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ISOLATION AND CHARACTERIZATION OF DIFFERENT  
AGGREGATES OF LIPID FROM BOVINE MILK

by

Ankur Jhanwar

A thesis submitted in partial fulfillment  
of the requirements for the degree

of

MASTER OF SCIENCE

in

Nutrition and Food Sciences

Approved:

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Logan, Utah

2009

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## ABSTRACT

Isolation and Characterization of Different Aggregates  
of Lipid from Bovine Milk

by

Ankur Jhanwar, Master of Science

Utah State University, 2009

Major Professor: Dr. Robert E. Ward  
Department: Nutrition and Food Sciences

Bovine milk fat globules naturally vary from less than 0.2  $\mu\text{m}$  to 15  $\mu\text{m}$  in diameter. Milk has at least two distinct distributions of fat globules. While the majority (~90%) of globules in milk are of the smaller distribution (average diameter of 0.4  $\mu\text{m}$ ), virtually all the fat is carried in the larger globules (average diameter 3.5  $\mu\text{m}$ ). This distribution suggests some compositional and/or functional significance might exist between the two populations of fat globules, which may be related to origin of these globules in the lactating cell.

Milk fat globules have a unique structure, composed of a core droplet of non polar lipids (triacylglycerol) surrounded by a lipid bilayer membrane known as milk fat globule membrane (MFGM). Other than MFGM, there is another source of membrane that has been identified in skim milk. It has been hypothesized that this skim milk membrane (SMM) is derived from MFGM, but

little data are available to support this idea, and the membrane may also have alternate origins.

In this study, different aggregates of lipids (small and large fat globules, SMM, skim milk) from milk were isolated and characterized for their lipid contents. Isolation of small and large fat globules fractions was verified by laser diffraction particle size analysis. The lipids were extracted from isolated different lipid aggregates and individual classes were separated using thin layer chromatography. Lipids were transesterified to fatty acid methyl esters and analyzed by gas chromatography-mass spectrometry.

The results indicate that there are some compositional differences between native milk fat globule membranes of different sizes. For example, the total phospholipid fraction of small fat globules (SFG) contained significantly more unsaturated C18:1n9 and C18:2n6 than large fat globules (LFG). Conversely, sphingomyelin composition of SFG contained less C18:1n9 and C18:2n6cc, but more long chain fatty acids C22:0, C23:0, and C24:0. Phosphatidylethanolamine composition of SMM contained more C17:1 than SFG and LFG. The composition of C18:1n9 in triacylglycerol increased with fat globule size. Clear differences were also found in lipid profile of SMM and small and large fat globules from milk. Composition differences between SMM and native milk fat globules of different sizes suggest that origin of this membrane material in skim milk might have some different source than that of MFGM.

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Ankur Jhanwar

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LIST OF SYMBOLS,  
ABBREVIATIONS, AND DEFINITIONS

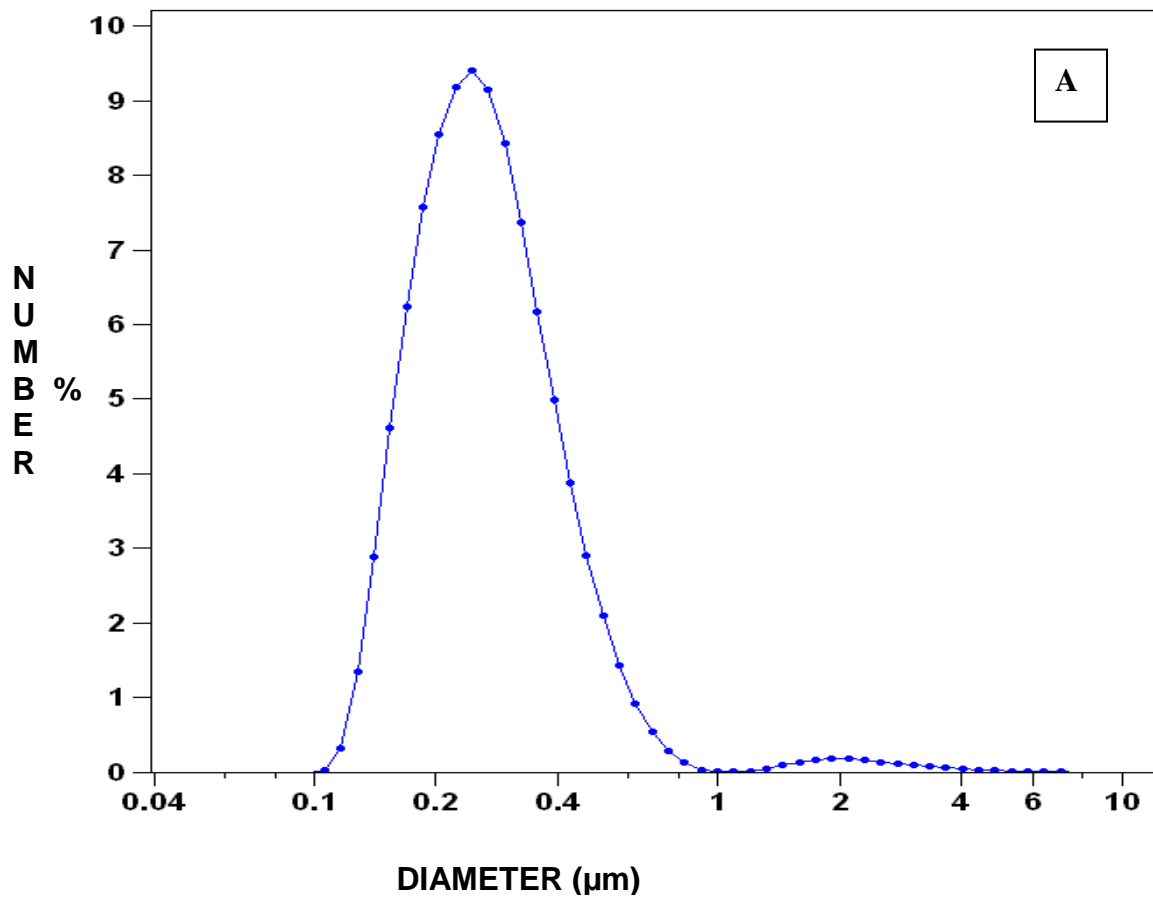
CE	Cholesterol Esters
DG	Diacylglycerols
FAMES	Fatty Acid Methyl Esters
FFA	Free Fatty Acids
GC-MS	Gas Chromatography-Mass Spectrometry
LFG	Large Fat Globules
MFGM	Milk Fat Globule Membrane
NaCl	Sodium Chloride
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PL	Phospholipids
PUFA	Polyunsaturated Fatty Acids
SFG	Small Fat Globules
SM	Sphingomyelin
SMM	Skim Milk Membrane
TAG	Triacylglycerols
TLC	Thin Layer Chromatography

