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Quantitation of Fatty Acids in Milk by Gas Chromatography

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ABSTRACT

This study investigated a reliable method for quantitation of fatty acids (FA) in milk by gas chromatography. Milk samples were treated with $CHCl_3/CH_3OH$(2:1, v/v) to extract the fats. A transesterification reaction using fat plus $AcCl/CH_3OH$(1:10, v/v) as a reagent to prepare fatty acid methyl esters (FAME) was carried out at 60°C for 90 min. The recoveries of FAME derived from standard FA were 98.2±3.2% for the high concentration group; 97.5±4.1% for the low concentration group. When FAME derived from each milk sample was injected into a gas chromatograph, a total of 9 corresponding FA were separated and detected. The FA portion was found to constitute more than 85% of total fat. The content of C4-C12 short-chain FA was also found to be slightly different between the various brands of marketing pasteurized milk and milk powder (reconstituted milk). The content of various FA in long-life milk is essentially similar to pasteurized milk; in flavored milk obvious differences were found due to a variety of additives blended. Raw cow and goat milk contain more short-chain FA than human milk.

Key words : Fatty acids, FAME, milk, gas chromatography.

INTRODUCTION

An important component in milk is lipid. When water is removed from milk, the total solid is classified into two parts, namely fat and solids-non-fat. Generally, the fat content in milk has been reported to be more than 3%[1]. Fat contributes to the main flavor of milk, thus various brands of milk sold in market is priced proportionally based on the content of fat. The milk fat is triglycerides (TG)——the esters formed by glycerol and FA. FA in milk fat are mainly even numbered saturated and unsaturated, normal straight carbon chains. The major saturated FA are[2]: palmitic acid, stearic acid, myristic acid, butyric acid, while the unsaturated are represented by oleic acid.

There are many well-established methods in separation and determination of FA in milk, including: titration[3], spectrophotometry[4], column chromatography[5], thin layer chroma-

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tography[6], gas chromatography[7], and high presssure liquid chromatography[8]. However, gas chromatography has proved to be the most important tool with respect to speed and accuracy[9].

The objective of this study[10] was to quantify FA in milk and to investigate the differences in the content of FA in pasteurized or reconstituted milk by a modified gas chromatographic method. FA were converted to their methyl esters (FAME) by transesterification in acetyl chloride/methanol solution for better manageability. Finally, a survey was conducted by analyzing 9 FA in various dairy products sampling from local food stores. The quantitative analyses of goat and human milk by GC were also investigated.

MATERIALS AND METHODS

I. *Materials*

(I). *Sources of Milk*

Raw cow and goat milk was supplied by Dairy Farm of National Taiwan University. Name brands of pasteurized milk were: Wei-Chuan (W), Tai-Ta (T'), Foremost (F), Kun-Chuan (K). Name brands of milk powder were: Anchor (A), Oak (O), Red Cow (R), Klim (C), Nestle(N) and Wei-Chuan (W). Other milk products were: Wei-Chuan(W), Tai-Nong (T), Kun-Chuan (K) and Foremost (F). These were purchased from local food stores during October, 1986.

(II). *Reagents*

1. Chloroform, methanol, 95% ethanol, sodium chloride, ammonium hydroxide, diethyl ether: Wako Pure Chemical Industries Ltd. (Japan), extra pure reagent grade. Acetyl chloride: E. Merck, reagent grade.

2. Silica gel 60(70-230 mesh ASTM) Art No.77344: E. Merck product.

3. Authentic samples: Butyric acid, caproic acid, caprylic acid, capric acid, lauric acid, myristic acid, palmitic acid, stearic acid and oleic acid: E. Merck, reagent grade.

4. Reference standards: n-Heptanoic acid and methyl n-heptanoate (MH): E. Merck, reagent grade.

5. Internal standards: Ethyl p-hyroxybenzoate (EHB) and butyl p-hydroxybenzoate (BHB): E. Merck, reagent grade.

II. *Instruments and Operating Conditions*

A gas chromatograph of Hitachi 263-30 Model equipped with a flame ionization detector was used to analyze FAME. The separation was performed on a 2m×3mm ID column packed with 3% OV-101, 80/100 mesh Chromsorb W-HP. Each time 10 μl of a sample was injected into GC at a programmed column temperature from 80°C to 280°C using N_2 as a carrier gas at a flow rate of 40 ml/min.

