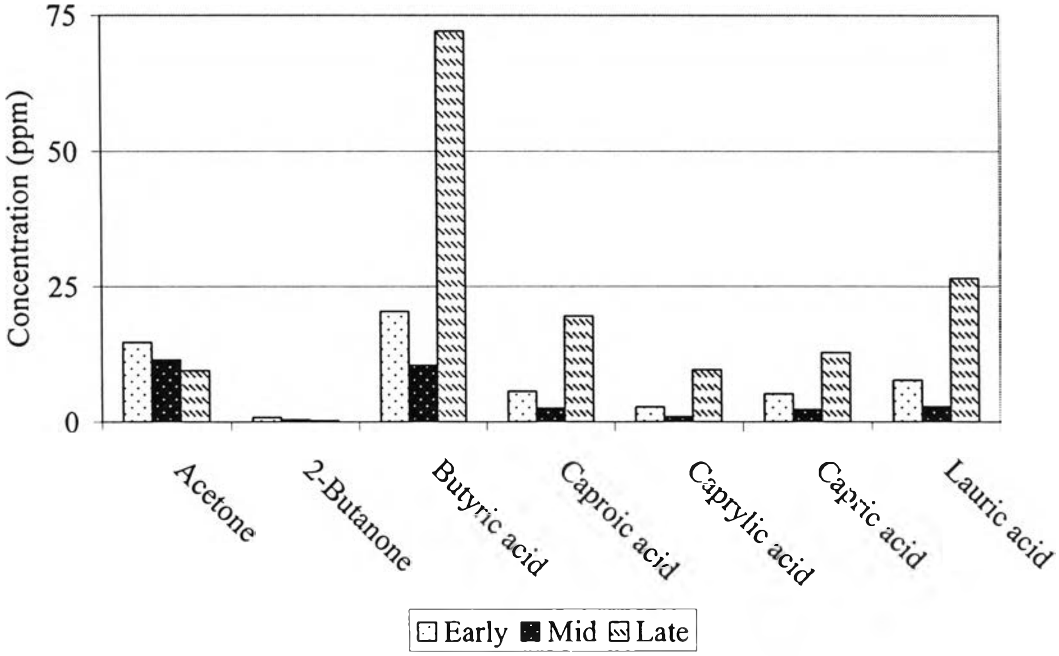

Figure 4.8 Effect of lactation stage to volatile components in cow's milk.

The amount of the short-chain fatty acids (C_4-C_{12} atom) showed similar the same tendency, namely, the concentration decreased from early lactation to mid lactation (20.398 ppm to 10.439 ppm, 5.631 ppm to 2.554 ppm, 2.743 ppm to 0.999 ppm, 5.153 ppm to 2.304 ppm and 7.690 ppm to 2.859 ppm for butyric acid, caproic acid caprytic acid, capric acid, and lauric acid, respectively). In late lactation, the concentration of short-chain fatty acid increased to 72.028 ppm, 19.518 ppm, 9.587 ppm, 12.751 ppm, and 26.497 ppm for butyric acid, caproic acid, caprylic acid, capric acid, and lauric acid, respectively. For this reason, at the end of lactation, the spontaneous lipolysis tends to increase producing free fatty acids. In addition, the concentrations of butyric acid, caproic acid, and lauric acid exceed the flavor threshold levels in late lactation. Therefore, milk collected during late lactation, tends to have off-flavor.

4.5.3 Influence of Feedstuff on Volatile Component in Cow's Milk

Both quality and quantity of feeds have influences on milk composition, affecting the volatile components in cow's milk. The results of feedstuff contributing to volatile components were shown in Appendix B, Table 4.6 and Figure 4.9.

Table 4.6 The concentrations (ppm) and P-values of volatile components in cow's milk from various feedstuffs.

