EFFECT OF MODERN AND TRADITIONAL METHODS OF PREPARATION

ON THE COMPOSITION AND FLAVOR PROFILES OF GHEE

By

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ABSTRACT OF THE THESIS

Effect of Modern and Traditional Methods of Preparation on the Composition and

Flavor Profiles of Ghee

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Ghee is a clarified milk-fat product originally from India. Ghee is prepared traditionally by culturing whole milk with lactic acid bacteria, prior to further processing steps. Industrially ghee is made by directly heating cream separated from whole milk. This research focuses on comparing chemical profiles of ghee made from different sources of milk and by different methods of preparation.

Raw (non-pasteurized, non-homogenized) organic-grass fed cow milk and regular diet fed cow milk was obtained from Birchwood Farms, PA and Readington Farms, NJ respectively. Bacterial culture typical to 'Dahi' or Indian-style yogurt was obtained from Danisco. Ghee samples were made using these materials by three different methods (Direct cream method, Traditional method and Cultured cream method).

Fatty acid profiles were elucidated using the FAMEs method using GC-MS. Non-saponifiable matter was analyzed using GC-Ms as well. Headspace volatiles

were analyzed to elucidate differences in flavor profiles. Sensory evaluation was carried out on ghee made from grass-fed cow milk to identify whether the methods of preparation had an impact on the aroma profile. The results from all the analyses were compared for the different samples.

Non-detectable differences were found in the fatty acid profiles and the non-saponifiable fractions of the different ghee samples. Cholesterol content in each sample was calculated. Method of preparation did not affect the chemical profiles of ghee significantly. Concentration of fatty acids was not affected by the source of milk or the method of preparation. This was analyzed by doing a two-factor ANOVA (Analysis of Variance). However, significant differences were found in their aroma profiles (based on sensory evaluation), instrumental color measurements and headspace volatile profiles. Thus the source of milk or method of preparation did not affect the fatty acid profiles of ghee however, method of preparation influenced the sensory properties and the flavor profiles.

ACKNOWLEDGEMENT

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

1. Introduction

1.1 Ghee

'Ghee' comes from the Sanskrit word 'Ghrita' (meaning 'sprinkle' (in order to make pure) or by another definition it means 'bright' or 'brighten'). Its origin can be traced back to 1500 B.C. Ghee is a kind of clarified butter (or dehydrated milk fat), that is indigenous to South Asia. It is widely used in India and the Indian subcontinent (Nepal, Bangladesh, Sri Lanka and Pakistan).

Clarified milk-fat products, similar to ghee, are used in other parts of the world including the Middle East (Roghan), Africa (Samna), Uganda, Ethiopia and Sudan (De, 2001). In French cuisine, this is called *beurre noisette*, translated as "hazelnut butter" and known as *brown butter* in English (Childe, 1970).

Ghee has a distinct nutty flavor and a rich texture at room temperature. Its unique flavor and grainy texture is what sets it apart from butter oil and other milk fat products. As seen in Fig. 1.1, ghee is a semisolid fat at room temperature and has a high smoke point (~250°C) (Bockisch, 1998). Owing to these unique properties it is a fat of choice for high temperature cooking and frying. It is also used as a spread on breads or added to rice preparations. Moreover, it is the major source of animal-origin fat for predominantly lacto-vegetarian Indians. Ghee has a shelf life of about a year at room temperature; hence it is an

ingenious way to store milk fat in the tropical climates of the Indian sub-continent for long periods of time (Singh, 2011).

Figure 1.1: (Left) Ghee at room temperature. (Right) Freshly made ghee, still molten.

Ghee, like any other oil or fat, can be characterized by the following physico-chemical properties:

- Method of manufacture

- Source of milk (different milch animals)

- Diet of the cattle

- Breed and stage of lactation of the animal

- Geographic location of breeding cattle

- Time of the year (seasonal variation)

Ghee is characterized by a melting point range of 28 $^{\circ}$C – 44 $^{\circ}$C and specific

gravity of 0.93-0.94. The butyro-refractometer reading for ghee, which is commonly used for ascertaining the purity of fats and oils, ranges from 40-45 (Fryer, 1920). Other characteristics such as Reichert Meissl value, Polenske value and Iodine value are defined in order to determine the degree of saturation in ghee (De, 2001). Adulteration of ghee, specifically with hydrogenated vegetable fats, is a major concern in the Indian market and hence these values are monitored and regulated. Variation in the degree of saturation in ghee from natural milk-fat profile (known standard) is a clear indicator of adulteration with other animal/vegetable fats. The general chemical composition of ghee is as follows: (Rajoria, 2003).

Component	Content
Milk fat	99-99.5%
Moisture	Less that 0.5%
Non-saponifiable matter	0.5-1%
Solids not fat (charred casein, salts, etc.)	Traces
Free fatty acids	Maximum 2.5%

Table 1.1: Typical composition of ghee (Rajoria, 2003).

There are more than one methods of preparation of ghee. We shall consider all of these in detail, as one major aspect of this research is to compare the fatty

acid and flavor profiles of ghee made by different methods. Each of these methods involves the following basic steps:

- Separating cream from non-homogenized whole milk

- Clarifying the milk fat (by heat treatment) to get rid of all moisture and obtaining dehydrated milk fat.

Figure 1.2 is a general representation of the basic steps in ghee preparation.
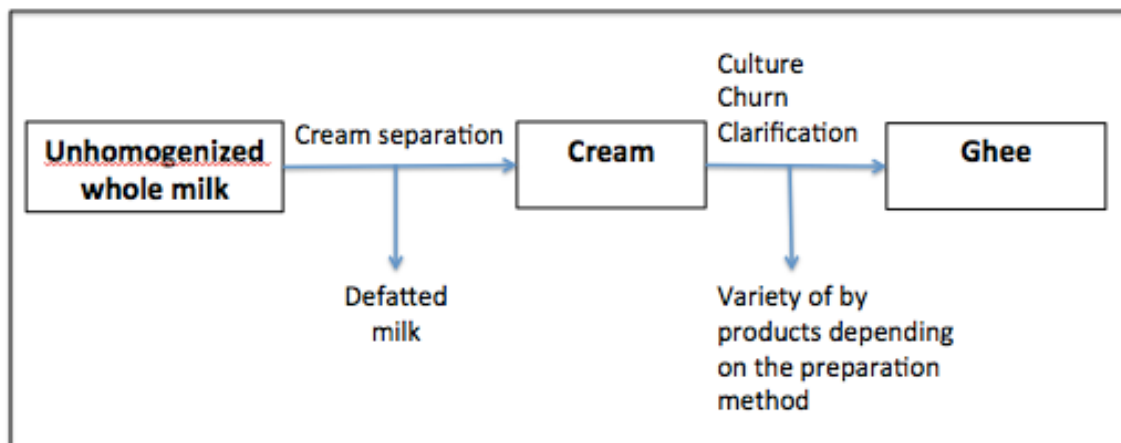
Figure 1.2: Basic Steps in Ghee Preparation (Generalized)

1.2 Background

1.2.1 Importance of ghee

Ghee has played a major role in the Indian culture and diet. Several medicinal properties of ghee have been reported in Ayurveda (traditional Indian medicine) and it is believed to be a coolant, digestive aid, capable of increasing mental power, curative of ulcers, eye diseases, improve vision, make skin radiant and even increase longevity. All of these claims have not been substantiated by modern science. Research has been done on evaluation of ghee based formulation for wound healing activity (Prasad, 2006). Few other studies demonstrating effects of herbal medicines compounded in ghee base have been published. For example, Effect of *Bramhi ghrita* (ghee fortified with the herb *Bacopa monnieri*) on the central nervous system (Achliya, 2005), sedative and anticonvulsant activity of *Unmadnashak ghrita* (ghee containing a mixture of several ayurvedic herbs) (Achliya, 2004), anti-inflamatory activity of *Jatyadi ghrita* (ghee containing a mixture of several specific ayurvedic herbs) (Fulzele, 2002). Several other studies have been done where efficacy of ghee as a drug and/ or drug carrier has been studied. Research has been done on the effect of dietary ghee on blood lipids in rats (Kumar, 1998). Ghee has shown to lower serum prostaglandins and secretion of leukotrienes by rat peritoneal macrophages (Kumar, 1999). Apart from its health benefits ghee has been given prime importance in religious rituals and has been considered a status symbol and sign of wealth, traditionally.

'Sneha Kalpana' is an ayurvedic procedure for preparation of oleaginous medicine by mixing 'kalka' (drugs/ nutraceutical powders) and 'dravya' (liquid material). The liquid material is usually a liposomal drug delivery medium. Ghee is considered to be a very good vehicle for delivery of nutraceuticals and is hence used as a base for a variety of Ayurvedic medicinal preparations (Singh, 2011). This phenomenon has been studied by Neetu singh and Anand Chaudhary (Singh, 2011) in an attempt to bridge the gap between traditional wisdom and current trend of drug delivery systems. The study states that "ghee is a versatile drug carrier, which can be used to control retention of entrapped drugs in the presence of biological fluids, control vesicle residence in the systemic circulation or other compartments in the body, and enhance vesicle uptake by target cells. It is biodegradable, biologically inert, weakly immunogenic, produces no antigenic or pyrogenic reactions, and possess limited intrinsic toxicity."

When considering ancient references, an important aspect that needs to be considered is that all the properties attributed to ghee are based on the premise that it is made using the traditional method and from grass-fed cow milk. Mr. Sandeep Agarwal of Pure India foods (NJ, USA) advocates this strongly and manufactures ghee made from the milk of organic grass-fed cows for the American market.

The technique of manufacturing ghee dates beyond recorded history. As mentioned earlier, ghee has a long shelf life and it is a very convenient way to

store milk fats in the tropical climate. Earlier, almost every household in India had cows and buffalos. There was an excessive supply of milk at home. The milk was usually boiled (for pasteurization) and used throughout the day. At the end of the day, the remaining milk containing cream was then warmed and transferred to earthen pots used for making yogurt. The porous walls of these pots served as a reservoir of culture consisting of Lactic acid bacteria. Thus the excess milk was cultured and prevented from spoilage. The yogurt (Dahi) was then consumed as is, and the excess yogurt was churned to yield butter and buttermilk. Butter obtained this way (called makkhan/loni), stays good for about 3-4 days when stored in cool water. After repeating this process for 3-4 days, enough butter would be accumulated. This butter was then heated until it boiled. Completion of the process was tested by sprinkling water in the boiling ghee. The instant crackling of water due to flash evaporation, indicated temperature above 100 ^{o}C. The clear fat obtained at the end of this process is ghee. The liquid ghee is then strained (to separate the solid residue) and cooled to obtain ghee. This is the traditional method or 'desi' method of making ghee.

During the heating stages, all of the water is evaporated. The fat (ghee) then starts heating above 100 ^{o}C rapidly. The amount of time ghee is heated beyond this point is responsible for imparting ghee its characteristic flavor, color and texture, due to caramelization of the solids-not-fat in the butter. In southern parts of India, slightly darker ghee is preferred whereas in the northern parts, a lighter version is preferred (Ganguly, 1972).

Ghee is traditionally stored in silver containers and has a shelf life of almost a year at room temperature. In summary, ghee has the following applications:

- As a spread for breads/ toast

- Flavoring for rice

- Shallow/ deep frying

- High temperature frying

- Ayurvedic medicinal preparations (as a medicine itself or more often as a vehicle for drug delivery)

- Religious rituals and customs.

1.2.2 Market value

India is the world's largest producer of dairy products by volume, accounting for more than 13% of total milk production. For the year 2014 fluid milk production in India has been estimated to reach a record of 140.6 million tons, owing to increased demand for milk and dairy products and rising consumer income (USDA GAIN, 2013). India also has the world's largest dairy herd.

It is interesting to note that, ghee ranks second to fluid milk, in the variety of milk products consumed in India. Of the total amount of milk products consumed in India, other than fluid milk, ghee constitutes over 50%. It is clear from these statistics that ghee manufacturing industry in India is large and tons of milk is

used for ghee preparation every year (IUF, 2011). Table 2 represents the percentage fraction of total milk produced, used in preparation of different milk products, in India.

Product	Percentage
Fluid Milk	46.0%
Ghee	27.5%
Butter	6.5%
Yogurt	7.0%
Khoa (partially dehydrated condensed milk)	6.5%
Dehydrated (powdered) milk	3.5%
Paneer (Indian Cottage cheese)	2.0%
Others (cream, ice cream, etc.)	1.0%

Table 1.2: Fraction of total milk produced in India used for making different milk products in 2009.

(http://cms.iuf.org/sites/cms.iuf.org/files/Indian%20Dairy%20Industry.pdf)

Apart from the traditional method, three other methods have been used to prepare ghee industrially, to increase yield, facilitate ease of scale up and reduce labor. These methods are:

i) Direct cream method

ii) Creamery butter method

iii) Pre-stratification method

(Note: These methods are explained in detail in Section 3.3). Currently, direct cream and creamery butter methods are employed for large-scale production of ghee. These methods do not include inoculation of the milk with bacteria prior to separation of fats as done in the traditional method. However, these methods have a higher yield of ghee and are hence the method of choice for industrial manufacture of ghee (Ganguly, 1972).

1.2.3 Role of Bacteria

What distinguishes the traditional method of preparation of ghee from the modern methods is the fermentation step with lactic acid bacteria. Usually, a cocktail of bacteria is used to culture milk. Lactic acid bacteria species indigenous to Indian style yogurt are *Streptococcus thermophilus, Lactobacillus delbrueckii subsp. Bulgaricus, Lactobacillus lactis subsp.* and *Lactis biovar. Diacetylactis*, (Maqsood, 2013).

Lactic acid bacteria digest the sugar (lactose) in milk and convert it to lactic acid. As more and more lactic acid is generated the pH of milk drops from about 6-6.7 to 4. Once the pH drops below 4.6, casein coagulates, resulting in a thickened, acidic, fermented milk product. Also, acetaldehyde is formed as a by-product, giving yogurt its characteristic taste (http://food.oregonstate.edu/learn/milk.html, 2012).

Bacteria are known to add methyl branches to fatty acid chains. Free fatty acids are toxic for bacteria; hence bacteria add a methyl branch on the free fatty acids resulting in odd carbon chain fatty acids (with a methyl side chain), that don't exist naturally in animal and plant lipids (Kinderlerer, 1993).

Also, the bacteria in the rumen play an important role in the breakdown of ingested fatty acids. Bacteria have structural lipids that have odd number of carbons and are hence capable of synthesizing odd carbon fatty acids (Tamime, 2009). All these factors lead to the presence of odd carbon fatty acids in the milk. Traditional ghee samples, which were screened as a part of preliminary experiments, showed presence of such odd carbon fatty acids.

1.2.4 Milk from Grass-fed Cows Vs. Conventional-diet-fed Cows

The primary role of milk is to provide a source of energy and growth to the neonate. It is a balanced mix of carbohydrates (mainly lactose), proteins (mainly casein), fats, minerals, vitamins, etc. Since this research involved studying the fatty acid profiles of ghee, we will focus on milk lipids.

Concentration of lipids in milk in animals ranges from 8-33 g/L in Lemurs to up to 502-533 g/L in Harp seals. Cow milk has about 33-47 g/L lipids. In cow milk more than 98% of the lipids are triacylglycerols. The rest of the lipid fraction consists of diacylglyceroils, monoacylglycerols, free fatty acids, phospholipids, sterols, fat-

soluble vitamins and flavor compounds. Typical composition of cow milk lipids is shown in Table 1.3 (Huppertz, 2009).

Lipid Class	% of Total Lipids
Triacylglycerols	98.3
Diacylglycerols	0.3
Monoacylglycerols	0.03
Free fatty acids	0.1
Phospholipids	0.8
Sterols	0.3
Carotenoids	Trace
Fat-soluble vitamins	Trace
Flavor compounds	Trace

Table1.3: Composition of different classes of lipids in cow milk

The fatty acids of animal milk are a result of uptake of fatty acids from the food intake and the fatty acids synthesized as a result of *de novo* synthesis in the mammary glands of the animal. It is important to note here that, fatty acids with carbon numbers 4-14 arise from the *de novo* synthesis whereas fatty acids with higher carbon number arise from lipids taken up by the blood stream either as a

result of digestion or body fat mobilization (Parodi, 2009). Hence, the composition of milk lipids is primarily influenced by the diet of the cows. Proportion of 18 carbon fatty acid (C18) in cow milk is greatly influenced by the fat content of the feed for the cows (Grummer, 1991).

In conventional/ regular diet fed cows, the diet typically consists of grains (mainly corn) and grain silages (grains that have been harvested, stored, and fermented), hays, and haylages (like alfalfa, clover, or sorghum and their fermented versions), soymeal, oilseed meals (such as cotton seed, sunflower seeds), etc. corn gluten, distillers grains, soybean hulls, citrus pulp, molasses, beet pulp, and other ingredients (Chiba, 2009). This kind of animal feed is often termed as Total Mixed Ration or TMR. Any of the above components may be combined together to make a TMR feed. The purpose of TMRs is to provide animals with a consistent and balanced dietary food source that is available all round the year (Linn, 2011).

Research studies show nutritional advantages of milk and meat products obtained from 100% grass-fed cows. Advantages include more omega-3 fats, better ratios of omega-6 to omega-3 fats, increased amounts of conjugated linoleic acid (Dhiman, 1999).

Three organizations offer certification for grass-fed foods in the United States: the American Grassfed Association (AGA), the Food Alliance (FA), and the U.S. Department of Agriculture (USDA).

1.3 Methods of Manufacture

1.3.1 Traditional Method

This method is commonly known as the Desi method. In India, this method is still employed in manufacturing ghee at homes and small-scale dairy industries. Excess full fat milk is often boiled for preservation and further cultured with bacteria to make yogurt and extend the keeping quality. This yogurt is churned to separate butter and buttermilk. The butter thus obtained is called cultured butter, desi butter or makkhan (in Hindi). This butter is then sold as is or heated to remove all moisture to obtain ghee.

Due to lack of efficiency, low yield and required amount of labor and handling, this method is not employed for large-scale production of ghee. Desi ghee has a poorer keeping quality, owing to unhygienic practices and cross-contamination of bacterial cultures with yeasts and fungi during yogurt making (Ponnusamy, 1994), compared to modern methods. However ghee made by this method has very low amount of residue (solids-not-fat) due to the removal of water-soluble solids through churning prior to clarifying the butter. Also traditionally made ghee arguably has a better flavor and general customer preference. Whether this is true or not was one of the objectives of this thesis.

The steps in the preparation of ghee by traditional (desi) method, as used in the laboratory, are summarized in the flow chart in Fig. 1.3.

Preparation of Ghee by Traditional Method
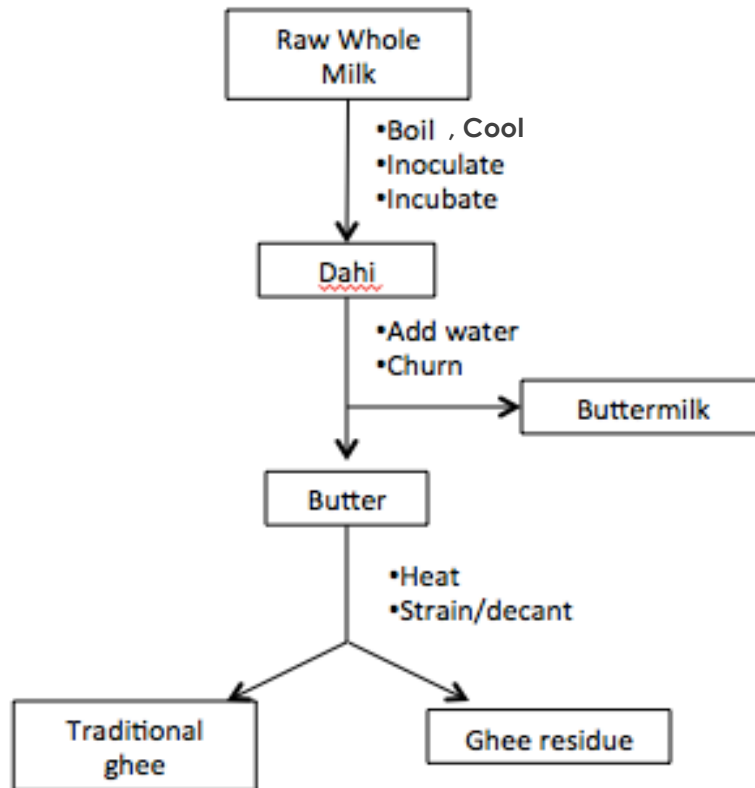
Figure 1.3: Traditional method of ghee preparation

1.3.2 Direct Cream Method

This is the simplest and most efficient method for preparation of ghee. In the direct cream method of ghee preparation, cream is separated from non-homogenized, unpasteurized whole milk using a centrifugal cream separator. Centrifugal force separates the cream or fat from the milk. Skimmed milk (almost fat-free) is separated at the bottom, which can be pasteurized and sold as

skimmed milk. The cream is subjected to heating until all the moisture in it is evaporated and filtered to separate pure ghee from the residue.

The steps for this process are summarized in Fig. 1.4.

Preparation of Ghee by Direct Cream Method

Raw Whole Milk

•Separate

Cream   Skim milk

•Heat
•Strain/decant

Ghee residue   Ghee
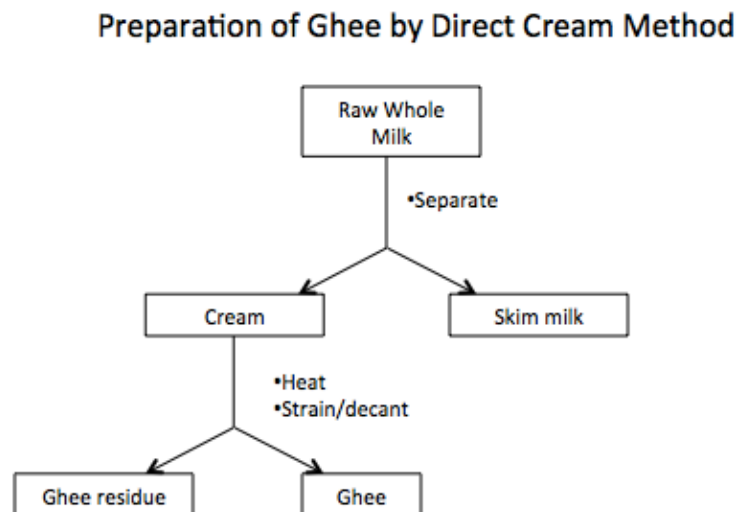
Figure 1.4: Direct cream method of ghee preparation

1.3.3 Creamery Butter Method

This is another method employed for large-scale production of ghee. In this method, cream is separated from non-homogenized raw whole milk using a centrifugal cream separator. This cream is then allowed to incubate at room temperature till it naturally curdles and attains a pH of desired acidity. The curdled cream is then churned with water to yield buttermilk and 'creamery

butter'. This butter is then clarified by heating. Molten ghee is decanted and filtered to separate the ghee residue. Steps involved in the preparation of ghee by cultured cream method are summarized in Fig. 1.5.

Preparation of Ghee by Creamery Butter Method
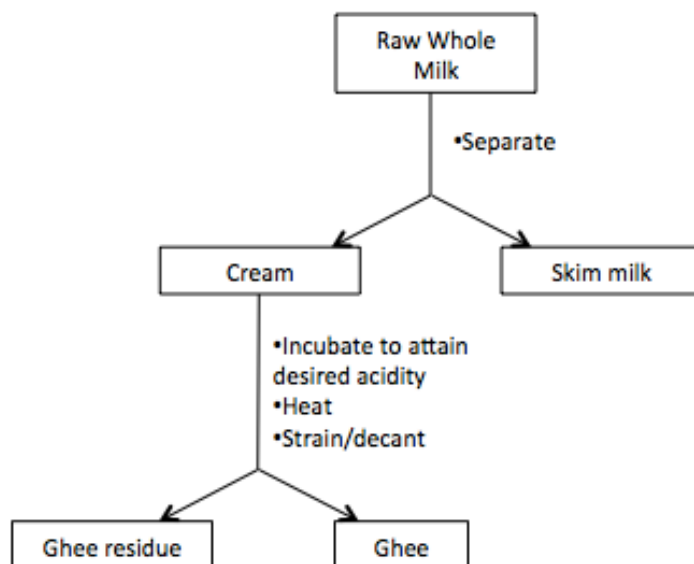
Figure 1.5: Creamery butter method of ghee preparation

1.3.4 Pre-stratification Method of clarification

This is just a clarification method employed industrially. When butter is heated and maintained at $80\,^{\circ}C - 85\,^{\circ}C$ for 15-30 minutes, it separates into three distinct layers. The top frothy layer consists of denatured proteins and the bottom layer consists of solids-not-fat and 80% of the moisture present in the butter. In pre-stratification method, the bottom buttermilk layer is removed before the

temperature of the middle layer is raised to clarification temperatures (above 100 ^{o}C) (Sserunjogi, 1998). This method just facilitates separating fats from moisture and solids-not-fat prior to clarification at high temperatures. This increases the yield of ghee and provides for energy conservation.

2 Hypothesis, Rationale and Objectives

2.1 Hypothesis

We hypothesized that the bacterial fermentation of cream would have an impact on the fatty acid profile of ghee. Also we expected differences in overall chemical and flavor profiles of ghee owing to different preparation methods and milk sources. Moreover, it was expected that the sensory characteristics of the ghee made by different methods of preparation and the two sources of milk will be different.

2.2 Rationale

This comparative study will help in understanding the differences between the chemical composition, flavor profiles and sensory qualities of ghee prepared by different methods. This will clarify whether or not fermentation with lactic acid bacteria has a significant impact on the chemical quality of ghee as well as whether change in feed affects the fatty acid composition.

This research should help us get a step closer to settling the debate as to whether traditionally made ghee is has any advantages over ghee made by direct cream method and in case a significant difference is found, it would provide as a lead in further research into the field. It was hoped that we will be able to determine if there is any chemical difference between the ghee

prepared by different methods. It was not the intent of this study to determine the health effects of ghee prepared by different methods.

2.3 Objectives

The specific objectives of this research were:

- To prepare ghee samples using two different sources of milk namely, milk from organic grass-fed cows and milk from regular diet-fed cows and by three different methods namely, direct cream method, traditional method and cultured cream method

- To analyze and compare the fatty acid profiles of the ghee samples

- To analyze and compare head space volatiles of the ghee samples in order to elucidate flavor profiles

- To do sensory evaluation of the ghee samples prepared by different methods of preparation.

3. Materials and Methods

3.1 Materials and Equipment

3.1.1 Raw Milk

Since the preparation of ghee requires separation of cream (fats) from the whey, non-homogenized milk was required. Hence, non-pasteurized, non-homogenized, whole milk was obtained. The sale of raw milk is banned in the state of New Jersey. Raw milk from regular diet-fed cows was donated by Readington Farms (Whitehouse Station, NJ) with special request from Rutgers and permission from the authorities at Readington Farms (to use this milk for the purpose of this research and not for consumption). Raw organic grass-fed cow milk was purchased from Birchwood farms (Newtown, PA). The sale of raw milk is permitted in the state of Pennsylvania.

Both kinds of milk was brought in plastic gallon-size containers, a day prior to sample preparation and stored in the cold room at 4 ^{o}C overnight. The milk was removed from the cold room, right before preparation of samples and processed as per the experimental design.

3.1.2 Bacterial Cultures

Considering the origin of ghee, it was necessary to use bacterial cultures indigenous to Indian yogurt cultures (Dahi culture). A special cocktail of bacteria

YO-MIX 905 LYO (specific for 'Dahi' or Indian-style yogurt) was provided by Danisco (New Century KS, USA) as shown in Fig. 3.1. This culture contains the following bacteria: *Streptococcus thermophilus, Lactobacillus delbrueckii subsp. Bulgaricus, Lactobacillus lactis subsp. Lactis biovar. Diacetylactis*. It was obtained in freeze-dried form and was refrigerated until use.

Figure 3.1: YO-MIX 905 Bacterial culture for 'Dahi' (Indian style-yogurt) packet (left), Freeze dried culture powder (contents of packet, right)

Each packet contains 50 DCU (DuPont Culture Units) of culture. Weight corresponding to 50 DCU changes per batch and is mentioned on each packet. For the purpose of this experiment, a usage level of 20 DCU/100 L of milk/cream was used and weight corresponding to this usage level was used based on the volume of sample being handled each time (on an average, 0.016 g of freeze-dried culture was added to 500 ml of milk).

3.1.3 Cream Separator

For the Modern and Cultured cream methods of ghee preparation, cream was separated from the whole milk using a centrifugal cream separator (Fig. 3.2). This technique to separate milk fat from the milk is based on two criteria:

- Milk fat is dispersed in the form of small globules

- The density of fat differs significantly from the surrounding serum (difference in density ~48 kg/m^3)

Because of lower density, fat globules experience buoyancy force. Upon rotation at high speeds, milk is pulled outward against the walls of the separator and the cream, which is lighter, collects in the middle and moves upwards. The cream and milk then flow out of separate spouts resulting in separation of cream from the top and skimmed milk from bottom of a centrifugal cream separator. Industrially cream separation is most commonly done using centrifugal cream separators.

Viscosity of the milk greatly influences the velocity of fat globules. Hence separation efficiency depends on the viscosity of the milk. Increased viscosity reduces the efficiency of separators; hence milk is heated to a certain temperature. The optimal temperature for industrial separators is about 57 ^{o}C. For the lab scale bench top separator used in our experiments, cream separation was tested at different temperatures. When the milk is too cold, cream does not separate at all. At very high temperatures, viscosity of the whey and cream both reduces and separation is not efficient. 40 ^{o}C – 45 ^{o}C was determined to be the

most suitable temperature for separation. This is the point where viscosity of cream is higher than that of the milk and the two can be separated because of this gradient (Gunsing, 2009).

Figure 3.2: Centrifugal cream separator

3.1.4 Incubator and culturing

According to the recommended inoculation and incubation conditions by Danisco the cream was inoculated at 42 ^{o}C and incubated at 38 ^{o}C for 8 hours. The required amount of culture (corresponding to the use level of 20 DCU/ 100 L) was weighed using a sensitive weighing balance. The culture was added to the cream and mixed for about 10-15 minutes using a hand-held blender.

For the cultured cream method of ghee preparation, the cream separated using the cream separator was used and for the traditional method, the cream separated by boiling was used as a substrate. Culture was added to both kinds

of cream, mixed and the cultured cream was transferred to glass jars with lids. The jars were then incubated overnight for 8 hours in a Yamato ADP-31 vacuum oven (Fig. 3.3), operated as an incubator (without vacuum) at 38 $^{\circ}$C.

Figure 3.3: Yamato ADP-31 Vacuum oven (operated as incubator)

3.1.5 Blender and churning

At the end of the incubation period, the glass jars were removed and kept at room temperature just prior to churning. An industry grade blender with a 4.5 HP GE motor was used for churning (Fig. 3.4, left). Approximately 500 g of yogurt was taken at a time in a blender with about 1 L of cold water. Cold water was added for more efficient separation of butter, as cold water helps the fats solidify and easier to clump together. On blending, the oil in water emulsion of the cream yogurt is reversed to that of water in oil (butter). Thus the lighter butter separates on the top (Fig. 3.4, right).

The cultured cream was blended at high speed with cold water for about 5 minutes. This procedure was carried out on both kinds of cultured creams and butter thus obtained was separated and collected in a stainless steel vessel for clarification.