The GC-MS analyses were performed using a Minnigan TSQ46-C equipped with an EI-Autotune. A column of 15m×0.247mm ID, packed with SE-54, was used. The temperature of injector was maintained at 300°C and the programmed column temperature was adjusted from 60°C to 240°C using He as a carrier gas at a flow rate of 0.2 ml/min.

III. *Standard Solutions*

(I). *FA Stock Solution*

FA with carbon chains: C4:0, C6:0, C8:0, C 10:0, C12:0, C14:0, C16:0, C18:0 and C18:1 [Material I.(II).3] were taken in a weight of 5. 0, 10, 20, 40, 50, 80, 100, 150 and 200mg, respectively. All FA were placed in a 10-ml volumetric flask and 1.0ml of a reference standard, n-heptanoic acid (30mg/ml in $CHCl_3$) was added. The stock solution was diluted with $CHCl_3$ to the meniscus. The concentrations of FA solution were 0.50, 1.0, 2.0, 4.0, 5.0, 8.0, 10, 15 and 20mg/ml, respectively.

(II). *Reference Standard Solution*

An amount of 300 mg n-heptanoic acid were weighed and placed in a 10-ml volumetric flask, to which $CHCl_3$ was added to the meniscus. The concentration of the reference standard was 30 mg/ml.

(III). *Internal Standard Solution*

One gram of ethyl p-hydroxybenzoate or butyl p-hydroxybenzoate was weighed separately and placed in a 10-ml volumetric flask. By adding $CHCl_3$ to mark, the internal standard solution was obtained with a concentration of 100 mg/ml.

IV. *Reconstituted Milk*

Twelve grams of milk powder (each brand) were weighed in a 250-ml flask. Distilled water was added until the solution weighed exactly 100 g. The solution was stirred until the powder was becoming homogeneous.

V. *Methods*

(I). *Extraction of Fat*

Folch's method[11] was applied by using $CHCl_3/CH_3OH$ (2:1, v/v) to extract liberated FA and TG in milk. Human milk was an exception by adding the solvents and the reagent, $CHCl_3/CH_3OH$ and $AcCl/CH_3OH$ (for methylation) simultaneously without employing the step of extraction.

(II). *The Preparation of FAME for GC Analysis*

Five grams of a milk sample was weighed accurately in a 10-ml volumetric flask. After the reference standard, p-heptanoic acid (1 ml, 30mg /ml) was added, the sample was diluted with $CHCl_3$/MeOH (2:1, v/v) to the meniscus. The glycerides were extracted by a separatory funnel for 5 min and centrifuged at 3,000 rpm for 15 min. The lower layer of the solution was collected. Two ml of the solution was pipetted to a flask and washed with 2 ml of 0.0145 mM NaCl solution. Subsequently, 1 ml of the solution was pipetted to a sample vial and 1.0 ml of AcCl/MeOH (1:10, v/v) was added. The vial was stoppered and sealed with a paraffin film and subject to transesterification in a steam bath at 60°C for 90 min. The sample was ready for GC analysis after 10 μl of ethyl p-hydroxybenzoate or 30 μl of butyl p-hydroxybenzoate (an internal standard) was added.

VI. *Calibration Curves*

The prepartion of FA calibration curves for GC analysis represented into two groups:

(I). *High Concentration Group*

One ml of FA stock solution with a concentration of 1.0, 2.0, 4.0, 8.0, 10, 15 and 20 mg /ml was pipetted and transferred separately to a sample vial. One ml of acetyl chloride/methanol reagent (1:10, v/v) was added to the vial, stoppered and sealed with a paraffin film. The sample vial was placed in a steam bath (60°C) for 90 min allowing for complete transesterification. Subsequently, 30 μl internal standard, butyl p-hydroxybenzoate solution was added. Ten μl of the sample was injected into GC; the obtained peak height ratio (the amount of FAME relative to internal standard) was plotted versus the amount of FA. An optimal straight line was obtained by a linear regression analysis.

(II). *Low Concentration Group*

One ml of FA stock solution with concentration of 0.50, 1.0, 2.0, 4.0 and 5.0 mg/ml was pipetted and transferred separately to a sample vial. After the same treatment as in high concentration group, 10 μl ethyl p-hydroxybenzoate solution were added as an internal standard. The remaining steps were identical to those in Method VI. (I).