Volatile Component	Feedstuff		*P-Value*
	Grass and Fodder	Corn Silage	
Acetone	14.314	18.544	*0.0574*
Butanone	0.571	2.859	*0.0000*
Butyric acid	22.359	30.155	*0.5119*
Caproic acid	6.315	8.043	*0.6068*
Caprylic acid	3.019	3.404	*0.7752*
Capric acid	5.292	5.421	*0.9467*
Lauric acid	8.861	8.968	*0.9716*

Dairy cows were fed with different feedstuff (grass or hay and corn silage). Milk from dairy cows fed with corn silage gave higher amount of volatile compounds than milk from dairy cows fed with grass or hay (Figure 4.9).

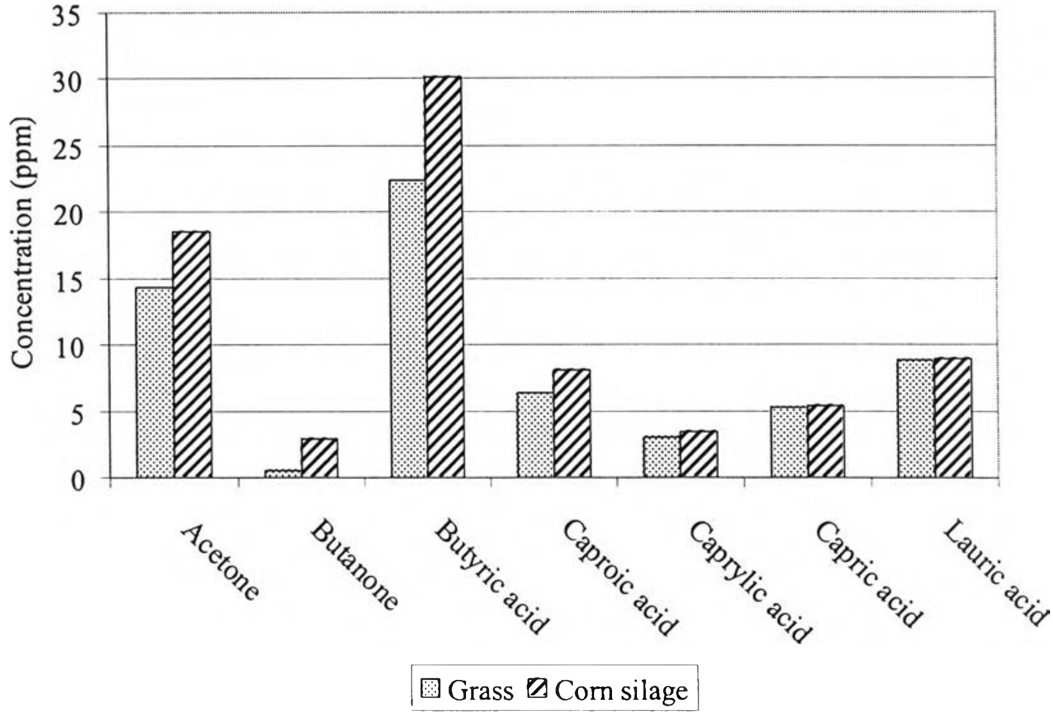

Figure 4.9 Effect of feedstuff to volatile components in cow's milk.

Major differences were observed in the output of 2- butanone. Because there was a highly significant ($P<0.05$) for 2- butanone which related to the characteristic of associated feed. The presence of other volatile compounds were no significant effect ($P>0.05$). The concentration of butyric acid in milk from dairy cow fed with corn silage exceeded flavor threshold level, indicating off-flavor milk cause by butyric acid.

4.5.4 Influence of Farm Environment on Volatile Components in Cow's Milk

Environmental factor also plays important role by affecting milk composition. Milk samples were collected from dairy cows grazed in different farms (Farm B is a cement house, easy to clean, while farm C is a terrain house, difficult to clean). The results of volatile components defected in milk samples collected from the two sites are shown in Appendix B, Table 4.7 and Figure 4.10.