## INTRODUCTION

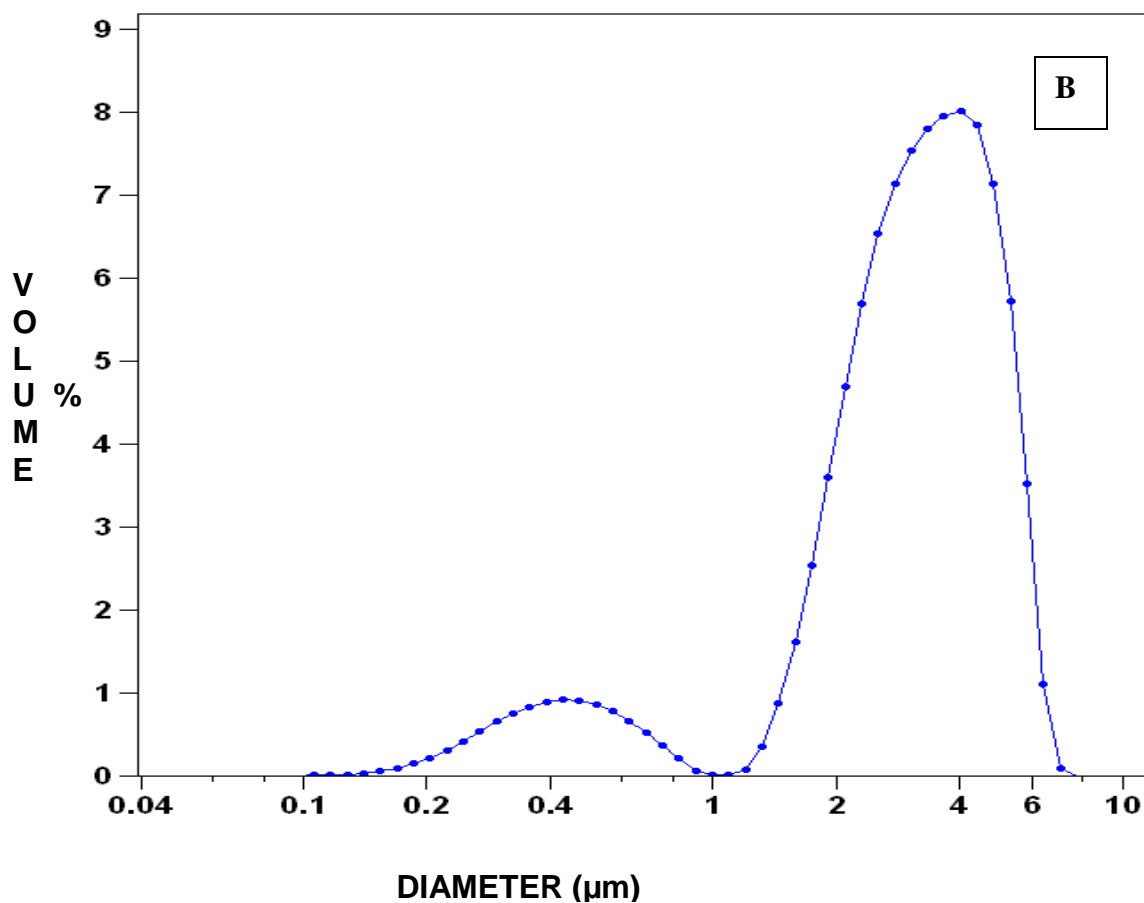
Fat is present in the milk in the form of droplets of micron size, with diameters ranging from less than 0.2  $\mu\text{m}$  to about 15  $\mu\text{m}$ , known as native milk fat globules. Bovine milk has at least two distinct distributions of fat globules [Figure 1], the average diameter of small fat globules is 0.44  $\mu\text{m}$  and the average diameter of large globules is 3.51  $\mu\text{m}$ . While the majority of globules in milk are present in the smaller distribution (98.5%) [Figure 1.A], the larger fat globules represent most of the fat volume (90%) [Figure 1.B]. This distribution suggests some compositional and functional significance might exist between two populations. However, to date, the fatty acid profile of different lipid classes of the small fat globules has not been reported.

Milk fat globules have a unique structure, composed of a core droplet of non polar lipids (triacylglycerol) surrounded by a lipid bilayer membrane known as milk fat globule membrane (MFGM). The structure of fat globules is due to a unique secretion process in the mammary gland. Milk fat triacylglycerol is synthesized in the smooth endoplasmic reticulum (SER) and forms small droplets which bud off the SER and transcytose towards the apical surface of the cell. Numerous small lipid droplets fuse together and form big droplets as they move toward the apical membrane. At the apical surface of the cell, the lipid droplet passes through the membrane and in the process is enveloped in a bilayer of cell surface membrane. Thus, each fat globule is coated in a bilayer of plasma membrane which originates from the secretory cell. This membrane (MFGM) is

composed of phospholipids, cholesterol, enzymes and membrane proteins  
(Heid and Keenan, 2005).



**Figure 1:** Milk fat globule size distribution A) by number, B) by volume



**Figure 1:** Continued.

The unique nature of the lipid secretion process, the bimodal distribution and the vast surface area of MFGM suggests potential nutritional significance of the structure. The composition and structure of bovine milk fat have been reviewed extensively (Morrison, 1970; Jensen and Newberg, 1995; Jensen, 2002). Bovine milk lipids are largely composed of triacylglycerols (TAG); however, there are also minor amounts of diacylglycerols (DG),



monoacylglycerols, free fatty acids (FFA), phospholipids and sterols. The main classes of lipids present in bovine milk are shown in Table 1.

Triacylglycerols account for about 98% of the total fat and have a major and direct effect on the properties of milk fat, such as hydrophobicity, density and melting characteristics. Phospholipids (PL) account for only 0.8 % of milk lipids. However, they play a major role in milk due to their amphiphilic properties. Most of the phospholipids (65%) are found in the milk fat globule membrane (MFGM), whereas the rest remain in the aqueous phase (MacGibbon and Taylor, 2006). Major classes of phospholipids are phosphatidyl choline (PC), phosphatidyl ethanolamine (PE) and sphingomyelin (SM). They comprise about 90% of the total phospholipids and are present in similar proportions, between 25 to 35% of total phospholipids. Phosphatidylserine and phosphatidylinositol are other class of phospholipids which comprise the remaining 10% of total phospholipids (MacGibbon and Taylor, 2006). Phospholipids play an important role in structure of cell membrane and in cell signaling. Specific polar lipids (such as sphingomyelin) or their metabolites are also recognized to have a number of positive health effects relating to immune function, heart health, brain health and cancer (Vesper et al., 1999).

Other than milk fat globule membrane, there is another source of membrane that has been identified in skim milk. Ultracentrifugation of skim milk results in a thin cream layer on the top, and a casein pellet at the bottom. In addition, some fluffy appearing material is visible just above the casein pellet, which was shown to contain membrane material (Stewart et al., 1972). Around

55-75% of membrane material in skim milk is recovered in this fluffy fraction. It has been hypothesized that this skim milk membrane is derived from MFGM, however little data are available to support this idea, and the membrane may also have alternate origins. The source of this membrane material in skim milk is yet to be confirmed.

**Table 1.** Main classes of lipids in milk<sup>a</sup>

Lipid class	Amount (% , w/w)
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

<sup>a</sup>MacGibbon and Taylor, 2006.

The composition of milk fat is of great importance, not only for technological and sensory properties of many dairy products but also from nutritional aspects. Bimodal distribution of fat globule size in bovine milk suggests some

compositional and functional significance might exist between two distinct populations of fat globules. Thus, isolation and characterization of the fatty acid composition of small vs. large native milk fat globules could allow a better understanding of milk fat. Identifying the composition of these different sizes of fat globules could allow the development of products with better control of technological processes and with new sensory properties. Moreover, it would bring new insights into the intracellular origin of milk fat globules of various sizes.

Hypothesis of this study are:

1. Lipid compositions of native small and large fat globules are different
2. Comprehensive lipid analysis of skim milk membrane will indicate whether or not it is derived from the milk fat globule membrane.

The research aims addressed in this thesis are:

1. Isolation of distinct aggregates of lipid from bovine milk; SFG, LFG, and SMM.
2. Characterization and comparison of fatty acid profile of different lipid classes (TAG, DG, FFA, CE, PL, PC, PE, and SM) extracted from SFG, LFG, and SMM.

## LITERATURE REVIEW

### Origin and Secretion of Milk Fat Globules

Milk fat globules are composed of a core droplet of non polar lipids (rich in triacylglycerol) surrounded by a monolayer of polar lipids and then by a lipid bilayer membrane. The outer membrane is composed of phospholipids, cholesterol, enzymes and membrane proteins. This milk fat globule membrane that surrounds the milk fat droplets is derived from the apical plasma membrane of the secretory cells in the lactating mammary glands. The secretion of the fat globules of milk from mammary epithelial cells seemingly occurs by a unique process and is unlike the exocytotic mechanism used by other cell types to secrete lipids (Heid and Keenan, 2005).