Figure 3.4: (left) Blender, (right) Butter floating at the top of the blender jar after churning

3.1.6 GC-MS

Gas chromatography, when combined with mass spectrometry, is a powerful analytical tool in identification and quantization of the unknown compounds. GC separates volatile or semi volatile compounds with good resolution. Mass spectrometer provides detailed structural information of the compounds, by

breaking down molecules which can then be identified by the molecular weights of the fragments each molecule typically breaks down into. In this research, the GC-MS system was employed for analysis of the following:

- Fatty Acid Methyl Esters (FAMEs)

- Non-saponifiable fraction

- Head-space volatile compounds

3.1.6.1 Gas Chromatography

Gas chromatography (GC) is a common technique used in analytical chemistry to separate compounds that can be vaporized without decomposition. A mixture of such compounds when injected into a GC column is carried through the column with the aid of a carrier gas. The column is coated with adsorbent material. Depending on the adsorption affinity of different compounds in the mixture, they will elute out of the column at different times. A detector at the end of the column detects when a compound is eluted. A program then generates a plot of absorbance vs. retention time. This is called a chromatogram. A typical chromatogram for the saponifiable fraction of ghee is shown in Fig. 3.5. Each peak on the chromatogram represents a specific compound and the area under the peak corresponds to the relative amount of that particular compound in the sample mixture. The four prominent peaks seen in the chromatogram are myristic, palmitic, stearic and oleic acid (respectively from left to right at approximately the 16th, 18th, 22nd and 23rd minutes, refer to section 4).
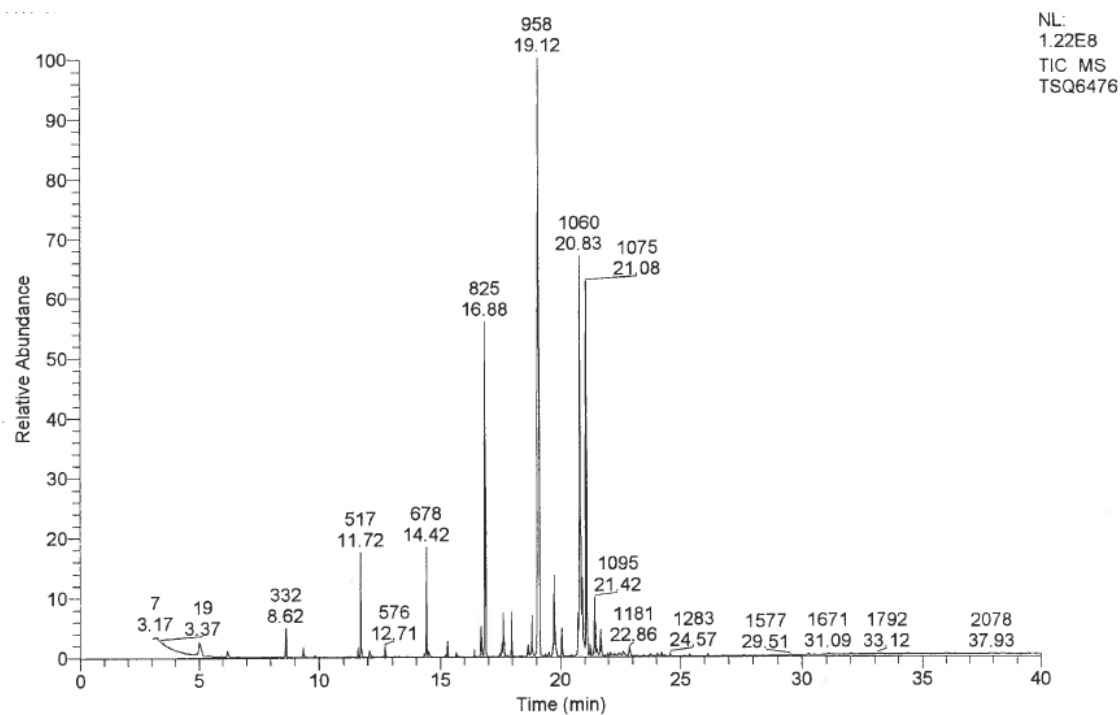
Figure 3.5: Chromatogarm for saponifiable fraction of ghee

Each compound has a unique retention time. The chromatogram can then be matched with a chromatogram of a reference standard and the compounds can be identified. More sophisticated method is to couple the GC with Mass spectrometry for identification of each compound. The area under each peak on a chromatogram represents the relative concentration of each compound in a given sample.
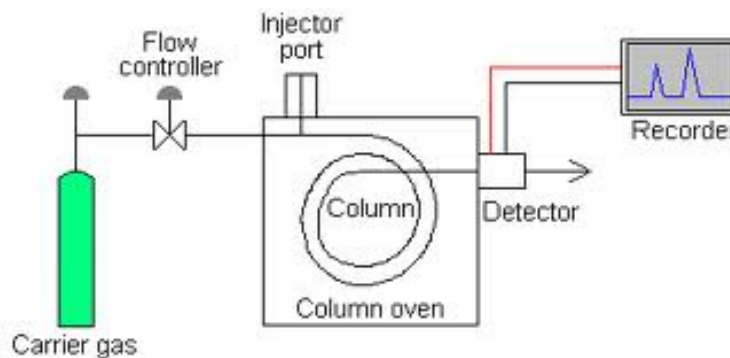
Figure 3.6: Schematic representation of Gas Chromatography

Figure 3.6 shows a schematic representation of GC. Explained below are the GC conditions that need to be tailored according to the sample. Selection of different conditions of GC is very crucial in order to get the best separation of compounds in the sample that is to be analyzed.

Column selection

Choice of the right column is very critical in order to obtain optimum separation of compounds in Gas Chromatography. The essential factors to be considered for selection of column are:

- Internal diameter (I.D.)

- Film thickness (of stationary phase)

- Length of column

- Phase polarity

Phase polarity controls the ability of the column to separate the components of the sample. A polar column is used for separation of components with polar functional groups. Separation efficiency of the capillary column is achieved by decreasing the

internal diameter of the column. However internal diameter dictates the sample holding capacity of the column. Low column I.D. will result in very low sample capacity and better resolution, higher I.D. will result in increased sample holding capacity and poor separation of components (poor resolution).
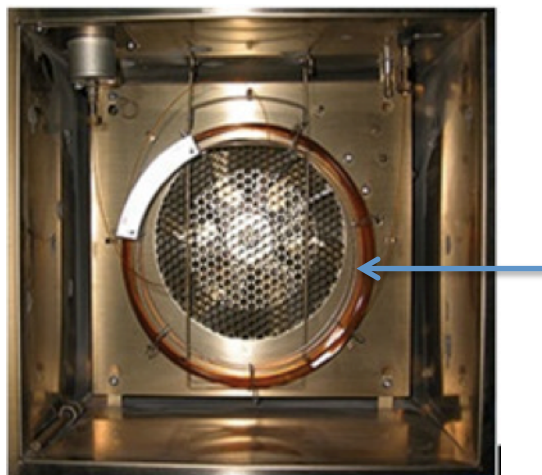
Figure 3.7: Column (marked by pointer) inside the GC oven

Figure 3.7 shows the column placed inside the GC oven. Columns with reduced film thickness will provide sharper peaks, reduced column bleed and improved signal to noise ratio. Reduced film thickness also allows increased operating temperature for the column. Selecting the length of the column is a compromise between speed and head pressure on one hand, and peak resolution on the other hand. Longer the length of the column, higher is the peak resolution. However there are practical limits to increasing the length of the column. Typical length of GC columns is 15 m - 30 m. For samples containing complex mixtures, columns of up to 150 m length have been used.

Sample Injection

GC injection port consists of a rubber septum situated at the head of the column, through which a syringe needle is inserted to inject the sample. The temperature of the port is higher than the boiling point of the least volatile component of the analyte. This ensures vaporization of the sample.

A calibrated micro-syringe is used to inject sample into the vaporization chamber of the GC instrument. The temperature of the sample injection chamber is set according to the requirement of the specific analysis.

The injections are made in the split or split less mode depending on the specific application. Split mode allows delivering only a fraction (depending on the set split ratio) of the sample into the injection port. Figure 3.8 shows the working of the split and splitless mode of injection.
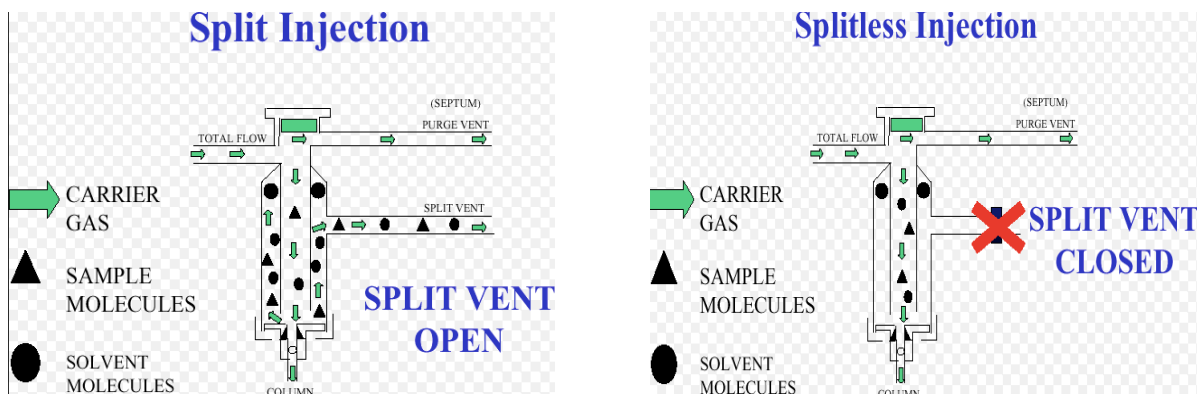
Figure 3.8: (left) Split injection mode of operation and (right) Splitless injection mode of operation of the injection port.

Carrier gas

The selection of the carrier gas depends on the type of detector used in the analysis, sample matrix and purity of the gas. The carrier gas linear velocity or the flow rate has an important role in influencing retention time and efficiency. The efficiency and the reproducibility of the analysis are obtained by controlling the head pressure of the carrier gas. The pressure adjustment depends on the type of the gas, length of the column and the column temperature. In our case helium was used as a mobile gas phase in the GC-MS analysis. Helium has low density and diffuses the solutes rapidly and improves the rate of mass transfer in the mobile phase, thus regulating the column efficiency.

Column Temperature

The rate at which the analyte travels through the column is directly proportional to

the temperature of the column. Higher the column temperature, faster the elution rate of the analyte. However, higher temperature leads to lesser interaction of analyte with the stationary phase, as a result of which the retention characteristics of the analyte are affected. The use of a temperature control program allows heating of the oven at a controlled rate, thus reducing peak broadening and improving retention times of the solute. The temperature program for initial and final temperatures with a fixed rate of temperature rise was set as per the requirements of the different samples analyzed. The variable temperature program allows gradual elution of different compounds and hence efficient separation.

3.1.6.2 Mass Spectrometer

Mass Spectrometer is a tool used for identifying individual compounds. Once compounds are fed to the mass spectrometer upon separation from a mixture, using a tool such as GC, the compound is ionized and broken down in several fragments. Every molecule has a typical pattern of molecular weights of the fragments that it is ionized into. This pattern is depicted in a mass spectrum which is a plot generated by the MS unit. Compounds can be identified accurately on the basis of their respective mass spectrums. For example, Fig. 3.9 shows a mass spectrum for cholesterol. The different peaks are the fragments that are generated upon ionization. The figure also shows the chemical structure of cholesterol.
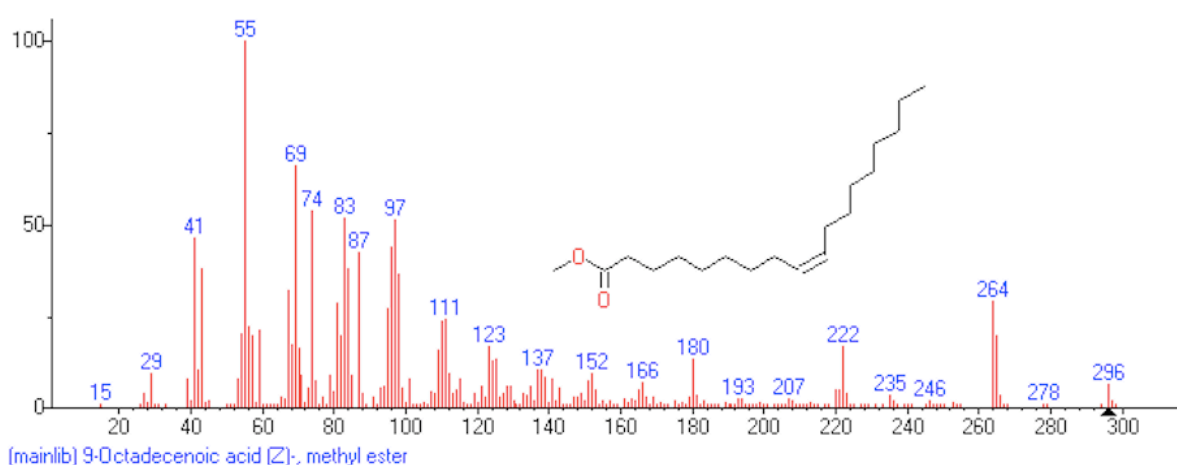
Figure 3.9: Mass spectrum: Methyl Oleate

The different components of the mass spectrometer are: Ion Source, Mass Analyzer and Detector.

Ion Source

The ion source converts the gaseous analyte molecules into ions by bombarding them with a beam of electrons. Both electron ionization and chemical ionization modes were used during our experiments. Chemical ionization helps in identifying the accurate molecular weight as the analyte does not fragment and the molecular ion remains intact. The electron ionization mode is operated with a scanning range of 35-750 amu (atomic mass units), while the chemical ionization mode with a 100-600 amu scanning range.

Mass Analyzer

Different types of mass spectrometers exist based on different mass analyzers they use. The mass analyzer separates the ions according to m/z (mass/charge) ratio.

For the purpose of analyzing the saponifiable and non-saponifiable fractions, the Finnigan Mat TSQ 7000 Mass spectrometer (MS) was used. This MS uses a quadrupole analyzer (Fig. 3.10). This facility is managed by Dr. Tom Hartman at the department of Food Science, Rutgers. The sample in the gaseous state (separated by GC) is bombarded with a beam of electrons (ion source) to produce the molecular ion of the original molecule. Since the molecular ion is unstable it breaks into fragments.
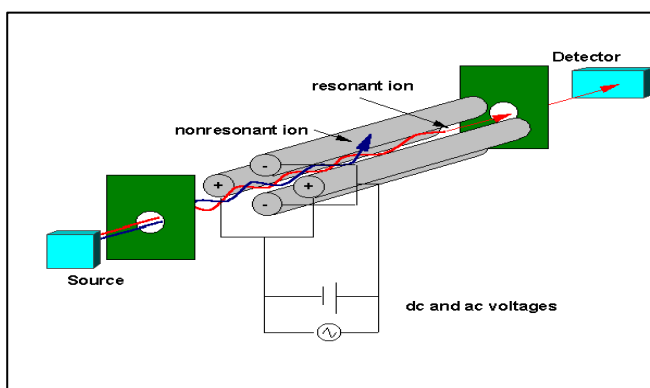
Figure 3.10: Schematic diagram of a Quadrupole mass analyzer

The fragmented ions are then separated by their m/z ratio and detected by the detector. The ion signal is then converted into mass spectrum, which is a plot of m/z vs. intensity of the fragments.

For the purpose of analysis of headspace volatiles, the Finnigan MAT MS-8230 was used. This mass spectrometer uses the magnetic sector mass analyzer. In this MS, ions leaving the ion source are accelerated to a high velocity. The ions then pass through a magnetic sector in which the magnetic field is applied in a direction

perpendicular to the direction of ion motion. When acceleration is applied perpendicular to the direction of motion of an object, the object's speed remains constant, but the object travels in a circular path. Therefore, the magnetic sector follows an arc; the radius and angle of the arc vary with different ion optical designs. A magnetic sector alone will separate ions according to their mass-to-charge ratio. Schematic representation of magnetic sector analyzer is shown in Fig. 3.11.
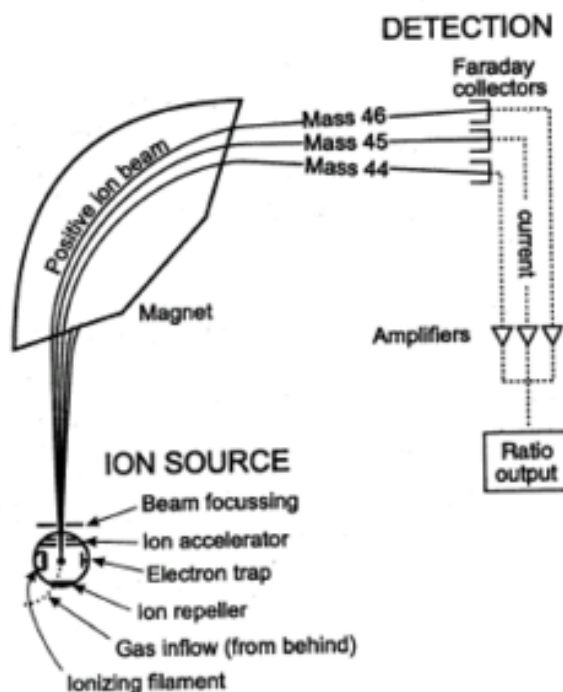
Figure 3.11: Magnetic Sector mass analyzer

3.1.7 37 FAME mix standard

The 37 component FAME mix standard by Supelco was ordered from Fisher Scientific (Catlog no. 47885-U). Figure 3.12 shows the list of components in the standard mix as provided by Supelco.

The standard comes in a glass ampule. The concentration of the standard is 10 mg mix in 1 ml of methylene chloride. The concentration of experimental test samples was adjusted to this concentration by dissolving the obtained sample in corresponding volume of methylene chloride in order to get 10 mg of fatty acid methyl esters per ml of solvent (Explained in section 3.4).

This Data Sheet Contains Important Information About The Product.

Supelco™ 37 Component FAME Mix
Catalog No. 47885-U

This fatty acid methyl ester (FAME) mixture is carefully prepared by weight. The weight percentage of each component is indicated. Each ampule contains 10mg/mL of the FAME reference standard mix in methylene chloride.

Column: **SP™-2560, 100m x 0.25mm ID, 0.20µm film**
Cat. No.: **24056**
Oven: 140°C (5 min) to 240°C at 4°C/min
Carrier: helium, 20cm/sec
Det.: FID, 260°C
Inj.: 1µL, 260°C, split 100:1

Component	Weight %
1. Butyric Acid Methyl Ester (C4:0)	4%
2. Caproic Acid Methyl Ester (C6:0)	4%
3. Caprylic Acid Methyl Ester (C8:0)	4%
4. Capric Acid Methyl Ester (C10:0)	4%
5. Undecanoic Acid Methyl Ester (C11:0)	2%
6. Lauric Acid Methyl Ester (C12:0)	4%
7. Tridecanoic Acid Methyl Ester (C13:0)	2%
8. Myristic Acid Methyl Ester (C14:0)	4%
9. Myristoleic Acid Methyl Ester (C14:1)	2%
10. Pentadecanoic Acid Methyl Ester (C15:0)	2%
11. cis-10-Pentadecenoic Acid Methyl Ester (C15:1)	2%
12. Palmitic Acid Methyl Ester (C16:0)	6%
13. Palmitoleic Acid Methyl Ester (C16:1)	2%
14. Heptadecanoic Acid Methyl Ester (C17:0)	2%
15. cis-10-Heptadecenoic Acid Methyl Ester (C17:1)	2%
16. Stearic Acid Methyl Ester (C18:0)	4%
17. Elaidic Acid Methyl Ester (C18:1n9t)	2%
18. Oleic Acid Methyl Ester (C18:1n9c)	4%
19. Linolelaidic Acid Methyl Ester (C18:2n6t)	2%
20. Linoleic Acid Methyl Ester (C18:2n6c)	2%
21. Arachidic Acid Methyl Ester (C20:0)	4%
22. γ-Linolenic Acid Methyl Ester (C18:3n6)	2%
23. cis-11-Eicosenoic Acid Methyl Ester (C20:1)	2%
24. Linolenic Acid Methyl Ester (C18:3n3)	2%
25. Heneicosanoic Acid Methyl Ester (C21:0)	2%
26. cis-11,14-Eicosadienoic Acid Methyl Ester (C20:2)	2%
27. Behenic Acid Methyl Ester (C22:0)	4%
28. cis-8,11,14-Eicosatrienoic Acid Methyl Ester (C20:3n6)	2%
29. Erucic Acid Methyl Ester (C22:1n9)	2%
30. cis-11,14,17-Eicosatrienoic Acid Methyl Ester (C20:3n3)	2%
31. Arachidonic Acid Methyl Ester (C20:4n6)	2%
32. Tricosanoic Acid Methyl Ester (C23:0)	2%
33. cis-13,16-Docosadienoic Acid Methyl Ester (C22:2)	2%
34. Lignoceric Acid Methyl Ester (C24:0)	4%
35. cis-5,8,11,14,17-Eicosapentaenoic Acid Methyl Ester (C20:5n3)	2%
36. Nervonic Acid Methyl Ester (C24:1)	2%
37. cis-4,7,10,13,16,19-Docosahexaenoic Acid Methyl Ester (C22:6n3)	2%

Figure 3.12: Components of the 37 component FAME standard mix by Supelco.

The manufacturer (Supelco) provided a chromatogram of the standard mix to indicate the elution times and order of the compounds (Fig. 3.12 and Fig. 3.13).
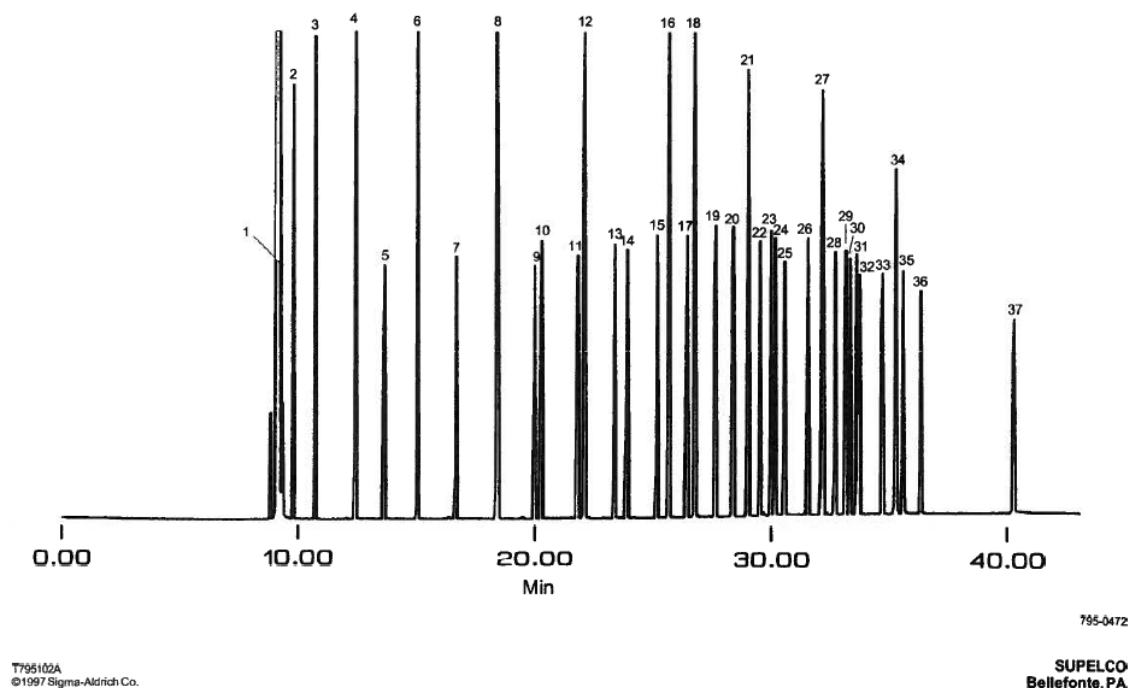
Figure 3.13: Chromatogram of the 37 component FAME mix standard, both provided by Supelco.

For our purposes, the ampule for the standard mix was carefully broken and the contents were transferred into a 4 ml borosilicate pre-cleaned glass vial. The vial was labeled using the spare labels provided and frozen until time of injection. In our experiments, the standard was handled like any other sample and 1 µL of the standard was injected in to GC at the same conditions as that of the samples. The chromatogram generated by the GC used for the experiments (Fig. 3.14) was compared to the one provided by Supelco (Fig. 3.13) visually, and the peaks were

identified. The identification was verified using Mass spectrometry results. Identification of compounds using GC and MS results was done with Dr. Tom Hartman's help.

Thus the retention times for corresponding compounds were standardized with the GC conditions used for the rest of the samples. The sample chromatograms could then be easily compared to this chromatogram for identifying compounds from different retention times.

Figure 3.14: Chromatogram of the 37 component FAME mix generated on the lab GC under the same conditions used for experimental samples.

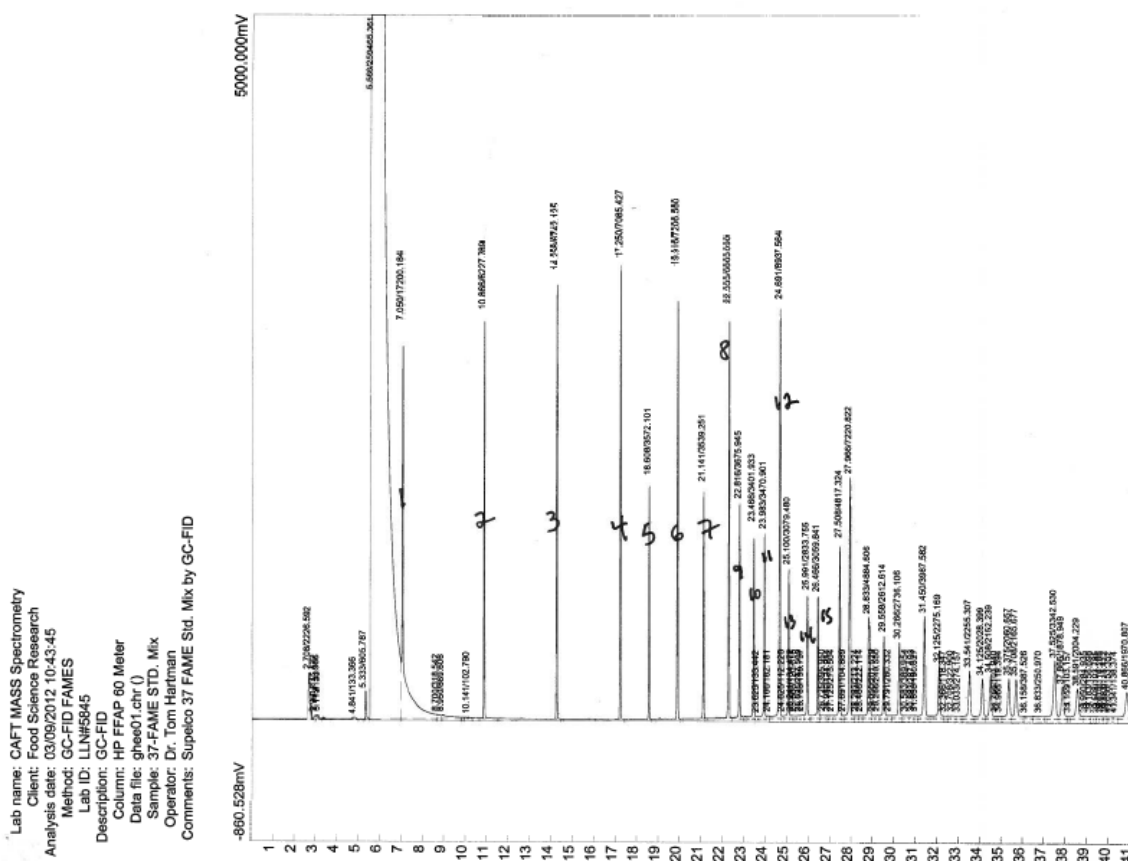## 3.2 Experimental Design

The selected methods of preparation of ghee were direct cream method, traditional method and cultured cream method as described earlier. In direct cream method there is a single cream separation step prior to clarification and the milk fats are least processed. Cultured cream method is a hybrid between the direct cream and traditional methods, designed to help observe and decipher changes found, if any, due to the additional steps in traditional method of ghee preparation when compared to direct cream method.   Figure 3.15 shows how the three methods compare.

Modern Method

- Separate Cream
- Clarify

Ghee

Cultured Cream

- Separate cream
- Culture
- Churn yogurt
- Clarify Butter

Ghee

Traditional Method

- Boil milk
- Separate cream
- Culture
- Churn yogurt
- Clarify Butter
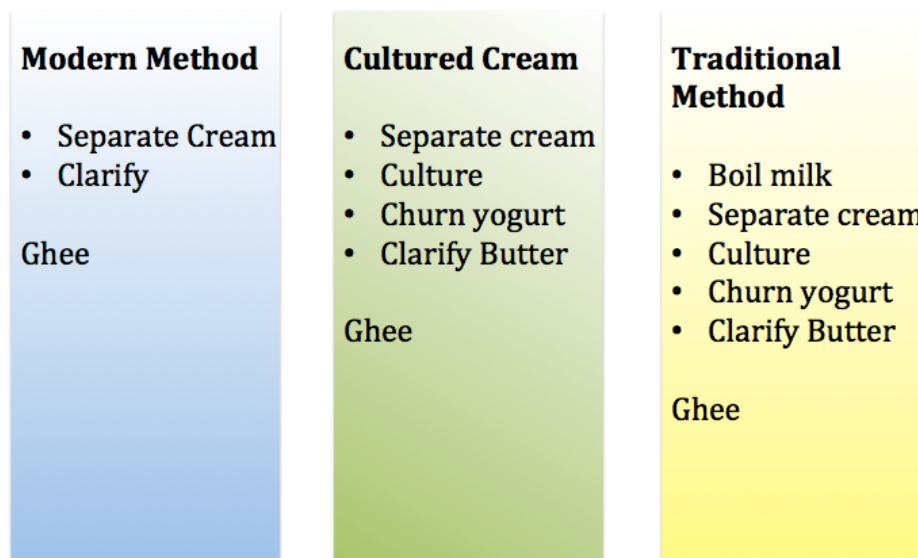
Ghee

Figure 3.15: Comparison of different methods of ghee preparation

Another aim of this research was to compare changes in fatty acid profiles of ghee depending on the source of milk, i.e., Organic grass-fed cow milk and regular diet-

fed cow milk. Hence, three methods of preparation and two sources of milk were used to give six distinct samples for testing. All six samples were made thrice with milk bought in three different batches over a period of three months in winter (seasonal variation was avoided and milk was assumed to be consistent, since source and diet remained constant throughout the experiment). Figure 3.16 and Table 3.1 shows the six distinct samples obtained.

Figure 3.16: Six samples used for the experiments

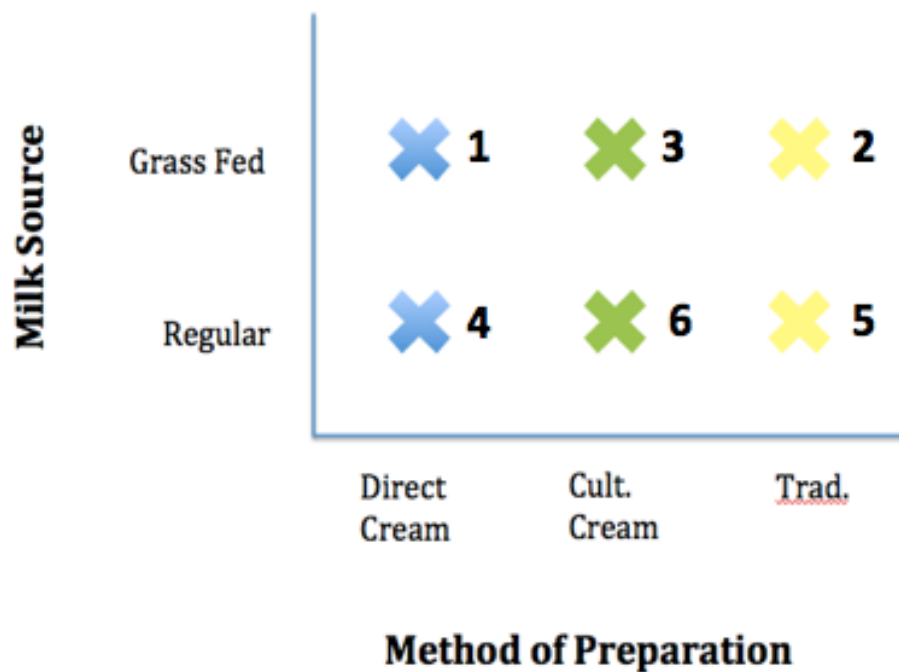| Sample No.	Ghee sample specification
1	Organic grass-fed milk, Modern method
2	Organic grass-fed milk, Traditional method
3	Organic grass-fed milk, Cultured cream method
4	Regular milk, Modern method
5	Regular milk, Traditional method
6	Regular milk, Cultured cream method

Table 3.1. Ghee sample numbering

Each ghee sample was saponified (refer to section 3.4.1) to separate the saponifiable fraction for the Fatty Acid Methyl Esters (FAMEs) assay that separated and identified the fatty acids of the samples. The resultant non-saponifiable fraction was analyzed as well. For the purpose of sensory analysis, samples made from Organic grass fed milk were analyzed for sensory quality by a panel of evaluators. Headspace volatiles were analyzed for these samples to elucidate flavor profiles and correlate the same with the sensory study results.