Table 4.7 The concentrations (ppm) and P-values of volatile components in cow's milk from various farm environments.

Volatile Component	Location		*P-Value*
	Cement House (Farm B)	Terrain House (Farm C)	
Acetone	17.183	23.969	*0.0294*
Butanone	2.805	4.896	*0.0419*
Butyric acid	18.918	16.819	*0.7263*
Caproic acid	4.941	4.406	*0.7397*
Caprylic acid	2.126	2.372	*0.7609*
Capric acid	3.566	3.436	*0.9194*
Lauric acid	6.934	5.018	*0.4998*

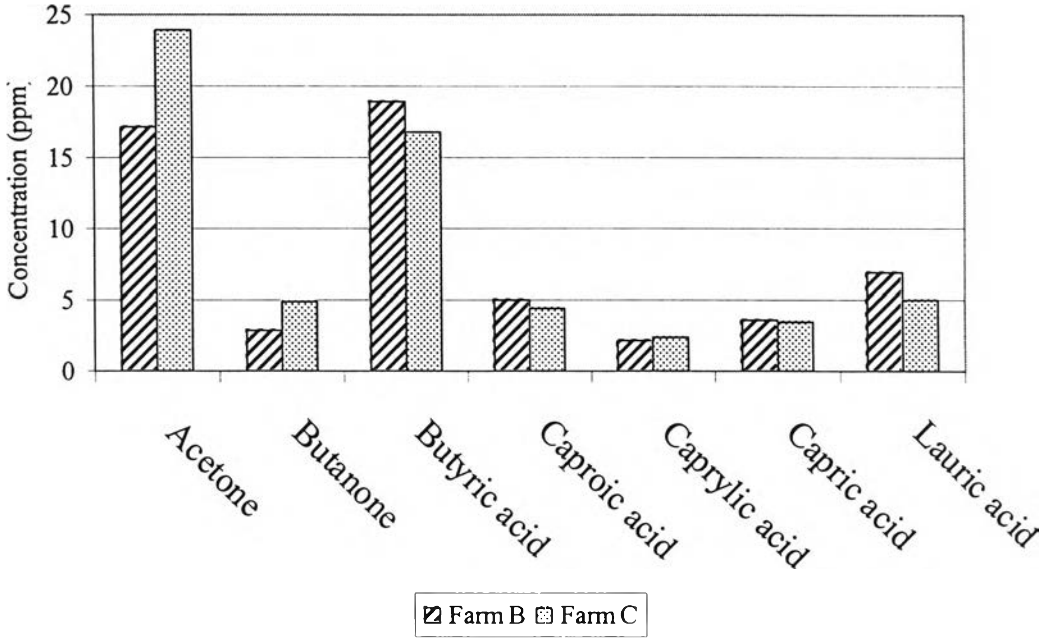

Figure 4.10 Influences of farm environment on volatile components in milk.

The amount of acetone and 2-butanone in cow's milk from farm B were less than farm C (acetone: 17.183 ppm and 23.969 ppm, 2-butanone: 2.805 ppm and 4.896 ppm, Farm B and C, respectively). Higher significant ($P<0.05$) were observed on farm acetone and 2-butanone concentrations. However, the environmental effect is not clear because dairy cows from farm B were younger than dairy cows from farm C. It is well established that cow age could contribute to the amount of acetone and butanone in milk resulting in the characteristic cowy flavor. The results showed non-significant ($p>0.05$) of farm environment on the amount of short chain fatty acids concentrations. However, we observed higher short-chain fatty acids in milk sampled collected from Farm B except for caprylic acid.

4.6 The Result of % Fat in Cow's Milk

Appendix C and Table 4.8 showed all data of the milk sample the %FAT nearly in Table 2.1. In Thailand, the amount of milk fat is same in general milk.

Table 4.8 Result of factors contributing to % FAT in cow's milk produced in Thailand.