Precursors of milk fat globules are formed in the endoplasmic reticulum and are transported through the cytosol as small lipid droplets covered by monolayer of polar lipids and proteins (Dylewski et al., 1984). Milk lipid globule precursors appear in the cytosol as droplets ranging in diameter from less than 0.5 to more than 4  $\mu\text{m}$  (Dylewski et al., 1984; Deeney et al., 1985). Droplets appear to grow in volume by fusing with each other, giving rise to larger droplets, termed cytoplasmic lipid droplets (Heid and Keenan, 2005). Droplet fusion has been reconstituted in a cell-free system (Valivullah et al., 1988). In this cell free system droplet fusion was promoted by calcium, gangliosides and by an as yet uncharacterized high-molecular- weight protein fraction from cytosol. While small droplets fuse readily, larger cytoplasmic lipid droplets did not fuse in the cell-free

system. The reasons larger, cytoplasmic lipid droplets do not fuse with each other are not apparent but may be related to some compositional differences between the coat material on micro- and cytoplasmic lipid droplets (Dylewski et al., 1984; Deeney et al., 1985). While evidence supports the view that increase in volume of lipid droplets occurs through fusions of microlipid droplets with each other and with cytoplasmic lipid droplets, it is not known if this is the only mechanism supporting droplet growth (Heid and Keenan, 2005). Observations suggest that micro lipid droplets 1) may be secreted from cells directly as the very small milk lipid globules, 2) may fuse with each other to form larger droplets or cytoplasmic lipid droplets, 3) may fuse with cytoplasmic lipid droplets to provide materials for growth of these precursors of large milk lipid globules (Deeney et al., 1985).

At the apical plasma membrane, the lipid droplets are secreted from the epithelial cells into the avolear lumen. During the unique secretion process, the droplets are progressively enveloped in the plasma membrane up to the point where the lipid droplet become pinched off from the cell completely surrounded by plasma membrane. This process was first described by Bargmann and Knoop (1959), who observed that lipid droplets approach closely to or contact the apical plasma membrane and are gradually enveloped in plasma membrane up to the point where they are dissociated from the cell, surrounded entirely by plasma membrane. This process was studied by several other groups and became the widely accepted mechanism of milk fat globule secretion (Patton and Keenan, 1975; Mather and Keenan, 1983, 1998; Keenan et al., 1988; Keenan and Patton,

1995). This portion of the cell membrane, which enveloped the globule during the extrusion process, is known as the milk fat globule membrane.

While general overview of the steps leading to the fat globules of milk has been extensively studied and described, virtually nothing is known about the potential physiological benefits of the unique structure of milk fat globules.

### **Isolation of Fat Globules and Measuring Fat Globule Size Distribution**

#### **Isolation of distinct distribution of fat globules**

Milk fat is predominantly present in spherical droplets which range in diameter of less than 0.2  $\mu\text{m}$  to about 15  $\mu\text{m}$ . Bovine milk has at least two distributions of fat globules [Figure 1]. Small fat globules with  $<1 \mu\text{m}$  in diameter by far are most numerous (98.5%) [Figure 1.A], but large fat globules (LFG) in the range of 1-10  $\mu\text{m}$  in diameter account for 90% of the volume of milk lipid [Figure 1.B] (Mulder and Walstra, 1974). This distribution suggests some compositional and functional significance might exist between two populations. The small native fat globules are expected to alter the functionality because they contain more MFGM and would differ slightly in composition (Timmen and Patton, 1988). Therefore, technologies have been developed to separate native milk fat globules of different sizes.

***Method of centrifugation.*** Traditional procedure for fat globule isolations involve repeated cycles of centrifuging to obtain globules and subsequent redispersion of them in fresh buffer to eliminate other milk components (Brunner,

1965). This process is time consuming and may affect the globule membrane structure, including partial churning of the globules. It has been shown that 85% of the xanthine oxidase and alkaline phosphatase activities are removed from bovine milk fat globules by four successive water washes (Zittle et al., 1956).

In 1986, Patton and Huston published a new and novel method for isolation of milk fat globules. They performed the comparison between the results obtained from old technique of fat globule isolation and their new method. By the old procedure, fresh milk was centrifuged at  $2,000 \times g$  and ambient temperature for 15 min. Recovered globule layer was resuspended in appropriate medium (buffer, saline, or water), contents were made to original volume and centrifuged as before. This washing process was repeated two or more times and the final globule layer was obtained and analyzed. In the new method, globules are centrifuged out of the milk and through an overlying buffer layer. Using this method, they recovered from human milk samples purified globules by centrifuging the milk at  $1,500 \times g$  for 20 min after deposition under suitable quantity of buffer. Their method is simple, less manipulative and yields purified globules in less time, which can be dispersed more satisfactorily than those by the traditional method. They compared the results from both old and new methods. They found that protein, phospholipids and cholesterol contents of globules by the two methods were quite similar. They also showed that the method can be applied satisfactorily to cow's and goat's milks.

Timmen and Patton (1988) used differential centrifugation method to prepare small and large fat globule- enriched fractions from raw, whole, bovine

milk, with mean globule diameters of 1.77 and 3.17  $\mu\text{m}$ , respectively.

Centrifugal separation segregates the larger ones into cream and the smaller ones with the skim milk (Mulder and Walstra, 1974). They centrifuged the milk in glass tubes plugged at the bottom end with rubber stoppers. Following centrifugation, cream layer was hardened by putting tubes in ice water and skim milk was decanted from the bottom by removing the stoppers. Remaining cream layer was used as sample of larger globules. Smaller globules fraction was obtained by centrifuging skim milk at  $33,000 \times g$  at  $4\text{ }^{\circ}\text{C}$  for 1 h and subsequently suspending thin cream layer in water. The Authors reported differences in fatty acid composition of obtained two distinct fractions of fat globules, which are discussed in later section of this literature review.

***Method of gravity separation.*** Ma and Barbano (2000), reported a method of gravity separation of native milk fat globules into seven different size fractions according to difference in density. Milk fat has a lower density than the skim phase; therefore, fat globules tend to rise under the influence of gravity (Walstra, 1995). Their study was focused towards determining effects of time and temperature on changes of fat globule size distribution and fat content in milk fractions during gravity separation. In lieu of centrifugal separation, they subjected fat globules to gravity separation in vertical columns and characterized the size distribution as a function of height. Seven different fractions were collected from bottom to top of separation columns after 2, 6, 12, and 48 h successively. With increased time, the bottom fraction was enriched in smaller fat globules (volume mean diameter at  $4\text{ }^{\circ}\text{C}$ ,  $1.16\text{ }\mu\text{m}$ ) and large fat globules



(volume mean diameter at 4 °C, 3.48 µm) were moved to the top fraction.

There were significant effects of time of separation, fraction number, and time by fraction interaction on both particle size and fat content. The Bottom most fraction had the lowest fat content at each separation time. At 48 h, the fat content of this fraction was as low as that of skim milk, about 0.2% and 58.8% (weight based) of the total fat ended up in the top 5 ml cream layer. The trend of fat content change in the bottom six layers over time was consistent with changes of fat globules size distribution.

O'Mahony et al. (2005) used a 2-stage gravity separation method to obtain different fat globule size distributions from milk for the manufacture of Cheddar-type cheeses. A two-stage gravity separation scheme was developed for fractionating raw, whole bovine milk into fractions enriched in small or large fat globules. In the primary stage, milk was allowed to separate under quiescent conditions for 6 h at 4 °C. Skim milk, thus obtained from separation was drained via tap in another vessel. The remaining fraction, i.e., the cream phase (cream-1) was removed and stored overnight, at 4 °C. Duration of secondary separation stage was 18 h at 4 °C, after which semi skim milk was drained and supernatant cream-2 retained. 'Cream-1' and 'Cream-2' were used as large fat globules and small fat globules, respectively. The volume mean diameter of fat globules in fraction enriched in small fat globules and large fat globules were 3.45 and 4.68 µm, respectively. Fat content of each fraction was measured using Gerber methods according to Bradley et al. (1992). The small fat globules fraction had 3.55% fat and large fat globules had 11.33% fat, compared to 4.00% fat for the

original whole milk. The specific surface area of fat globules in small fat globules milk was significantly greater than that of the large fat globule milk, but not significantly different from that of the control milk. Cheddar cheeses were manufactured using each of the 3 milks (whole milk, small and large fat globules fractions). Rennet coagulation properties of milks and the evolution of free fatty acids in the cheeses during ripening were compared. The maximum value of storage modulus, which is an index of stiffness of the gel, was significantly higher for rennet gels formed from small fat globule milk than from large fat globule milk in cheese making. They also found that the use of milk enriched in large fat globules resulted in a significant increase in the rate of liberation of free fatty acids during ripening.