3.2.1 Cultured cream method

If the traditional method and modern method of ghee-making are compared, it is observed that traditional method involves initial boiling of milk as well as culturing with bacteria that the modern method does not require. Cultured cream method was thus devised during the course of this research to evaluate the effect of the additional steps in the traditional method on the chemical profile of ghee. It is a hybrid version of the two methods. The idea was to be able to design a more

efficient, easier and higher-yield technique of making ghee maintaining the traditional procedure of 'culturing' with lactic acid bacteria. Figure 3.17 shows the steps in preparation of ghee by cultured cream method.

Preparation of Ghee by Cultured Cream Method
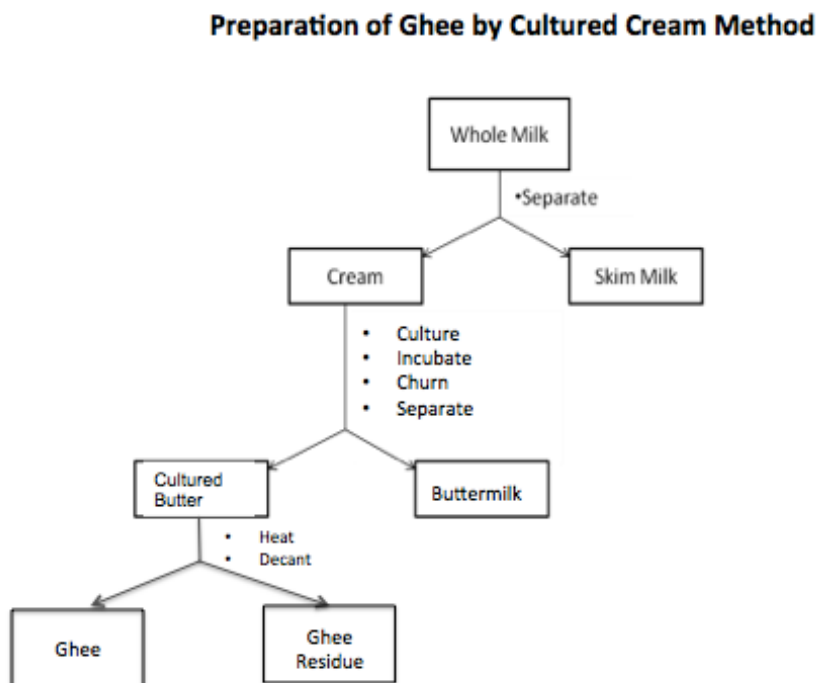
Figure 3.17: Cultured cream method of ghee preparation

3.3 Methods

3.3.1 Sample preparation

3.3.1.1 Traditional method

In this research, the traditional method of ghee making was modified in order to be carried out consistently in the lab. Milk was boiled and then cooled to room temperature. The top part was taken into a glass jar and volume measured. It was

then inoculated with Danisco's YO-MIX-900-LYO (freeze dried culture cocktail of lactic acid bacteria) at 42 $^{\circ}$C. After inoculation, the jar was incubated at 38 $^{\circ}$C for 10 hours. At the end of the incubation period, the yogurt was churned with cold water using an industry grade high-speed blender. Butter was separated at top. Butter was removed carefully from the top and weight was measured. This butter was then heated slowly in a heavy bottom stainless steel vessel using a lab burner and temperature was recorded with respect to time. The heating process was continued till the temperature reached 120 $^{\circ}$C. At this point almost all the moisture had evaporated and clear yellow liquid was obtained. Solids-not-fat from the butter settled at the bottom of the pan. It was carefully strained using a strainer with filter paper. The strained liquid (ghee) was then stored in labeled, pre-cleaned glass vials at 0 $^{\circ}$C until further use.

This method was used to make samples with three different batches of milk (each in triplicates) for regular diet-fed cow milk and organic grass-fed cow milk.

3.3.1.2 Direct Cream Method

In this research, non-homogenized milk was warmed to 40 $^{\circ}$C. This milk was then fed to the centrifugal cream separator. Cream thus obtained was collected and weighed. It was then directly heated in a heavy bottom stainless steel vessel using a lab burner, till all the moisture was lost and temperature reached 120°C. Temperature was monitored with respect to time using thermocouples. At the end of the process, ghee was filtered using a strainer with a filter paper. Modern ghee

samples were thus made with three different batches of milk (each in triplicates) for regular diet-fed cow milk and organic grass-fed cow milk.

3.3.1.3 Cultured cream method

For the purpose of this experiment, non-homogenized milk was warmed to about 38 ^{o}C (close to body temperature for efficient cream separation). This milk was then fed to the centrifugal cream separator. Cream thus obtained was collected, weighed and transferred to a glass jar. It was then inoculated with YO-MIX-900-LYO (freeze dried culture cocktail of lactic acid bacteria) at 42 ^{o}C. After inoculation, the jar was incubated at 38 ^{o}C for 10 hours. At the end of the incubation period, the mixture was churned with cold water using an industry grade high speed blender. Butter was separated at top. Butter was removed carefully from the top and weight was measured. This butter was then heated slowly in a heavy bottom stainless steel vessel using a lab burner and temperature was monitored using thermocouples. The heating process was continued till the temperature reached 120 ^{o}C. Ghee was decanted using a strainer with filter paper. The strained ghee was then stored in labeled, pre-cleaned glass vials at 0 ^{o}C until further use. Ghee by the cultured cream method was made from both regular diet-fed cow milk and organic grass fed cow milk with three different batches of milk.

3.3.2 Boiling Milk

For the traditional method to prepare the ghee samples, raw milk was boiled in order to separate the cream. The milk was boiled in a stainless steel vessel on a

burner over low flame. It was then cooled to room temperature and the top layer of cream was separated and stored in a glass jar. Figure 3.18 shows the top part of the milk after boiling (Left), yellow fat globules trapped in a network of casein can be seen (Right).

Figure 3.18: Milk boiling (left), fat globules trapped in casein network seen in top view of boiled milk cooling at room temperature (right)

The time temperature data for the boiling of milk was recorded using thermocouples inserted in the milk during boiling (Fig. 3.19) and a data acquisition system. When the temperature reaches 100°C the milk rises as it starts to boil (indicated in the graph shown in Fig. 3.20). Heating was stopped at this point and the foam was allowed to settle and cool, before removing the top cream layer.

Figure 3.19: Thermocouples (shown by pointer) used for recording temperature

profile during milk boiling

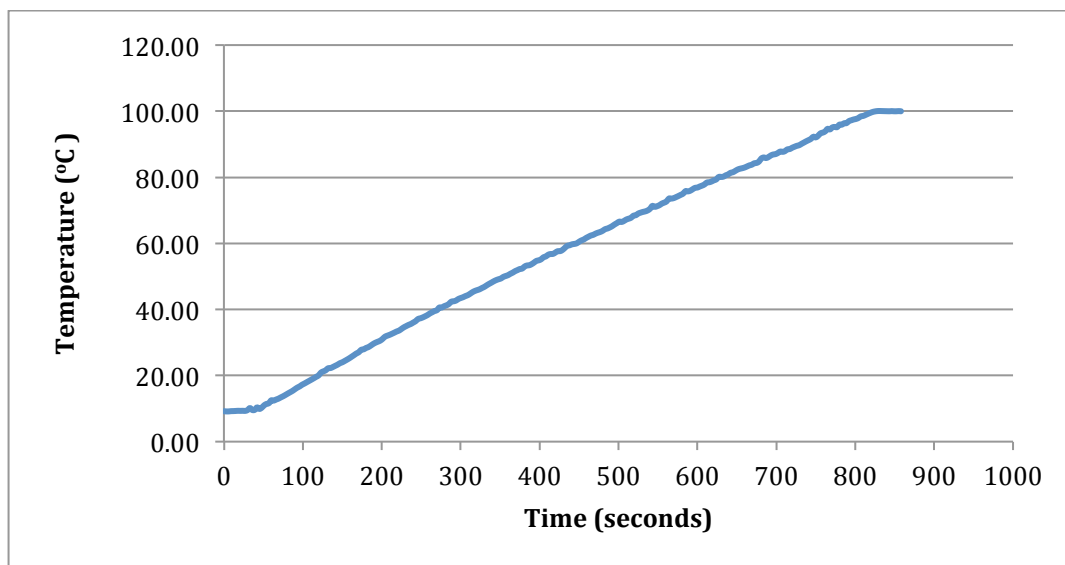Figure 3.20 shows a graph of Temperature vs. time recorded during boiling of milk.

Figure 3.20: Temperature of milk during heating up to boiling

3.3.3 Heat Clarification

With respect to ghee-making, clarification is the process of heating butter/ cream, in order to get rid of most of the moisture in it to obtain dehydrated milk fats (ghee/ butter oil). The process can be broken down into three stages, each separated by effervescence. During the first stage, the temperature of the butter/cream slowly rises from room temperature to 100 ^{o}C. At 100 ^{o}C, the water starts boiling. This point is marked by the first effervescence (De, 2001). The temperature remains constant at 100 ^{o}C till all the water has been evaporated. Then the temperature rapidly rises above 100 ^{o}C and a light bubbling occurs. This is the second effervescence as a result of the boiling of the fats. Temperature at different times was recorded by suspending a thermocouple in the sample during clarification, as shown in Fig. 3.21.

Figure 3.21: Thermocouples used for recording temperature data for clarification of butter when making ghee.
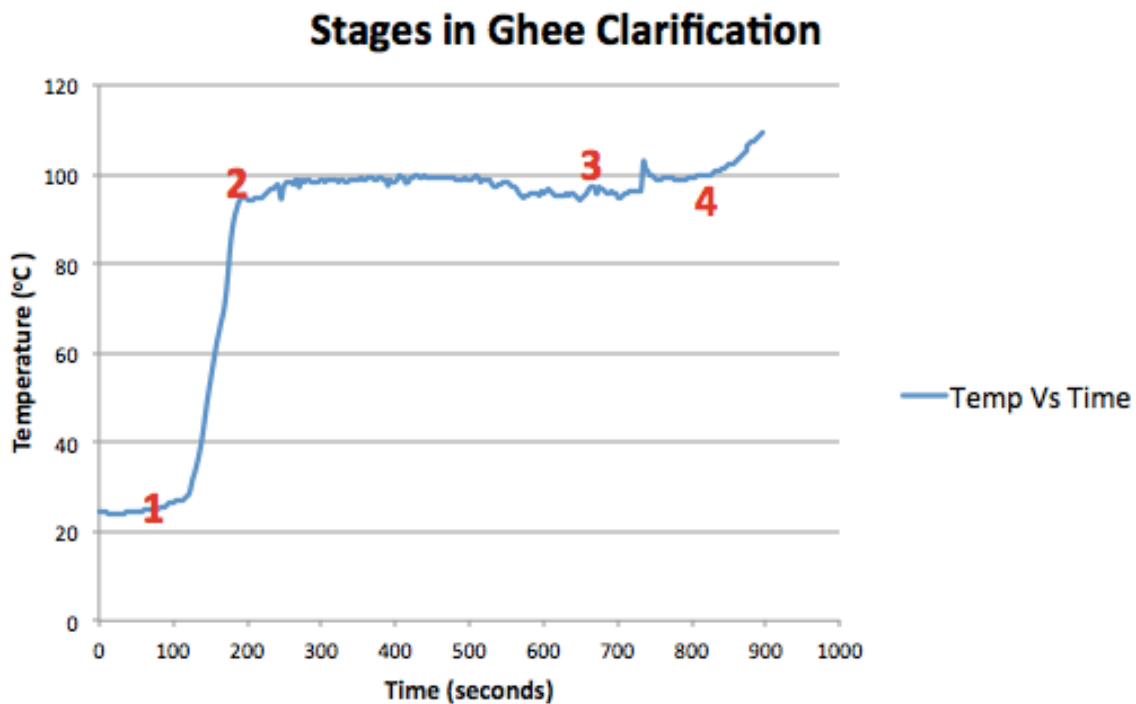
Figure 3.22: Temperature profile of ghee clarification

Figure 3.22 shows the temperature changes over time during clarification of ghee.

The points on the graph are as follows:

1. Butter/ cream at room temperature

2. First effervescence

3. Solids-not-fat settle down, beginning of second effervescence

4. Second effervescence

Figure 3.23 shows photographs taken at each of these four points from left to right.

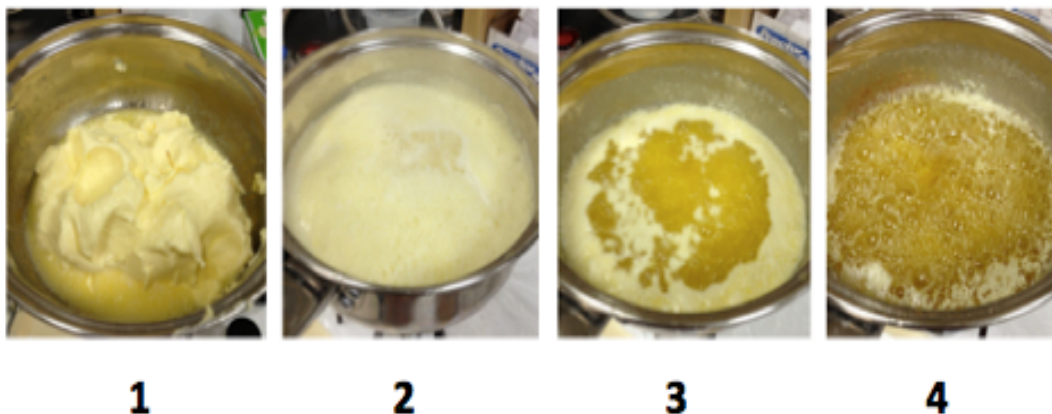Note the foamy first effervescence and a clear bubbling second effervescence.

Figure 3.23: Different stages in ghee clarification from left to right: Butter at room temperature, first effervescence, beginning of second effervescence and second effervescence.

For making ghee, depending on individual taste, the process is carried out for a longer time, till it reaches high temperatures to get a darker ghee. For the purpose of this experiment, the process was stopped when the ghee reached 110 ^{o}C.

For the cultured cream and traditional methods of ghee preparation, the butter obtained after churning was clarified using the procedure mentioned above. For the ghee preparation using modern method, cream separated using the cream separator was directly heated and clarified.

3.3.4 Filtration

The clarified butter contains milk solids that are not fats. These solids are water soluble, and once all the water is evaporated during the clarification, they separate

out of solution and settle at the bottom. These need to be filtered out and separated from the ghee. A stainless steel strainer was used for this purpose along with a coffee filter, as shown in Fig. 3.24. All ghee samples were filtered after clarification.

Figure 3.24: Filtration of ghee at end of clarification (left) freshly made ghee (right)

Ghee residue left after filtration consists of about 25.8% crude protein, 50.8% fat, 12.3% lactose, 8.98% ash, 0.88% calcium, 0.50% phosphorus (Arumugam, 1989). The residue was discarded and not used for further analysis.

3.3.5 Storage

The ghee was stored in clean, labeled glass vials (Fig. 3.25). The vials were frozen in a freezer (temperature ~ -10 ^{o}C) almost immediately to avoid stratification of fats at room temperature. The vials were removed from the freezer and thawed at room temperature as and when required for further analysis.

Figure 3.25: Labeled glass vials filled with freshly prepared ghee samples

3.4 Analyses

3.4.1 Analysis of Saponifiable and non-saponifiable fractions

Sample preparation

Saponification: 200 mg of homogenous solid ghee sample was measured into the bottom of Pyrex heavy-duty round bottom centrifuge tubes. 5 ml of 5% alcoholic KOH was added to the sample. The tubes were then heated in a heating block at 80 ^{o}C for one hour. Tubes were vortexed occasionally. After one hour the tubes were taken out of the heating block and allowed to cool. This is the saponified sample.

Hexane extraction of non-saponifiables

30 ml hexane was added to the saponified sample and the tubes were centrifuged at 3000 rpm for 30 min. This extraction was repeated twice and

hexane layer (bottom) was transferred to fresh tubes labeled 'non-saponifiable fraction'.

Purification and preparation of saponifiable fraction:

After hexane extraction, the bottom fraction was transferred to clean Pyrex heavy-duty round bottom centrifuge tubes. 1 ml concentrated HCl was added to each tube to neutralize unreacted KOH and the solution was tested for strong acidity with a pH paper. 5 ml methylene chloride was added followed by centrifugation at 3000 rpm for 30 minutes. Methylene chloride layer separates at the bottom.

The methylene chloride layer that has the saponified matter dissolved in it was carefully transferred to pre-cleaned glass vials. Weights of empty vials were individually noted, beforehand.

Each sample was dried by blowing the solvent with nitrogen, under a hood. Weight of dried sample was noted and the solids were suspended in 4 ml methylene chloride and concentration was calculated. Volume equivalent of 1 mg of sample was transferred to fresh 4 ml vials.

Diazomethane reagent preparation

Diazomethane reagent is used to methylate fatty acids in order to synthesize

fatty acid methyl esters. The detailed procedure for making the reagent is as follows:

Materials required

- 1-methyl 1-3-nitro-1-nitrosoguanidine (MNNG), 97% purity from Sigma Aldrich

- Methylene chloride HPLC grade from Fisher scientific, ACS reagent grade

- Sodium Hydroxide from Fisher Scientific

- Glacial acetic acid (ACS reagent grade) 99.7% from Fisher Scientific

- Screw capped borosilicate glass vial (15 ml)- fitted with gas-tight, teflon faced silicon septum closure from Supelco Co.

- 5 ml, 20 guage Luer-lok tip disposable Plastipak syringe from Beckton-Dickinson

- 12-inch length of 0.53 mm i.d. deactivated fused silica tubing from Scientific Instrument Services, Inc.

20 ml of Methylene chloride was taken in a pyrex heavy duty glass tube. A 6 N NaOH solution in distilled water was prepared and the disposable syringe was filled with this solution. About 500 mg of Nitrosoguanidine was sealed into a large glass vial with the sealed septum. This vial was placed in a dry ice bath. One end of the silicone tubing was pushed through the septum of the vial containing MNNG. The other end of the tubing end was immersed in the methylene chloride tube. Care was taken that the tube is submerged all the way to the bottom of this

tube. Once this setup was ready, the NaOH filled syringe needle was inserted into the septum of the MNNG vial and NaOH was injected drop by drop into the vial. As the NaOH comes into contact with the MNNG, a violent reaction occurs producing Diazomethane gas. The setup is shown in Fig. 3.26.
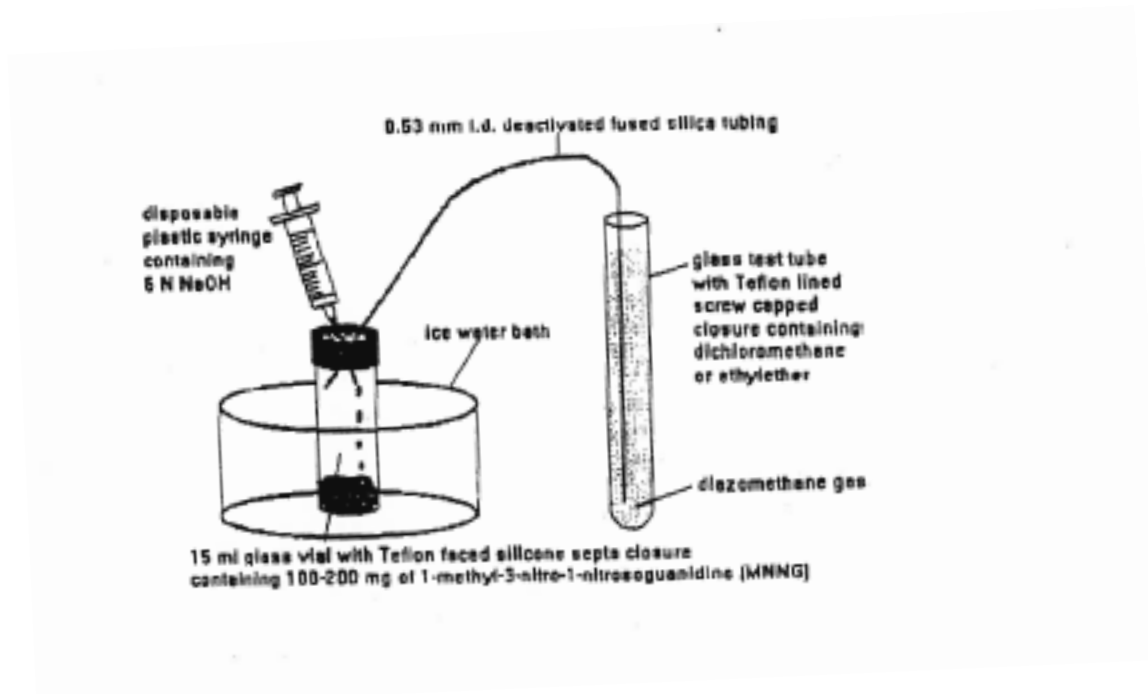

Figure 3.26: Preparation if the diazomethane reagent

This gas travels through the tubing into the Methylene chloride tube. Injection of NaOH was continued until there is no further reaction with MNNG. The methylene chloride solution becomes deep yellow-orange indicating formation of a saturated solution of diazomethane. The end of the silica tubing was removed from this tube and it was capped tightly. This is the methylation reagent. It was made fresh right before use every time. Although the lifetime of this reagent is

that of about a week, it is strongly recommended that it be generated fresh and used the same day due to its hazardous nature.

Extreme precaution was exercised while conducting this procedure. Necessary protective gear was worn. The precursors MNNG and diazomethane are known mutagens and carcinogens. Diazomethane is extremely flammable and can explode if exposed to heat sources and sparks. The exhausted MNNG vial and remnant methylation reagent were disposed properly after use.

Methylation of saponified samples

About 2-3 ml of methylation reagent was added to the vials containing 1 mg of saponified fatty acids. It was then allowed to react at room temperature for an hour. At the end of the reaction, the fatty acids are methylated to their corresponding methyl esters and the samples are ready to be injected in the GC-MS.

3.4.1.1 Analysis of saponifiable fraction

1 microliter of the methylated saponifiable fraction was injected into Varian GC 3400 with flame ionization detector on split mode (split ratio 10:1). The column selected for this analysis was a HP-FFAP capillary column of 50 m length. The I.D. (internal diameter) of the column was 0.32 mm with a film thickness of 0.52 µm. The temperature of the injector port was set at 220 $^{\circ}$C. Column temperature was initially set at 40°C for 3 minutes followed by a steady rise at 10 $^{\circ}$C/minute till

it reached a final temperature of 240 ^{o}C. This was achieved using the temperature-programming feature. Carrier gas used was Helium with a head pressure of 20 psi. The detector temperature was set at 250 ^{o}C and make up gas (Helium) was used at a flow rate of 30 ml/min. The injection port septum was changed and the column was baked out before analysis to avoid contamination.

Purification and preparation of non-saponifiable fraction:

The hexane from the hexane extracts of non-saponifiable fraction was evaporated using rotary evaporator. At the end of evaporation, the rotavac flask was rinsed with methylene chloride, to dissolve the non-saponifiable matter from the walls of the flask. The solution was transferred to fresh tare-weighed borosilicate capped glass vials labeled as 'non-saponifiable fraction'. The vials were then blown to dryness under nitrogen; weight of dry matter was measured and re-suspended in methylene chloride to get a concentration of 1 mg/ml of non-saponifiable in methylene chloride. These samples were ready for injection into GC-MS.

3.4.1.2 Analysis of non-saponifiable fraction

1 µl of the prepared non-saponifiable fraction sample was injected into Varian GC 3400 with flame ionization detector on split-less mode. The column selected for this analysis was a ZB-5MS column of 30 m length with 0.32 mm internal diameter. The temperature of the injector port was set at 300 ^{o}C. Column temperature was initially set at 40 ^{o}C for 3 minutes followed by a steady rise at

10 °C/minute till it reached a final temperature of 320 °C. This was achieved using the temperature-programming feature. Carrier gas used was Helium with a head pressure of 10 psi. The detector temperature was set at 250 °C and make up gas was used at a flow rate of 30 ml/min. The injection port septum was changed and the column was baked out before analysis to avoid contamination.
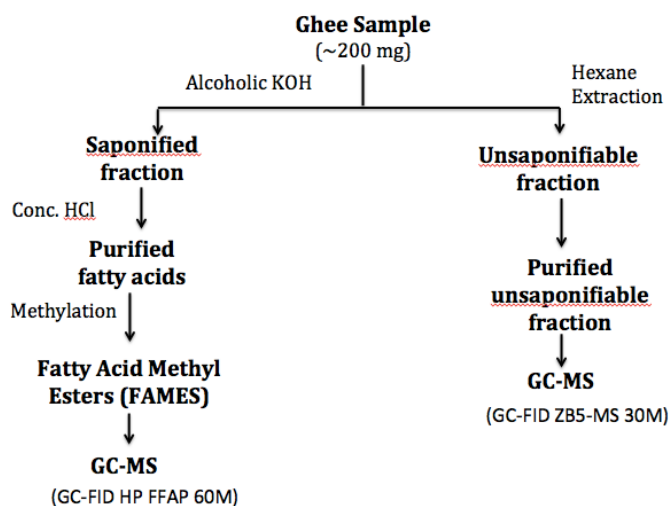
Figure 3.27:Treatment of ghee samples for analysis of saponifiable and non-saponifiable fractions

Therefore, in summary, each ghee sample was saponified and separated into two distinct fractions, which were further purified, processed and analyzed. The chart in Fig. 3.27 summarizes the treatment of each ghee sample, for analysis of saponifiable and non-saponifiable fractions.

3.4.2 Headspace volatiles analysis

1 g of ghee sample was taken in a glass vial and sealed with a silicon cap (Fig. 3.28). The sample was spiked with an internal standard comprising of Benzene, Toluene and Naphthalene. The vial was then baked at 100 ^{o}C for an hour. This helped the volatile compounds in the sample and the internal standard to volatilize and build up in the headspace of the vial.
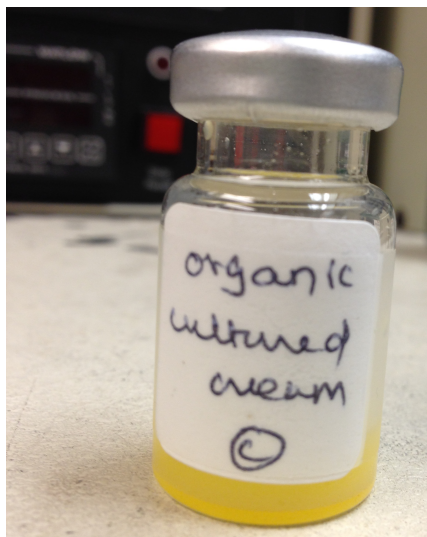
Figure 3.28: Sealed glass vial with ghee sample

At the end of baking, the built-up headspace gas was sucked using a syringe and injected into the GC. Output from the GC was fed to the MS connected in series. Figure 3.29 shows a typical chromatogram for a ghee headspace volatile sample.

```
Analysis Name: DU00:(300,300)FM45451.DAT;1        Max. Spec: 1350
Operator: DR. TOM HARTMAN
SPC:60V ■Frq:25.00 kHz ■NSAM: 6    ■Res:  1000■Date: 23-JAN-13  15:51
RTOL: 700.0MMU ■Mass:    35-   351  ■Cycle:1.318sec ■Instr:
Commission: GHEE HEADSPACE
SAMPLE 2, ORGANIC TRADITIONAL, 1.0450G/100C/30MIN/10UG IS/HS-GC-MS
```
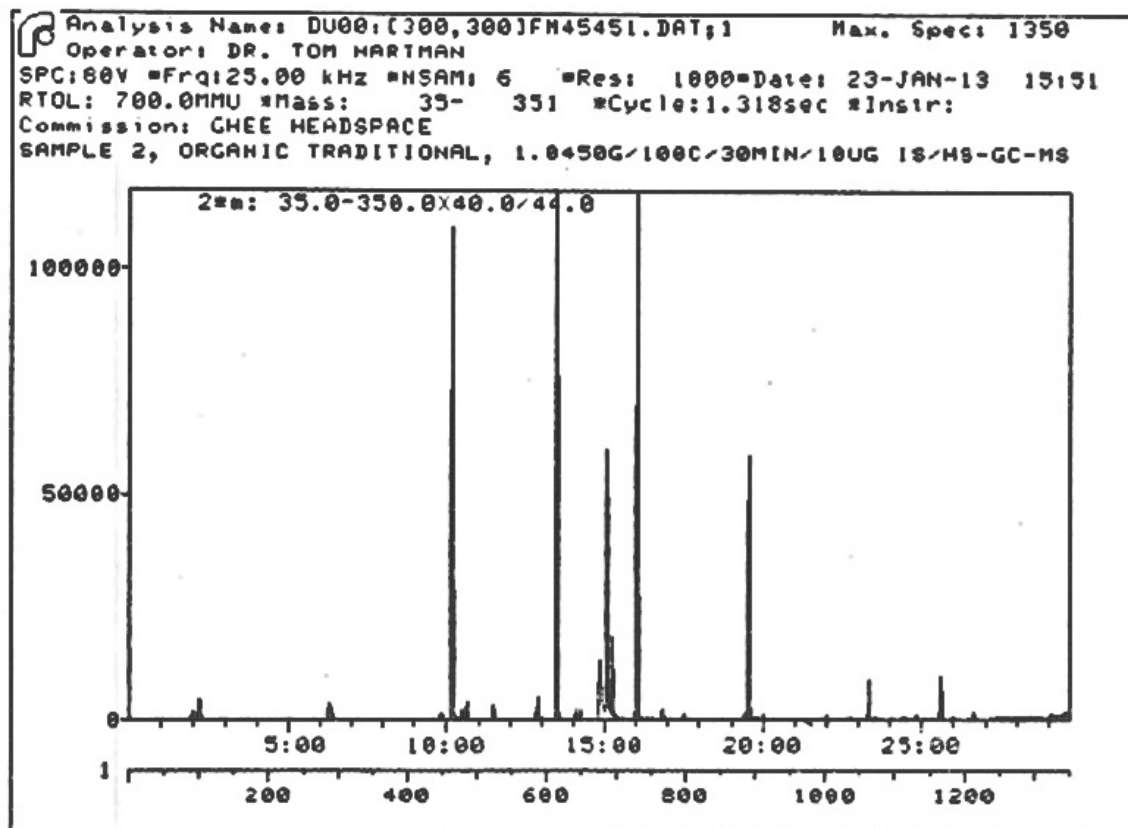
Figure 3.29: Typical chromatogram of headspace volatiles sample of ghee

3.4.3 Sensory Evaluation

Difference from control test was performed in order to determine if a difference in aroma profiles of ghee made by different methods. The following resources were used for the sensory evaluation:

- Ghee samples made from organic grass-fed cow milk by all three methods (direct cream, traditional and cultured cream method)
- Glass vials
- Labels
- Panel members

- Ballot sheet

- SAS program for statistical analysis

Method

The purpose of the 'difference from control' test is to determine the degree of difference between samples, compared to a control (Kemp et al., 2009). This test requires a minimum of 50 participants. The study was conducted in Dr. Beverly Tepper's lab (Sensory evaluation lab, Rutgers University, NJ) with an untrained panel members consisting primarily of faculty, staff and students. The age group of the panel members ranged from 20 years to 55 years. Analyzing the difference in aroma profiles as a result of different methods of preparation of ghee was the purpose of this study. Hence with Dr. Tepper's advice, only the three ghee samples made from organic grass-fed cow milk were used. Also, for the purpose of this evaluation, ghee made by direct cream method, being the least processed sample, was treated as the 'control' sample.

The control was then compared against three test samples: traditionally made ghee sample, ghee made by cultured cream method and blind control (ghee made by direct cream method). All the test samples were marked with a random 3-digit code. A total of 55 subjects participated in the study. Each subject was presented with a labeled control sample and one test sample marked with the 3-digit code (Fig. 3.30). The blind control helps to establish a base line for the rest of the test samples and reduce error, as most blind controls will get a non-zero score due to individual variability (Lawless, 2003).