Factor	% FAT
Lactation Number	
1	4.15
2	4.17
3	4.13
4	4.70
5	4.77
Stage of Lactation	
Early	4.07
Mid	3.45
Late	4.27
Feedstuff	
Grass and fodder	4.04
Corn silage	4.23
Environmental	
Cement House (Farm B)	3.78
Terrain House (Farm C)	4.29

%FAT was found to increase in the forth and the fifth lactation because the dairy cows became mature and wholesome of milk can produce of higher milk fat. In the study of lactation stage, the milk fat in mid lactation is the lowest because milk sample were collected in the summer time when average temperature was higher than other study stage. Then the dairy cow produced decreasing milk fat. In fact, the milk fat slightly increased when lactation stage change from early lactation to late lactation. The milk from dairy cow fed corn silage has higher milk fat than the milk from dairy cow fed grass and fodder, because the grass and fodder in Thailand is low quality feedstuff. When the dairy cow fed grass and fodder as roughage, low milk fat was produced. In the case of farm environment factor, milk fat in farm B is less than milk fat in farm C because some milk was sampled in higher temperature than other milk samples. Then the dairy cow produced decreasing milk fat and the cow age of farm B is lower than the cow age of farm C. However, milk fat in mid lactation and Farm B is low because some milk was sampled in higher temperature than other milk samples.

4.7 The Results of % w/w FFA/FAT in Milk Produced in Thailand

From Appendix C, all data of milk samples have the % w/w FFA/FAT nearly in Table 2.5. Data were analyzed using the one-way analysis of variance. The variances are lactation number, stage of lactation, feedstuff, and environmental factor. The significant differences were considered by P-values. If P-value is less than 0.05, then it is considered to be significantly differing.

4.7.1 Influence of Lactation Number on Fatty Acid in Cow's Milk

The variability of milk depends on many factors; lactation number is one parameter affecting milk composition such as volatile components and fatty acids composition. The results of lactation number on medium chain fatty acid composition were compiled in Table 4.9 and showed Figure 4.11.

Table 4.9 P-values and % w/w FFA/FAT in cow's milk from various lactation numbers.

Fatty acid	Lactation Number					*P-Value*
	1	2	3	4	5	
C14:0	6.82	6.51	8.80	7.24	16.33	*0.2194*
C14:1	1.17	0.58	0.64	0.28	0.53	*0.4811*
C16:0	19.02	17.46	21.31	19.80	20.55	*0.1015*
C16:1	1.81	1.68	1.41	0.79	1.43	*0.8705*
C18:0	14.77	9.06	10.59	11.45	12.17	*0.0688*
C18:1	8.83	6.37	8.21	8.39	7.83	*0.2071*
C18:2	0.82	1.09	0.90	0.42	0.40	*0.8060*
C18:3	0.00	0.39	0.00	0.00	0.00	*0.4857*

The medium chain fatty acids (C14-18 atoms) were observed in milk. The amount of medium chain saturated fatty acids also changed in different lactation. C14:0 concentrations increased slightly in more lactation number. Concentrations of C16:0 and C18:0 significantly changed between lactation number ($P<0.05$) no trend was observed.