**Membrane technology.** St-Gelais et al. (1997) used a proprietary milk fat fractionation process for the manufacture of low-fat Cheddar cheese from milks enriched in small or large fat globules. They reported the diameter of small and large globules as 1.6 and 2.4  $\mu\text{m}$ , respectively. Cheese made from milk containing primarily large fat globules was scored significantly higher for texture, flavor and color than cheese made from milk containing primarily small fat globules.

Membrane microfiltration, in association with centrifugal separation, has been employed for the fractionation of milk fat globules. Gouedranche et al. (2000) separated milk fat in small globules (diameter lower than 2  $\mu\text{m}$ ) and in large globules (diameter higher than 2  $\mu\text{m}$ ) by a patented process using special ceramic microfiltration membranes. They performed some transformations in

drinking milks, yogurts, sour cream, camembert, Swiss cheese, and butters from milks of which the fat content was adjusted either by reference cream or by creams issued from the small or large fat globules fractions. These authors reported that except for butter, use of milks containing small fat globules led to more unctuous products and more finely textural characteristics versus products made with reference creams or with mainly large fat globules.

A different group has conducted work into separation of fat globules using cross flow microfiltration. Cream samples containing native milk fat globules of different sizes, ranging from diameter ( $d_{4,3}$ ) 1.5 to 7.34  $\mu\text{m}$  were obtained using a patented microfiltration process with raw whole milk by Briard et al. (2003). The purpose of their study was to characterize the compositional differences among natural milk fat globules of various sizes from two different seasons. The differences that they noted in fatty acid composition of small and large fat globules from two different seasons are described below in last section of the literature review.

### **Measurement of fat globule size distribution**

The determination of particle-size distribution seems very straightforward in the case of fat globules, because the particles may be considered as homogeneous spheres, which only differ in size. Nevertheless, accurate determination of the size distribution causes many problems. Different methods have been employed for measuring the size distribution of milk fat globules, such

as ordinary and fluorescence microscopy, photomicrography, spectroturbidometry, Coulter counter, and laser light scattering technique.

**Microscopy.** Microscopy allows the viewing and measuring of each individual particle, but many problems of accuracy, reproducibility and ease of operation arise. Also the smallest globules can easily escape notice (Walstra et al., 1969). Timmen and Patton (1988) measured particle size of isolated small and large milk fat globules fractions using camera-equipped Leitz Orthomat microscope. In preparation of samples they diluted the sample with warm water and mixed with 5% solution of gelatin in warm water. They used 1790 × total magnification to measure fat globule diameters. This method is not adequate in accounting huge numbers of very small globules (Walstra et al., 1969).

Another microscopy technique is a method of coloring the milk fat globules by fluorescence, as was first shown by King (1955). This technique of fluorescence microscopy was utilized by Scolozzi et al. for quantification and size distribution assessment of milk fat globules. Fat globules from fresh ewe's milk were identified by staining with the fluorescent dye acridine orange (Scolozzi et al., 2003). The mean number of fat globules was determined using the Burkert chamber and an analyzer system (Quantimet 500, Leica Ortomat) connected to fluorescence microscope. Fluorescence with a 40 × objective lens was used to identify and measure single globules having a diameter greater than 1.2 μm. Values for globules with diameter < 1 μm were estimated from the cumulative volume of milk fat. The globules have a specific color, and non-fat materials are usually invisible or have a different color. This technique is superior than normal

microscopy as due to the clear contrast, the possibility of overlooking small globules is very much reduced. However, microscopic counting and measurement is difficult, tedious and time consuming, and the results show poor reproducibility, therefore the method is not suitable for routine analysis (Walstra et al. (1969).

**Coulter counter.** An alternative to sizing by microscopy is the Coulter principle, i.e., the change in impedance when an oil droplet passes through an orifice (Walstra and Oortwijn, 1969; Walstra et al., 1969). Cornell and Pallansch (1966) determined the particle volume and number of fat globules in milk using the electrical sensing-zone principle employed in the Coulter Counter. Walstra et al. (1969) reported that Coulter counter gives more reproducible results as compare to microscopic methods, however it is a complicated instrument, thus handling and interpretation of the results require much care and knowledge.

**Spectroturbidimetry.** Spectroturbidimetry is another technique to measure fat globule size distribution. From the turbidity at several wavelengths, the fat content of the milk and refractive index of the fat, a specific turbidity spectrum can be calculated. It yields information on the size distribution by graphical comparison with theoretically computed spectra. The application of this technique in determining the size distribution was discussed by Walstra (1968). Although this method is very simple and rapid, the size of globules with a small mean diameter ( $<0.6 \mu\text{m}$ ) cannot be determined (Robin and Paquin, 1991). Robin and Paquin (1991) developed a technique using photon correlation spectroscopy to measure the average diameter and the relative dispersion of fat

globules in a milk model emulsion (sodium caseinate, butter oil, and a lipophilic emulsifier).

***Laser light scattering.*** More recently, laser light scattering particle size analyzers have been used in several studies to measure size distribution of fat globules in various types of milk (Ma and Barbano, 2000; Michalski et al., 2001, 2006; Briard et al., 2003; Wiking et al., 2004; Fauquant et al., 2005; Michalski et al., 2005a, 2005b; O'Mahony et al., 2005). Laser light scattering particle size analyzer, with two laser sources, allowing the characterization of micronic as well as submicronic populations. To prevent artifacts regarding submicronic globules, the casein micelles (usually ~ 150 nm) are dissociated by diluting the sample in 35 mM EDTA (pH 7) prior to measurement.

O'Mahony et al. (2005), determined fat globules size in milk using a static laser light-scattering technique to measure the size of large and small fat globules obtained from milk by 2-stage gravity separation. Volume mean diameter of small and large fat globules obtained by gravity separation at 4 °C after 24 h was 3.45  $\mu\text{m}$  and 4.68  $\mu\text{m}$ , respectively.

Size distribution of fat globules in human colostrum, breast milk, and infant formula were measured by laser light scattering using Mastersizer 2000 by Michalski et al. (2005b). They measured milk fat globule size distribution in colostrums and transitional human milk in comparison with fat globules of mature milk and infant formula. In mature milk, the milk fat globules diameter was 4  $\mu\text{m}$  on average and increase with advancing lactation, whereas the droplets in infant formula measured 0.4  $\mu\text{m}$ .

### **Isolation and characterization of skim milk membrane**

Besides milk fat globule membrane (MFGM), membrane material may be recovered from skim milk. Plantz et al. (1973) used differential ultracentrifugation method to concentrate membrane fragments and microvillus like sacs from bovine skim milk. They found that the ratios of the free cholesterol, lipid phosphorus, and triacylglycerol contents of the fraction were similar to those for plasma membrane of the lactating cell. On ultracentrifugation of skim milk supernatant, they obtained a “fluff” layer (membrane rich material of skim milk) that overlay the pelleted casein micelles. After doing lipid analysis of this fluff fraction they reported that triacylglycerols with traces of free cholesterol and phospholipids might be released from the original membrane fraction to the supernatant. They concluded the co-identity of skim milk membrane material and plasma membrane of lactating mammary cell. But the source of this skim milk membrane material is still unknown.

