CHAPTER III

EXPERIMENTAL

3.1 Instrument and Equipments

3.1.1 GC 6890 was equipped with Flame Ionization (FID) from Agilent Technologies, USA.

3.1.2 GC 6890N was equipped with 5973 inert Mass Selective Detector from Agilent Technologies, USA.

3.1.3 7693 Auto sampler was used in fatty acid analysis. Agilent Technologies, USA.

3.1.4 HP-FFAP (Nitroterephthalic acid modified polyethylene glycol), 25 m × 320 μm × 0.5 μm film thickness from Agilent Technologies, USA.

3.1.5 HP-5ms (5% phenyl methyl siloxane), 30 m × 250 μm × 0.25 μm film thickness) from Agilent Technologies, USA.

3.1.6 11 mm Pre-drilled Septa from Supelco, USA.

3.1.7 0.75 mm i.d. SPME Injection Sleeve from Supelco, USA.

3.1.8 Solid Phase Micro Extraction Holder (Manual) from Supelco, USA.

3.1.9 SPME fibers from Supelco, USA.

- 75 μm Carboxen™ PDMS
- 2 cm - 50/30 μm DVB/Carboxen/PDMS Stable flex

3.1.10 Gastight syringe 1.00 mL, Hamiton Company, USA.

3.1.11 Micro syringe 10.00 μL, Hamiton Company, Switzerland.

3.1.12 Headspace vial, 20 mL and 60 mL from Agilent Technologies, USA.

3.1.13 20 mm Aluminium Crimp caps and grey butyl septa from Agilent Technologies, USA.

3.1.14 Clear vial 2 mL with Crimp cap

3.1.15 11 mm Aluminium Crimp cap with septum from Agilent Technologies, USA.

3.1.16 Crimper 11 mm and 20 mm from Agilent Technologies, USA.

3.1.17 Decapper 11 mm and 20 mm from Agilent Technologies, USA.

3.1.18 Thermostated water bath from Memmert, Germany.

3.1.19 Sand bath

3.1.20 Hot plate from Schott,

3.1.21 Oven from Memmert, Germany.

3.1.22 Volumetric pipettes 5.00 mL, 10.00 mL and 50.00 mL

3.1.23 Graduated pipettes 2.00 mL and 10.00 mL

3.1.24 Graduated Cylinders 10.00 mL and 50.00 mL

3.1.25 Separatory funnels 100 mL

3.1.26 Beakers 25 mL, 50 mL and 250 mL

3.1.27 Desiccator

3.1.28 Balance from Metler

3.1.29 Round bottom flasks 50 mL

3.1.30 Condenser

3.1.31 Clear vial 20 mL with screw cap

3.1.32 Dropper

3.1.33 Micro-pipettes and tips

3.1.34 Stand

3.1.35 Clamp

3.1.36 O-ring

3.1.37 Thermometer

3.1.38 Milli-Q, Ultrapure Water Systems, with Millipak® 40 Filter Unit 0.22 μm, model Millipore ZMQS 5 V00Y, Millipore, USA.

3.2 Chemical Reagents

3.2.1 Standard Chemicals

- Butyric acid, Caproic acid, Caprylic acid, Capric acid, and Lauric acid were purchased from Fluka Chemika, Switzerland.

- Mixed Standard of Fatty Acid Methyl Ester (FAMEs) C_4–C_{24} when (the purity and percent weight of each component is presented in Table 3.1) was purchased from Supelco, USA.

Table 3.1 purity and %weight of each standard in mix standard.