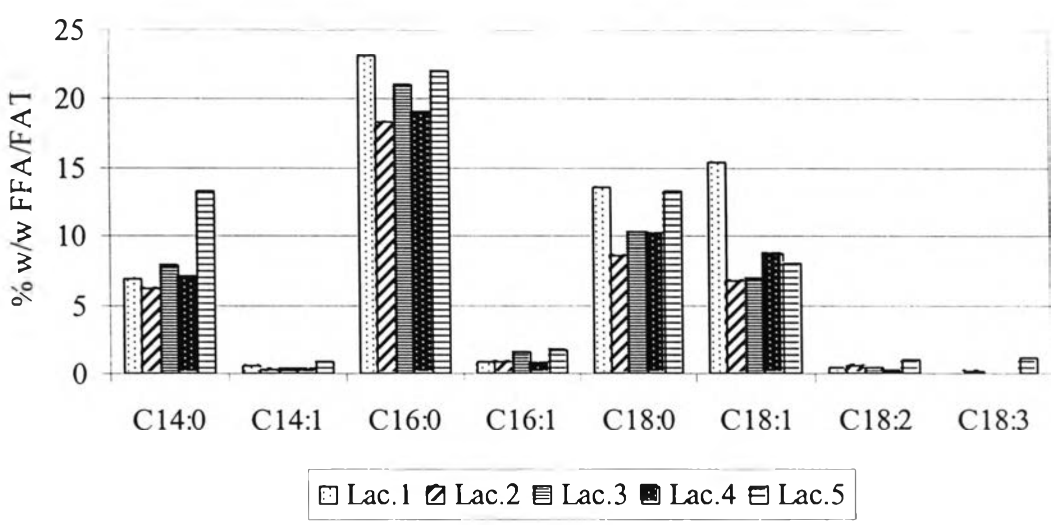

Figure 4.11 Influence of lactation number on fatty acids in cow's milk.

4.7.2 Influence of Stage of Lactation on Fatty Acids in Cow's Milk

The tendency of amount of the medium-chain fatty acid and, amount of the short-chain fatty acid are the same. The amount of medium-chain fatty acids were shown in Table 4.10 and Figure 4.12

Table 4.10 P-values and % w/w FFA/FAT in cow's milk from various lactation stages.

Fatty acid	Lactation stage			*P-Value*
	Early	Mid	Late	
C14:0	8.063	6.217	9.912	*0.018*
C14:1	0.000	0.111	0.306	*0.286*
C16:0	21.853	16.591	22.779	*0.020*
C16:1	0.000	0.177	0.659	*0.306*
C18:0	9.650	9.160	11.121	*0.485*
C18:1	7.993	6.719	7.419	*0.621*
C18:2	0.000	0.381	0.298	*0.614*
C18:3	0.000	0.000	0.000	-

The amount of medium-chain saturated fatty acid decrease from early lactation to mid lactation and increase to late lactation (from 8.063% to 6.217% to 9.912% for C14:0, from 21.853% to 16.591% to 22.779% for C16:0, and from 9.650% to 9.160% to 11.121% for C18:0). The medium chain unsaturated fatty acids were found in mid and late lactation, except C18:1. The tendency of amount of C18:1 is similar to saturated fatty acid because C18:1 was produced by desaturation of C18:0 to C18:1 (cis-9).