The panel members were thus given three pairs of samples, one pair at a time. Each pair consisted of a label control and one of the three samples (labeled with a three-digit code) in a random order.
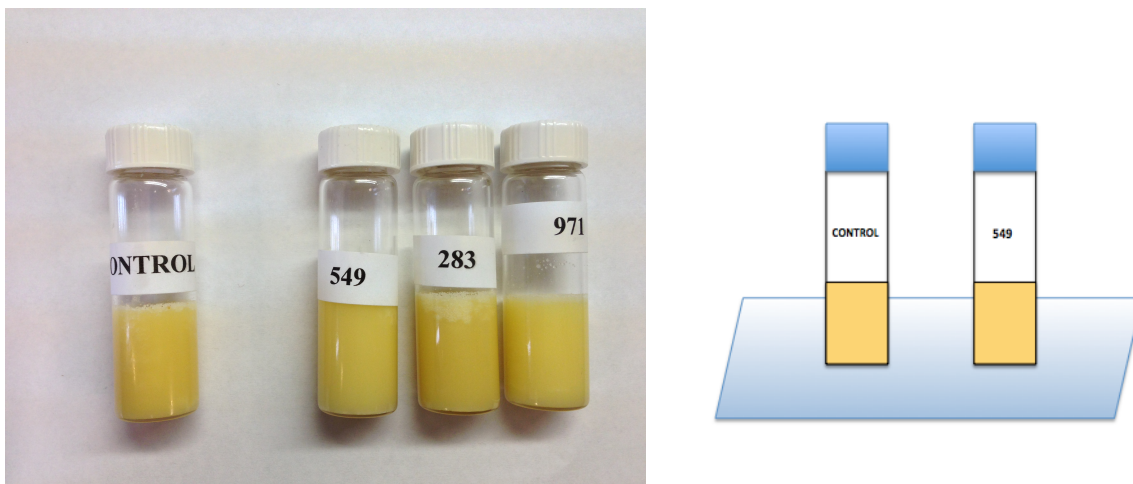
Figure 3.30: Labeled vials with ghee samples for sensory study (left), manner of presenting samples to subjects participating in the sensory study (right)

Individual participants were asked to open the vials and smell the samples. They were then asked to rate the degree of the difference between the test sample and the labeled control. The verdict was recorded on the ballot sheet as shown in Fig. 3.31. To interpret the results, the boxes were given scores from 0-8 (left to right) and scores were calculated depending on how further from control the test sample was perceived. 5 sets of samples were alternated as they were given to participants, providing a 20-minute rest period for every vial between two tests in order for the volatile compounds to build up in the headspace.

Subject ID:_____   Date:_____

You will receive pairs of samples to smell. Please smell the sample marked "Reference" first and then the sample with the 3-digit code. Assess the overall difference between the samples, and then mark that difference by placing an "X" in the boxes below.

Please open the bottle and smell it, then put the cap back on as soon as you are finished smelling it. Also, please allow time between samples to allow for a change to be perceivable.

When you are finished with the first pair, please turn on your signal light to indicate to the server that you are ready for the next set of samples.

Pair 1: Reference /_____
How different are the two samples?

No Difference   Extremely different

Pair 2: Reference /_____
How different are the two samples?

No Difference   Extremely different

Pair 3: Reference /_____
How different are the two samples?
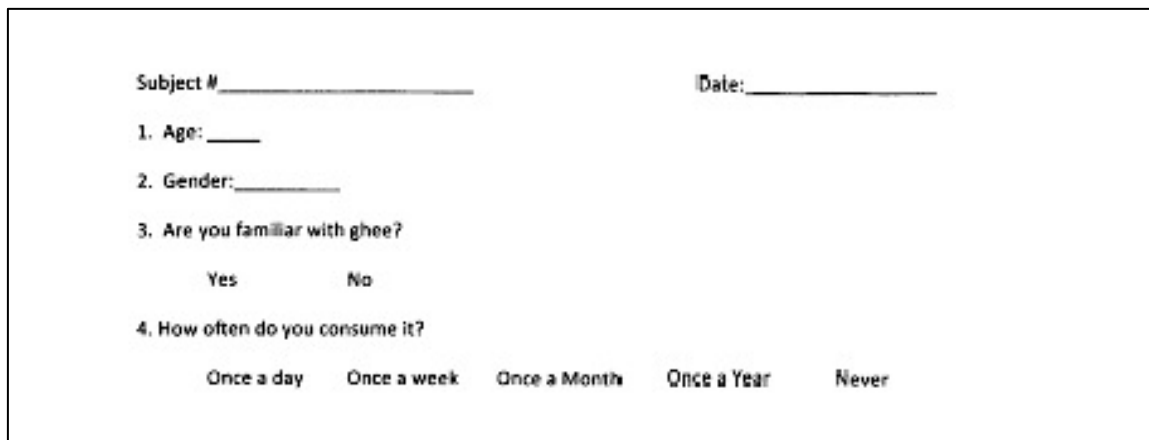
No Difference   Extremely different

You are now finished with this part of the study. Please turn your booth light on to indicate to the experimenter that you are done. Thank you for your participation.

Figure 3.31: Sample ballot sheet used for the 'difference test' for sensory analysis

Additionally, a basic questionnaire was filled by each participant to record their familiarity with and frequency of consumption of ghee (Fig. 3.32). A score of 0 or 1 (No and Yes) was accounted for in the final results in order to test whether familiarity made any difference with perception of difference.

Subject #_____   Date:_____

1. Age: _____

2. Gender:_____

3. Are you familiar with ghee?

      Yes   No

4. How often do you consume it?

      Once a day   Once a week   Once a Month   Once a Year   Never

Figure 3.32: Sample questionnaire used for sensory study

Results were recorded in a spreadsheet and statistical analysis of the data was done using SAS.

3.4.4 Colorimetric analysis

Color of the ghee samples prepared by different methods was measured using a CR – 410 Konica Minolta chroma meter as shown in (Fig. 3.33, left). The instrument was calibrated using a white D65 standard disc (Y = 94.7, x = 0.3156 and y = 0.3319). Ghee samples were placed in a circular clear plastic dish that perfectly fits the top of light port of the instrument. L*, a* and b* values were then measured and recorded for all the samples. The CIELAB color space is shown in

the (Fig. 3.33, right).
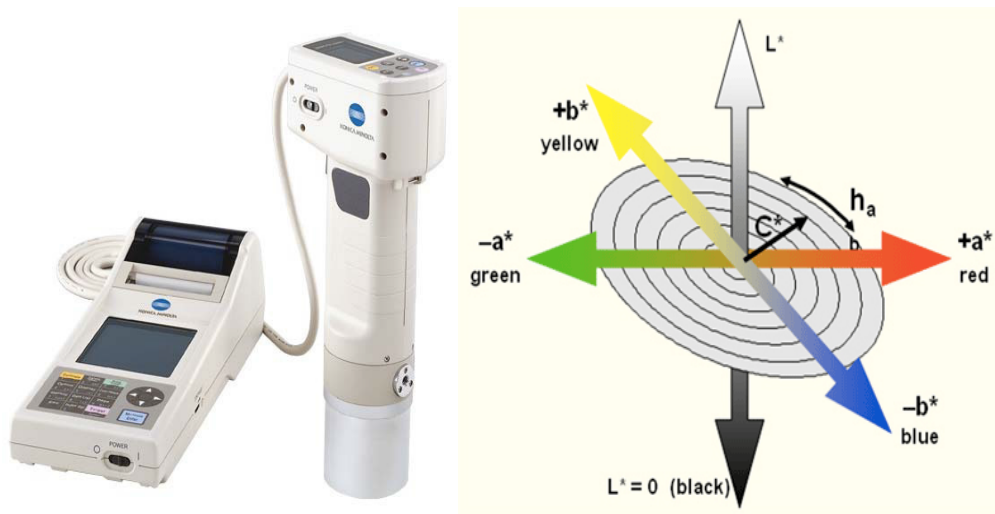
Figure 3.33: Konica Minolta chroma meter (left) and CIELAB color space

4. Results and Discussion

The results from the different analyses performed on the six different ghee samples (two milk sources and three methods of preparation) are presented and discussed in this chapter. Differences in the fatty acid profiles, Cholesterol content, flavor profiles, aroma profiles (sensory) and color measurement were evaluated and compared across the six samples.

4.1 Fatty Acid Profiles

Based on the mass spectrum obtained for the saponifiable fraction samples and the 37 FAME mix standard, using Mass spectrometry, peaks separated by GC were identified. Each fatty acid peak on the mass spectrum was correlated to the corresponding peak on the chromatogram. This was done for all the peaks on all the chromatograms. Once the peaks were identified, concentration of each compound was calculated on the basis areas under the peak in the chromatogram. The chromatograph generated by the software includes vales for area under the peak for each peak.

4.1.1 Overall Fatty Acid Profiles

Percentage concentration of each fatty acid was calculated with respect to total fatty acid content. Area under the peak for each fatty acid was divided by total area under all fatty acid peaks was used for this calculation.

Concentration of fatty acid 'X'=_____Area under the peak for fatty acid 'X'_____
Σ (Area under each peak on the chromatogram)

A total of 20 different fatty acids were identified in every sample. The overall fatty acid profile of all the samples was similar consisting of this set of 20 fatty acids in varying amounts over a limited range of concentrations as summarized in Table 4.1. Some of the peaks that are unidentified are either impurities, or in amounts below detectable limits.

Fatty acid	name		Conc. Range (%)
C4	butanoic	butyric	0.8-1.6
C6	hexanoic	caproic	1.6-2.4
C8	octanoic	caprylic	1.3-1.7
C10	decanoic	capric	2.9-3.7
C10:1			0.26- 0.36
C11	undecanoic acid		0.3-0.4
C12	dodecanoic	lauric	3-4.5
C13	tridecanoic acid		0.2-0.46
C14	tetradecanoic	myristic	12.5-13.5
C14:1		myristoleic acid	0.4-1
C15	pentadecanoic acid		0.4-1
C15:1	cis-10-pentadecenoic acid		0.25-0.45
C16	hexadecanoic	palmitic	31-35
C16:1	hexadecenoic acid	Palmitoleic	1.5-4
C17	heptadecanoic	margaric (daturic)	0.6-1.1
C17:1			0.3-0.5
C18	octadecanoic	stearic	10-14
C18:1		oleic acid	18-25
C18:2		linoleic acid	0.9-1.5
C18:3		linolenic acid	0.6-1.2

Table 4.1: Relative percentage concentration ranges of different fatty acids of ghee.

Myristic acid, palmitic acid, stearic acid and oleic acid are the major fatty acids of milk fat (highlighted in Table 4.1). They make up for about 80% of the total fats (Tamime, 2009).

Figure 4.1 shows a cluster diagram of the four major fatty acids for the 6 distinct samples.
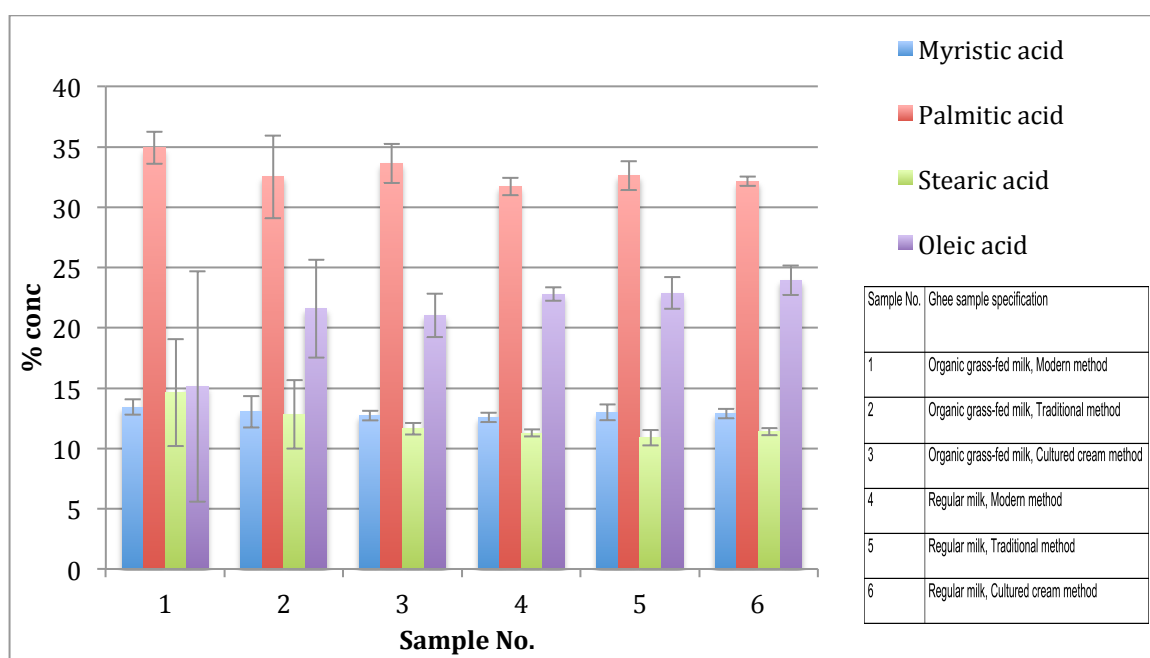
Figure 4.1: Comparison of concentration profile of the four major fatty acids of ghee in the six experiment samples (refer to key).

4.1.2. Individual Fatty Acids

Concentration of each fatty acid was compared across the six samples. Statistical analysis using two-factor ANOVA revealed no significant difference. All

samples showed high P-values indicating that the differences based on processing method and source of milk were statistically insignificant. The following sections present results for individual fatty acids.

Butyric acid

It is the smallest fatty acid detected in all samples. It was found in the range of 0.6% to 2%. Figure 4.2 shows C4 concentrations for the 6 different samples tested. Milk fat is the sole source of butyric in the diet. butyric acid is a potent anti-cancer agent that inhibits cell proliferation and induces differentiation and apoptosis. (Kurita-Ochiai, 2001).
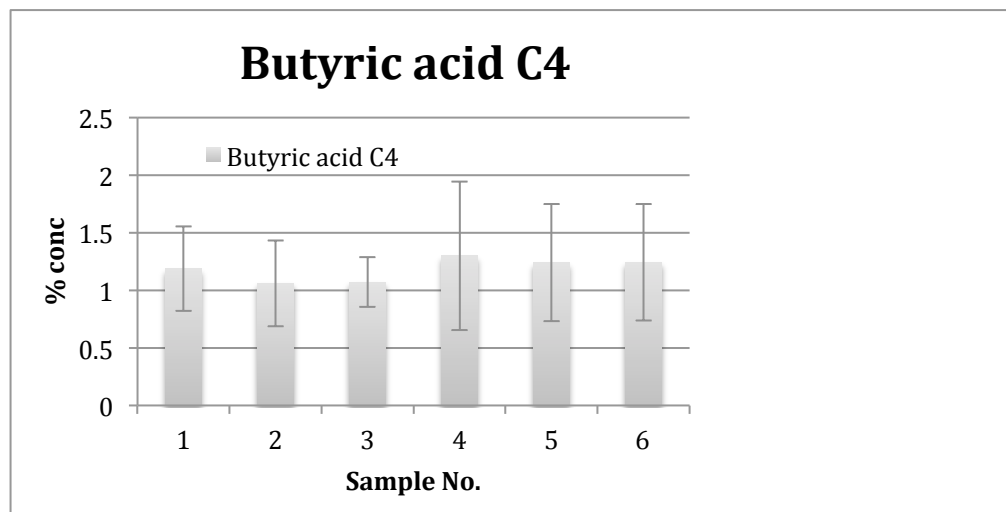
Figure 4.2: Comparison of C4 fatty acid in the six samples

C4

Source	Sum Sq.	d.f.	Mean Sq.	F	Prob>F
Process	0.03271	2	0.01636	0.09	0.9133
Source	0.10671	1	0.10671	0.6	0.4531
Error	2.50847	14	0.17918		
Total	2.64789	17			

Table 4.2: Two factor ANOVA for C4 fatty acid (factor 1: source of milk;

factor 2: method of preparation)

The two-factor ANOVA result tables show that the changes in concentration levels of these fatty acids between samples is not statistically significant and not related to difference in processing treatment or source of milk.

Myristic acid

This 14 carbon fatty acid was detected in both saturated (myristic) as well as monounsaturated (myristoleic) forms. Myristic acid is one of the four major fatty acids in milk and is found at a high concentration of 11.6% -14.2%.

Myristic acid C14
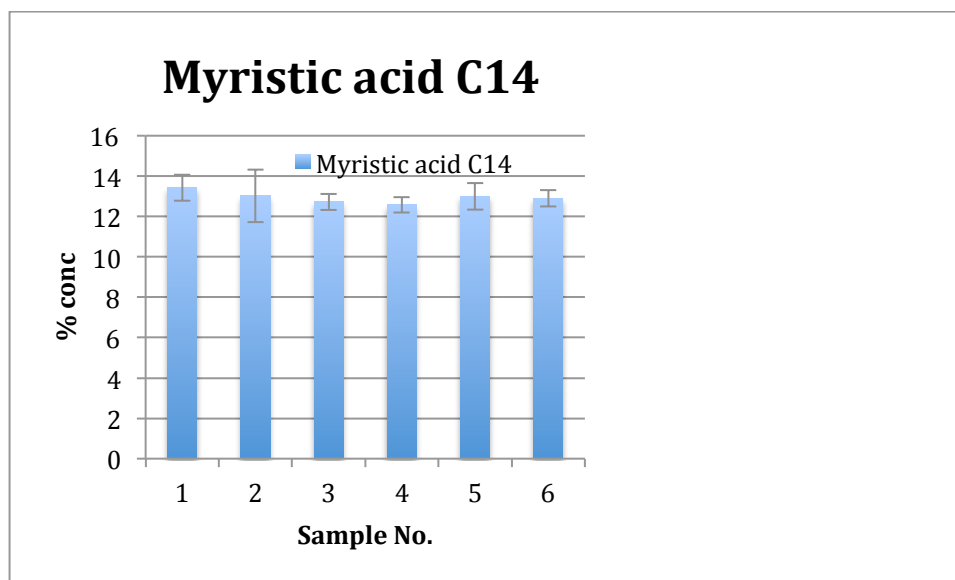
Figure 4.3: Comparison of C14 fatty acid in the six samples

```
C14
 Source   Sum Sq.  d.f.  Mean Sq.   F    Prob>F
-----------------------------------------------------
 Process  0.15391   2   0.07695   0.16  0.8568
 Source   0.2415    1   0.2415    0.49  0.4953
 Error    6.89623  14   0.49259
 Total    7.29164  17
```

Table 4.3: Two factor ANOVA for C14 fatty acid (factor 1: source of milk;

factor 2: method of preparation)

Palmitic acid

This is another one of the four major fatty acids of milk. It is the most abundant

fatty acid present in milk. It was detected at levels ranging from 28% -36%. The

differences in the concentrations of this fatty acid were tested for statistical

significance using ANOVA. As seen from the ANOVA results in Table 4.10, the

low P-value suggests the processing technique or the source of milk did not affect the concentration of C16 in the ghee.

Although differences were found in concentration of C16:1 (0.3-5.7%) over the six samples tested, the difference wasn't significant and/or related to the variable parameters of processing technique and source (Fig. 4.10).
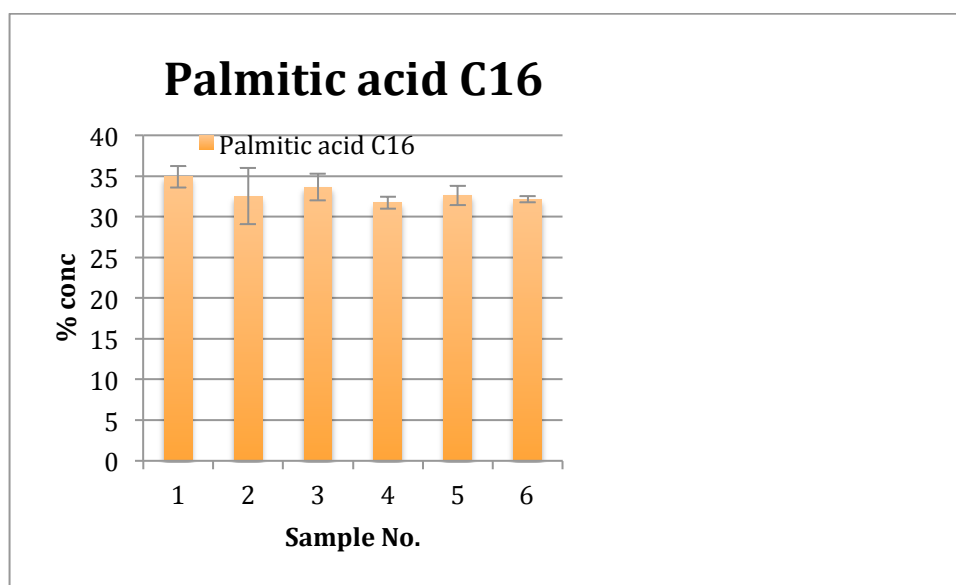
Figure 4.4: Comparison of C16 fatty acid in the six samples

```
C16
 Source   Sum Sq.   d.f.   Mean Sq.    F     Prob>F
-------------------------------------------------------
 Process   1.6933     2     0.8466    0.26   0.7716
 Source    10.567     1     10.567    3.3    0.0909
 Error     44.864    14     3.2046
 Total     57.1243   17
```

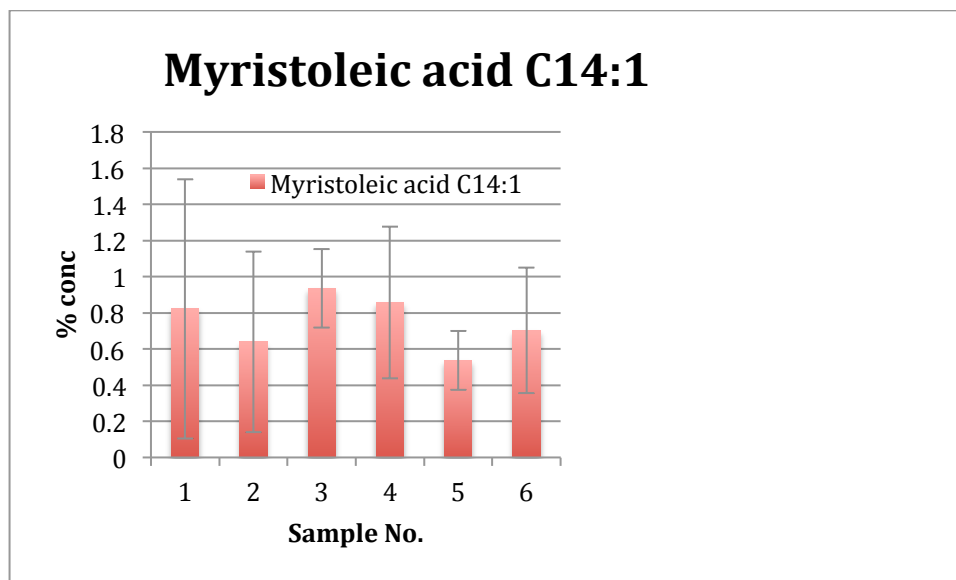Table 4.4: Two factor ANOVA for C16 fatty acid (factor 1: source of milk; factor 2: method of preparation)

Stearic acid and Oleic acids

The third major fatty acid in milk, stearic acid, typically found between 10-20%. The fourth and last major fatty acid of milk is oleic acid and was detected 4-26%. Large variations were found in the concentrations of these fatty acids in the six samples tested. Two-factor ANOVA revealed a high for the variable of processing method, indicating it did not have any effect on the concentration of stearic acid in the final product. The variable of milk source yielded a comparatively lower P-value indicating difference in stearic acid concentrations based on source of milk. However the P-value was still high (0.09 and 0.07 for C18 and C18:1 respectively.) to establish statistical significance.

Saturated fatty acids of milk have been demonized for increasing the cholesterol levels. However different saturated fatty acids have different extent of contributing to this effect. Short chain fatty acids butyric, caproic and caprylic acids and medium chain fatty acids capric and stearic have no significant effect on cholesterol levels. However myristic, lauric and palmitic acids do raise cholesterol levels. Myristic acid being the most potent. However these three fatty acids increase the level of HDL cholesterol (Daley, 2010).
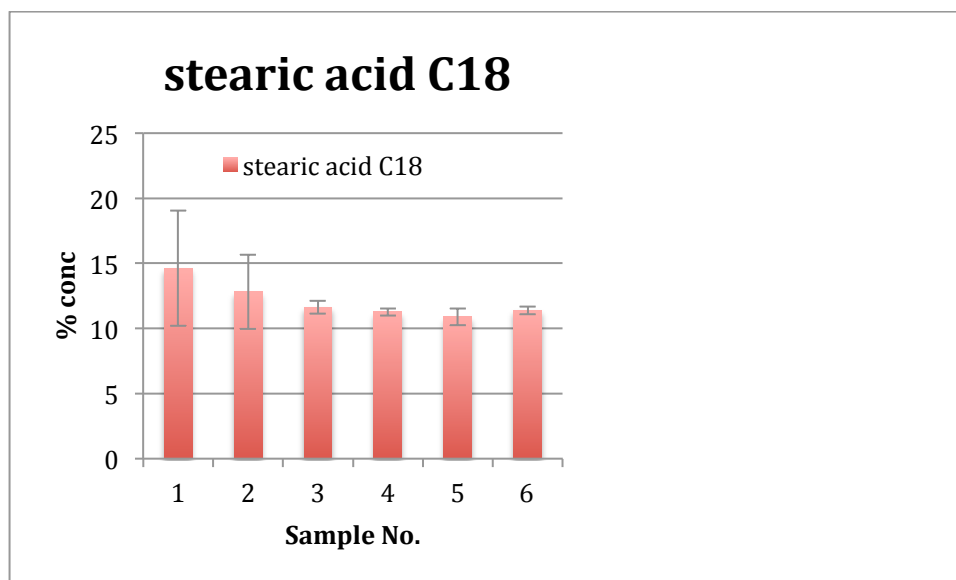
Figure 4.5: Comparison of C18 fatty acid in the six samples

```
C18
 Source    Sum Sq.   d.f.   Mean Sq.    F     Prob>F
 ------------------------------------------------------
 Process    6.7403     2     3.3701    0.73   0.4987
 Source    15.0963     1    15.0963    3.28   0.0918
 Error     64.4926    14     4.6066
 Total     86.3292    17
```

Table 4.5: Two factor ANOVA for C18 fatty acid (factor 1: source of milk;
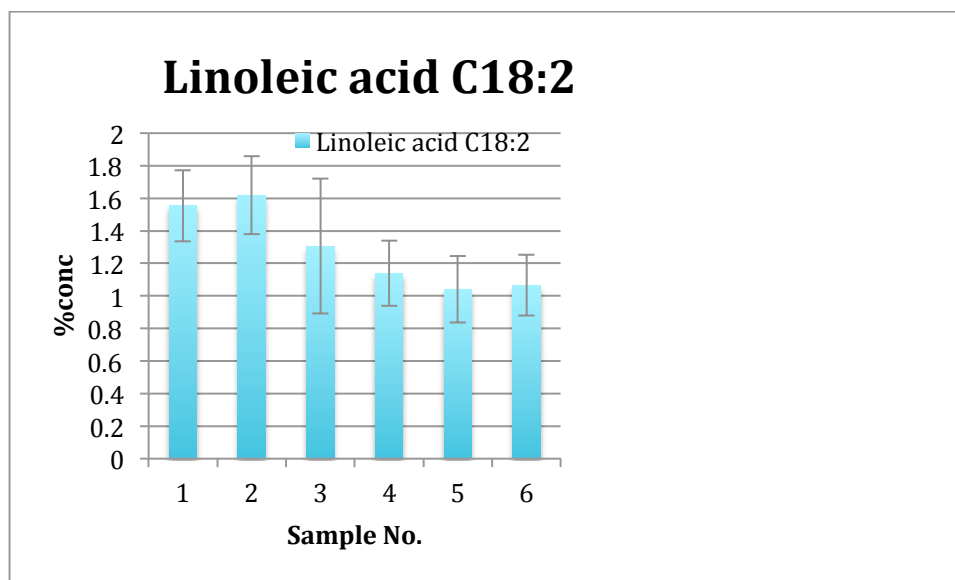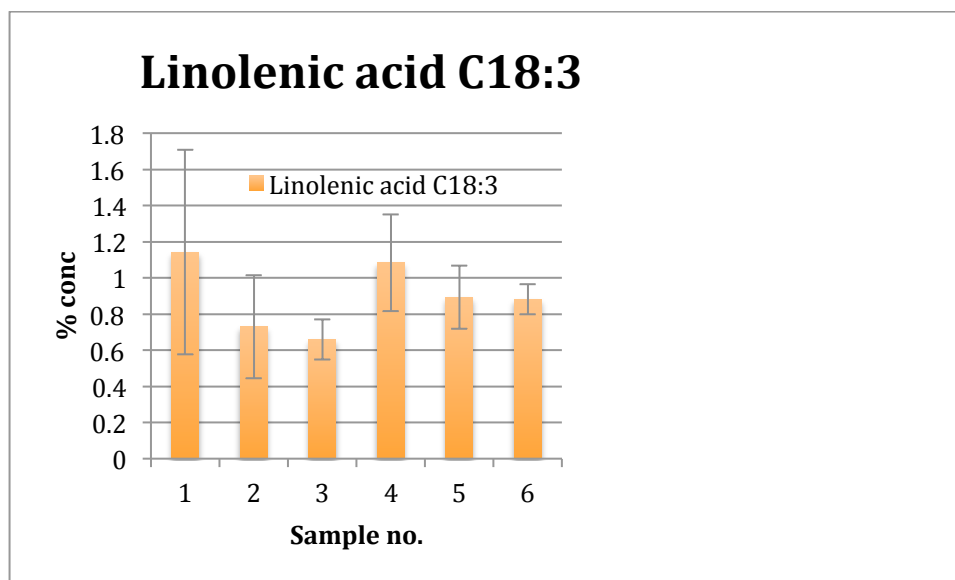
factor 2: method of preparation)
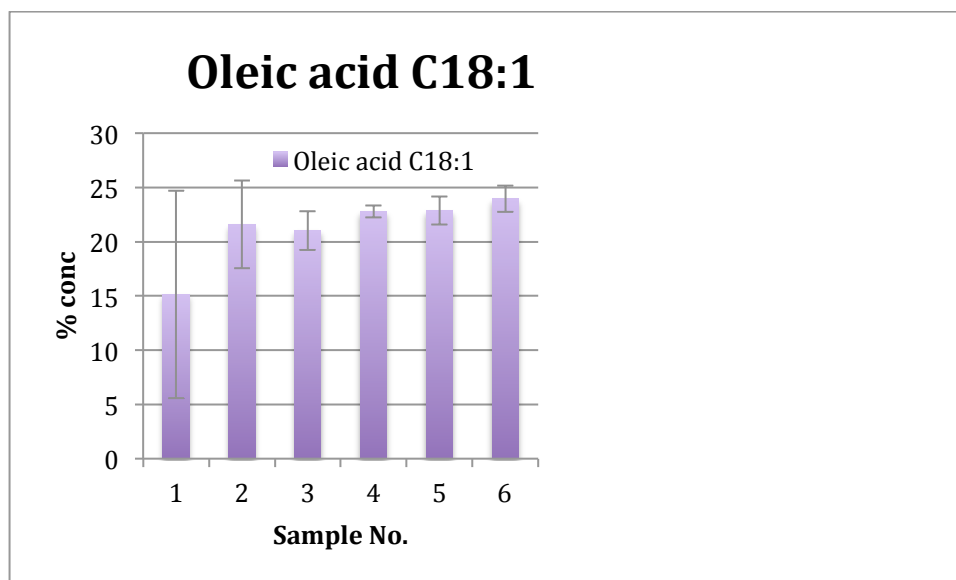
Figure 4.6: Comparison of C18:1 fatty acid in the six samples

```
C18.1
 Source   Sum Sq.  d.f.  Mean Sq.    F    Prob>F
------------------------------------------------------
 Process   46.203   2   23.1016   1.23  0.3211
 Source    70.503   1   70.5027   3.76  0.0728
 Error    262.213  14   18.7295
 Total    378.919  17
```

Table 4.6: 2-factor ANOVA for C18:1 fatty acid (factor 1: source of milk;

factor 2: method of preparation)