Analyte	Purity	Weight (%)
Butyric acid methyl ester (C4:0)	99.9	3.996
Caproic acid methyl ester (C6:0)	99.8	3.993
Caprylic acid methyl ester (C8:0)	99.9	3.995
Capric acid methyl ester (C10:0)	99.6	3.996
Undecanoic acid methyl ester (C11:0)	99.9	1.997
Lauric acid methyl ester (C12:0)	98.2	3.993
Tridecanoic acid methyl ester (C13:0)	99.0	1.997
Myristic acid methyl ester (C14:0)	99.9	3.993
Myristoleic acid methyl ester (C14:1)	99.6	1.996
Pentadecanoic acid methyl ester (C15:0)	99.9	2.001
Cis-10-Pentadecanoic acid methyl ester (C15:1)	99.0	1.998
Palmitic acid methyl ester (C16:0)	99.9	5.992
Palmitoleic acid methyl ester (C16:1)	99.8	1.997
Heptadecanoic acid methyl ester (C17:0)	99.9	1.996
Cis-10-Heptadecanoic acid methyl ester (C17:1)	99.1	1.996
Stearic acid methyl ester (C18:0)	99.9	3.998
Oleic acid methyl ester (C18:1)	99.9	3.994
Elaidic acid methyl ester (C18:1)	99.9	1.997
Linoleic acid methyl ester (C18:2)	99.9	1.998
Linoelaidic acid methyl ester (C18:2)	99.9	1.999
Linolenic acid methyl ester (C18:3)	99.9	1.998
Gamma-Linolenic acid methyl ester (C18:3)	99.9	1.998
Arachidic acid methyl ester (C20:0)	99.9	3.994
Cis-11-Eicosenoic acid methyl ester (C20:1)	98.2	2.001
Cis-11,14-Eicosadienoic acid methyl ester(C20:2)	99.9	1.996
Cis-8,11,14-Eicosatrienoic acid methyl ester (C20:3)	95.8	2.089
Cis-11,14,17-Eicosatrienoic acid methyl ester (C20:3)	99.6	2.003
Arachidonic acid methyl ester (C20:4)	99.6	2.004

Table 3.1 (continued).

Analyte	Purity	Weight (%)
Cis-5,8,11,14,17-Eicosapentaenoic acid methyl ester (C20:5)	99.9	1.996
Heneicosanoic acid methyl ester (C21:0)	99.9	1.999
Behenic acid methyl ester (C22:0)	99.3	3.993
Erucic acid methyl ester (C22:1)	99.9	1.998
Cis-13,16-Docosadienoic acid methyl ester (C22:2)	99.9	
Cis-4,7,10,13,16,19-Docosahexaenoic acid methyl ester (C22:6)	98.1	2.014
Tricosanoic acid methyl ester (C23:0)	99.7	1.999
Ligoceric acid methyl ester (C24:0)	99.9	3.993
Nervonic acid methyl ester (C24:1)	99.9	2.003

3.2.2 Organic Solvent

Petroleum ether and Anhydrous diethyl ether were obtained from J.T. Baker Chemical Company, Deventer, Holland. Methanol, Ethanol, Hexane, Acetone and 2-Butanone were analytical grade obtained from E. Merck, Darmstadt, Germany.

3.2.3 Other Reagents

Ammonium hydroxide (J.T. Baker Chemical Company, Deventer, Holland), Boron trifluoride/Methanol 14% (Sigma-Aldrich Company, Steinhiem, Germany), Sodium chloride and Anhydrous Sodium sulfate (E. Merck, Darmstadt, Germany).

3.3. Preparation of Standard Solutions

3.3.1. The Mixture of Volatile Compounds Standard Solution

3.3.1.1 The 1000 ppm stock solution of each compound was prepared by weighing capric acid and lauric acid 50 mg of each standard in 60 mL clear headspace vial with butyl septum. Next added 50 mL of Milli Q water into the headspace vial by pipetting. Then, pipette acetone, 2-butanone, butyric acid, caproic acid, and caprylic

acid (63.30 μL, 62.20 μL, 52.20 μL, 54.00 μL, and 55.00 μL, respectively) from standard of each component. Finally, the vial was rapidly closed with aluminium crimp cap. This mixture standard was stored in refrigerator.

3.3.1.2 The stock standard solution of mixture volatile compounds at ten fold flavor threshold value was prepared by weighing capric acid 6.5 mg and lauric acid 4.5 mg in 60 mL clear headspace vial with butyl stopper and diluting them with 50 mL Milli Q water. Then acetone, 2-butanone, butyric acid, caproic acid, and caprylic acid (50.63 μL, 49.69 μL, 13.0 μL, 7.55 μL, and 5.49 μL, respectively) were added. The vial was rapidly closed with aluminium crimp cap. The mixture standard solution was stored in refrigerator.