Stewart et al. isolated a lipid fraction by two stage ultracentrifugation of skim milk (Stewart et al., 1972). During the first stage, skim milk was ultracentrifuged at  $135,000 \times g$  for 1 h at  $4^\circ C$  and a small amount of “fluffy” material on top of casein pellet was collected. In the second stage, pooled solution of this fluff fraction was centrifuged at  $45,000 \times g$  for 1 h at  $4^\circ C$  to recover skim milk material in the fluff layer that overlay the pelleted casein micelles. They showed by electron microscopy that this fluff fraction is composed of membrane material, and speculated the majority of which arises from shed microvilli. During milk secretion, the Golgi vesicle membrane appears to fuse with

and add to the apical plasma membrane. When fat globules emerge, they removed membrane from this cell surface. However, the Golgi vesicles containing all the non fat phase of milk would require a greater flux from the mammary cell than would the fat globules. Thus more membrane would be added to the apical cell surface than would be removed. Some membrane out of this excess membrane could be sloughed off and they suggested that this excess membrane is lost to the milk possibly in the form of sloughed microvilli.

Membrane bound vesicles, open vesicular structures, and tubular sacs, microvillus in nature, were observed in thin sections of this material. Preliminary analysis of several "fluff" layer preparations for lipid phosphorus and cholesterol indicated that 55 to 75% of membrane material in the skim milk was recovered in "fluff" layer. Intramammary infusions of  $^{14}\text{C}$ - palmitate had shown that skim milk phospholipids become labeled more promptly and intensively than those of the MFGM (Patton and Keenan, 1971). MFGM is plate-like in appearance when negatively stained (Keenan et al., 1970), but negative stain of this material obtained from skim milk revealed flattened vesicles varying in shape from sacs to tubular structures. These morphological and radioactive data do not support the concept that skim milk lipoprotein arises by disintegration of the MFGM. Thus, the nature or function of the particles observed on the membrane isolated from skim milk is not known.



## Extraction and Characterization of Lipids

Timmen and Patton (1988) extracted lipid from small and large fat globules (average diameter 1.5 and 3  $\mu\text{m}$ , respectively) by the Rose-Gottlieb procedure (1975) as modified by Walstra and de Graaf (1962). Triacylglycerols of lipid samples were isolated by thin layer chromatography. The bands of triacylglycerols were detected by exposing the silica plate to iodine vapor. Isolated lipids were transesterified to methyl esters using sodium methoxide in methanol (Christopherson and Glass, 1969). Resulting methyl esters were analyzed by gas chromatography. The results were expressed as wt. % of each fatty acid in relation to the total fatty acids. They found that small milk fat globules have a slightly different composition from the larger ones. Small globules were found to contain less short-chain fatty acids in their triacylglycerols, also small fat globules contain less stearic acid (C18:0) and more oleic acid (C18:1). Brunner also found more unsaturated C18 fatty acids in very small globules (Brunner, 1965).

Briard et al. (2003) extracted lipid from freeze dried samples of small (1.5  $\mu\text{m}$ ) and large fat globules (7.34  $\mu\text{m}$ ) isolated from milk obtained in winter and spring seasons. They used ethanol, NaCl solution and mixture of hexane and diethyl ether as solvents to extract total lipid. Gas chromatography was used to analyze lipid samples followed by transesterification of lipid to methyl and butyl esters. In both winter and spring, they reported significantly more C12:0, C14:0, C16:1 and less C18:0 in total lipid of small fat globules compared to large fat globules. They observed difference in composition of small and large globules

was season dependent. They found that in winter, small fat globules contain significantly less C18:1 and C18:2 and more C8:0, C10:0 and C16:0 than in large globules. Conversely, there was significantly more C18:1 and C18:2 in small globules than in large globules in spring milk. They proposed, in spring milk, the higher oleic and linolenic fatty acids proportion in small globules can be accounted for by their higher MFGM proportion in total fat, but it can be assumed that there is also more unsaturated fatty acids in the fat core. Timmen and Patton (1988) also found more oleic acid in fat core triacylglycerols of small fat globules (average diameter 1.5  $\mu\text{m}$ ) from skim milk. Tverdokhleba (1957), on the other hand found that small globules contain less oleic acid. They hypothesized that these differences might be related to the origin of milk fat globules of different sizes in the lactating cell.

Isolation of different populations of native milk fat globules according to their size (3 and 6  $\mu\text{m}$  for small and large fat globules, respectively) and characterization of their content and profile in the different CLA isomers was performed by Michalski et al. (2005a). Extraction and analysis of lipid was done in the same manner as described above in Briard et al study (Briard et al., 2003). They found that total CLA content was higher in the small fat globules fraction as compared to the large fat globules fraction. Their fatty acid results suggest that predominant CLA isomer in cow milk is the cis-9, trans-11, which represents 80% of total CLA isomers in the study. Smaller globules had around 7 % more cis-9, trans-11 isomer as compared to large globules fraction regarding total CLA isomers, and at least 37% more cis-9, trans-11 quantity in total fat.

Fauquant et al. (2007) obtained MFGM from isolated small (3.2  $\mu\text{m}$ ) and large fat globules (6.3  $\mu\text{m}$ ) for characterization of phospholipids and sterols and compared to untreated milk fat globules (4.4  $\mu\text{m}$ , original milk globules without any microfiltration treatment). After the separation of total lipids from the MFGM using the Bligh and Dyer technique (Bligh and Dyer, 1959), phospholipids were separated from neutral lipids using a silica cartridge BakerBond SPE (Juaneda and Rocquelin, 1985). The different PL and neutral lipid classes were separated by thin-layer chromatography. They found that the proportion of PL in the total MFGM lipids was higher in the MFGM of LFG than in that of SFG. There were less unsaturated fatty acids in PL of the MFGM from SFG than those from the untreated milk fat globules (UFG). The total fatty acid composition of PL extracted from the MFGM fractions has been analyzed previously by the same group Fauquant et al and no significant differences have been found between SFG and LFG fractions (Fauquant et al., 2007). However, in this study they found some differences in the MFGM of SFG and that of UFG. They showed in the results that SFG phospholipids contain more saturated fatty acids than LFG and than UFG. Monounsaturated fatty acids were more in phospholipids of LFG as compared to SFG and were found least in UFG. Finally, UFG was found to contain more polyunsaturated fatty acids than LFG and than SFG. Thus, the PL of the MFGM from SFG tended to be less unsaturated than those from untreated fat globules. Their study revealed no significant differences regarding the composition of the PL and sterol species in the MFGM of fat globule fractions selected by microfiltration of raw mixed milk. However, the PL fractions of the

MFGM from SFG tended to contain more sphingomyelin than the untreated MFGM. Minor bioactive sterols were also detected in treated globules, especially lanosterol and phytosterols.

Fauquant et al. (2005) characterized differences regarding fatty acid composition of the MFGM and the triacylglycerol core among differently sized milk fat globule fractions (average diameter 3 and 6  $\mu\text{m}$ ). Lipids from the MFGM and triacylglycerol core were extracted using the Bligh and Dyer technique (Bligh and Dyer, 1959). Silica cartridge (BakerBond SPE) was used to separate MFGM phospholipids from residual triacylglycerols following the method of Juaneda and Roquelin (1985). Recovered lipids were transesterified to fatty acids methyl esters and analyzed by gas chromatography. Data was reported by calculating relative compositional difference for a given fatty acid in small fat globules compared to large fat globules. They observed less C18:1 and C18:2 in the membrane of small fat globules compared to large fat globules; however, these differences were not significant. Thus, they believed that the differences in total fatty acid composition between small fat globules and large fat globules observed previously by Briard et al. (2003) should not be due to fatty acid compositional differences among the milk fat globule membranes. Relative compositional difference between the triacylglycerol cores of small fat globules and large fat globules showed that there was always significantly more C12:0, C14:0, C14:1, C16:0, C16:1, C21:0, C20:3n-3 and less C18:0 and C20:5n-3 in small fat globules compared to large fat globules. SFG were also found to contain more CLA, C20:1n-9 and C20:4n-6 and less C20:0, but these results were not

significant, may be due to their low amount in triacylglycerols (< 0.1%) and their great variations. Only small and non-significant differences were observed for C18:1 and C18:2 (content in SFG< LFG). They explained the reason of their different results than other studies (Timmen and Patton, 1988; Briard et al., 2003) in regard of oleic and linolenic acid contents by mentioning that differences were might be due to the difference in fat globule size studied.