VII. *Sample Analysis*

(I). *Recovery of FAME*

The percent recovery of the esters was calculated according to the following formula.

$$\text{Recovery}(\%)=\frac{\text{Peak height of FAME/Peak height of IS}}{\text{Peak height of MH/Peak height of IS}}\times 100\%$$

where

MH=Authentic sample of methyl p-hydroxybenzoate and

IS=Internal standard

(II).*Content of FA in Milk*

The content of FA in milk was calculated as following:

$$\text{Content of FA (mg)}=\frac{C\times M}{R}$$

where

C=Amount of FA (mg) obtained from the

calibration curve

M=Multiple of dilution

R=The % recovery of methyl ester of the reference standard (n-heptanoic acid)

VIII. *Determination of Fat and Total Solid*

Roose-Gottieb method[12] was adopted for quantitation of fat in milk. Five grams milk sample was weighed and placed in a Majonnier tube. Two ml of concentrated ammonia water, 10 ml of 95% ethanol and 10 ml of ethyl ether were added in succession. The mixture was agitated for 30 seconds and allowed to stand vertically for 1 hour. The supernatant was decanted to a 100-ml beaker. The process was repeated by adding 10 ml each of ethyl ether and petroleum ether to the tube. The supernatant was also decanted to the beaker heated at 75°C on a heating plate to evaporate solvents until nearly dry. Subsequently, it was placed in an oven at 90-100 °C for complete dessication until a constant weight was obtained.

RESULTS AND DISCUSSION

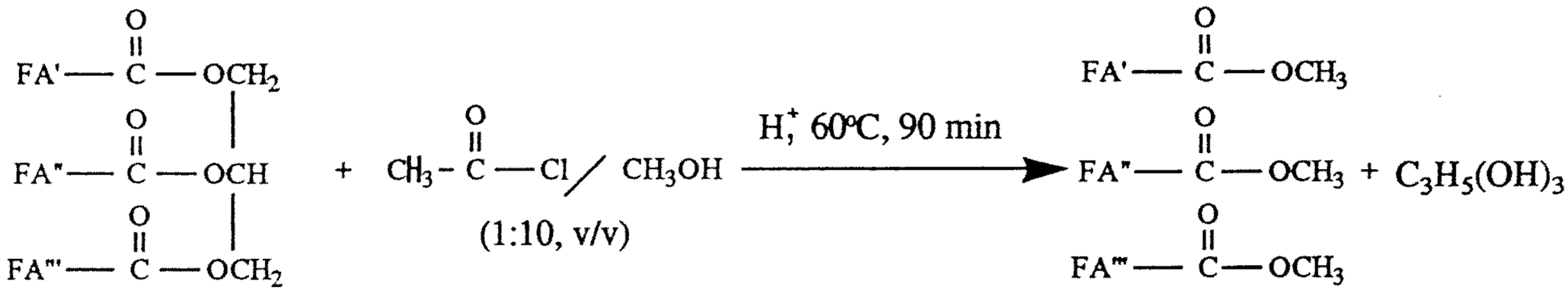
Preparation and Characterization of FAME

Prior to the analysis by gas chromatography, all FA must be converted to the derivatives of higher volatility and lower polarity. The best choice was by methylation of FA to their methyl esters which exhibit the advantages of good yields[7], shorter retention time and lower boiling points[13]. For the sake of safer and easier manipulation, the reagent acetyl chloride in methanol[14] was used to carry out the transesterification (Scheme 1). The proton released from step 1 could be used as a catalyst to liberate FA from glycerides, immediately followed by step 2-methylation to produce FAME. In a search of the optimum reaction conditions with respect to reaction time (Fig.1) and the concentrations of acetyl chloride (Fig.2), it was found that a complete appareant recovery of FAME could be reached after 80 minutes in a water bath at 60°C with acetyl chloride in methanol (8%, v/v). In the methylation of FA, acetyl chloride in methanol (10%, v/v) with 90 minutes reaction time was actually performed for ensuring the complete recovery of the products.

The structures of the obtained FAME were analyzed by GC-MS: The common features of alkyl cleavage producing $[C\text{-}OCH_3]^+$ with m/z 59; acyl cleavage producing $[OCH_3]^+$ with m/z 31; McLafferty rearrangement producing $[CH_3\text{-}COCH_3]^{\cdot +}$ with m/z 74 were observed and correctly identified to be methyl esters. The

1.

2.



Scheme 1. The formation of FAME by transesterificaion

molecular ions, M^+ with m/z 102, 130, 158, 186, 214, 242, 270, 298, 296, were identified in sequence to be the methyl ester of butyric acid, caproic acid, caprylic acid, capric acid, lauric acid, myristic acid, palmitic acid, stearic acid, oleic acid, respectively.