3.3.2 The Mixture of FAMEs Stock Standard Solutions in Hexane

The 2000 ppm stock standard solutions of mix C_4 – C_{24} was prepared by dissolving 100 mg of mix C_4 – C_{24} and diluting them to the mark with hexane in 50.00 mL.

3.4 Solid Phase Microextraction (SPME) Optimization

In this work, the volatile components in cow's milk were extracted. The extraction condition must be optimized for the suitable extraction method. Several variables affect to the extraction efficiency. For examples, the type of fiber coating, the extraction temperature, extraction time and sample volume.

3.4.1 Preparation of Standard Solutions

The mixture stock standard solutions of volatile component at a concentration of ten fold flavor threshold level was diluted to flavor threshold level with commercial pasteurized milk in 20 mL headspace vial fitted with butyl septum and aluminium cap. These solutions were used in SPME optimization. Due to there was no deodorized milk a sample blank was prepared by adding 1.00 mL of Milli Q water to 9.00 mL of pasteurized milk in 20 mL headspace vial fitted with butyl septum and aluminium cap. No organic solvent and preparation standard solutions with

pasteurized milk were decreased the matrix effect of milk composition in real sample and avoided interference from organic solvents.

3.4.2 The Study of SPME Fibers

In order to optimize SPME fiber used in this work, the coating of SPME fibers were studied. Typically, the chemical nature [polarity and volatility (molecular weight)] of target analyte determines the type of suitable coating.

Two SPME fibers were evaluated in the study: of CAR/PDMS and DVB/CAR/PDMS. The CAR/PDMS phase is suitable for volatile compound while the DVB/CAR/PDMS phase is suitable for higher molecular weight substances than the CAR/PDMS. The standard solutions in Section 3.4.1 were placed in thermostatic water bath at 45°C. After 10 min equilibrating, each fiber was exposed in the headspace in the vial for extraction of volatile compounds 20 min, and then immediately desorbed in the gas chromatograph injector. The fiber was held in the injector for 10 min. GC condition is shown in Table 3.2. Each extraction was repeated three times. The results are shown in Section 4.1.1.

Table 3.2 The gas chromatographic conditions for analysis of volatile components.

GC Parameters	**GC Condition**
Injector	Spit mode; Split ratio 10:1 , 250°C
Analytical Column	25m × 0.32 mm × 0.5 film thickness HP-FFAP (Nitrophthalic acid modified polyethylene glycol) Capillary column
Temperature program	50°C (2 min) to 220°C (5 min) at 6°C /min
Detector	Flame Ionization Detector (FID), 250°C
Flow rate	
Carrier gas (He)	1.0 mL/min
H_2	40.0 mL/min
Air	450.0 mL/min
Make up gas (N_2)	45.0 mL/min

3.4.3 The Study of Sample Volume

In headspace SPME, the sensitivity loses for volatile components when the headspace/sample volume ratio (phase ratio) increases. In addition, the change in phase ratio affects not only sensitivity but also equilibration times because of change in headspace volume. However, in pratice, the sample volume is limited to vial size. Then, the sample volume was studied.

From the result in Section 3.4.2, one could either choose CAR/PDMS fiber or DVB/CAR/PDMS fiber. The mixture stock standard solution of volatile component at a concentration of ten-fold flavor threshold level was diluted to flavor threshold level with commercial pasteurized milk into 20 mL headspace vial fitted with butyl septum and aluminium cap. Final volume of the mixture solution is 5 and 10 mL for this study. Then the vial placed in thermostatic water bath at 45°C. After 10 min, the fiber was exposed in the headspace of the vial for 30 min, and then instantly desorbed in the GC injection port. The fiber was held in the injection port for 10 min. GC condition is shown in Table 3.2. Each sample volume was repeated three times. The results are shown in Section 4.1.2.