Other fatty acids

Figures 4.7 - 4.15 represent the data for the rest of the fatty acids that were

identified in the samples. The respective tables show the statistical data for the
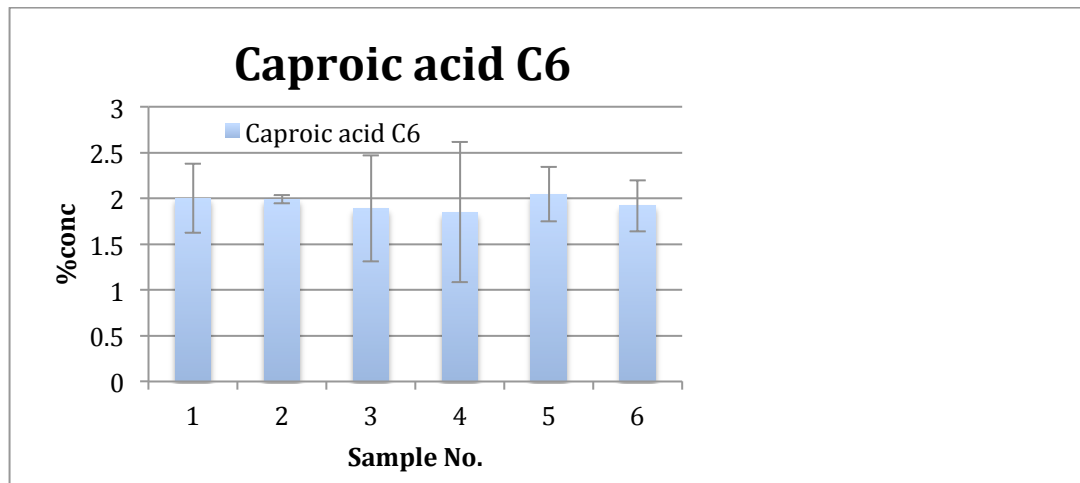
Figure 4.7: Comparison of C6 fatty acid in the six samples

```
C6
 Source   Sum Sq.  d.f.  Mean Sq.   F    Prob>F
 -----------------------------------------------------
 Process  0.04449   2    0.02224   0.12  0.8844
 Source   0.00243   1    0.00243   0.01  0.909
 Error    2.51214  14    0.17944
 Total    2.55906  17
```

Table 4.7: Two factor ANOVA for C6 fatty acid (factor 1: source of milk;

factor 2: method of preparation)
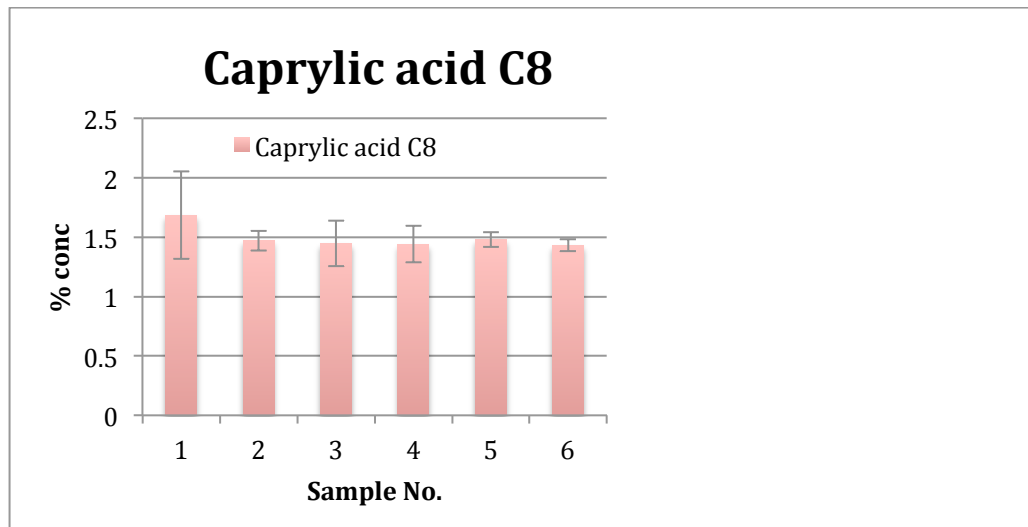
Figure 4.8: Comparison of C8 fatty acid in the six samples

```
C8
 Source   Sum Sq.  d.f.  Mean Sq.   F    Prob>F
-----------------------------------------------------
 Process  0.04924   2   0.02462  0.72  0.5029
 Source   0.03098   1   0.03098  0.91  0.3566
 Error    0.47719  14   0.03408
 Total    0.5574   17
```

Table 4.8: Two factor ANOVA for C8 fatty acid (factor 1: source of milk; factor 2: method of preparation).
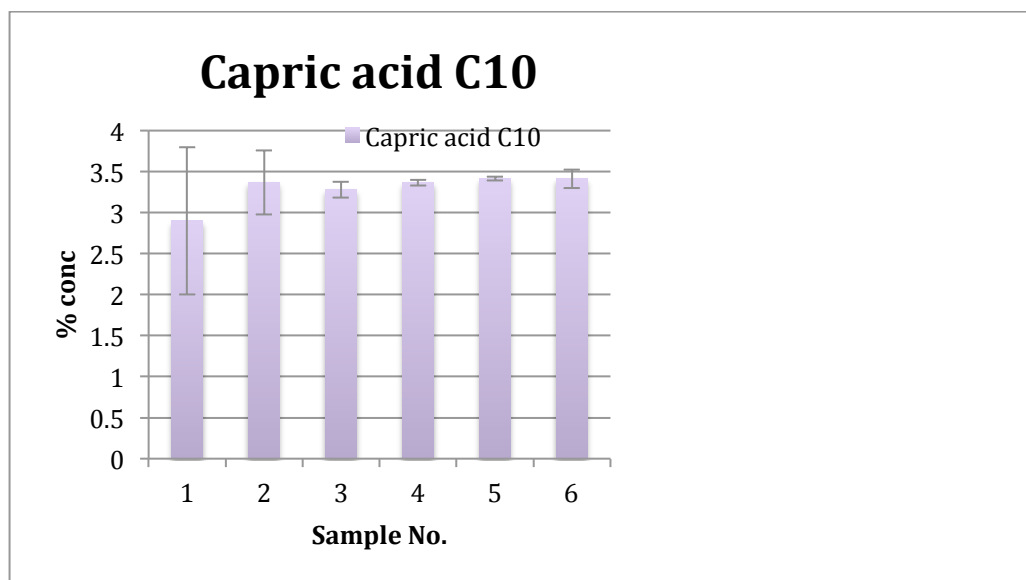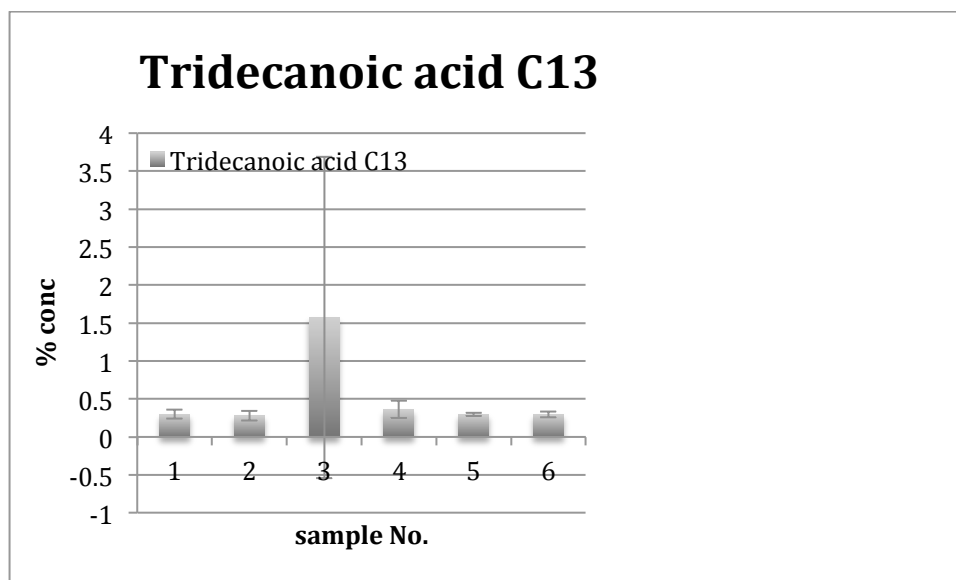
Figure 4.9: Comparison of C10 fatty acid in the six samples

C10

Source	Sum Sq.	d.f.	Mean Sq.	F	Prob>F
Process	0.226	2	0.113	0.75	0.4922
Source	0.20469	1	0.20469	1.35	0.2645
Error	2.12051	14	0.15146		
Total	2.5512	17			

Table 4.9: Two factor ANOVA for C10 fatty acid (factor 1: source of milk;

factor 2: method of preparation)
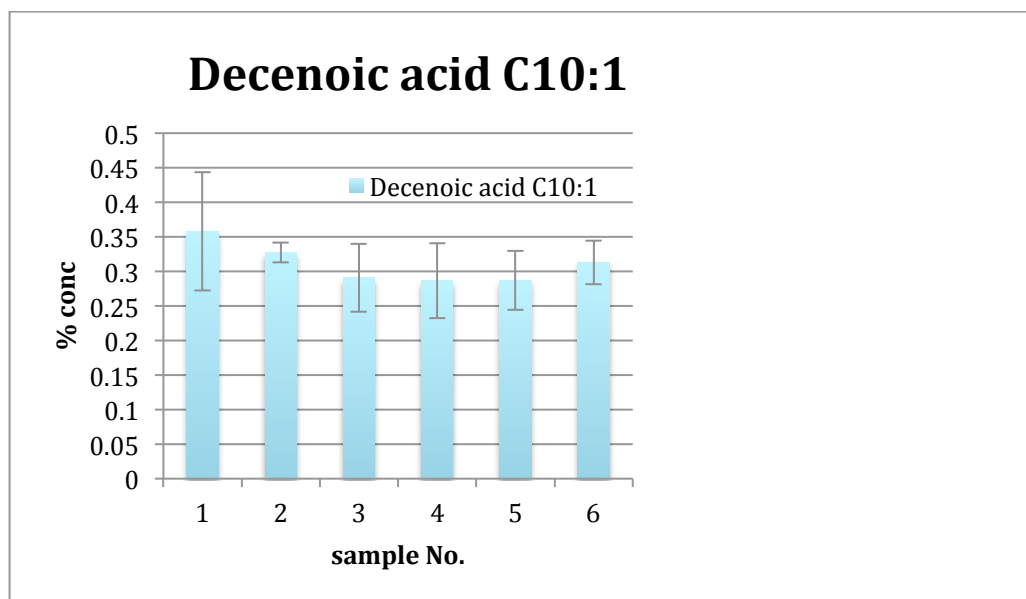
Figure 4.10: Comparison of C10:1 fatty acid in the six samples

```
C10.1
 Source   Sum Sq.  d.f.  Mean Sq.   F    Prob>F
 ----------------------------------------------------
 Process  0.00132   2   0.00066  0.24  0.7883
 Source   0.00405   1   0.00405  1.48  0.2435
 Error    0.03826  14   0.00273
 Total    0.04364  17
```

Table 4.10: Two factor ANOVA for C10:1 fatty acid (factor 1: source of milk;

factor 2: method of preparation)
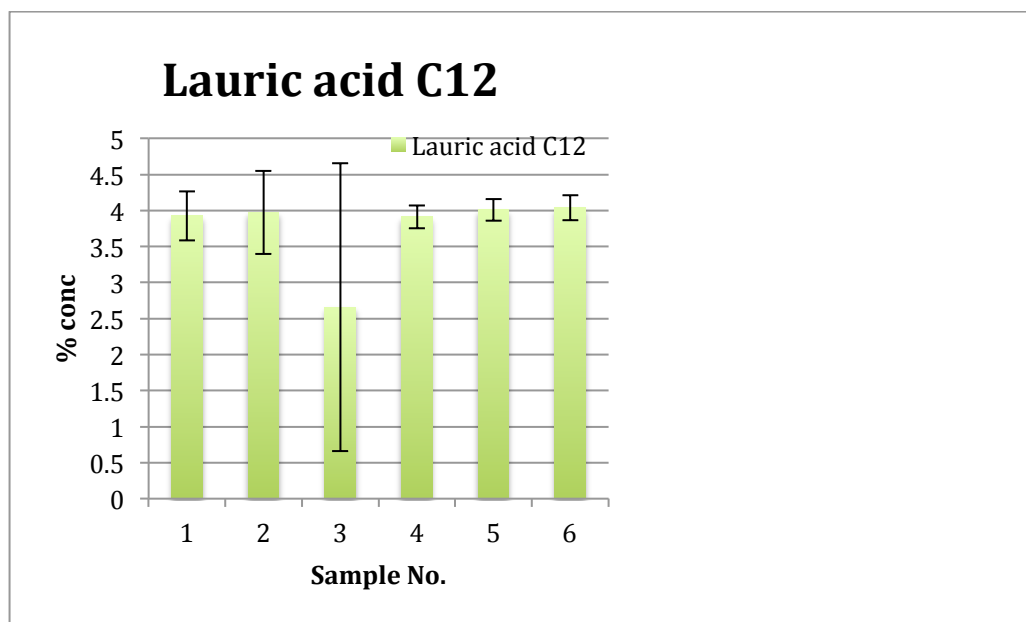
Figure 4.11: Comparison of C12 fatty acid in the six samples

In Fig. 4.7 for sample 3, large variation was due to experimental error in peak identification however the difference in concentration was verified for its statistical significance using ANOVA. It indicated no significant difference in levels of C12 with respect to processing method and source of milk.

```
C12
 Source   Sum Sq.  d.f.  Mean Sq.   F    Prob>F
-----------------------------------------------------
 Process   1.4918    2    0.74588   0.96  0.4076
 Source    0.9783    1    0.97831   1.26  0.2813
 Error    10.9067   14    0.77905
 Total    13.3767   17
```

Table 4.11: Two factor ANOVA for C12 fatty acid (factor 1: source of milk;
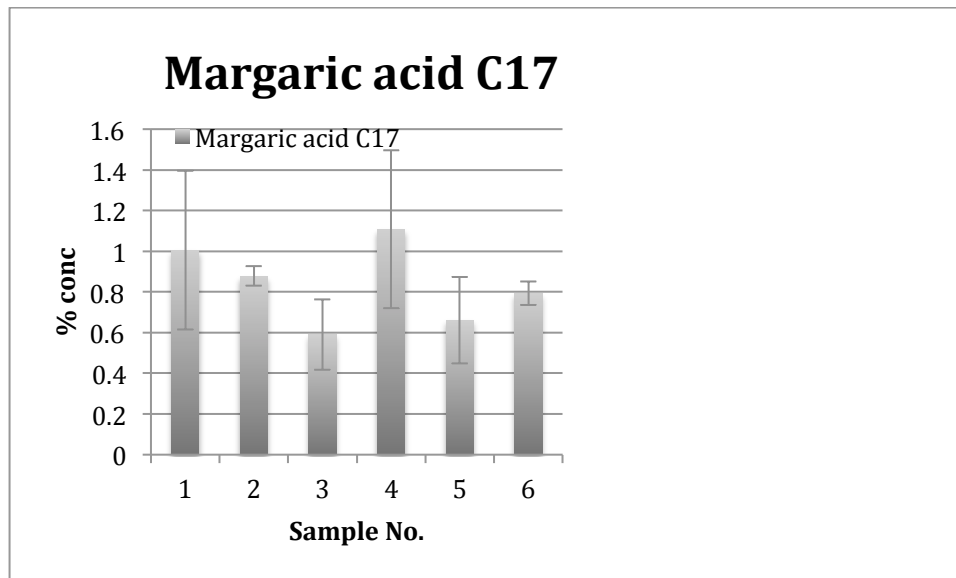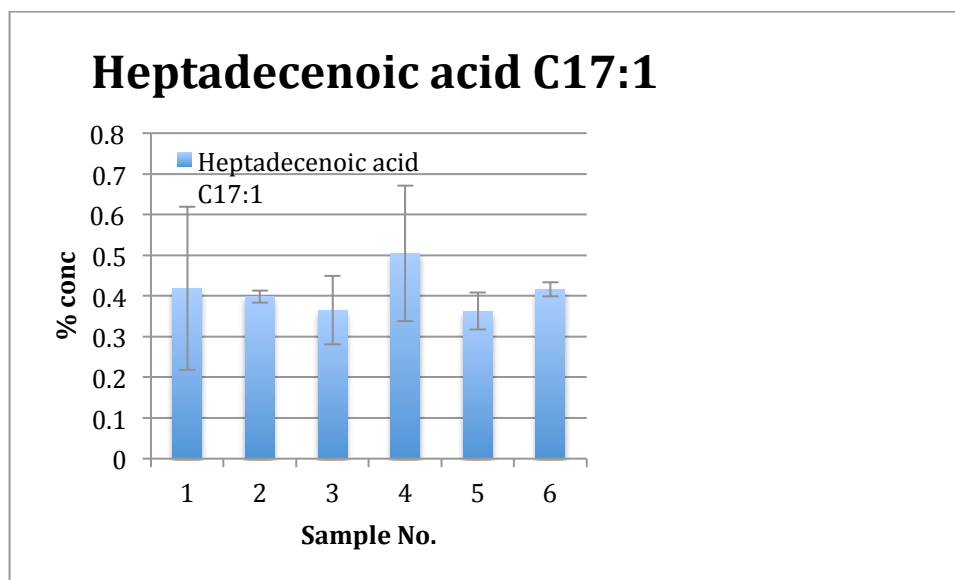
factor 2: method of preparation)

Figure 4.12: Comparison of C14:1 fatty acid in the six samples

```
C14.1
 Source   Sum Sq.  d.f.  Mean Sq.   F    Prob>F
-----------------------------------------------------
 Process  0.23439   2   0.11719   0.71  0.5104
 Source   0.04573   1   0.04573   0.28  0.6079
 Error    2.32405  14   0.166
 Total    2.60417  17
```

Table 4.12: Two factor ANOVA for C14:1 fatty acid (factor 1: source of milk;

factor 2: method of preparation)
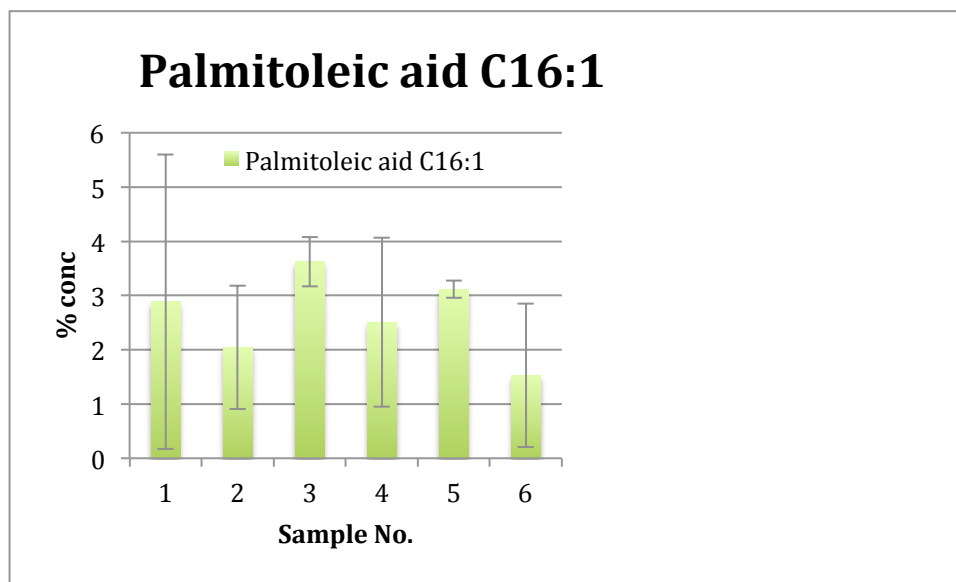
Figure 4.13: Comparison of C16:1 fatty acid in the six samples

```
C16.1
 Source   Sum Sq.  d.f.  Mean Sq.    F    Prob>F
----------------------------------------------------
 Process   0.0553   2   0.02764   0.01  0.9886
 Source    0.988    1   0.98798   0.41  0.5315
 Error    33.6084  14   2.4006
 Total    34.6517  17
```

Table 4.13: Two factor ANOVA for C16:1 fatty acid (factor 1: source of milk;

factor 2: method of preparation)

Figure 4.14: Comparison of C18:2 fatty acid in the six samples

```
C18.2
 Source   Sum Sq.  d.f.  Mean Sq.    F    Prob>F
-------------------------------------------------------
 Process  0.09371   2   0.04686    0.75  0.4898
 Source   0.75711   1   0.75711   12.14  0.0036
 Error    0.87297  14   0.06235
 Total    1.72379  17
```

Table 4.14: Two factor ANOVA for C18:2 fatty acid (factor 1: source of milk;

factor 2: method of preparation)

Figure 4.15: Comparison of C18:3 fatty acid in the six samples

C18.3

Source	Sum Sq.	d.f.	Mean Sq.	F	Prob>F
Process	0.41912	2	0.20956	2.64	0.1063
Source	0.05306	1	0.05306	0.67	0.4271
Error	1.11026	14	0.0793		
Total	1.58244	17			

Table 4.15: Two factor ANOVA for C18:3 fatty acid (factor 1: source of milk;

factor 2: method of preparation)

4.1.3 Presence of Odd-carbon Chain Fatty Acids (branched odd chain fatty acids)

Milk fat contains a series of branched chain fatty acids that have odd number of carbon atoms (Jensen, 2002). Since animals and plants cannot synthesize this kind of fatty acid, the origin cannot be de novo synthesis in mammary glands or the

feed. The source can be traced to the structural lipids of certain rumen bacteria. These branched fatty acids are said to have anti-cancer properties (Yang, 2000).

Our hypothesis was that fermentation of fats with bacteria would lead to an increase in the odd-carbon chain fatty acids. This, however, was not observed in the experimental results. Figures 4.16 - 4.21 show the comparison of levels of different branched fatty acids in the six experimental samples. The ANOVA analysis tables provided alongside the charts show no significant difference in their levels was found as a result of difference in processing technique. Therefore, fermentation or no fermentation (by lactic acid bacteria) as a part of the process method had no effect on the final concentration of these fatty acids. In other words there was no further increase in the concentrations of odd carbon chain fatty acids in the fermented samples and hence it can be deduced that these fatty acids are exclusively contributed by bacteria in the rumen and not the lactic acid bacteria used for culturing the milk to make yogurt.
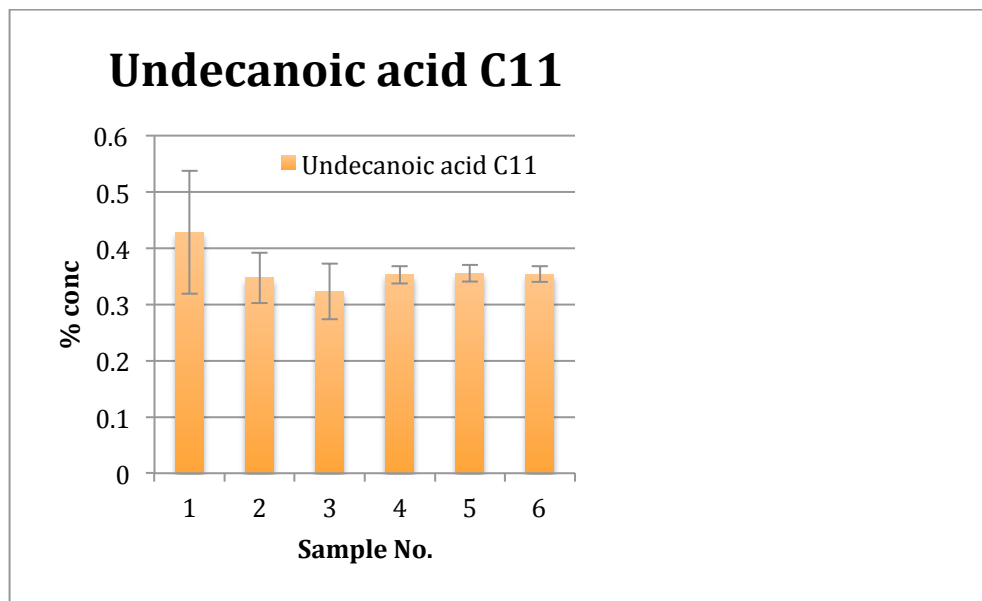
Figure 4.16: Comparison of C11 fatty acid in the six samples

```
C11
 Source   Sum Sq.  d.f.  Mean Sq.   F    Prob>F
------------------------------------------------------
 Process  0.00866   2   0.00433   1.4   0.2798
 Source   0.00067   1   0.00067   0.22  0.6496
 Error    0.0434   14   0.0031
 Total    0.05272  17
```

Table 4.16: Two factor ANOVA for C11 fatty acid (factor 1: source of milk;

factor 2: method of preparation)

Figure 4.17: Comparison of C13 fatty acid in the six samples

```
C13
 Source   Sum Sq.  d.f.  Mean Sq.   F    Prob>F
------------------------------------------------------
 Process   1.5598    2   0.77991   1.02  0.3864
 Source    0.7074    1   0.70744   0.92  0.3528
 Error    10.7212   14   0.7658
 Total    12.9885   17
```

Table 4.17: Two factor ANOVA for C13 fatty acid (factor 1: source of milk;

factor 2: method of preparation)
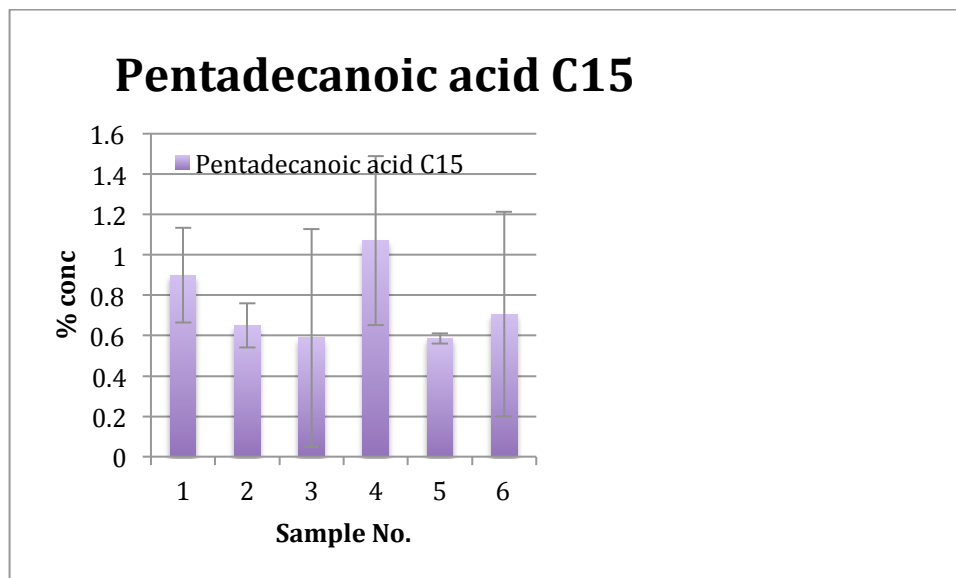
Figure 4.18: Comparison of C15 fatty acid in the six samples

```
C15
 Source   Sum Sq.  d.f.  Mean Sq.   F    Prob>F
------------------------------------------------------
 Process  0.23439   2   0.11719   0.71  0.5104
 Source   0.04573   1   0.04573   0.28  0.6079
 Error    2.32405  14   0.166
 Total    2.60417  17
```

Table 4.18: Two factor ANOVA for C15 fatty acid (factor 1: source of milk;

factor 2: method of preparation)
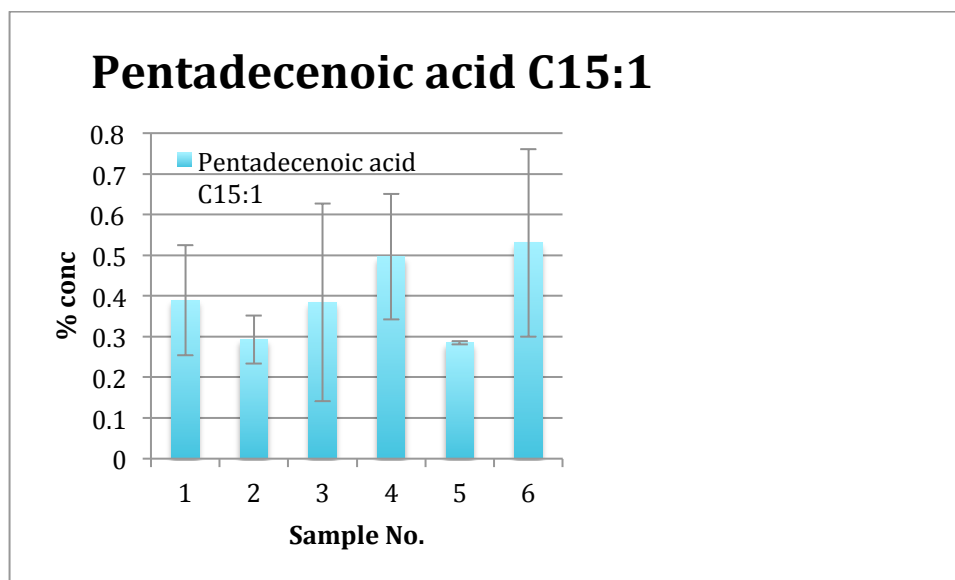
Figure 4.19: Comparison of C15:1 fatty acid in the six samples

```
C15.1
 Source   Sum Sq.  d.f.  Mean Sq.   F    Prob>F
----------------------------------------------------
 Process  0.10496   2    0.05248   2.2   0.1479
 Source   0.02993   1    0.02993   1.25  0.2818
 Error    0.33437  14    0.02388
 Total    0.46927  17
```

Table 4.19: Two factor ANOVA for C15:1 fatty acid (factor 1: source of milk;

factor 2: method of preparation)

Margaric acid C17

% conc vs Sample No.

Figure 4.20: Comparison of C17 fatty acid in the six samples

C17

Source	Sum Sq.	d.f.	Mean Sq.	F	Prob>F
Process	0.44283	2	0.22142	3.4	0.0625
Source	0.00401	1	0.00401	0.06	0.8076
Error	0.91101	14	0.06507		
Total	1.35786	17			

Table 4.20: Two factor ANOVA for C17 fatty acid (factor 1: source of milk;

factor 2: method of preparation)

Figure 4.21: Comparison of C17:1 fatty acid in the six samples

```
C17.1
 Source   Sum Sq.  d.f.  Mean Sq.   F    Prob>F
----------------------------------------------------
 Process  0.02338   2   0.01169   0.98  0.4003
 Source   0.0051    1   0.0051    0.43  0.5242
 Error    0.16731  14   0.01195
 Total    0.19579  17
```

Table 4.21: Two factor ANOVA for C17:1 fatty acid (factor 1: source of milk;

factor 2: method of preparation)

4.2 Cholesterol content

Figure 4.22 shows a typical chromatogram of the non-saponifiable fraction of ghee. The two major compounds detected by the analysis of non-saponifiable fraction were cholesterol and Squalene. The largest peak at retention time approximately 24 minutes is Cholesterol and the other peak at 23 min is Squalene. The area under the peak was calculated for cholesterol by integrating corresponding areas for all the peaks from retention time 28 minute onwards.

The non-saponifiable fraction of the samples consisted of approximately 95% Cholesterol and about 3-4% Squalene. Thus, each serving of ghee (1 teaspoon ~5 g) has about 22-38 mg cholesterol.
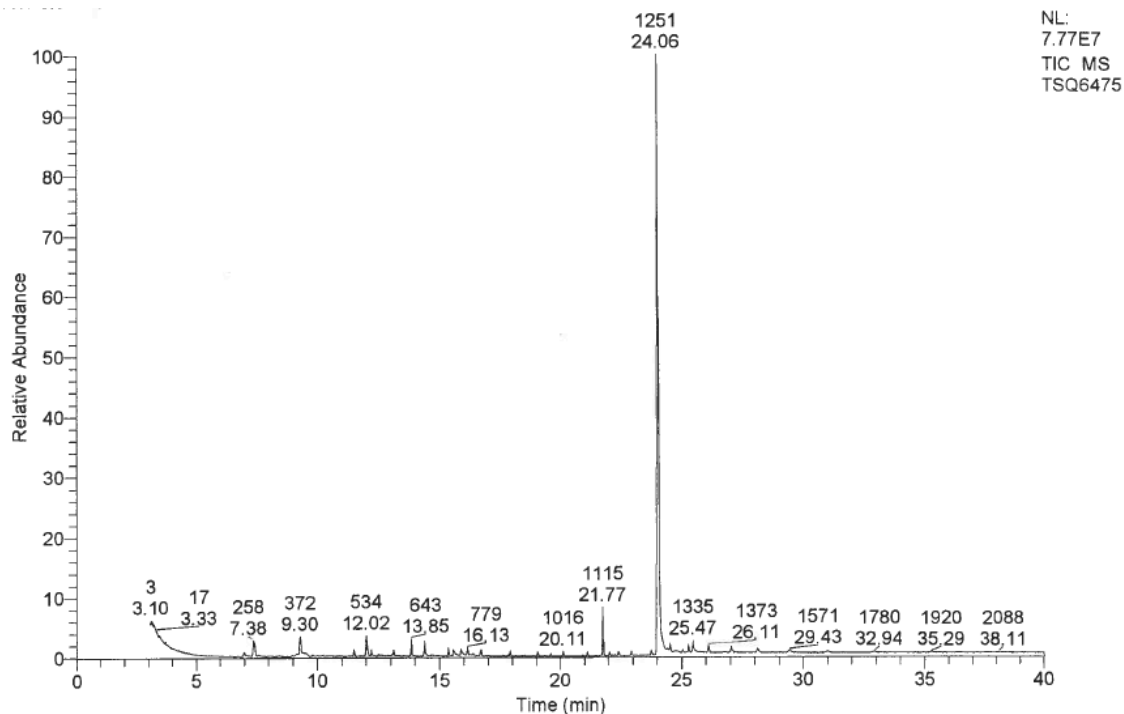
Figure 4.22: Typical chromatogram of non-saponifiable fraction of ghee

4.3 Headspace Volatiles

Overall 35 distinct flavor compounds were separated and detected in the headspace volatile matter of ghee samples using GC-MS. Each compound was found in varied amounts in each kind of sample. The variation was observed with respect to processing technique. Table 4.22 below summarizes the concentrations of the identified compounds in the organic grass-fed cow milk ghee samples by three methods of preparation, namely direct cream method, traditional method and cultured cream method.