Standard Calibration Curves

For the purposes of obtaining accurate GC analysis and the best peak separation, methyl n-heptanoate as a reference standard, ethyl p-hydroxybenzoate and butyl p-hydroxybenzoate as double internal standards were carefully tested and chosen. After methylation of the standard solution of FA plus n-heptanoic acid, a sample was injected into GC. A typical gas chromatogram of a standard solution of FAME is shown in Fig.3. A standard calibration curve was completed by plotting the peak height ratio of methyl ester of standard FA to methyl ester of reference standard as abscissa versus the weight (in mg) of FA as ordinate. Nine calibration curves were obtained with regression analyses as shown in Talbe 1.

Recovery of FAME

According to Guy's method[15] of calculating percentage recovery of FA, an internal standard was added to calibrate the loss of the reference standard after methylation. For better accuracy consideration, we divided the FA into

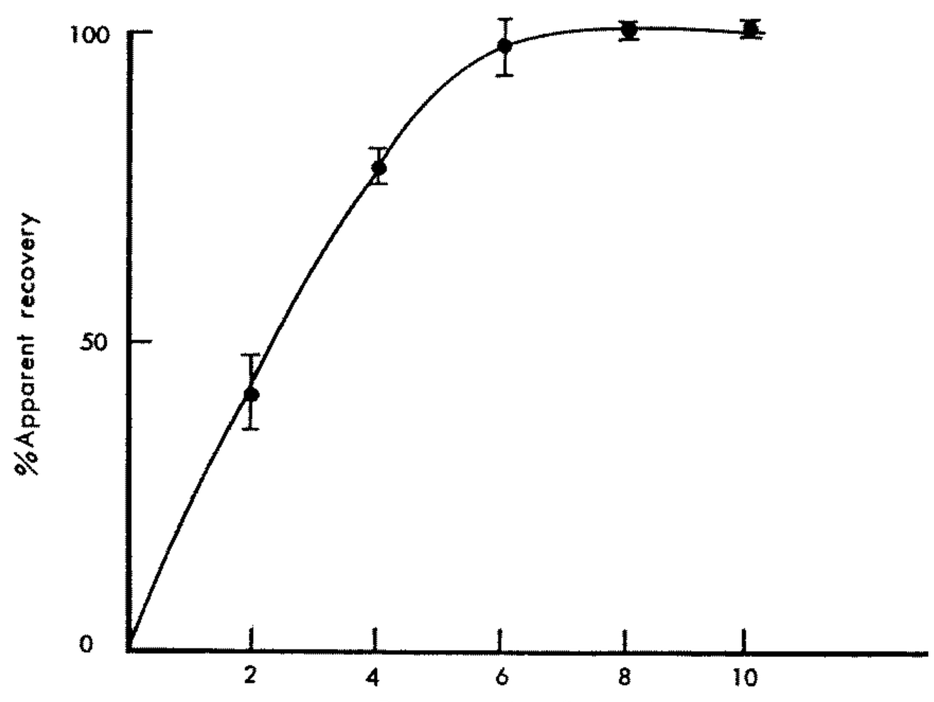


Figure 2. Percent apparent recovery of FAME as a function of acetyl chloride concentration.