3.4.4 The Study of Extraction Temperature

An increase in extraction temperature cause an increase in extraction rate but simultaneously a decrease in sensitivity. From the results in Section 3.4.2 and 3.4.3, we obtained suitable SPME fiber and suitable sample volume for the method. In this section, extraction temperature was studied by the results in Section 3.4.2 and 3.4.3. The mixture solution in Section 3.4.1 was equilibrated in thermostatic water bath at 30°C, 45°C and 60°C for 10 min. Then the fiber was exposed to the headspace for 20 min then immediately desorbed in the injection port of gas chromatograph in which was held for 10 min. GC condition is shown in Table 3.2. Each extraction was repeated three times. The results are shown in Section 4.1.3.

3.4.5 The Study of Extraction Time

In principle, the objective of the SPME experiment is to reach equilibrium in the system. At this condition, a variation of mass transfer dose not affects the final results. From the results in Section 3.4.2, 3.4.3, and 3.4.4, we obtained the SPME fiber, the sample volume, and the extraction temperature for the method. In this section, the extraction time was studied to determine the optimal exposure time of the fiber to the sample headspace. The fiber was held for several time periods in the headspace of the mixture solution in Section 3.4.1 (at a concentration of flavor threshold level of each standard). Then the mixture solution placed in thermostatic water bath at 45°C. The mixture solution was equilibrated for 10 min. Then, the fiber was exposed for 10, 20, 30, 45, 60, and 90 min, and instantly desorbed in the gas chromatograph injector. The fiber was held in the injector for 10 min in order to decrease the carry over effect on the next extraction. GC condition is shown in Table 3.2. Each extraction was repeated three times. The results are shown in Section 4.1.4.

3.5 The Study of Response Factor

For the quantitation analysis, the reporting of peak areas for SPME analyzes is common, but may be misleading as the binding affinity of compounds to SPME fibers can vary greatly. To determine the amount of each volatile compound, response factor were calculated. From the result in Section 3.4, we obtained the SPME condition. In this section, the response factor was evaluated; 1.00 mL of the 1000 ppm stock mixture standard solution was diluted to 10.00 mL with pasteurized milk in 20 mL head space vial fitted with butyl septum and aluminium cap. Due to there was no deodorized milk as a sample blank, the blank was prepared adding 1.00 mL of Milli-Q water added to 9.00 mL of pasteurized milk. These solutions were use in evaluation of response factor. The peak area differences between the samples with and without added standard solution were used to calculate response factor. Volatile component were extracted by the results in Section 3.4.2-3.4.5. GC condition was shown in Table 3.2. Desorption time for 10 min decreased carry over effect. Each extraction was repeated seven times. The results are shown in Section 4.2.

$$\text{Response Factor} = \frac{\text{Concentration of VC}}{[\text{PA of VC with SS} - \text{PA of VC without SS}]}$$

VC = Volatile component

PA = Peak area

SS = Standard solution

3.6 The Study of Calibration Curve of Mixed Standard of Fatty Acid Methyl Ester (FAMEs) C_4-C_{24} Solution

For the quantitation analysis of fatty acids in milk, the reporting of %w/w fatty acid per fat in milk (% w/w FFA/FAT) was recommended. Then the calibration curve of FAMEs plotted for the determination of fatty acid in mg/L. Change the concentration unit from mg/L to %w/w FFA/FAT is considered in detail later. Each concentration of mixed standard of fatty acid methyl ester (FAMEs) C_4-C_{24} solutions in Table 3.3 was run 5 points and each point run in triplicate. The peak areas of a function of concentration were plotted. Each point was the average of 3 runs summary of value of slope, intercept, and correlation coefficient (r^2) of FAME in mixed standard of FAMEs C_4-C_{24} by the condition in Table 3.4 was shown in Section 4.3 and calibration curve was shown in the APPENDIX.

The retention time of FAME was identified by comparison of the mass spectrum with those of the mass spectrum library Wiley 7. The results are showed in Section 4.3.

Table 3.3 The concentrations (mg/L) of some FAME in vary concentration of mixed standard FAMEs.