Flavor compound	Direct cream (conc. Ppm w/w)	Traditional (conc. Ppm w/w)	Cultured cream (conc. Ppm w/w)
Methyl Mercaotan	0.03	0	0
acetaldehyde	0.6	0.49	0.94
Furan	0.06	0	0
Propanal	0.22	0.14	0.1
Acetone	1.8	10.09	8.54
Metyl acetate	0.04	0.21	0.1
2-methylpropanal	0.44	0.4	0.04
2-methylfuran	0.15	0	0
Diacetyl	0.97	0.19	0.13
2-Butanone	0.17	0.65	0.5
chloroform	0	0	0.5
3-methylbutanal	0.66	0.2	0.02
2-methylbutanal	0.41	0.44	0.03
Formic acid	0.29	0	0
acetic acid	8.29	1.24	0.61
2-Pentanone	2.41	5.13	4.51
Methyl Butyrate	1.02	1.48	0.56
2,3-pentanedione	0.1	0.1	0.1
Acetol	2.05	0	0
acetoin	0.04	0.31	0.31
3- methyl butyric acid	0.1	0.05	0
2-hexanone	0	0.04	0.04

hexanal	0	0.04	0.05
pyrrole	0.09	0.1	0
furfural	2.1	0.15	0.03
2-Heptanone	1.79	4.63	4.41
Methyl Pentanoate	0.02	0.08	0
furfuryl alcohol	6.02	0	0
2-acetyl furan	0.13	0	0
cyclopent-2-en-1,4-dione	0.32	0	0
5 methyl furfural	0.22	0	0
gamma-butyrolactone	0.12	0	0
2(5H)-Furanone	0.68	0	0
2-Nonanone	0.23	0.43	0.36
2-undecanone	0.06	0.1	0.09

Table 4.22: Headspace volatiles in organic grass-fed cow milk ghee

In Table 4.22 the compounds highlighted in green are the compounds found only in the ghee prepared by direct cream method. Direct cream method ghee contained the most number of distinct flavor compounds and was more aromatic. These compounds are derivatives of components of solids-not-fat. This fraction either gets discarded during churning or is digested during fermentation. Hence derivatives of these components are absent in ghee samples made by cultured cream and traditional methods.

The compounds highlighted in yellow are the compounds that are absent in the ghee made by direct cream however are formed in the cultured ghee. These are products of fermentation.

The compounds highlighted in red are the compounds that are present in all three kinds of ghee however are significantly higher in amounts in the ghee made by traditional and cultured cream methods. These higher concentrations indicate that these compounds increased in amount upon fermentation.

Values for organic grass-fed cow milk ghee listed in Table 4.22 are used to correlate the results of this analysis to the sensory aroma analysis results. Table 4.23 shows the similar results obtained for regular diet fed cow milk ghee samples.

Flavor compound	Direct cream (conc. Ppm w/w)	Traditional (conc. Ppm w/w)	Cultured cream (conc. Ppm w/w)
acetaldehyde	0.39	0.46	0.85
Furan	0.05	0.01	0
Propanal	0.08	0.12	0.09
Acetone	2.16	7.29	13.12
methyl acetate	0	0.15	0.12
Ethanol	0.08	0	0
2-methylpropanal	0.08	0.18	0.04
2-methylfuran	0.06	0	0
Diacetyl	0.8	0.11	0
2-Butanone	0.27	0.58	0.92
3-methylbutanal	0.14	0.13	0.02
2-methylbutanal	0.09	0.11	0.02
acetic acid	2.81	1.3	1
2-Pentanone	1.23	2.79	5.05
Methyl Butyrate	0.68	1.19	0.72
2,3-pentanedione	0.48	0	0
Acetol	0.87	0	0
acetonin	0.1	0.09	0.22

Hexanal	0	0.05	0.04
pyrrole	0.07	0	0
furfural	1.99	0.13	0.15
2-Hexanone	0	0.04	0.06
2-Heptanone	1.76	3.31	4.88
furfuryl alcohol	4.89	0.1	0
2-acetyl furan	0.11	0	0
cyclopent-2-en-1,4-dione	0.29	0	0
5 methyl furfural	0.14	0	0
gamma-butyrolactone	0.13	0	0
2(5H)-Furanone	0.41	0	0
2-Nonanone	0.25	0.41	0.5
2-undecanone	0.05	0.1	0.09

Table 4.23: Headspace volatiles in regular diet fed cow milk ghee

4.4 Sensory analysis

Participants were presented with 3 pairs of samples one at a time. One of each pair was a labeled control and other was coded. Participants were asked to compare the coded sample with the control sample and rate the degree of difference between the two. Subjects checked the box on the ballot sheet depending on how far from control they found the test sample to be, as shown in the Fig. 4.23.

Pair 1: Reference / 283 (modern Vs modern)
How different are the two samples?

No
Difference

Extremely
different

Figure 4.23: Sample ballot for one of the three test samples. (Note: Sample identities (modern Vs. modern) revealed in the figure for representation was unknown to test participants)

The boxes were assigned scores from 1-9 (left to right). Scores were assigned to each test. In Fig. 4.23 for example, the second box is checked; hence that particular sample was given a score of '2' for degree of difference from control. Similarly, scores were assigned to basic questions for familiarity with ghee (yes/no: 1/0) and the frequency of consumption (never-daily: 0-4).

The scores were recorded for each participant. A total of 55 subjects (ages 18-60) participated. The means, standard deviation and standard errors were calculated for the three tests.

	Modern Vs Modern	Modern Vs Traditional	Modern Vs Cultured Cream
Mean	2.5272	4.5272	5.2909
Standard Deviation	1.7935	2.3242	2.4242
standard error	0.2418	0.3133	0.3268

Table 4.24: Statistical analysis results for sensory scores
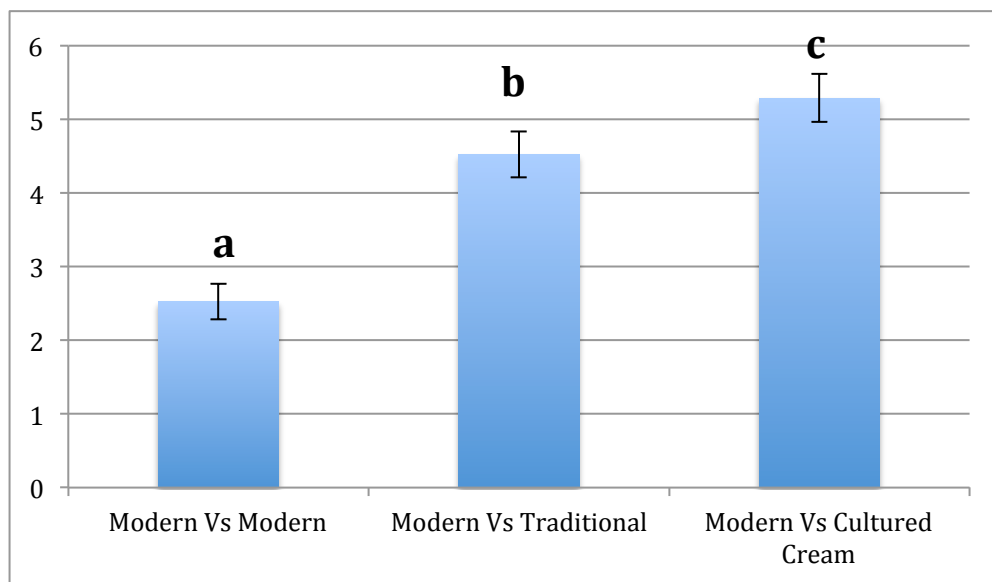
Figure 4.24: Degree of difference in aroma profiles perceived between samples and control (different letters a, b and c indicate statistical significance)

The data with scores for the questionnaire and difference from control test were compiled and a 3 way ANOVA (analysis of variance) was done to test the statistical significance of each set of readings. P-value of less than 0.0001 was obtained for each test. The Dunnett's test for rating was done to control the experimental error in comparing all the treatments against a control. It indicated that the error was lower than the threshold for each test.

Overall the sensory test indicated that the data were statistically significant and that subjects generally perceived an obvious difference in the aroma profiles of ghee made with different preparation methods. It is very important to note that

familiarity with ghee did not matter when it came to perceiving the difference in aroma.

Thus the sensory test supported our basic hypothesis that there exists a perceivable difference in sensory properties of ghee made by different preparation methods and the fact that the difference being fairly obvious did not depend on one's familiarity with ghee was validated.

4.5   Color measurements

Figure 4.25: Difference in color in (from left to right) direct cream method ghee, cultured cream method ghee and traditional method ghee.

As seen in Fig. 4.25, the direct cream method sample was the darkest followed by cultured cream method ghee and traditional method ghee (from left to right). This observation was asserted by the readings recorded with the Konika-Minolta colorimeter (Table 4.25).

Value	Direct Cream	Cultured Cream	Traditional
L*	$68.95^a \pm 0.1871$	$72.1^b \pm 0.2078$	$74.28^c \pm 0.6596$
a*	$0.81^a \pm 0.866$	$-2.39^b \pm 0.0435$	$-3.6^c \pm 0.1374$
b*	$48.46^c \pm 1.1172$	$49.66^c \pm 1.0923$	$50.41^c \pm 1.5519$

Table 4.25: Color measurement results (different letters a, b and c indicate statistical significance)

Here the L*, a* and b* scales indicate the following:

L* scale: Light vs. dark, a low number (0-50) indicates dark and a high number (51-100) indicates light.

a* scale: Red vs. green, a positive number indicates red and a negative number indicates green.

b* scale: Yellow vs. blue where a positive number indicates yellow and a negative number indicates blue.

5. Conclusions

Five different analyses were carried out to fulfill the objectives and verify or disprove the hypotheses. Table 5.1 summarizes the results and conclusions of all the analyses done for this research.

We hypothesized that the bacterial fermentation would have an impact on the fatty acid profile of ghee. This was not observed from the experimental results and could not be confirmed.

No.	Analysis	Results	Samples compared	Conclusion
1.	Saponifiables by FAMEs using GC-MS (Elucidation of Fatty acid profiles.)	No significant differences were found in the fatty acid profiles	All six samples	The source of milk or the method of preparation ghee does not affect the fatty acid profile of ghee.
2.	Non-saponifiables by GC-MS (Cholesterol content)	Ghee contains about 22-38 mg cholesterol per serving (1 tsp)	All six samples	Cholesterol content is fairly constant and is unaffected by source of milk or method of preparation of ghee.
3.	Headspace volatiles by GC-MS (Flavor profile)	35 distinct flavor compounds were isolated and concentrations were compared	All six samples	Flavor profiles of samples made by different method of ghee preparation were different. Certain compounds got eliminated during additional processing steps in traditional and

				cultured cream methods of preparation whereas other increased in amounts upon culturing with bacteria.
4.	Sensory analysis (Aroma profile)	Significant difference in aroma was perceived in each of the samples	Three types of Ghee made by organic grass fed cow milk	Results indicate that method of preparation influenced the aroma profile of ghee
5.	Color analysis	Significant difference was found in color. Ghee made by modern method being the darkest and that made by traditional method being the lightest	Three types of Ghee made by organic grass fed cow milk	It can be concluded that due to increased amounts of solids-not-fat in the ghee made by modern and cultured cream methods, more milliard browning products were formed creating a darker colored ghee.

Table 5.1: Summary of experimental results and conclusions

It was expected that the sensory characteristics of the ghee made by different methods of preparation and the two sources of milk will be different. This was supported by the data obtained from the headspace volatile analysis and the sensory analysis.

In summary,

1. Bacterial fermentation of cream did not have an impact on the fatty acid profile of ghee.

2. No significant differences were found in the overall fatty acid profiles of ghee owing to the method of preparation or source of milk.

3. The differences in headspace volatile profiles were significant for ghee made by the different methods of preparation.

4. These differences translated into differences in aroma profiles of ghee made by different methods preparation as evaluated in sensory studies.

6. Future Work

- The research can be conducted in a different season to assess seasonal variation in the milk source and how it affects the fatty acid profiles.

- It would be interesting to study other nutritional aspects, such as vitamin A and β-carotene content, of ghee made by different methods

- Further investigation may be done to study the structure of the odd carbon fatty acids in details.

- The strains and compositions of authentic dahi (yogurt) cultures vary from region to region. It would be interesting to test the impact of authentic bacterial cultures from different regions of India, in order to conclusively say that bacteria do or do not impact the fatty acid profile of ghee.

- Literature suggests that yogurt in India is often contaminated with yeasts and fungi and it would be interesting to study if that affects the sensory and chemical quality of ghee.

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8. Appendices

Appendix A

1. Saponifiable fraction chromatograms: 1st set

Saponifiable fraction chromatogram sample 1: Organic grass fed cow milk,

modern method

Saponifiable fraction chromatogram sample 2: Organic grass fed cow milk,

traditional method

Saponifiable fraction chromatogram sample 3: Organic grass fed cow milk,

cultured cream method

Saponifiable fraction chromatogram sample 4: regular milk, modern method

Saponifiable fraction chromatogram sample 5: regular milk, traditional method

Saponifiable fraction chromatogram sample 6: regular milk, cultured cream

method

2. Saponifiable fraction chromatograms: 2nd set

Saponifiable fraction chromatogram sample 1: Organic grass fed cow milk,

modern method

Saponifiable fraction chromatogram sample 2: Organic grass fed cow milk,

traditional method

Saponifiable fraction chromatogram sample 3: Organic grass fed cow milk, cultured cream method

Saponifiable fraction chromatogram sample 4: regular milk, modern method

Saponifiable fraction chromatogram sample 5: regular milk, traditional method

Saponifiable fraction chromatogram sample 6: regular milk, cultured cream method

3. Saponifiable fraction chromatograms: 3rd set

Saponifiable fraction chromatogram sample 1: Organic grass fed cow milk,

modern method

Saponifiable fraction chromatogram sample 2: Organic grass fed cow milk,

traditional method

Saponifiable fraction chromatogram sample 2: Organic grass fed cow milk, cultured cream method