## MATERIALS AND METHODS

### **Isolation of Different Aggregates of Lipid from Bovine Milk**

#### **Fractions with different sizes of native fat globules**

Whole milk was obtained from dairy plant, Nutrition and Food Sciences department, Utah State University. Small fat globules were isolated by combination of centrifugation and microfiltration of whole milk. In the process, whole milk was centrifuged at  $2,000 \times g$  at  $4\text{ }^{\circ}\text{C}$  for 20 min (IEC Centra CL3R, refrigerated centrifuge, rotor 243, Thermo Electron Corporation). Skim portion was collected from the bottom of centrifuge tube after removing cream layer from the top. An aliquot of skim milk was removed and kept for characterization of lipid profile. Remaining skim milk was subjected to microfiltration using combination of  $1.2\text{ }\mu\text{m}$  filter (Nitrocellulose isopore membrane, Millipore, Ireland) and  $1.0\text{ }\mu\text{m}$ ,  $47\text{ mm}$ , prefilter (Presep prefilter, Glass, GE Water and Process Technologies). Microfiltrate thus obtained was enriched in SFG with size less than  $1.2\text{ }\mu\text{m}$ . The retentate containing the remaining fat globules was discarded.

Large fat globules were isolated by the method of Patton and Huston (1986). In this method milk is mixed with sucrose (at a concentration of  $5\text{g}/100\text{ ml}$  of milk), and  $35\text{ ml}$  of this treated milk is layered beneath a  $15\text{ ml}$  of phosphate-buffered salt solution ( $0.14\text{ M NaCl}$  in  $0.01\text{M}$  phosphate buffer,  $\text{pH } 7.3$ ,  $15\text{ ml}$ ) in a  $50\text{-ml}$  plastic centrifuge tube. This delivery is accomplished by inserting a loaded pipette through the buffer to the bottom of the tube and then slowly draining

completely. The drained pipette is removed avoiding agitation. During subsequent centrifugation at  $1,500 \times g$  for 20 min, milk fat globules migrate to the top of the tube through the buffer, and are washed of skim contaminants in the process. This resulting globule layer is enriched in milk fat globules  $>1 \mu\text{m}$ . This layer was collected with the help of spatula and characterized as LFG in further analyses.

### **Recovery of membrane material from skim milk**

Skim milk was obtained by centrifuging whole milk and removing the cream layer. SMM was isolated using ultracentrifugation according to the method of Stewart et al. (1972). In the process, fresh whole milk was centrifuged at  $2,000 \times g$  for 15 min at  $4 \text{ }^\circ\text{C}$ . Skim milk was collected from the bottom of the tube. The skim milk was then centrifuged in 25-ml centrifuge tubes at  $135,000 \times g$  for 60 min at  $4 \text{ }^\circ\text{C}$ . The result was a clear supernatant with the exception of a thin cream layer on the top. At the bottom, just above the casein pellet, small amounts of fluffy material were observed. The top 15 ml of supernatant was removed by pipetting and the remaining 10 ml fluid was used to resuspend the fluff material and to gently remove any material from the face of the casein pellet. Pooled solution of this fluffy fraction was made by collecting the bottom fraction from all the centrifuge tubes. During subsequent centrifugation of this pooled fraction at  $45,000 \times g$  for 60 min at  $4 \text{ }^\circ\text{C}$ , fluffy material was found suspended at the bottom of the tube. The fluid at the bottom of the centrifuge tube was

collected to obtain this fluffy material. This fluffy fraction was designated SMM in further experiment.

Samples of SM, LFG, SFG, and SMM were extracted in duplicate from the same batch of whole milk to perform the analysis in duplicate. Three different batches of whole milk were used in the study to repeat the whole experiment three times.

### **Particle Size Measurements**

The size distribution of fat globule populations (small and large fat globules) was measured by laser diffraction particle size analysis using a Beckman Coulter LS 230 (software version 3.19). Casein micelles were dissociated by diluting samples in 35 mM EDTA, pH 7 prior to measurement. Standard parameters were calculated by the software with the following equations: volume average diameter  $d_{4,3} = \frac{\sum(v_i \cdot d_i)}{\sum v_i}$  (where  $v_i$  is the volume of globules in a size class of average diameter  $d_i$ ), volume-surface average diameter  $d_{3,2} = \frac{\sum v_i}{\sum(v_i/d_i)}$  and specific surface area  $S = 6 \cdot \rho^{-1} \cdot d_{3,2}^{-1}$ , where  $\rho$  is the milk fat density.

### **Lipid Extraction and Recovery of Different Lipid Classes**

#### **Lipid extraction**

Isolated fractions were freeze-dried and the dried samples were weighed out in glass tubes (100 mg for milk and LFG, 400 mg for SM, SFG, and SMM) according to their fat content. Internal standards of different lipid classes;



triacylglycerol, diacylglycerol, free fatty acids, cholesterol ester, phospholipid, phosphatidyl choline, phosphatidyl ethanolamine, and sphingomyelin were prepared in chloroform. 150  $\mu$ l of internal standard of each class was added to each sample and weights were recorded. Lipids were extracted by the method of Folch et al. (1957). According to this method, samples with internal standards were mixed with chloroform/methanol (2:1) to a final volume 20 times the volume of the dried sample (1 g in 20 ml of solvent mixture). After dispersion, the whole mixture was homogenized (sonicated) and then agitated for 15-20 min in an orbital shaker at room temperature. The homogenate was filtered (funnel with a folded filter paper) to recover the liquid phase. The solvent was washed with 0.2 volumes (4 ml for 20 ml solvent mixture) 0.9% NaCl solution. After vortexing 20 seconds, the mixture was centrifuged at 1,500  $\times$  g to separate the two phases. The lower chloroform phase containing lipids were collected and evaporated under a nitrogen stream, weighed, reconstituted in a small volume of chloroform and stored at -80°C until further analysis.

#### **Separation and recovery of different lipid classes**

Individual classes of extracted lipid were separated using thin layer chromatography (TLC). Extracted lipid from each sample was diluted by chloroform such that 20  $\mu$ l of solution contained 2.5 mg of lipid. Aliquots of 20  $\mu$ l were spotted on a 20  $\times$  20 cm silica gel 60 analytical plate (250  $\mu$ m layer) (Whatman Inc., Florham Park, NJ). Total lipid classes were separated by developing the plate in a solvent system containing hexane, diethyl ether, and

formic acid in the ratio 80:20:2, respectively. Whereas, individual classes of phospholipid were separated using a solvent system containing 100ml chloroform, 67ml methanol, 7 ml acetic acid, and 4 ml milli-Q water. Individual lipid spots on TLC plate were detected using saturated iodine vapor (example is shown in Appendix B, Figure B3). As I had already added specific internal standards for each different lipid classes during previous step of isolation of lipids, identification of individual lipid class band were easier. These individual lipid classes were recovered from the TLC plate in pre-weighted glass tubes with caps. Recovered lipid classes from total lipid TLC plate were: triacylglycerol, diacylglycerol, free fatty acid, phospholipid, and cholesterol ester. Phosphatidylcholine, phosphatidylethanolamine, and sphingomyelin were separated from phospholipids TLC plate.

### **Preparation and Analysis of Fatty Acid Methyl Esters**

Recovered individual classes of lipids were transesterified to fatty acids methyl esters (FAMES) using the method of Curtis et al. (2008) with slight modifications. In each tube 0.8 ml of hexane and 1.2 ml of 10% acetyl chloride in methanol was added. Tubes were capped, vortexed and placed in 100 °C oven for 40 min. After completion of incubation period, samples were removed and allowed to cool down to room temperature. After vortexing, 2 ml of 6% sodium carbonate solution and 0.4 ml of hexane was added in each tube. On subsequent vortexing and centrifugation of tubes two distinct phases were obtained. Top organic layer was removed and transferred to gas chromatography vials. Solvent

was evaporated under a nitrogen stream and fatty acid methyl esters were collected in 200  $\mu$ l of hexane and transferred to vial inserts. The samples were subsequently analyzed by gas chromatography-mass spectrometry (GC-MS). Standard curve was prepared by running commercially available FAMES standards on GC-MS equipment just before running samples.