FAMEs	Concentration of mixed standard FAMEs				
	400 ppm	800 ppm	1200 ppm	1600 ppm	2000 ppm
C14:0	15.9720	31.9440	47.9160	63.8880	79.8600
C14:1	7.9840	15.9680	23.9520	31.9360	39.9200
C16:0	23.9680	47.9360	71.9040	95.8720	119.8400
C16:1	7.9840	15.9680	23.9520	31.9360	39.9200
C18:0	15.9920	31.9840	47.9760	63.9680	79.9600
C18:1	15.9760	31.9520	47.9280	63.9040	79.8800
C18:2	7.9920	15.9840	23.9760	31.9680	39.9600
C18:3	7.9920	15.9840	23.9760	31.9680	39.9600

Table 3.4 The gas chromatographic conditions for analysis of FAMEs.

GC Parameters	**GC Condition**
Injector	Spit mode; Split ratio 10:1 , 250°C
Analytical Column	30m × 0.25 mm × 0.25 μm film thickness HP-5ms (5% phenyl methyl siloxane) Capillary column
Flow rate/Carrier gas	1.0 mL/min, Helium
Temperature program	150°C (4 min) to 250°C (5 min) at 4°C /min
Detector	Mass Selective Detector (MSD)
MSD condition	EI Voltages: 70 eV
	Scan mode: 45 to 400 m/z
	Solvent delay time: 2 min

3.7 Organoleptic Test

The milk sample was shaked very mildly for 1 min. A 30 mL portion was poured into 50 mL beaker. The color of milk sample was observed and the odor of milk sample was sniffed. The results are shown in Section 4.4.

3.8 Quantitative Analysis of the Volatile Component in Milk Produced in Thailand

From the result in Section 3.4.2-3.4.5, we were achieved the extraction condition for volatile component. The GC condition is shown in Table 3.2. Milk samples were collected from dairy farms and shown in Section 3.6. The quantity of volatile component is determined by multiplying the peak area with response factor for each compound. The results are shown in Section 4.5.

3.9 The Quantitation of Fatty Acid in Milk Produce in Thailand

3.9.1 Extraction of Milk Fat *(31)*

Weigh, to nearest mg, ca 10 g sample into 100 mL separatory funnel. Add 1.25 mL ammonium hydroxide and mix thoroughly. Add 10 mL ethanol and mix well. Add 25 mL diethyl ether and shake very vigorously for 1 min. Add 25 mL petroleum ether and repeat vigorous shaking. Let the mixture stand until the upper liquid layer is partically clear. Decant ether solution into a 100 mL beaker. Repeat extraction of the remaining liquid in separatory funnel, using 15 mL of each solvent. Evaporate solvents completely on sand bath at temperature that does not cause spattering or bumping. Dry fat in oven at 102±2 °C to constantly weight. Weigh cooled beaker without wiping immediately. Dissolved fat completely from the container using 15-25 mL warm petroleum ether, and dry, and weigh as before. Loss in weight = weight fat. The results are shown in Section 4.5.

3.9.2 Preparation of Methyl Ester Derivatives of Fatty Acids (FAMEs) *(31)*

Add fat from 3.8.1 to a round bottom flask and then add 0.5 N sodium hydroxide (NaOH) in methanol (see Table 3.5) and boiling chip. Attach condenser, and reflux until fat globules disappear (usually 10-15 min). Add 14% boron trifluoride in methanol (see Table 3.6) from graduated pipette through condenser and continue boiling for 2 min. Add 5 mL hexane through condenser and boil for 1 min longer. Turn off the heater, remove the condenser, and add several mL saturated sodium

chloride solution to float hexane solution through the neck of the round bottom flask. Transfer the upper hexane solution into vial and add small amount of anhydrous sodium sulphate to remove water. Dilute the hexane solution for GC analysis. GC condition is shown in Table 3.7.

Table 3.5 Weight of milk fat and volume of reagent.