Saponifiable fraction chromatogram sample 4: regular milk, modern method

Saponifiable fraction chromatogram sample 5: regular milk, traditional method

Saponifiable fraction chromatogram sample 6: regular milk, cultured cream

method

4. Non-saponifiable fraction chromatograms: 1st set

Non-saponifiable fraction chromatogram sample 1: organic grass-fed cow milk,

modern method

Non-saponifiable fraction chromatogram sample 2: organic grass-fed cow milk,

traditional method

Non-saponifiable fraction chromatogram sample 3: organic grass-fed cow milk,

cultured cream method

Non-saponifiable fraction chromatogram sample 4: regular milk, modern method

Non-saponifiable fraction chromatogram sample 5: regular milk, cultured cream

method

Non-saponifiable fraction chromatogram sample 4: organic grass-fed cow milk,

traditional method

Non-saponifiable fraction chromatogram sample 3: organic grass-fed cow milk,

cultured cream method

Non-saponifiable fraction chromatogram sample 4: regular milk, modern method

Non-saponifiable fraction chromatogram sample 5: regular milk, traditional

method

Non-saponifiable fraction chromatogram sample 6: regular milk, cultured cream

method

5. Headspace volatiles chromatograms: 1st set
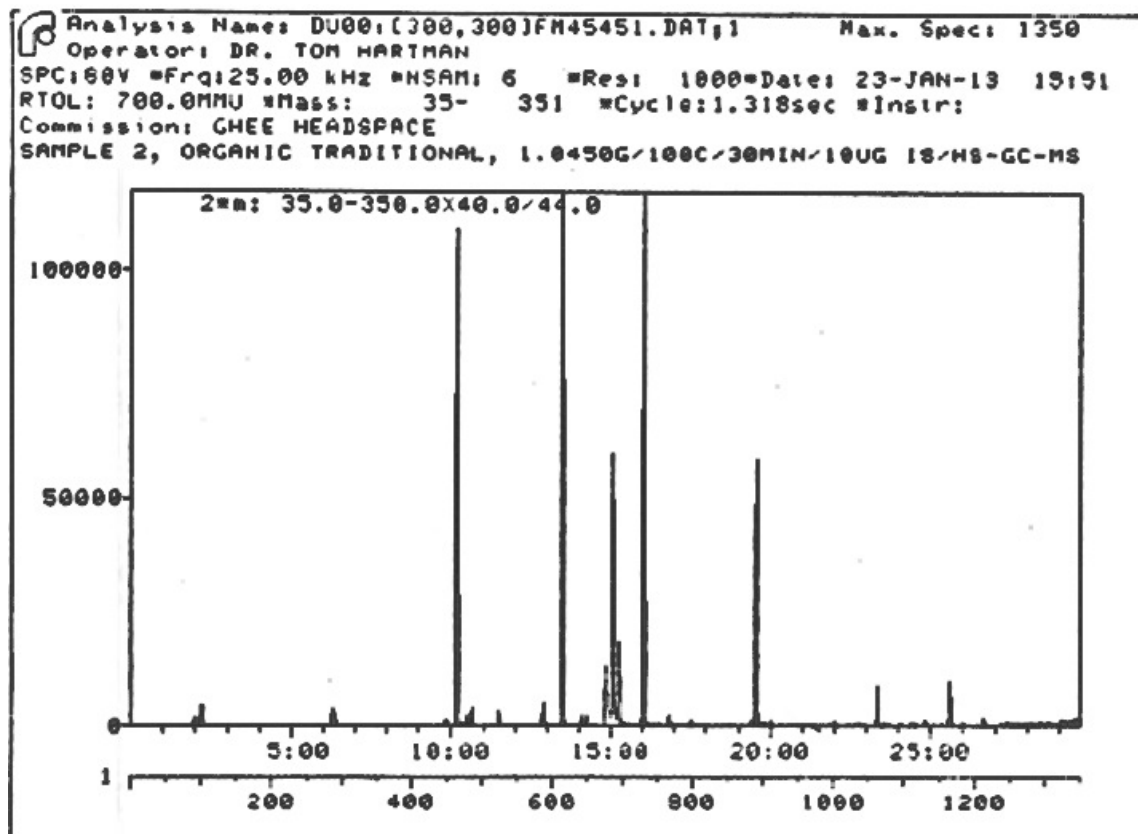

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SPC:60V =Frq:25.00 kHz =HSAM: 6    =Res:  1000=Date: 23-JAN-13   15:51
RTOL: 700.0MMU =Mass:      35-   351 =Cycle:1.318sec =Instr:
Commission: GHEE HEADSPACE
SAMPLE 2, ORGANIC TRADITIONAL, 1.0450G/100C/30MIN/10UG IS/HS-GC-MS
```

Headspace volatiles chromatogram: Organic grass-fed cow milk, traditional

method

Headspace volatiles chromatogram: Organic grass-fed cow milk, modern method
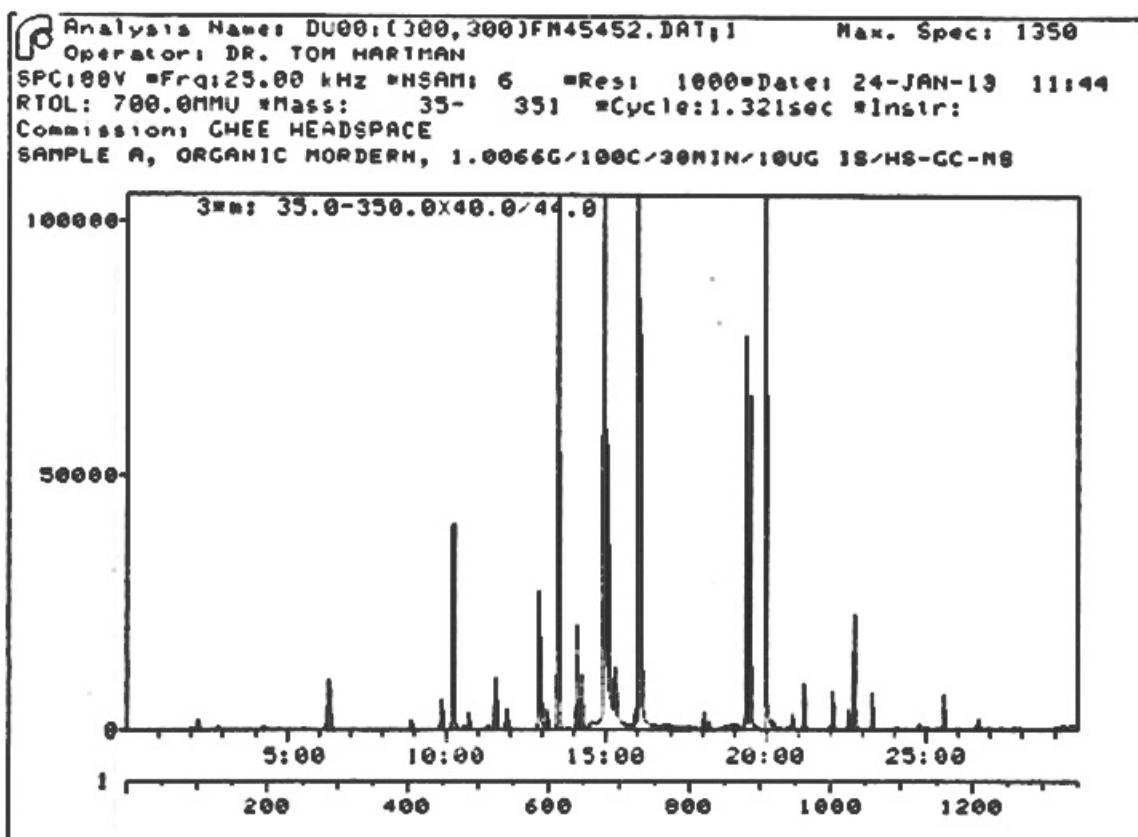
Headspace volatiles chromatogram: Organic grass-fed cow milk, modern method
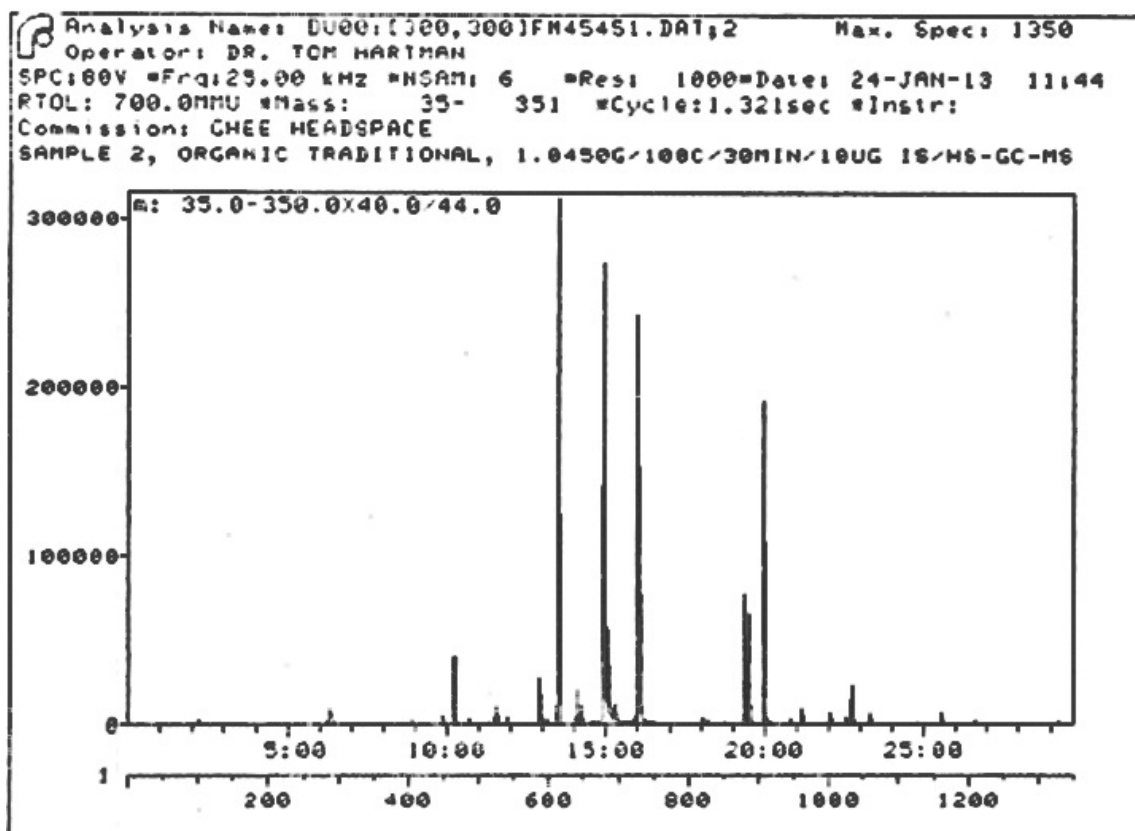
Headspace volatiles chromatogram: Organic grass-fed cow milk, traditional
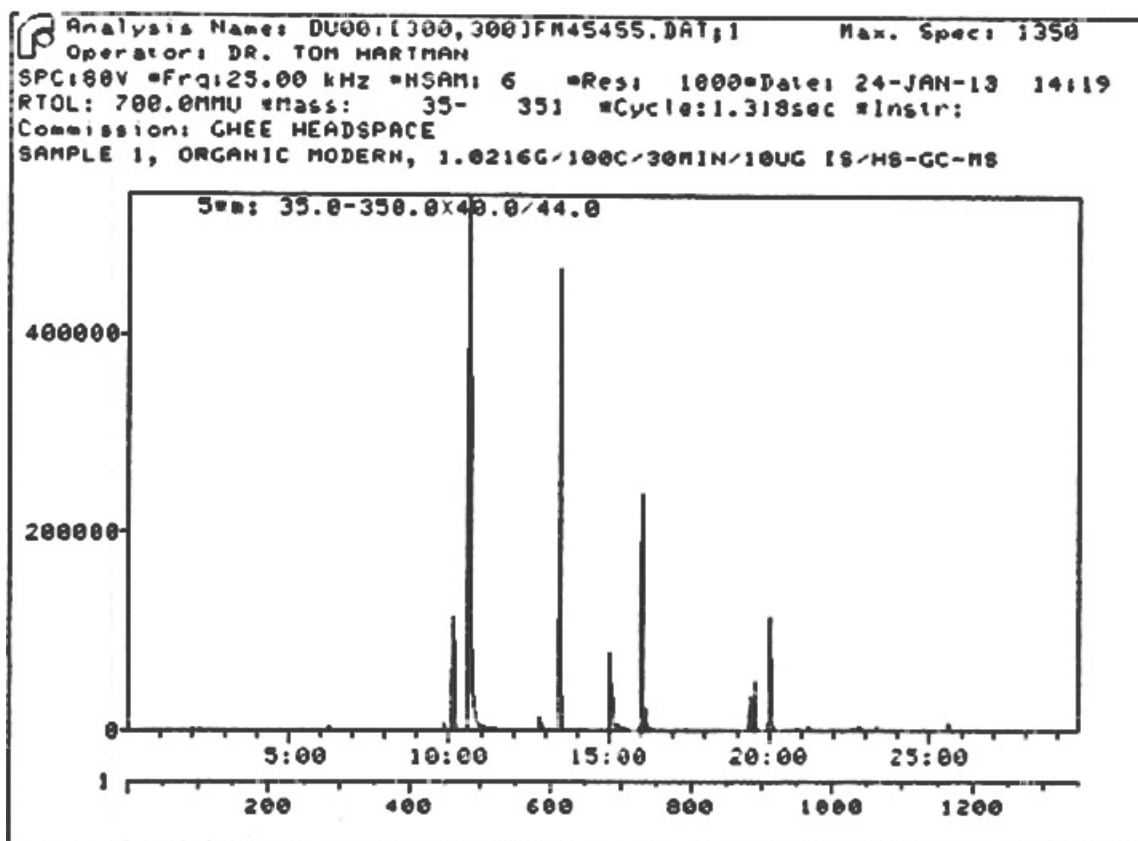
method

Headspace volatiles chromatogram: Organic grass-fed cow milk, modern
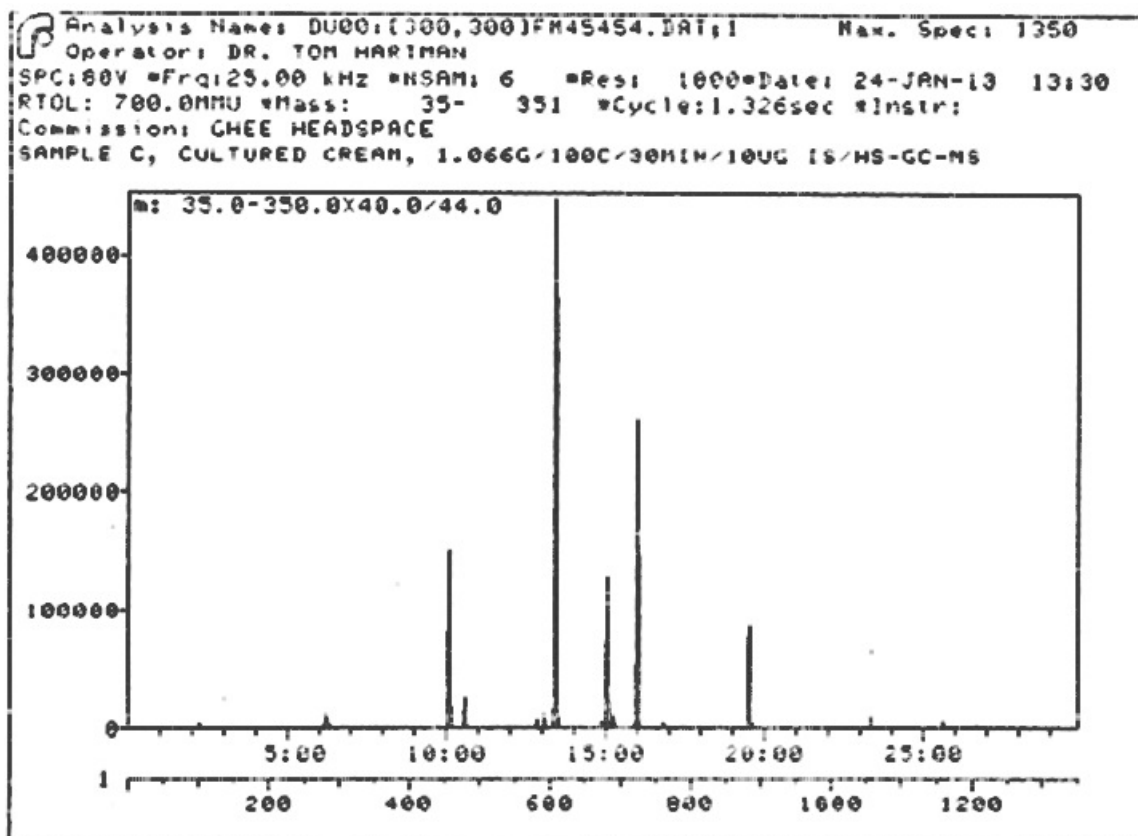
method

Headspace volatiles chromatogram: organic grass-fed cow milk, cultured cream

method

6. Headspace volatiles chromatograms: 2nd set
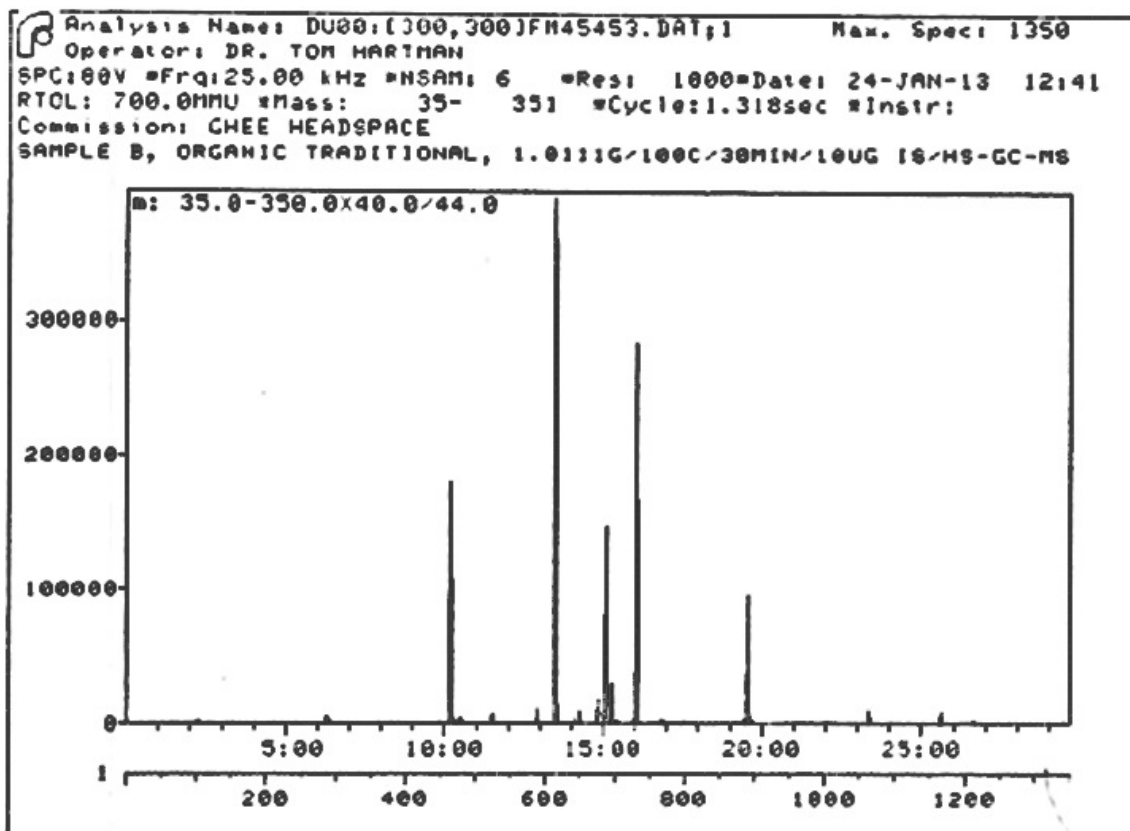
Headspace volatiles chromatogram: Organic grass-fed cow milk, traditional
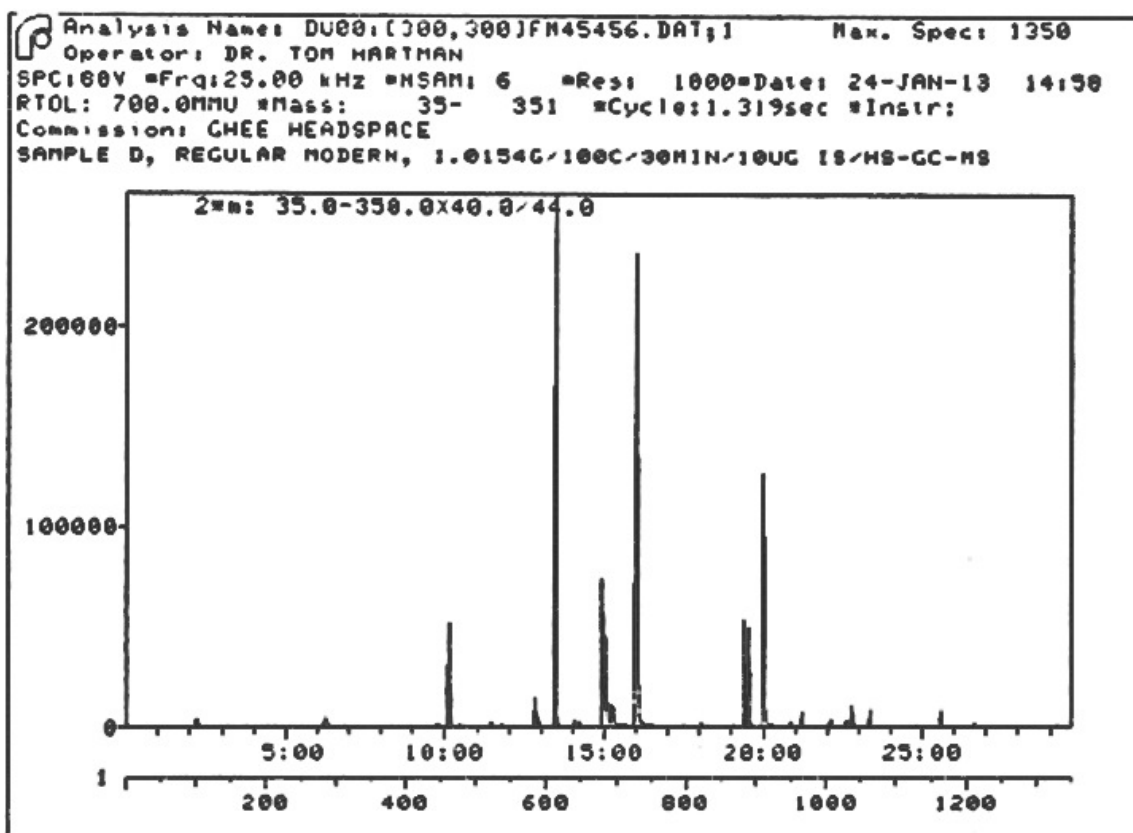
method

Headspace volatiles chromatogram: organic grass-fed cow milk, modern method
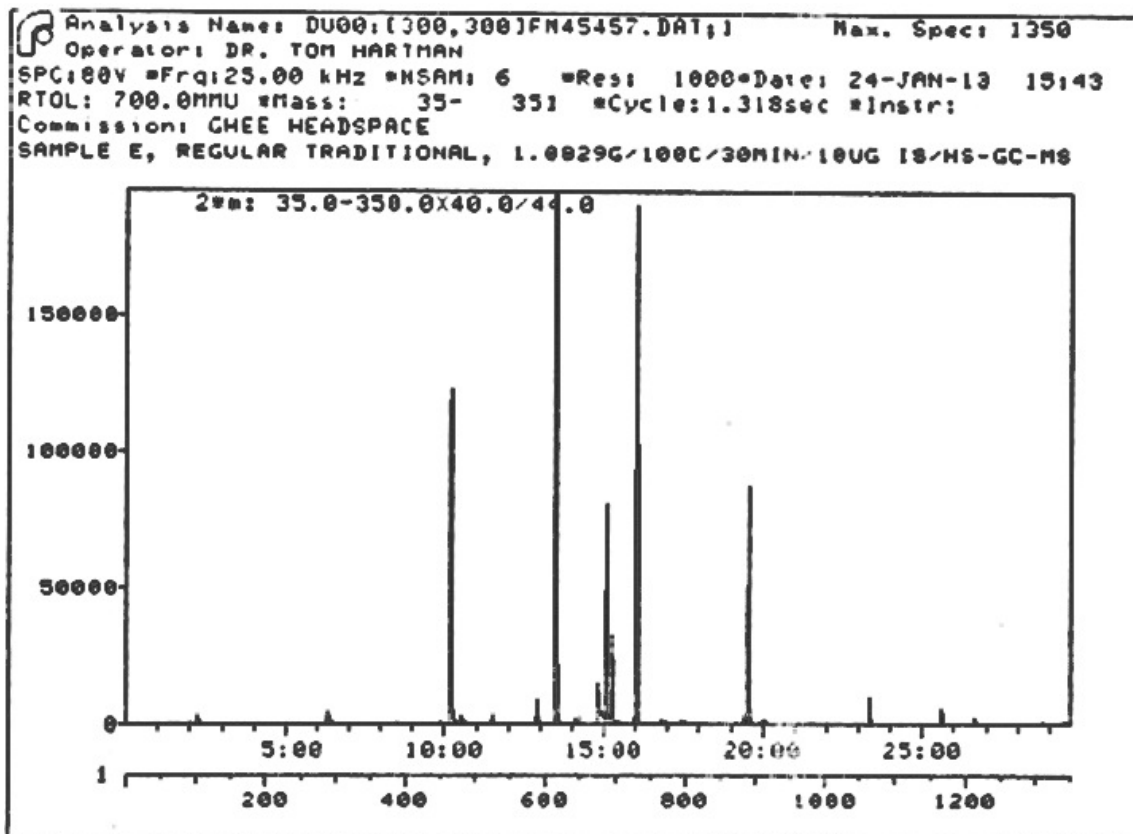
Headspace volatiles chromatogram: organic grass-fed cow milk, traditional