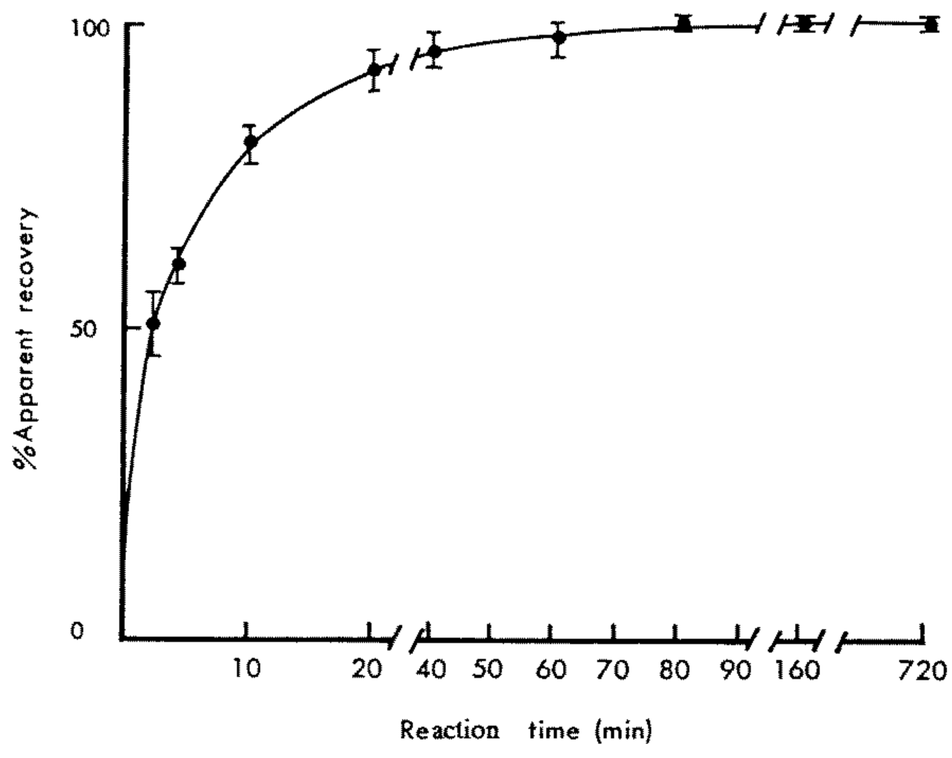


Figure 1. Percent apparent recovery* of FAME as a function of reaction time.

$$\%\text{Apparent recovery} = \frac{\dfrac{\text{Peak height of FAME}}{\text{Peak height of IS}}}{\dfrac{\text{Peak height of MH}}{\text{Peak height of IS}}} \times \frac{1}{\text{Recovery of C7:0 from methylation}} \times 100\%$$

*Expressed as Mean±S.D. of nine fatty acids

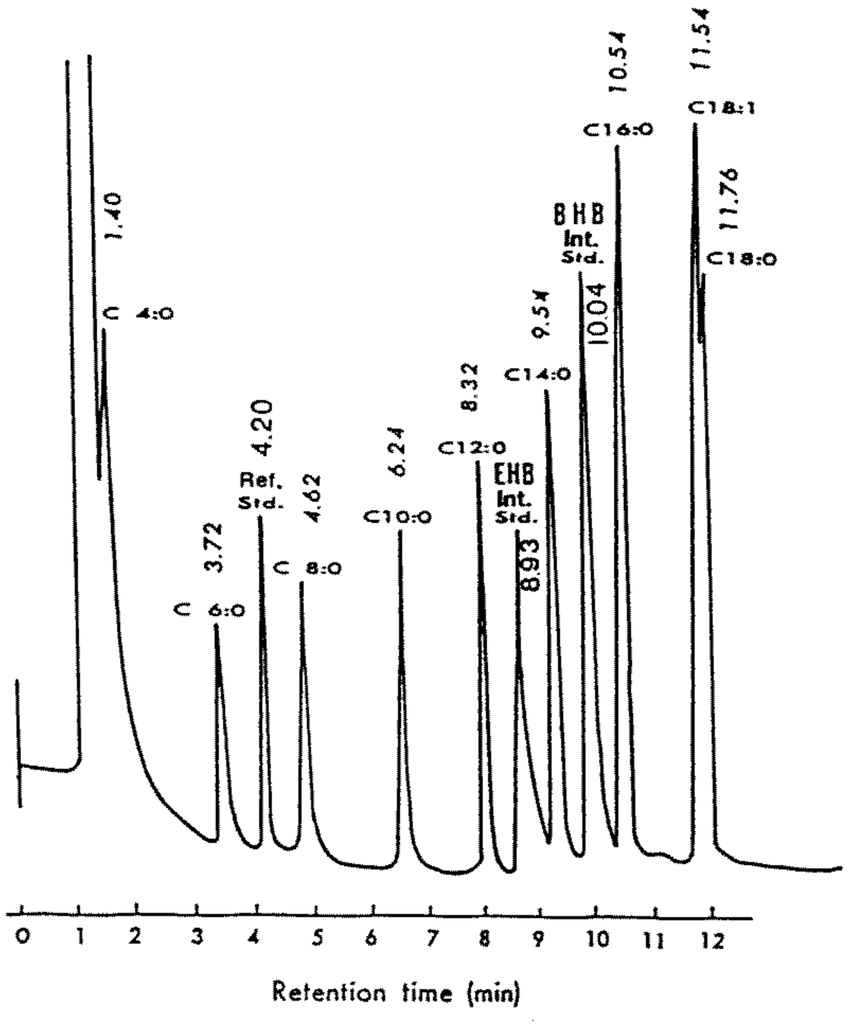


Figure 3. A Gas chromatogram of FAME derived from a standard fatty acids solution.