FAMES were analyzed by gas chromatography-quadrupole mass spectrometry using a Shimadzu QP2010MS equipped with an HP-88 capillary column (100m  $\times$  0.25mm  $\times$  0.2  $\mu$ m, Agilent Technologies, Santa Clara, CA). Experimental conditions were as follows: injection temperature 260  $^{\circ}$ C; injection mode splitless, carrier gas helium at a pressure of 230.9 KPa. Initial oven temperature 50  $^{\circ}$ C and this was held for 3 min post injection. The oven was ramped to 175  $^{\circ}$ C at a rate of 40  $^{\circ}$ C.min $^{-1}$  and held for 9 min, followed by a ramp to 250  $^{\circ}$ C at a rate of 3  $^{\circ}$ C.min $^{-1}$  and a 5 min hold. The transfer line was kept at 240  $^{\circ}$ C, the ion source at 250  $^{\circ}$ C, and ions were generated using electron impact at 70 eV. Total run time was 45.13 min. Data collection software (GCMS Solutions) was operated in simultaneous scan and single ion monitoring (SIM) modes. Full ion scans were conducted in the mass range from 40-350 m/z every 0.2 s alternating with SIM events monitoring the following ions 74, 87, 43, 41, 55, 69, 67, 81, 79, which were also on a 0.2-s interval.

FAMES were identified by comparison to authentic external standards. To establish the linearity of the detector response, a six point calibration was run with every sample set. The calibration standard contains 42 fatty acids representing most of the common species found in milk and dairy products. The

calibration levels and target ions for each analyte are shown in Table 2.

Percentage content of individual fatty acids was calculated with respect to total sum of fatty acids present by the formula: (Concentration of individual fatty acids/ sum of the concentration of all fatty acids - concentration of internal standard added)\*100

Compositional difference for a given fatty acid in SFG compare to LFG was calculated as (fatty acid % in LFG- fatty acid % in SFG). Similarly compositional difference between other samples (LFG-SMM, SFG-SMM) was also calculated.

### **Statistical Analysis**

An ANOVA was performed using SAS software version 9.0 (SAS Institute Inc.) to perform compositional comparisons between different aggregates of lipid (milk, large and small fat globules, skim milk and skim milk membrane) isolated from milk. All data were analyzed as two-way factorial designs with two replicates. Proc GLM was also run and least square means (LSmeans) were compared using Ryan-Einot-Gabriel-Welsch Multiple Range Test. Whole experiment was repeated three times.

**Table 2.** FAME calibration levels and respective target ions

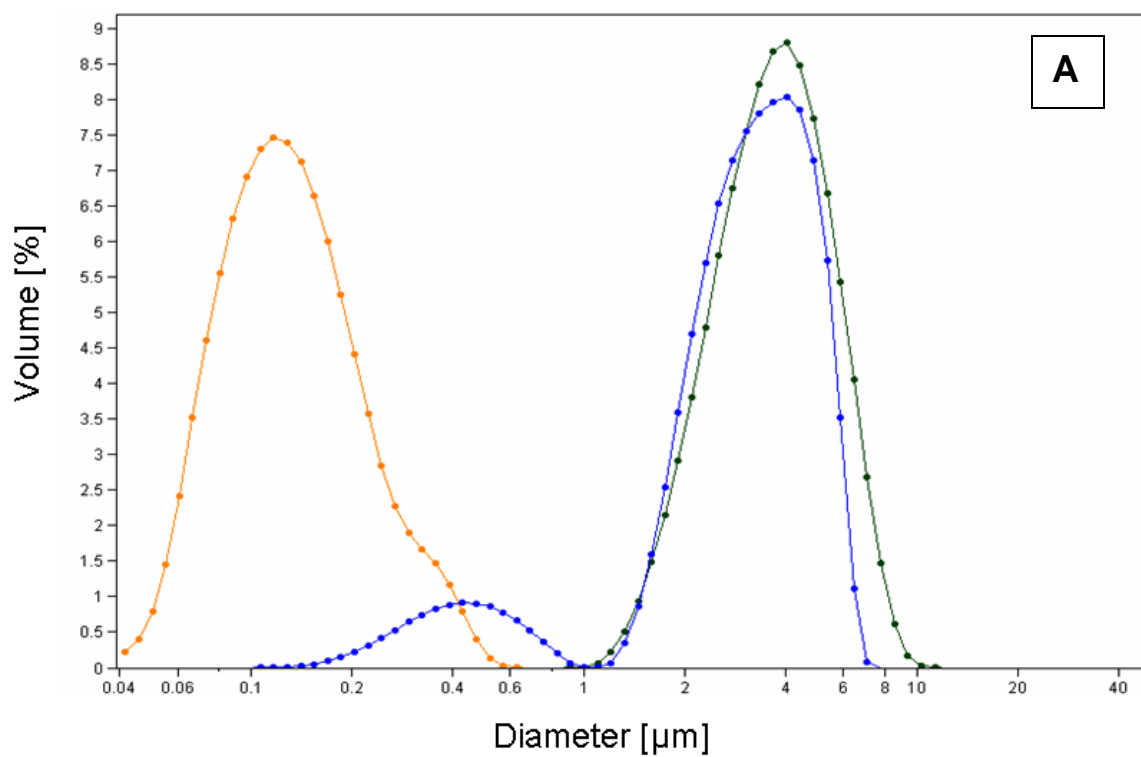
FAME calibration levels, all concentrations in ppm								
FAME	retention time	Level 1	Level 2	Level 3	Level 4	Level 5	Level 6	m/z
C10:0	12.921	0.01	0.05	0.25	0.5	1.25	2.5	74
C12:0	14.338	0.015	0.075	0.375	0.75	1.875	3.75	74
C14:0	16.499	0.035	0.175	0.875	1.75	4.375	8.75	74
C14:1T	17.203	0.0025	0.0125	0.0625	0.125	0.3125	0.625	55
C14:1	17.612	0.01	0.05	0.25	0.5	1.25	2.5	55
C15:0	18.016	0.005	0.025	0.125	0.25	0.625	1.25	74
C15:1T	18.773	0.0025	0.0125	0.0625	0.125	0.3125	0.625	55
C15:1	19.248	0.005	0.025	0.125	0.25	0.625	1.25	55
C16:0	19.61	0.095	0.475	2.375	4.75	11.875	23.75	74
C16:1T	20.39	0.005	0.025	0.125	0.25	0.625	1.25	55
C16:1	20.818	0.02	0.1	0.5	1	2.5	5	55
C17:0	21.474	0.005	0.025	0.125	0.25	0.625	1.25	74
C17:1T	22.408	0.005	0.025	0.125	0.25	0.625	1.25	55
C17:1	22.877	0.005	0.025	0.125	0.25	0.625	1.25	55
C18:0	23.463	0.04	0.2	1	2	5	10	74
C18:1T7	24.16	0.005	0.025	0.125	0.25	0.625	1.25	55
C18:1T9	24.252	0.005	0.025	0.125	0.25	0.625	1.25	55
C18:1T11	24.361	0.005	0.025	0.125	0.25	0.625	1.25	55
C18:1c7	24.545	0.0025	0.0125	0.0625	0.125	0.3125	0.625	55
C18:1c9	24.652	0.1	0.5	2.5	5	12.5	25	55
C18:1c11	24.832	0.005	0.025	0.125	0.25	0.625	1.25	55
C19:0	25.52	0.01	0.05	0.25	0.5	1.25	2.5	74
C18:2T	25.613	0.0025	0.0125	0.0625	0.125	0.3125	0.625	67
C19:1t7	26.248	0.0025	0.0125	0.0625	0.125	0.3125	0.625	55
C19:1t10	26.345	0.0025	0.0125	0.0625	0.125	0.3125	0.625	55
C18:2n6cc	26.53	0.015	0.075	0.375	0.75	1.875	3.75	67
C20:0	27.609	0.01	0.05	0.25	0.5	1.25	2.5	74
C18:3n6	27.792	0.0025	0.0125	0.0625	0.125	0.3125	0.625	79
C20:1t	28.41	0.0025	0.0125	0.0625	0.125	0.3125	0.625	55
C18:3n3	28.514	0.01	0.05	0.25	0.5	1.25	2.5	79
C20:1c	28.721	0.0025	0.0125	0.0625	0.125	0.3125	0.625	55
9-11 CLA	28.979	0.0025	0.0125	0.0625	0.125	0.3125	0.625	67
C20:2	30.657	0.0025	0.0125	0.0625	0.125	0.3125	0.625	67
C22:0	31.701	0.0025	0.0125	0.0625	0.125	0.3125	0.625	74
C22:1T	32.479	0.0025	0.0125	0.0625	0.125	0.3125	0.625	55
C20:3n3	32.649	0.0025	0.0125	0.0625	0.125	0.3125	0.625	79
C20:4n6	32.789	0.0025	0.0125	0.0625	0.125	0.3125	0.625	79
C22:1	32.863	0.0025	0.0125	0.0625	0.125	0.3125	0.625	55
C23:0	33.561	0.0025	0.0125	0.0625	0.125	0.3125	0.625	74
C22:2	34.707	0.0025	0.0125	0.0625	0.125	0.3125	0.625	67
C20:5 n3	34.807	0.0025	0.0125	0.0625	0.125	0.3125	0.625	79
C24:0	35.445	0.0025	0.0125	0.0625	0.125	0.3125	0.625	74
C24:1	36.537	0.0025	0.0125	0.0625	0.125	0.3125	0.625	55
C22:6n3	39.403	0.0025	0.0125	0.0625	0.125	0.3125	0.625	79