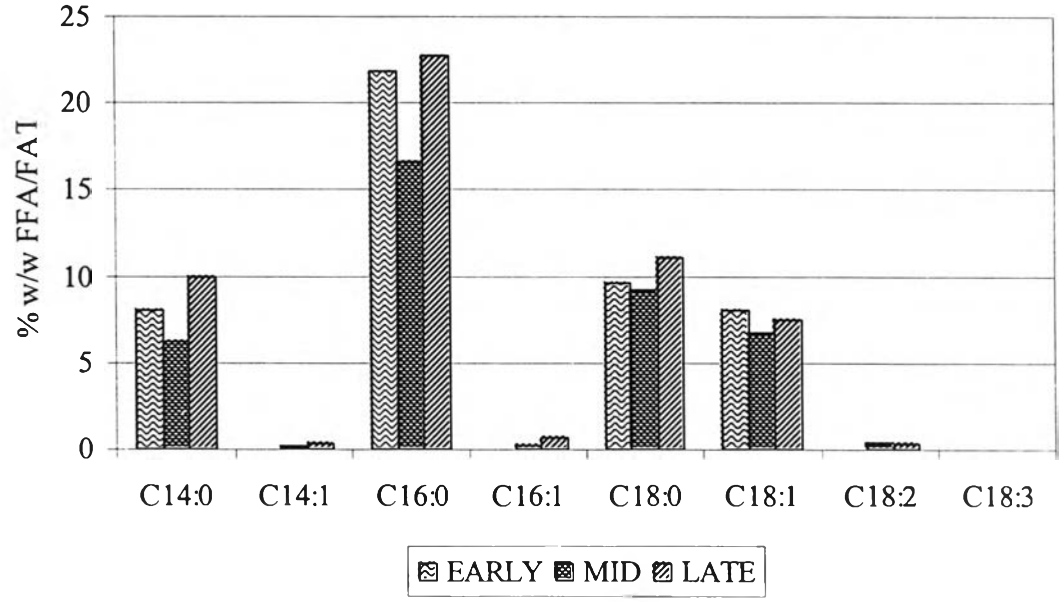

Figure 4.12 Effect of lactation stage to the fatty acids in cow's milk.

4.7.3 Influence of Feedstuff on Fatty Acids in Cow's Milk

The result of feedstuff contributing to fatty acids in cow's milk was shown in Table 4.11 and Figure 4.13.

Table 4.11 P-values and % w/w FFA/FAT in cow's milk from various feedstuffs.

Fatty acid	Feedstuff		*P-Value*
	Grass and Fodder	Corn Silage	
C14:0	7.23	6.72	*0.7060*
C14:1	0.82	0.65	*0.4698*
C16:0	16.36	16.31	*0.9844*
C16:1	1.44	1.14	*0.4472*
C18:0	9.69	8.34	*0.4617*
C18:1	6.61	6.33	*0.7710*
C18:2	0.65	0.33	*0.3669*
C18:3	0.40	0.18	*0.5472*

The medium-chain fatty acids composition (Figure 4.12) from dairy cows fed with grass or hay gave higher amount than from dairy cows fed with corn silage. The percentage of C14:0 (from 7.228% of grass or hay as feed to 6.721% of corn silage as feed), C14:1 (from 0.820% to 0.654%), C16:0 (from 16.305% to 16.363%), C16:1 (1.438% to 1.144%), C18:0 (from 9.689% to 8.337%), C18:1 (from 6.611% to 6.329%), C18:2 (from 0.653% to 0.329%), and C18:3 (from 0.396% to 0.185%). There was no significant (P>0.05) effect of feedstuff on the medium chain fatty acid in cow's milk.

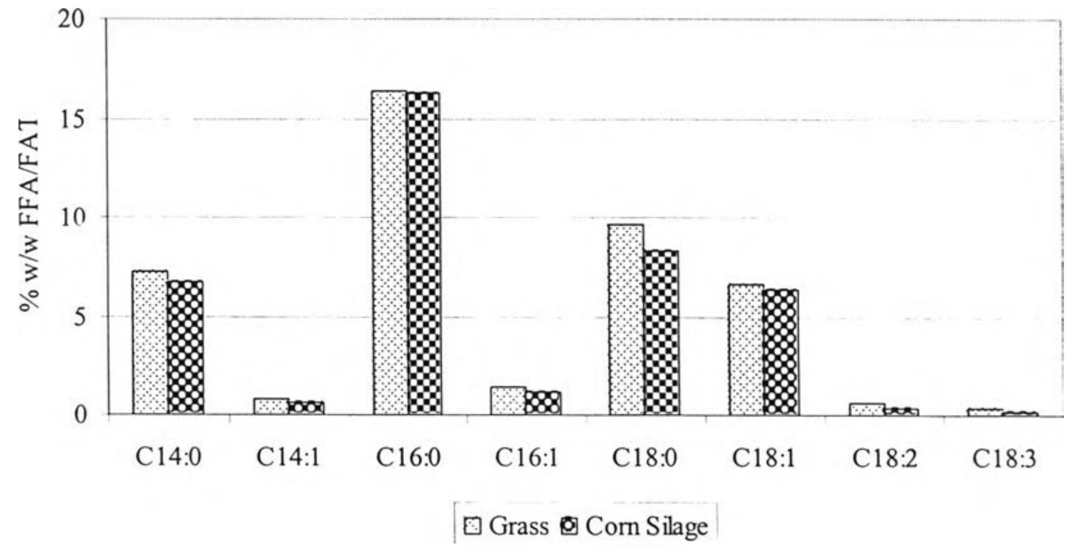

Figure 4.13 Effect of feedstuff to fatty acids in cow's milk.