Table 1. Regression equations obtained from the standard calibration curves of FAME

Calibration curve	Methyl ester of fatty acid	Regression equation	Correlation coefficient r
High concentration (1.0-20mg/ml)	C14:0	9.673x−0.078	1.000
	C16:0	9.233x−0.027	0.995
	C18:0	9.054x+0.098	0.997
	C18:1	9.448x+0.079	0.999
Low concentration (0.50-5.0mg/ml)	C 4:0	0.659x+0.021	1.000
	C 6:0	0.542x+0.010	0.994
	C 8:0	0.540x+0.063	0.998
	C10:0	0.644x+0.018	0.999
	C12:0	0.694x+0.014	0.999

Table 2. The composition of FA in pasteurized milk

Milk sample	W	T'	F	K	Mean±S.D.
Total solid[a]	12.0±0.6	12.8±0.7	12.2±0.8	12.0±0.8	12.5±0.7
Total fat[b]	3.4±0.1	3.4±0.2	3.8±0.3	3.6±0.2	3.6±0.2
C 4:0	2.8±0.9	2.8±0.7	3.0±0.2	2.7±0.1	2.8±0.6
C 6:0	1.5±0.2	1.5±0.2	2.0±0.2	1.6±0.2	1.7±0.2
C 8:0	1.5±0.2	1.4±0.2	1.6±0.1	1.6±0.0	1.5±0.2
C10:0	2.2±0.7	2.4±0.4	3.0±0.2	2.4±0.4	2.5±0.5
C12:0	3.0±0.2	3.3±0.4	3.8±0.2	2.9±0.2	3.3±0.3
C14:0	14.3±1.7	19.3±2.6	13.9±0.2	12.8±1.5	15.1±1.7
C16:0	34.6±3.5	34.1±4.1	29.0±0.4	30.4±3.6	32.0±3.2
C18:0	7.6±0.7	7.5±0.2	5.8±0.2	7.2±2.1	7.0±1.1
C18:1	21.4±1.9	22.1±0.9	18.5±0.0	20.2±1.5	20.5±1.3
Total[c]	88.9±10.0	94.4±9.7	80.6±1.7	81.8±9.6	86.4±9.1

a.b. Data expresseed as % w/w sample. (n=5)

c. Data expresseed as % w/w fat. (n=5)

high and low concentrartion groups by adding two internal standards simultaneously-butyl p-hydroxybenzoate for the calibration of the former; ethyl p-hydroxybenzoate for the latter. It was found that the recovery of FAME derived from the standard FA was 98.2±3.2% (n=5), c.v.=3.3% for the high concentration group; 97.5±4.1% (n=5), c.v.=4.2% for the low concentration group.

Quantitation of FA in Milk

Gas chromatography has been widely used in analyzing FA in dairy and related products up to recently[16-18]. By using the present gas chromatographic method to analyze the FA in pasteurized milk of different brands sold at the local food stores, the results are compiled in Table 2. The content of total solid averages 12.5±0.7% (w/w); total fat, 3.6±0.2% (w/w). A typical gas chromatogram of FAME in milk is shown in Fig.4. Table 3 lists similar analytical

results for various brands of milk powder (12.0g in 88.0 ml H_2O) in the form of reconstituted milk. The content of fat averages 3.4±0.6% (w/w of reconstituted milk). In the comparison of FA content in pasteurized and reconstituted milk, it was found that the former contains more C6:0, C8:0, C10:0, C12:0, C14:0; and less C4:0 and C18:0 than in the latter. In response to the heightened consumers' sense of demanding better quality of marketing foods today, an exhaustive search for reliable methods in quality control remains necessary. The same demand applies to how to evaluate percentage of blended reconstituted milk in the marketing pasteurized milk. Further examination of Tables 2 and 3 reveals that the variation in contents of shorter C chain (carbon number <10) FA in pasteurized and reconstituted milk seems to provide us a feasible means to evaluate the percentage of reconstituted milk in pasteurized milk sold at market. This area is presently under further investigation.