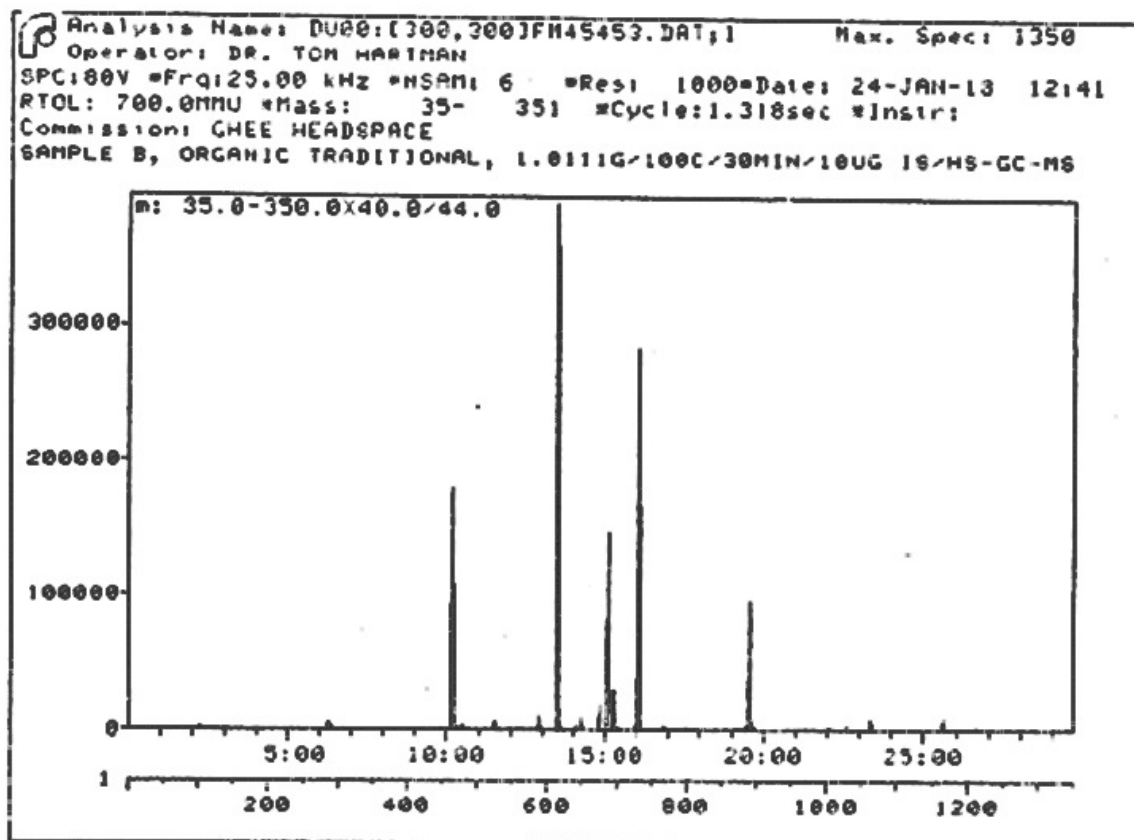
method

Headspace volatiles chromatogram: Organic grass-fed cow milk, traditional
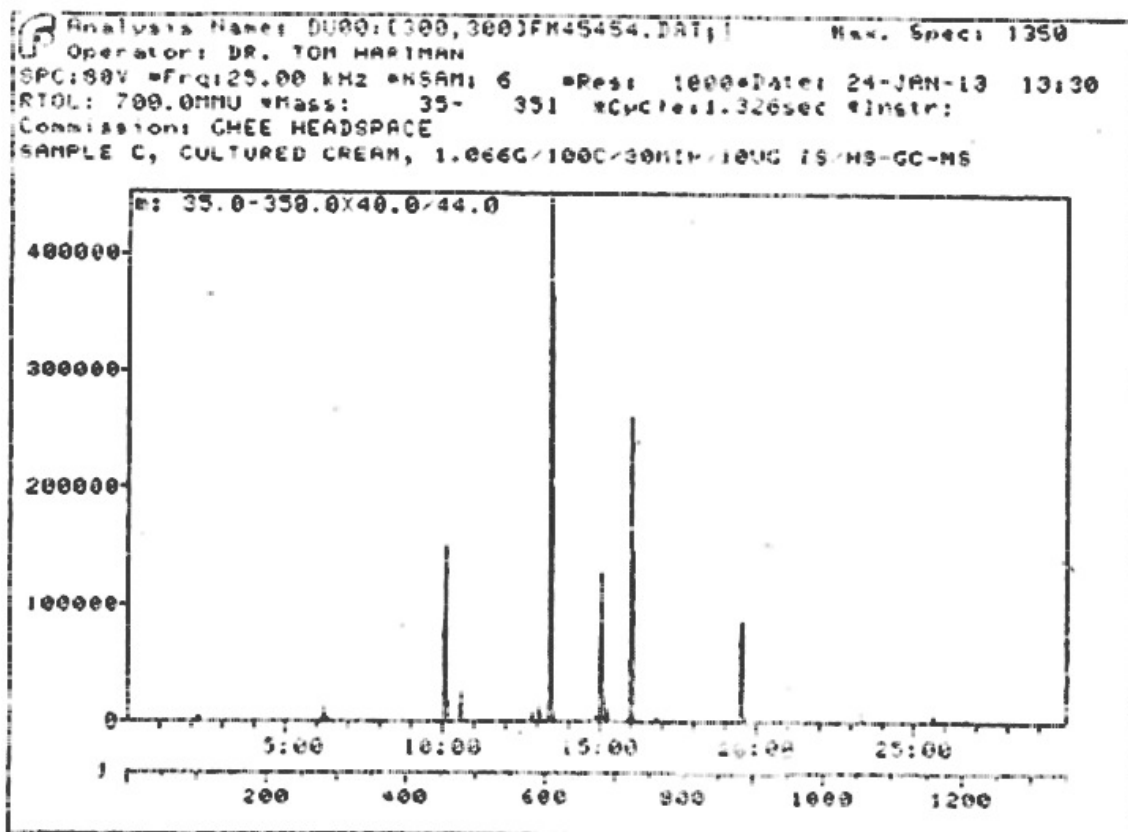
method

Headspace volatiles chromatogram: organic grass-fed cow milk, cultured cream

method

7. Headspace volatiles chromatograms: 3rd set
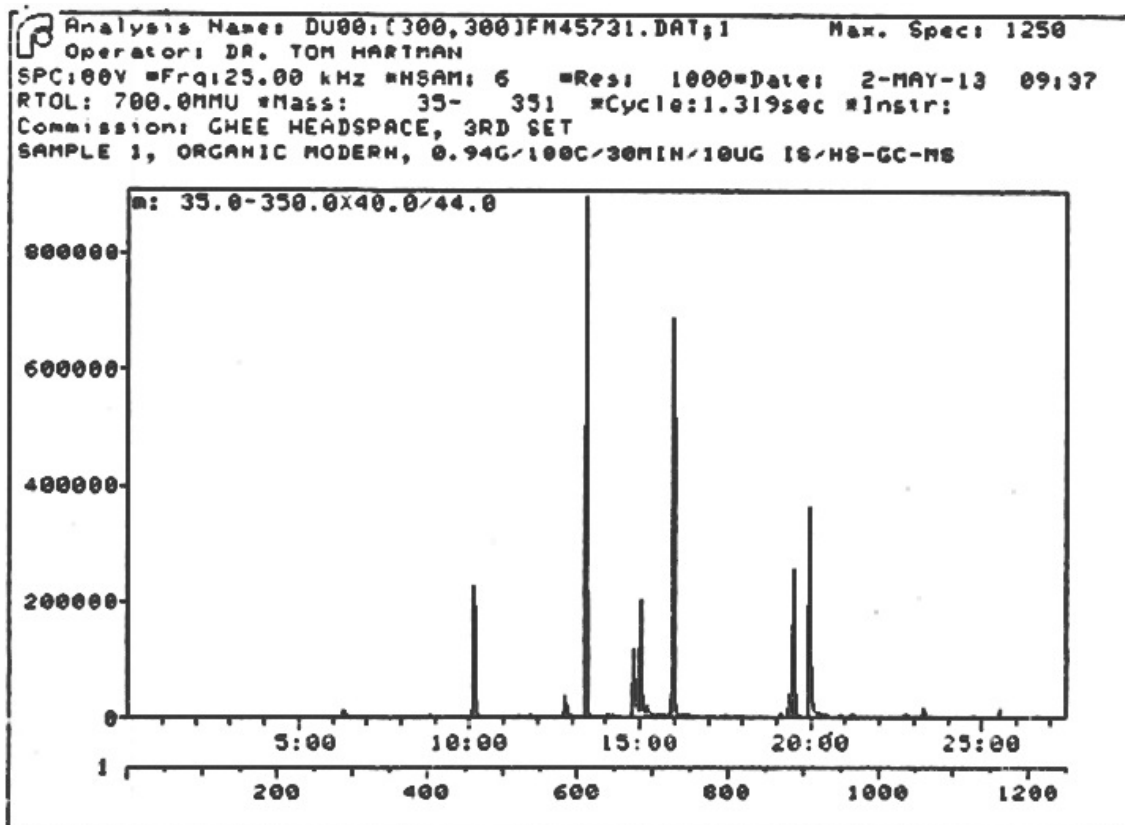
Headspace volatiles chromatogram: Organic grass-fed cow milk, modern method

7. Headspace volatiles chromatograms: 3rd set

Headspace volatiles chromatogram: Organic grass-fed cow milk, modern method
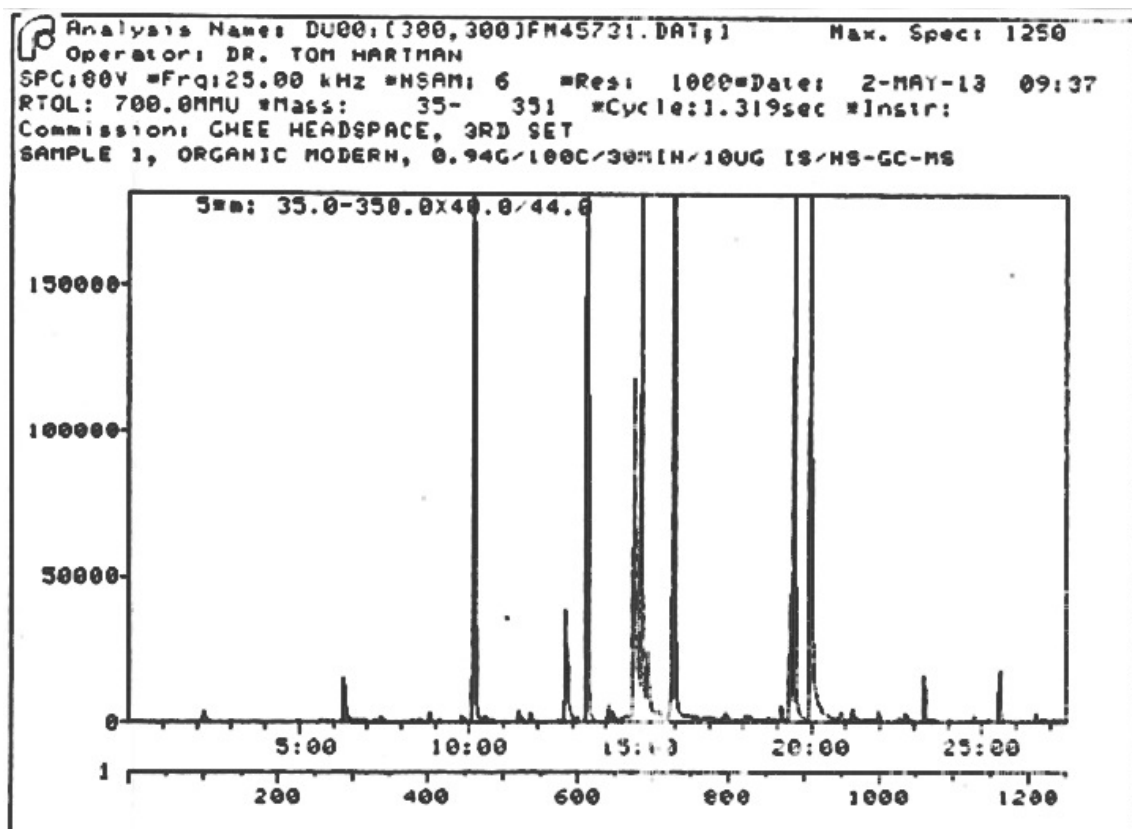
Headspace volatiles chromatogram: Organic grass-fed cow milk, modern method
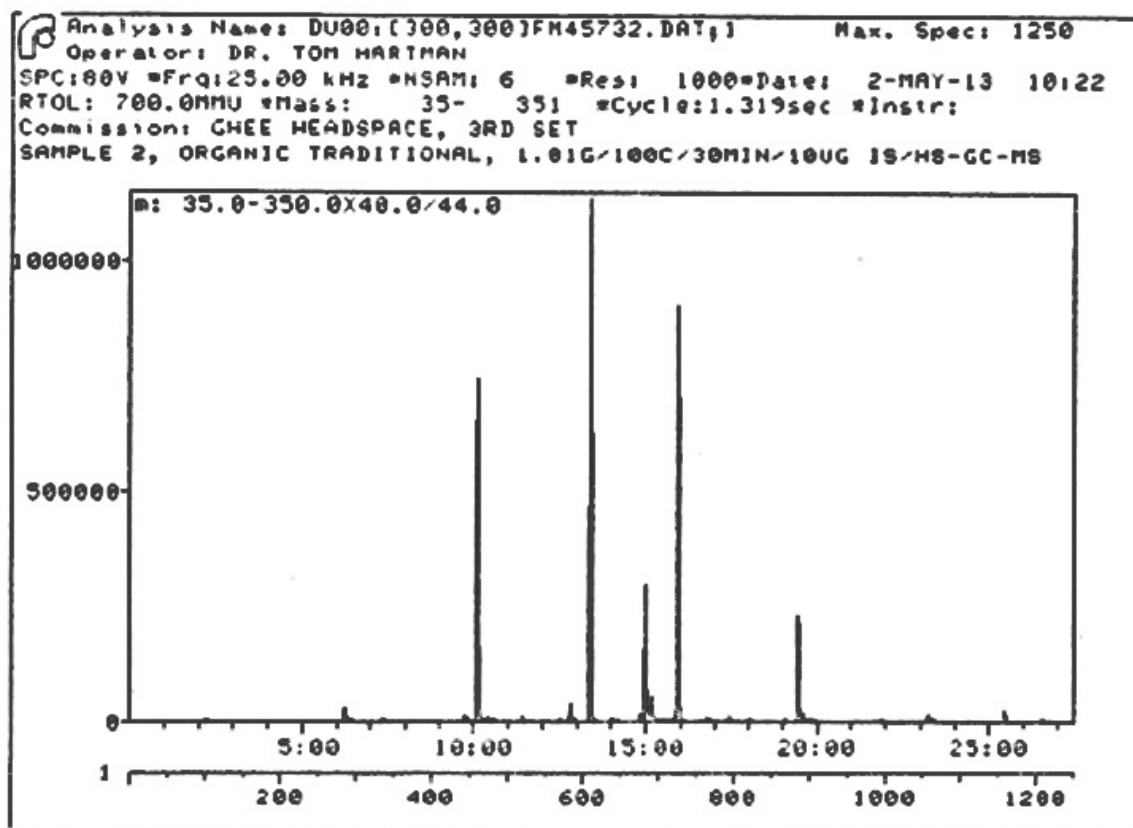
Headspace volatiles chromatogram: Organic grass-fed cow milk, traditional
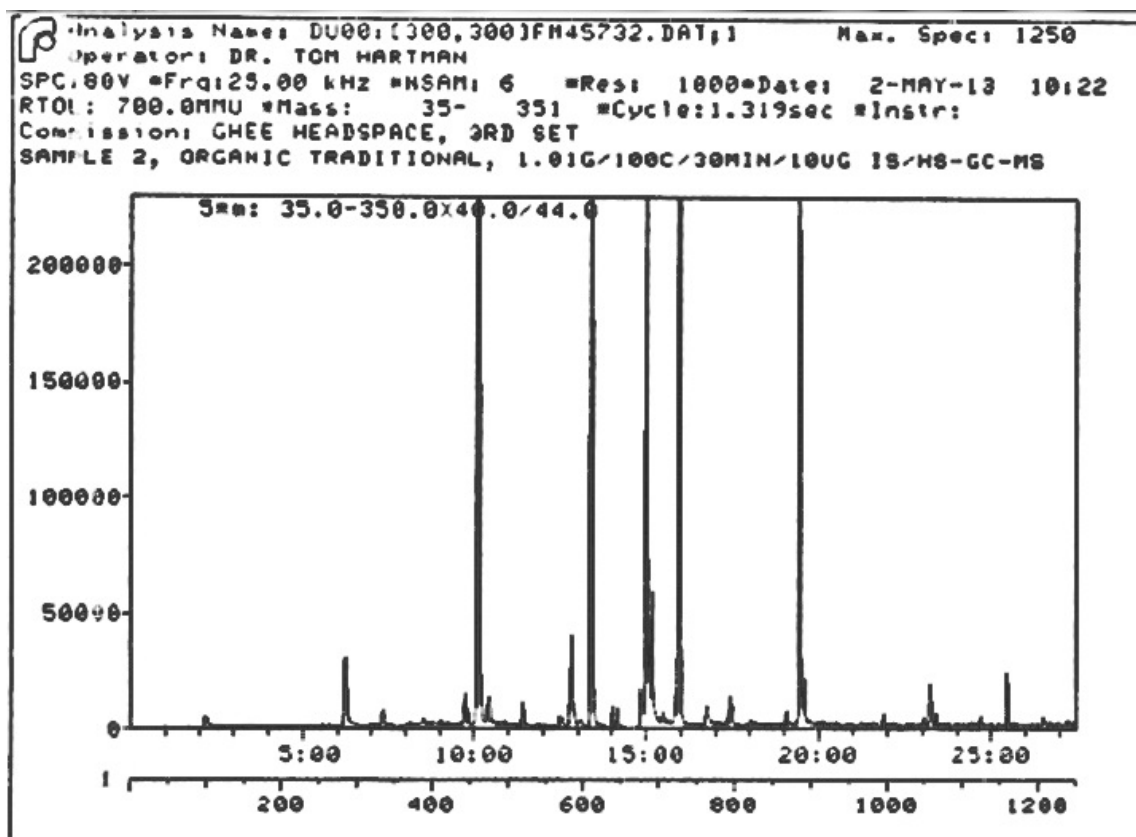
method

Headspace volatiles chromatogram: Organic grass-fed cow milk, traditional
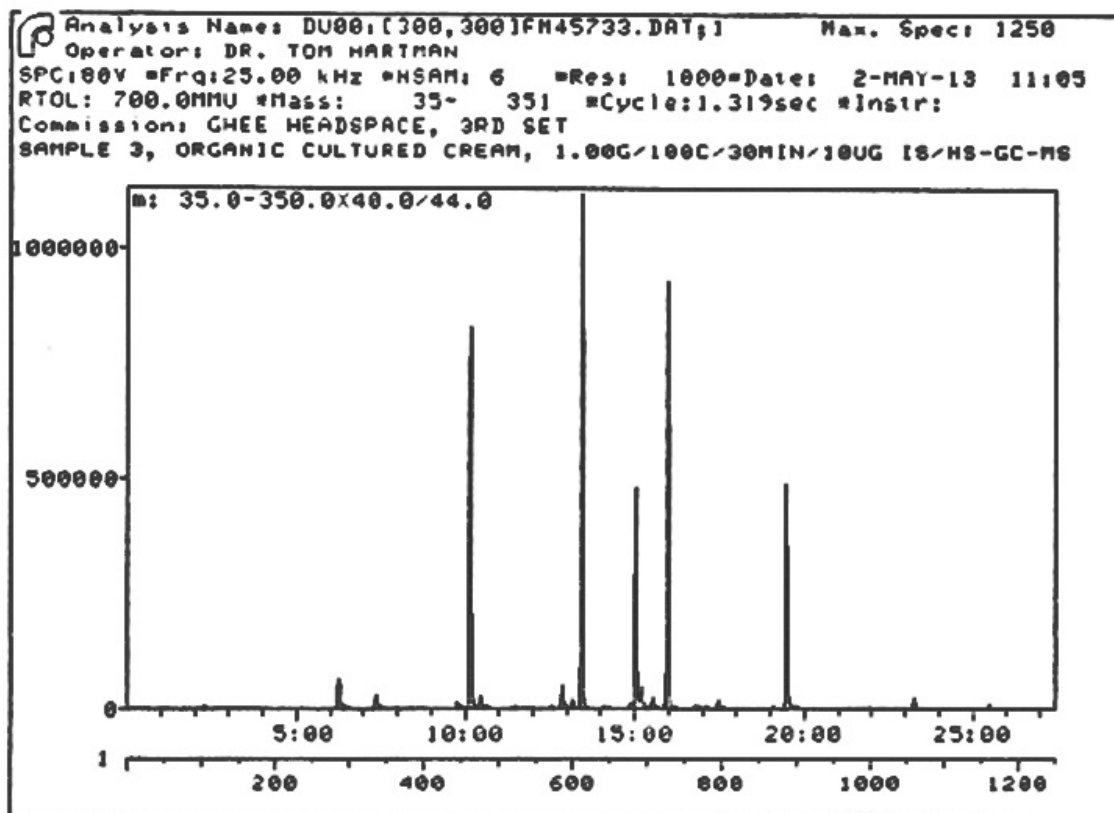
method

Headspace volatiles chromatogram: Organic grass-fed cow milk, cultured cream
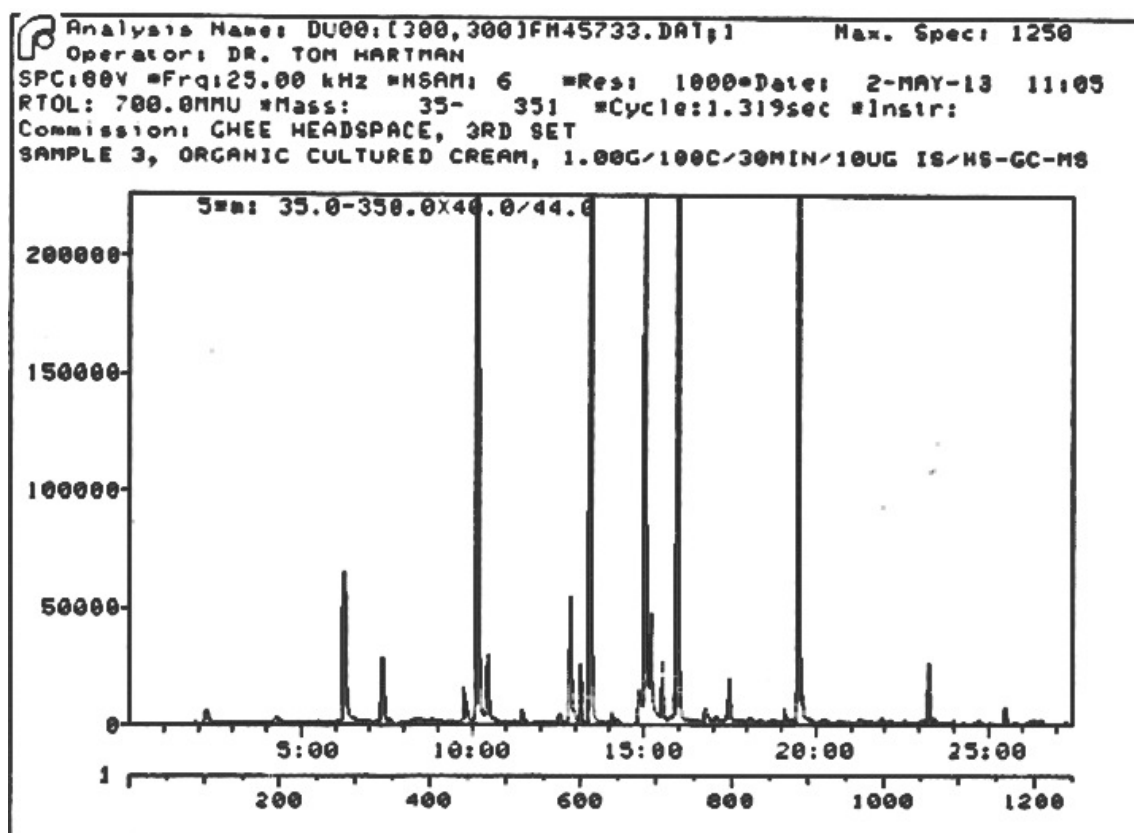
method

Headspace volatiles chromatogram: Organic grass-fed cow milk, cultured cream
method

Appendix B

1. Mass spectrums of fatty acids
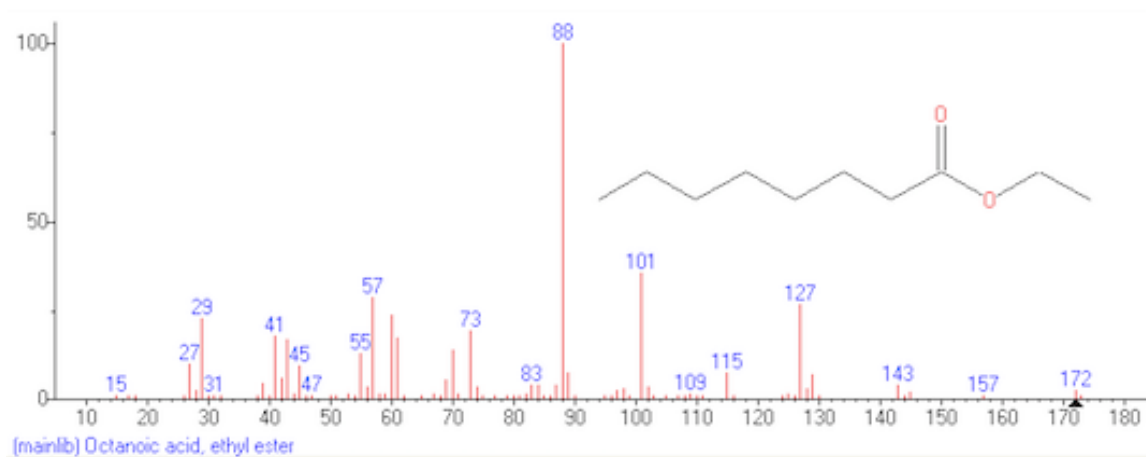
Octanoic acid, ethyl ester
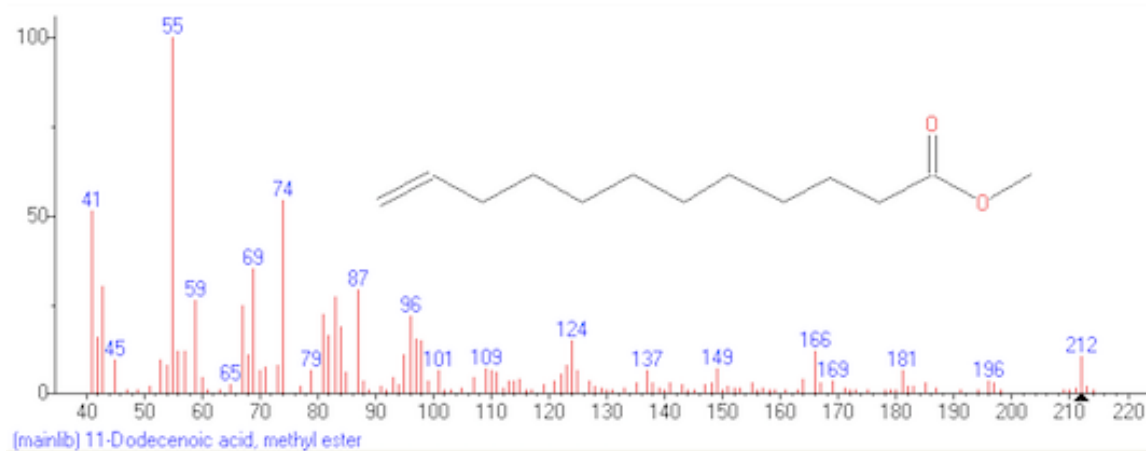
Dodecenoic acid, methyl ester
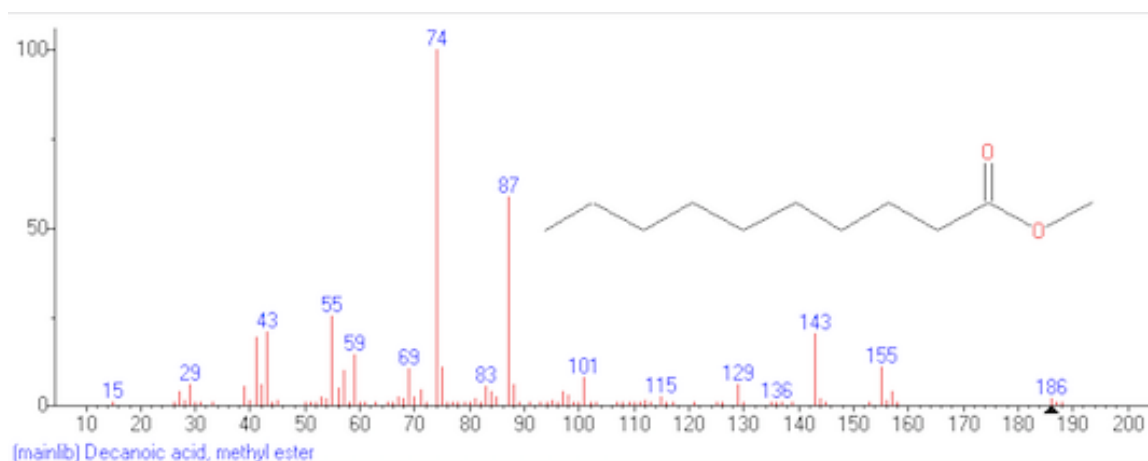
Decanoic acid, methyl ester
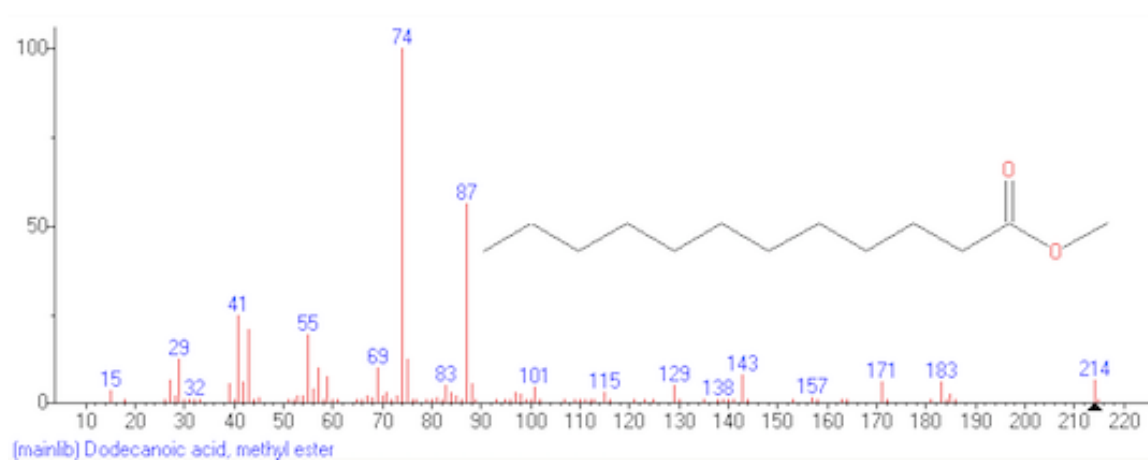
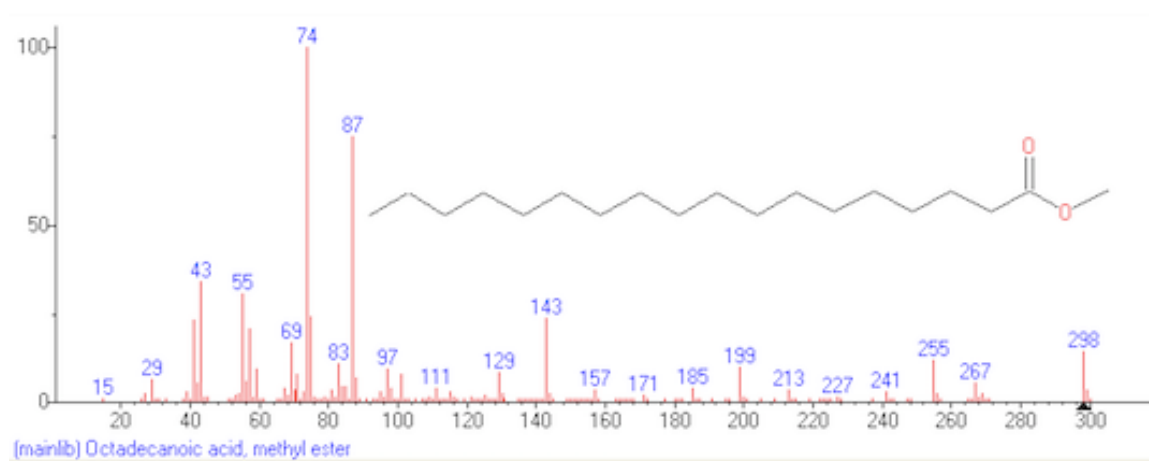Dodecanoic acid, methyl ester
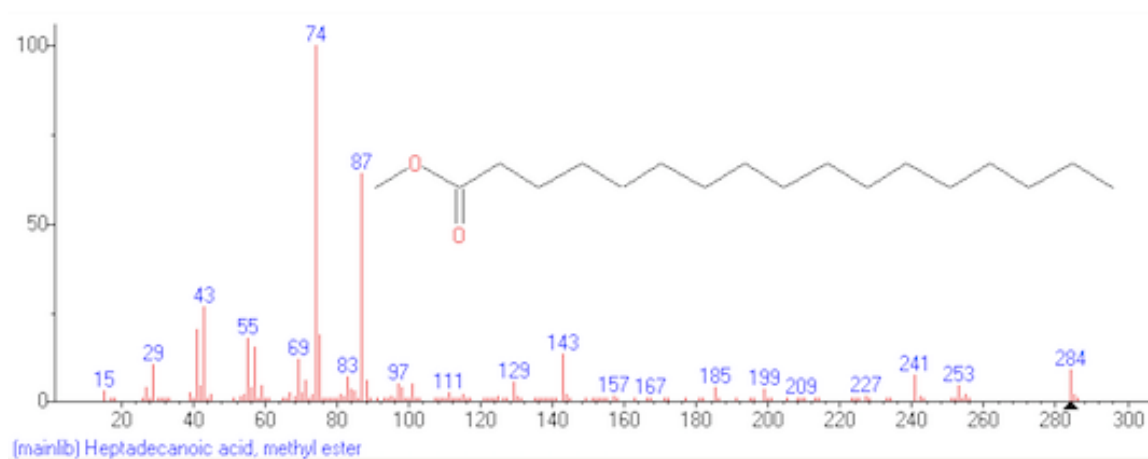
Heptadecanoic acid, methyl ester

Hexadecanoic acid, methyl ester
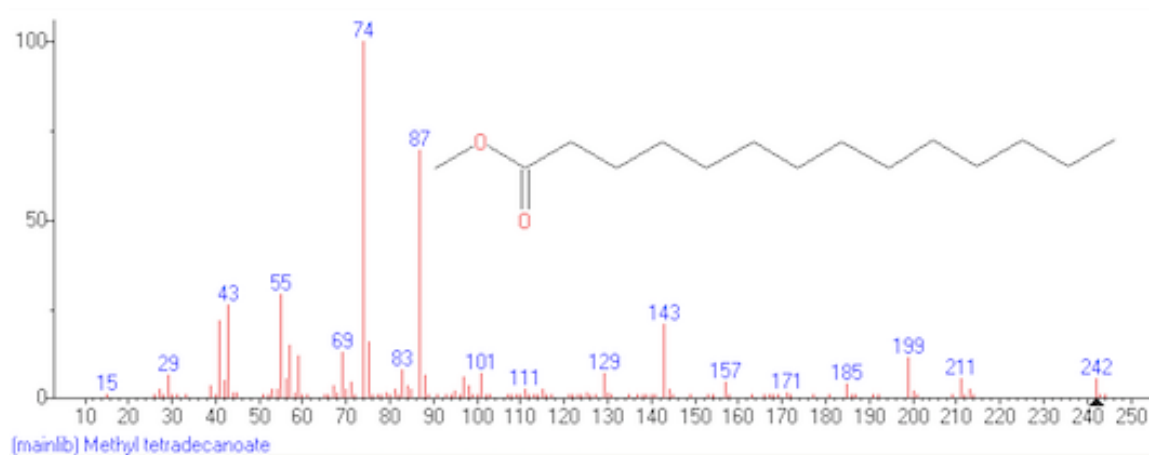
[mainlib] 9,12-Octadecadienoic acid (Z,Z)-, methyl ester
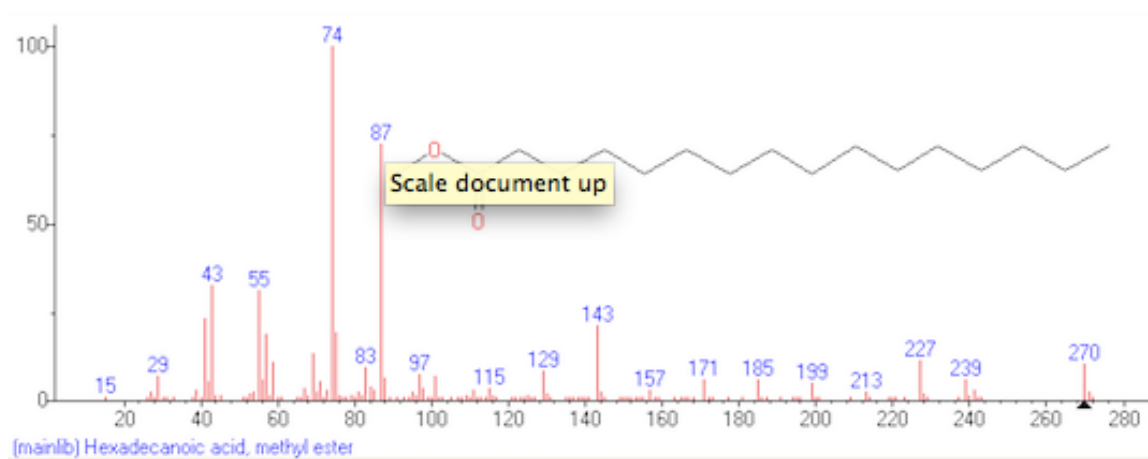
Octadecanoic acid, methyl ester
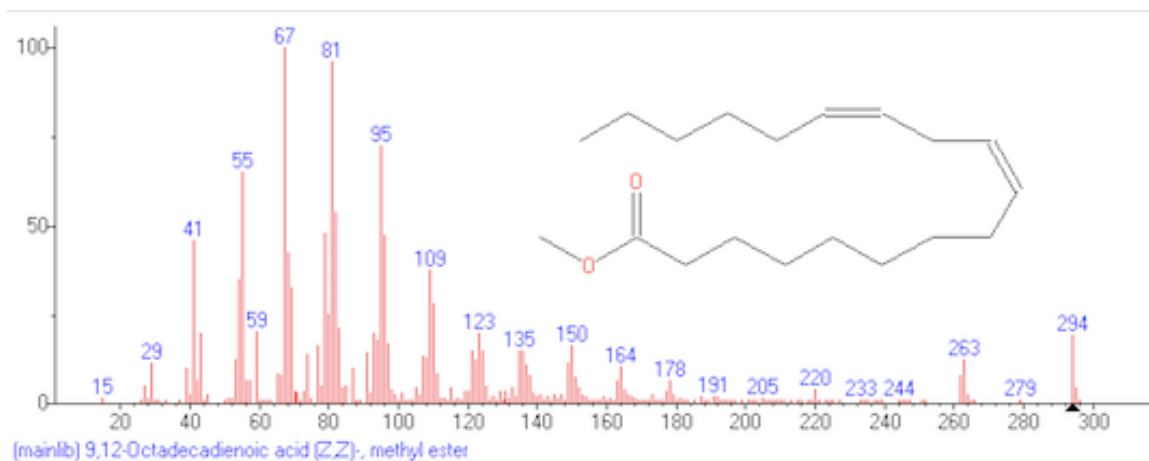
[mainlib] Nonanoic acid, methyl ester
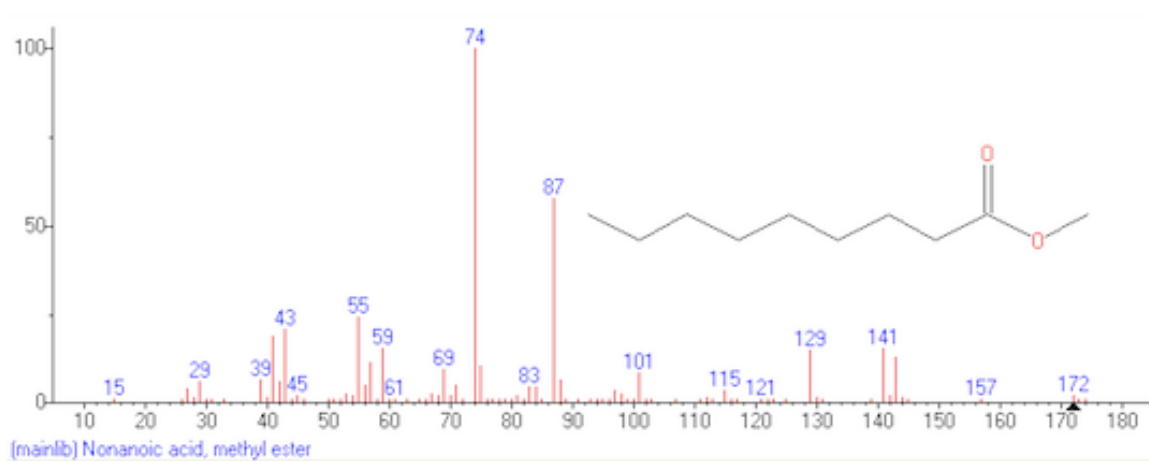
Nonanoic acid, methyl ester
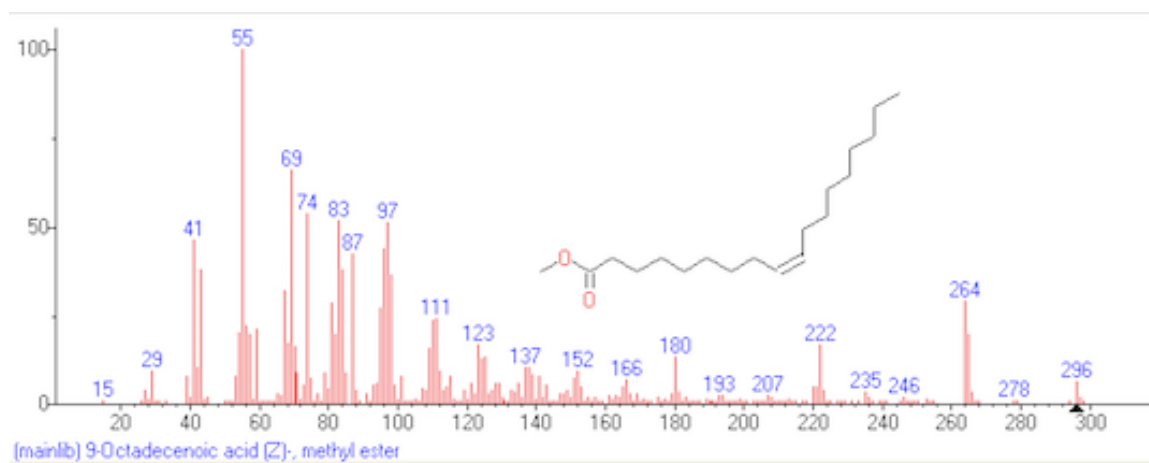
Octadecenoic acid, methyl ester
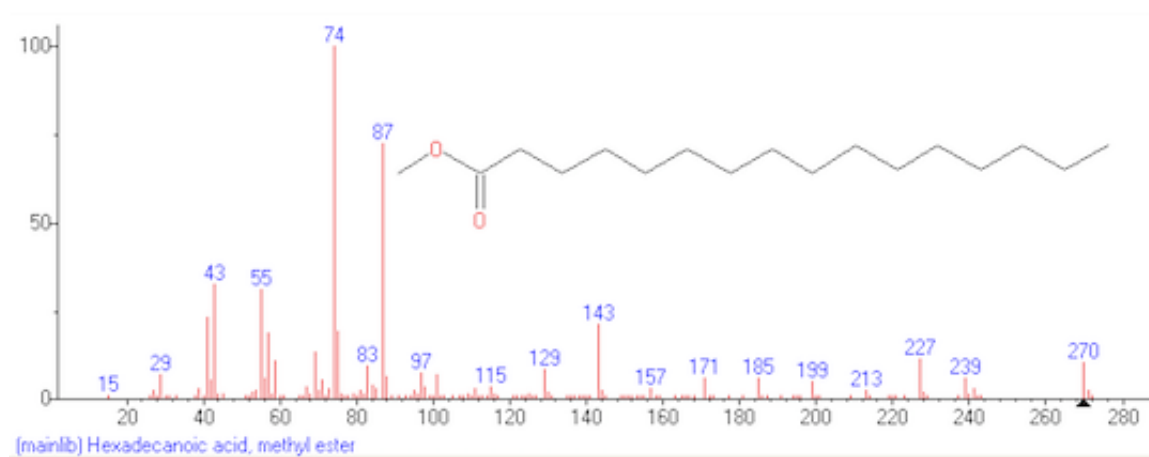
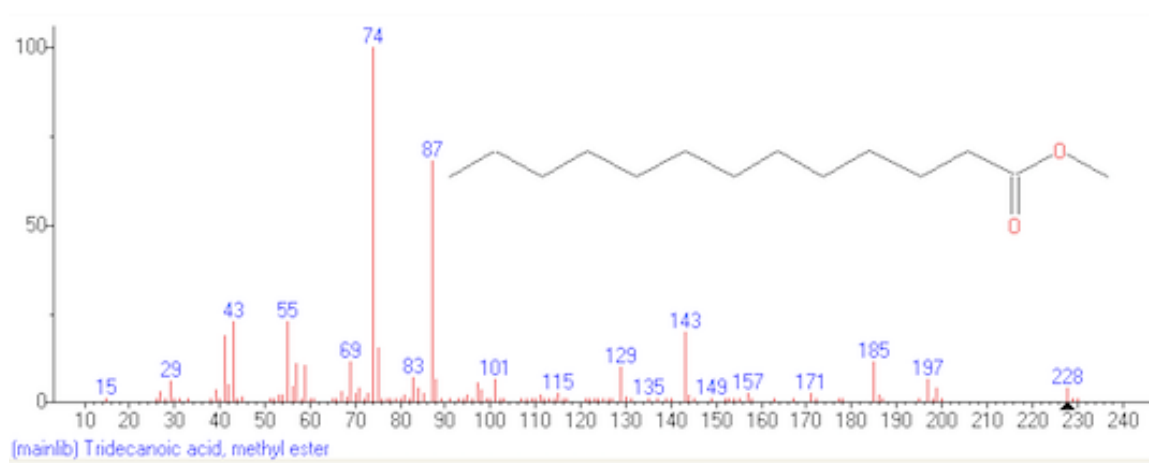Hexadecanoic acid, methyl ester
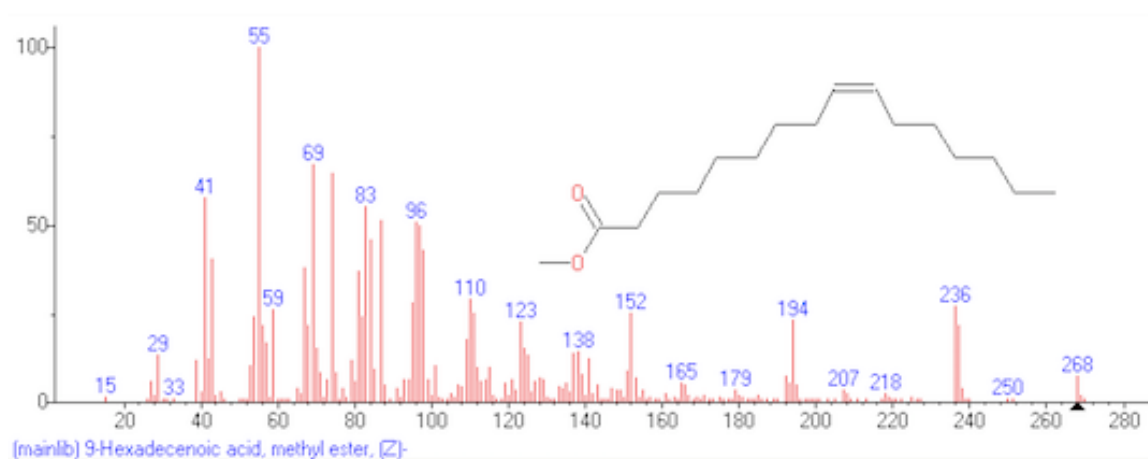
Hexadecenoic acid, methyl ester
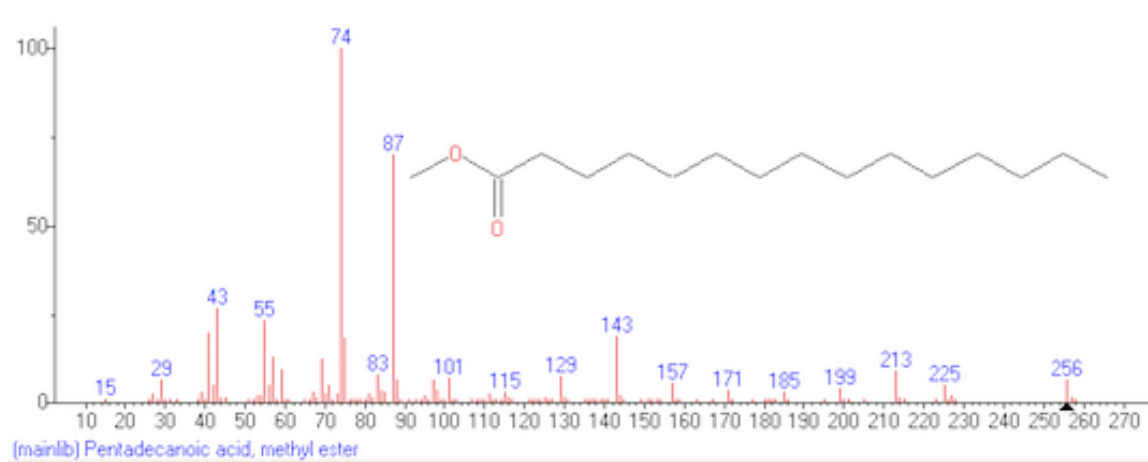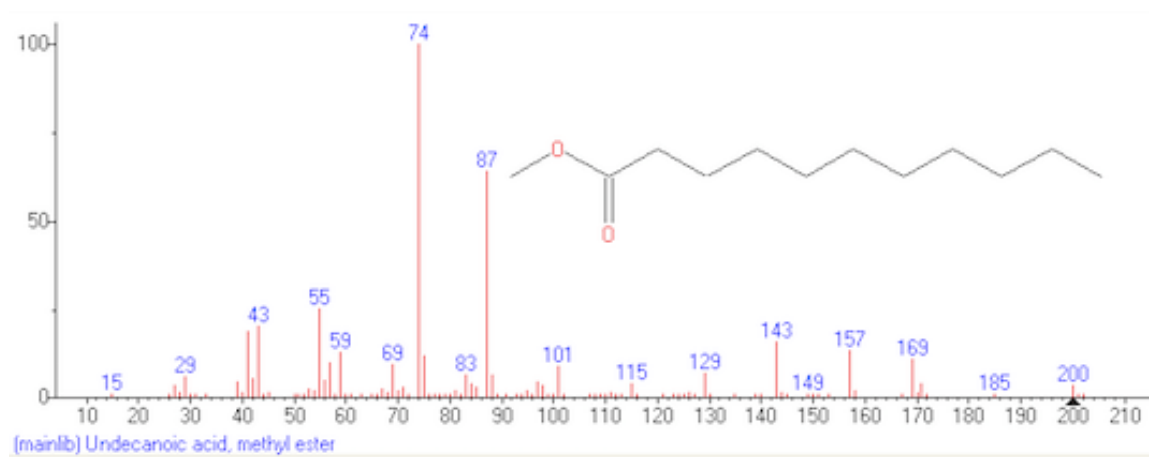

Pentadecanoic acid, methyl ester

Octadecanoic acid, methyl ester
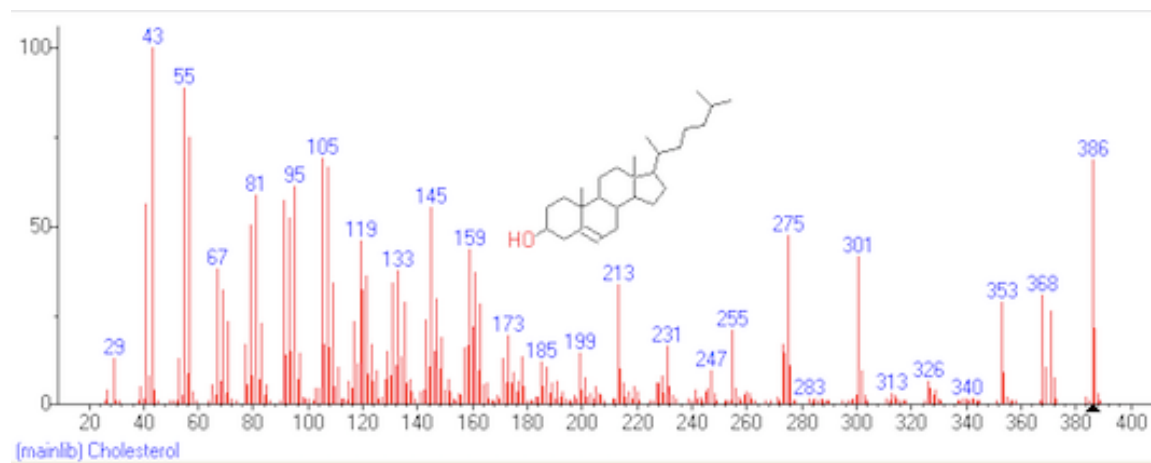

Methyl tetradecanoate

Tridecanoic acid, methyl ester

Undecanoic acid, methyl ester

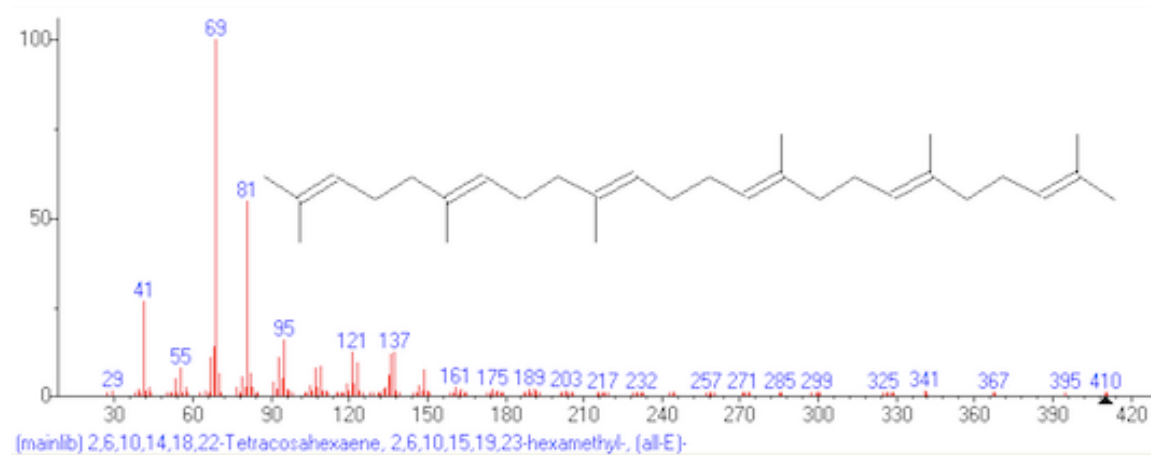## 2. Mass spectrums for cholesterol and Squalene

Cholesterol

Squalene