Sample (mg)	Round bottom flask (mL)	0.5 N NaOH in Methanol (mL)	BF_3 Reagent (mL)
100-250	50	4	5
250-500	50	6	7
500-750	100	8	9
750-1000	100	10	12

3.9.3 Calculation of % w/w Fatty Acid per fat in Milk Samples

The reporting of percentage of fatty acid per fat weight (%w/w FFA/FAT) in milk was used in this work. % w/w FFA/FAT was calculated by equation 2. The results were show in Section 4.6.

$$\%\ w/w\ FFA/FAT = \frac{C_i \times \text{Concentration Factor}}{1000} \times 0.1 \qquad \ldots (2)$$

C_i = Concentration of $FAME_i$ in mg/g

$$\text{Concentration Factor} = \frac{1000}{W_f}$$

W_f = Weight of fat in gram

3.10 Milk Samples

3.10.1 Preparation of Sample Bottles

Amber bottle was cleaned and baked at 180 °C for 3 hrs. Then it was cooled to room temperature in the oven and closed with the cover.

3.10.2 Sampling Method

First, dairy farms according to differences in feedstuff, characteristic, location and number of cows were chosen. Information of dairy cows showed in Section 3.7.2.1 to Section 3.7.2.8. Milk samples were collected every other week from each dairy cow after the milking of each dairy cow finished. Milk was stirred in milk tank and was taken to sample bottle form Section 3.7.1. Milk sample was stored at 0-4 °C until analysis. The experimental period covered the period of June 2004 to February 2005.

Farm A.

Location:	Bangkok, Thailand
Characteristic:	Cement house, easy to clean, installed ventilator
Breed:	75% up of Holstein-Friesian
Time period of milking:	5:00 a.m. and 4:00 p.m.
Feed stuff:	Grass and fodder as roughage

Farm B.

Location:	Chonburi, Thailand
Characteristic:	Cement house, easy to clean, installed ventilator
Breed:	75% up of Holstein-Friesian
Time period of milking:	5:00 a.m. and 4:00 p.m.
Feed stuff:	Corn silage as roughage

Farm C.

Location:	Chonburi, Thailand
Characteristic:	Terrain house, difficult to clean, wide and well ventilated
Breed:	75% up of Holstein-Friesian
Time period of milking:	5:00 a.m. and 4:00 p.m.
Feed stuff:	Corn silage as roughage

3.10.2.1 The Study of Number of Lactation Contributing to Volatile Component in Cow's Milk

5 lactation number (first to fifth lactation) were studied. Each lactation, milk sample were collected from 5 Holstein-Friesian cows. Some characteristics of dairy cows showed in Table 3.6.

3.10.2.2 The Study of Stage of Lactation Contributing to Volatile Component in Cow's Milk

The stage of lactation was consisted of early lactation (10-90 days in lactation), mid lactation (91-180 days in lactation), and late lactation (181-240 days in lactation). At each lactation stage, 4 Holstein-Friesian dairy cows in their second lactation (three cows) and fourth lactation (one cow) from farm A was used. They were fed with grass and fodder as roughage.

Table 3.6 Some Characteristics of dairy cows used in the study of lactation number contributing to volatile components in cow's milk.

Lactation number	Cow number	Farm	Stage of lactation
1	1	A	Early
	2	A	Mid
	3	B	Early
	4	B	Mid
	5	C	Early
2	1	A	Early
	2	A	Mid
	3	B	Early
	4	B	Mid
	5	C	Early
3	1	A	Early
	2	A	Mid
	3	B	Early
	4	B	Mid
	5	C	Early
4	1	A	Early
	2	A	Mid
	3	B	Early
	4	B	Mid
	5	C	Early
5	1	A	Early
	2	A	Mid
	3	B	Early
	4	B	Mid
	5	C	Early

3.10.2.3 The Study of Feedstuff Contributing to Volatile Component in Cow's Milk

Milk samples were collected from 20 Holstein-Friesian cows that were fed with roughage of grass and fodder in farm A, and 20 Holstein-Friesian cows that were fed with roughage of corn silage in farm B. In each group, some characteristics of 20 Holstein-Friesian dairy cows were shown in Table 3.7.

Table 3.7 Some Characteristics of dairy cows used in the study of feedstuff contributing to volatile components in cow's milk.