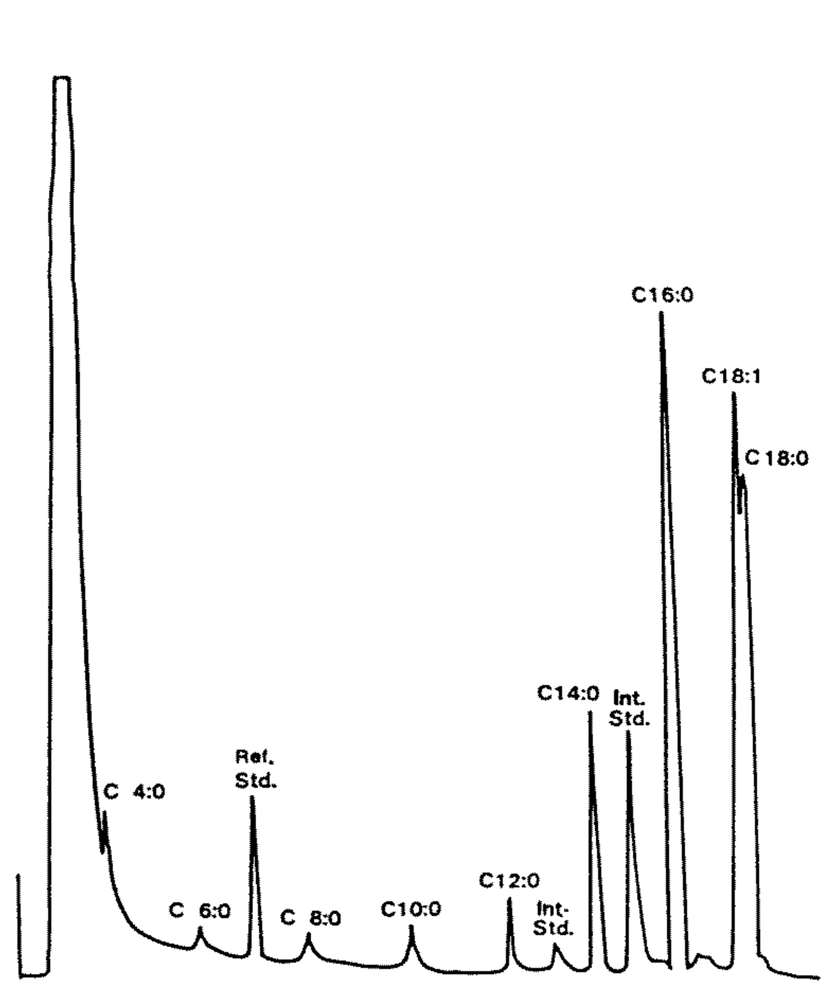


Figure 4. A Gas chromatogram of FAME in milk.

Furthermore, the GC method was employed to analyze FA in low-fat, long-life and flavored milk sampling from the food stores and the results are given in Table 4; or in human, raw cow and goat milk with results given in Table 5. In general, the percentages (w/w fat) of summing up 9 FA in all brands of the marketing milk or milk powder are similar, about 81 to 90% including 63 to 68% saturated FA and 18 to 22% unsaturated FA. Flavored milk is an

Table 3. The composition of FA in reconstituted milk

Milk sample	W	N	C	O	A	R	Mean±S.D.
Total solid[a]	12.0	12.0	12.0	12.0	12.0	12.0	12.0
Total fat[b]	3.4±0.3	3.9±0.4	3.2±0.8	3.3±0.8	3.3±0.9	3.3±0.3	3.4 ±0.6
C 4:0	5.3±0.5	4.9±0.2	5.0±0.2	3.1±0.2	3.8±0.6	5.4±0.2	4.6 ±0.4
C 6:0	1.1±0.2	1.0±0.2	1.2±0.2	1.2±0.2	0.8±0.2	1.2±0.2	1.1 ±0.2
C 8:0	0.9±0.2	0.6±0.0	0.7±0.0	0.9±0.2	0.6±0.2	0.7±0.0	0.7 ±0.1
C10:0	2.3±0.5	1.4±0.2	1.7±0.0	2.1±0.8	1.6±0.2	2.5±0.2	1.9 ±0.4
C12:0	2.5±1.1	2.7±0.2	3.0±0.5	2.8±0.9	2.6±0.4	3.2±1.0	2.8 ±0.8
C14:0	14.9±0.7	14.3±0.8	15.4±2.0	17.3±1.2	9.5±2.2	16.3±0.2	14.7 ±1.4
C16:0	34.1±3.2	29.8±3.3	32.5±1.7	37.3±3.5	26.3±1.6	31.7±1.7	32.0 ±2.7
C18:0	8.2±0.2	7.2±0.4	9.2±0.2	6.4±1.4	6.9±0.2	8.1±0.5	7.7 ±0.6
C18:1	20.7±0.5	19.3±1.2	24.0±0.5	17.7±3.0	19.6±0.4	21.9±1.5	20.5 ±1.4
TOTAL[c]	90.0±13.7	81.2±6.5	92.7±5.3	88.8±11.4	71.7±6.0	91.0±5.5	86.0 ±8.0

a. Data expresseed as 12.0% w/w (sample equivalent to 1g pasteurized milk)

b. Data expresseed as % w/w sample. (n=5)

c. Data expresseed as % w/w fat. (n=5)