4.7.4 Influence of Farm Environment on Volatile Component and Fatty Acids in Cow's Milk

The result of farm environment contributing to fatty acids in cow's milk was shown in Table 4.12 and Figure 4.14.

Table 4.12 P-values and % w/w FFA/FAT in cow's milk from various farm environments.

Fatty acid	Farm environment		*P-Value*
	Cement House (Farm B)	Terrain House (Farm C)	
C14:0	7.02	6.74	*0.7749*
C14:1	0.17	0.29	*0.5661*
C16:0	21.31	19.05	*0.5178*
C16:1	0.28	0.70	*0.2841*
C18:0	9.64	8.48	*0.4742*
C18:1	10.92	7.20	*0.2308*
C18:2	0.22	0.55	*0.2904*
C18:3	0.00	0.00	-

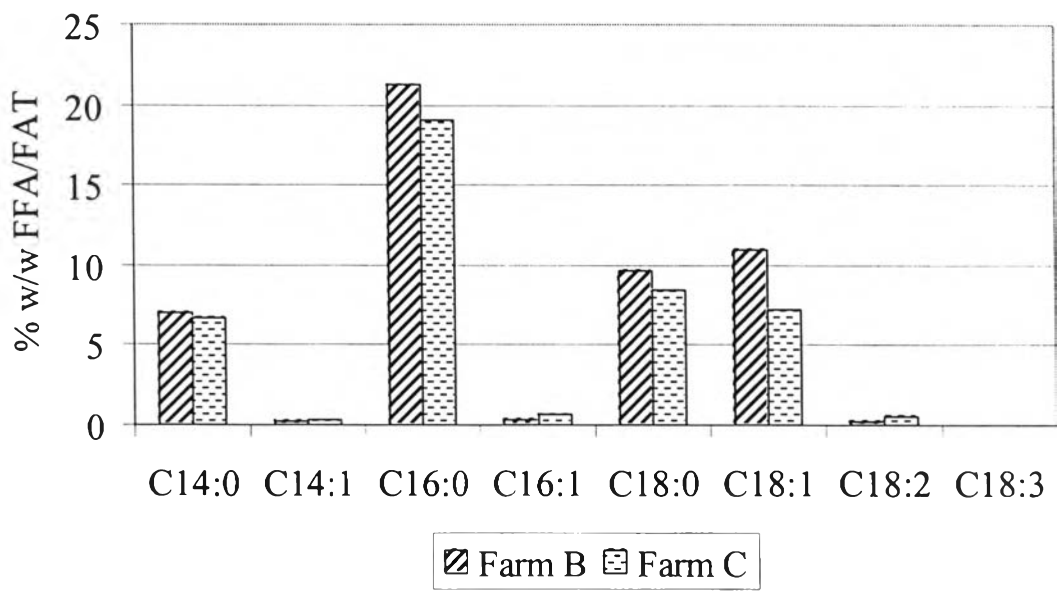

Figure 4.14 Influence of farm environment on fatty acids in milk.

Figure 4.14 showed influence of farm environment on the medium chain fatty acid. The amount of medium chain saturated fatty acid (C14:0-C18:0) in milk from farm B was higher than that in milk from farm C. The amount medium chain unsaturated fatty acid (C14-18 atoms) in milk from farm B was lower than that in milk from farm C, except C18:1. The tendency for amount of C18:1 is similar to saturated fatty acid because C18:1 is a product of desaturation C18:0 in ruminant. However, the farm environment on milk had no significant ($P>0.05$) effect of the amount of fatty acid.