Cow number [a]	Lactation Number	Stage of Lactation
1	1	Early
2	1	Early
3	1	Late
4	2	Early
5	2	Early
6	2	Early
7	2	Early
8	2	Mid
9	2	Mid
10	2	Mid
11	2	Mid
12	2	Late
13	3	Early
14	3	Early
15	3	Mid
16	3	Mid
17	4	Early
18	4	Mid
19	5	Mid
20	6	Mid

a: Number of cows from each Farm

3.10.2.4 The Study of Environmental Contributing to Volatile Component in Cow's Milk

11 Holstein-Friesian dairy cows raised in farm B and 11 Holstein-Friesian dairy cows raised in farm C were used. Milk samples were collected from them. In each group, some characteristics of 11 dairy cows were shown in Table 3.8.

Table 3.8 Some Characteristics of 11 dairy cows used in the study of farm environment contributing to volatile components in cow's milk.

Cow number [a]	Characteristics of dairy cow	
	Lactation Number	Stage of Lactation
1	1	Early
2	1	Early
3	2	Early
4	2	Early
5	2	Early
6	2	Early
7	2	Mid
8	2	Mid
9	3	Mid
10	5	Mid
11	6	Mid

a: Number of cows in each Farm

3.10.2.5 The Study of Lactation Number Affecting to Milk Fatty Acid Composition in Cow's Milk

Milk Samples were collected from 10 Holstein-Friesian dairy cows from five lactations. In each lactation was consisted of one cow in mid lactation (farm A), and another in early lactation (farm C) were selected. Some characteristics of dairy cows showed in Table 3.9.

Table 3.9 Some Characteristics of dairy cows used in the study of lactation number contributing to fatty acid composition in cow's milk.

Lactation number	Cow Number[a]	Farm	Stage of lactation
1	1	A	Mid
	2	C	Early
2	3	A	Mid
	4	C	Early
3	5	A	Mid
	6	C	Early
4	7	A	Mid
	8	C	Early
5	9	A	Mid
	10	C	Early

3.10.2.6. The Study of Stage of Lactation Affecting to Milk Fatty Acid Composition in Cow's Milk

The stage of lactation was consisted of early lactation (10-90 days in lactation), mid lactation (91-180 days in lactation), and late lactation (181-240 days in lactation). 4 Holstein-Friesian dairy cows in their second lactation (two cows) from farm B, third lactation (one cow) from farm C, and fourth lactation (one cow) from farm A was used.

3.10.2.7. The Study of Feedstuff Affecting to Milk Fatty Acid Composition in Cow's Milk

The influence of feedstuff on milk fatty acid composition was studied. Milk from 7 Holstein-Friesian dairy cows that were fed with roughage of grass and fodder (farm A), and 7 Holstein-Friesian dairy cows that were fed with roughage of corn silage (farm B) were collected. Some characteristics were shown in Table 3.10.

Table 3.10 Some characteristics of 7 Holstein-Friesian- cows, using in the study of feedstuff affecting to milk fatty acid composition.

Cow [a]	Lactation Number	Stage of Lactation
1	2	Mid
2	2	Late
3	2	Late
4	2	Late
5	4	Early
6	5	Mid
7	6	Mid

a: Number of cows in Farm A and Farm B

3.10.2.8. The Study of Environmental Affecting to Milk Fatty Acid Composition in Cow's Milk

Seven Holstein-Friesian dairy cows in Farm B and seven Holstein-Friesian dairy cows in Farm C were used. Milk samples were collected from them. In each group, lactation number and stage of lactation were shown in Table 3.11

Table 3.11 Some characteristics of 7 Holstein-Friesian dairy cows, using in the study of farm environment affecting to milk fatty acid composition.

Cow Number[a]	Lactation Number	Stage of Lactation
1	1	Early
2	1	Early
3	2	Early
4	2	Early
5	2	Mid
6	2	Mid
7	4	Early

a: Number of cows in Farm B and Farm C

3.11 Statistic Analysis.

All data were analyzed for significant different using Excel software. For each volatile component and fatty acids, one-way analysis of variance (one-way ANOVA) was performed with Excel software.