Table 4. The composition of FA in various milk products

Milk smple	W (Low-fat)	T (Bottled long-life)	W (Tetra pak long−life)	K (Flavored)	F (Chocolate flavored)	F (Fruit juice flavored)
Total solid[a]	12.1±0.6	11.1±0.7	11.4±0.6	13.6±0.5	12.9±0.7	12.7±0.8
Total fat[b]	1.9±0.3	3.2±0.2	3.3±0.4	3.7±0.3	2.7±0.3	2.8±0.6
C 4:0	3.9±0.4	4.6±0.3	4.5±0.9	−	1.7±0.0	−
C 6:0	1.6±0.4	0.7±0.0	0.8±0.0	−	−	0.2±0.1
C 8:0	1.6±0.0	0.4±0.3	0.7±0.0	−	3.0±0.7	2.3±0.6
C10:0	2.0±0.0	1.8±0.5	0.6±0.0	−	1.6±0.7	1.4±0.9
C12:0	2.5±0.0	2.8±0.3	2.0±0.2	−	34.6±2.3	30.3±5.6
C14:0	14.1±3.2	13.2±0.5	13.4±0.2	1.4±0.0	16.9±1.7	15.2±2.7
C16:0	35.1±3.2	31.9±1.0	33.5±0.5	17.7±1.2	8.1±0.7	9.0±2.9
C18:0	5.6±1.6	7.6±0.3	7.5±0.2	26.2±1.4	2.1±0.0	2.2±0.6
C18:1	19.6±2.0	21.6±0.8	21.4±0.7	38.5±2.1	5.9±0.3	6.3±1.8
TOTAL[c]	86.1±11.3	83.6±4.0	84.4±2.7	83.8±4.7	70.1±6.4	67.0±15.4

a.b. Data expresseed as % w/w milk sample. (n=5)

c. Data expresseed as % w/w fat. (n=5)

Table 5. The composition of FA in human milk, raw cow and goat milk

Fatty acid *	Human milk[a]	Cow milk[b]	Goat milk[b]
C 4:0	−	2.26±0.28	1.82±0.25
C 6:0	−	1.08±0.04	0.44±0.10
C 8:0	−	1.04±0.04	0.41±0.06
C10:0	1.03	1.43±0.13	1.26±0.18
C12:0	6.93	1.62±0.10	1.81±0.03
C14:0	3.32	6.50±0.42	0.48±0.04
C16:0	14.31	14.30±0.88	14.00±0.57
C18:0	4.76	3.02±0.9	3.26±0.35
C18:1	10.32	8.60±0.53	9.28±0.23
Total	40.67	39.85±2.61	32.76±1.81

* Expressed as mg/g sample

a. One determination

b. Mean±S.D. (n=5)

exception due to saturated, unsaturated FA or fruit juice added during processing. The contents of FA in cow or goat milk were also found to be approximately equal, while shorter C chain (C number<10) FA are not found (trace amount) in human milk. This study extends the potential application of GC to the FA analysis of commerical milk samples and is a reliable and reproducible method.

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以氣相層析法測定牛奶中脂肪酸含量

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摘　要

本研究係以氣相析法探討牛奶中脂肪酸定量之可行性。牛奶以氯仿/甲醇(2:1, v/v)萃取脂肪，以氯化乙醯/甲醇(1:10, v/v)在60°C經90分鐘後，轉酯反應完成，可製成脂肪酸甲基酯。以脂肪酸標準品製成甲基酯其回收率爲高濃度組，98.2±3.2%；低濃度組97.5±4.1%。將檢樣注入氣相層析儀分析結果：各類牛奶檢樣中檢出九種主要脂肪酸，佔總脂肪量85%以上。市售主要廠牌之鮮奶與奶粉中，C4-C12短鏈脂肪酸含量略有差異。保久乳中各類脂肪成分大致與鮮奶相同。而調味乳依廠牌不同，皆摻入添加物致脂肪酸含量有顯著差異。牛奶和羊奶較人奶含有較多短鏈脂肪酸。