## RESULTS AND DISCUSSION

### Particle Size Results

The size distributions of the original milk sample and the isolated large and small native fat globules used in this study is presented in Figure 2. The corresponding average diameters of these samples isolated from three different batches of milk are shown in Table 3.

Particle size analysis of the fat globule distribution in bovine milk revealed that the number of small fat globules (< 1.0 micron) represents around 98.5 % of total fat globules in whole milk, but covers only around 9% volume of total fat. Particle size distribution of isolated small fat globules did not exactly correspond to particle size of small fat globules (< 1.0 micron) of raw milk. However, the isolated small globules are distinct from the larger ones, compositionally. It should be noted that the small milk fat globules obtained, show almost no size distribution overlap with the largest globules. Conversely, in studies such as that by Fauquant et al. (2005), and Briard et al. (2003), some overlapping was observed between distributions of small and large fat globules fraction. Thus the means of isolation (combination of microfiltration and centrifugation for SFG, and treatment with sucrose and centrifugation for LFG) used in this study was a successful technique which allowed the collection of two extreme distribution of fat globules. Some experiments were also performed to support the fact that the small fat globules fraction obtained in this study is truly present in the whole milk. Results of these experiments are attached in Appendix B as Figure B1 and B2.



**Figure 2:** Particle size distribution of milk sample with isolated different extreme sizes of native milk fat globules A) by volume B) by number  
Blue line: original milk sample; orange line: SFG; green line: LFG

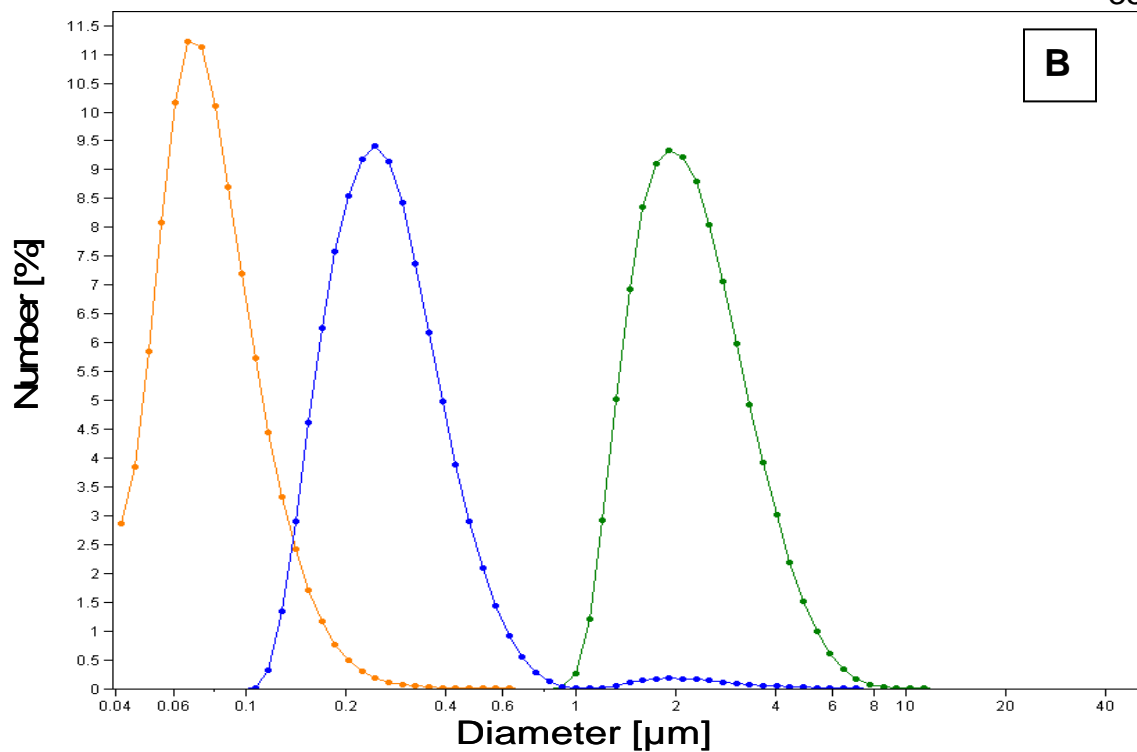


Figure 2: **Continued**

The result of the particle size analysis (bimodal distribution) of the fat globule distribution in bovine milk is similar to the results obtained in earlier studies with ewe milk (Scolozzi et al., 2003), human milk (Michalski et al., 2005b) and bovine milk (Fauquant et al., 2005). This indicates the distribution is a real feature, and not an instrumental artifact, as in these studies different techniques were employed to analyze the distribution of fat globules. Results are also similar to the analysis results of Michalski et al. (2006), wherein the same technique of laser light scattering by globules was used that we used for analysis of particle sizes. The average diameter of SFG used in this study was about 22 times



smaller than LFG and had a specific surface area up to 27 times larger. This size distribution from bovine milk has not been studied yet.

**Table 3.** Data of particle size distribution of small (SFG) and large (LFG) milk fat globules obtained from three different batches of milk. SFG and LFG with the same subscript originate from the same milk sample.

<b>Samples</b>	<b>D<sub>(3,2)</sub> [<math>\mu\text{m}</math>]</b>	<b>Mean, D<sub>(4,3)</sub> [<math>\mu\text{m}</math>]</b>	<b>Specific Surface area (<math>\text{m}^2/\text{ml}</math>)</b>	<b>% Fat volume &lt; 1<math>\mu\text{m}</math></b>	<b>% Fat volume &gt; 1 <math>\mu\text{m}</math></b>
Milk <sub>1</sub>	1.80	3.12	3.30	10.1	89.9
SFG <sub>1</sub>	0.12	0.15	50.91	100	0
LFG <sub>1</sub>	3.34	3.92	1.79	0	100
Milk <sub>2</sub>	1.94	3.37	3.09	9.33	90.67
SFG <sub>2</sub>	0.11	0.13	55.51	100	0
LFG <sub>2</sub>	2.85	3.40	2.10	0.08	99.92
Milk <sub>3</sub>	1.81	3.23	3.30	8.78	91.22
SFG <sub>3</sub>	0.11	0.14	51.23	100	0
LFG <sub>3</sub>	3.09	3.32	1.92	0.11	99.89

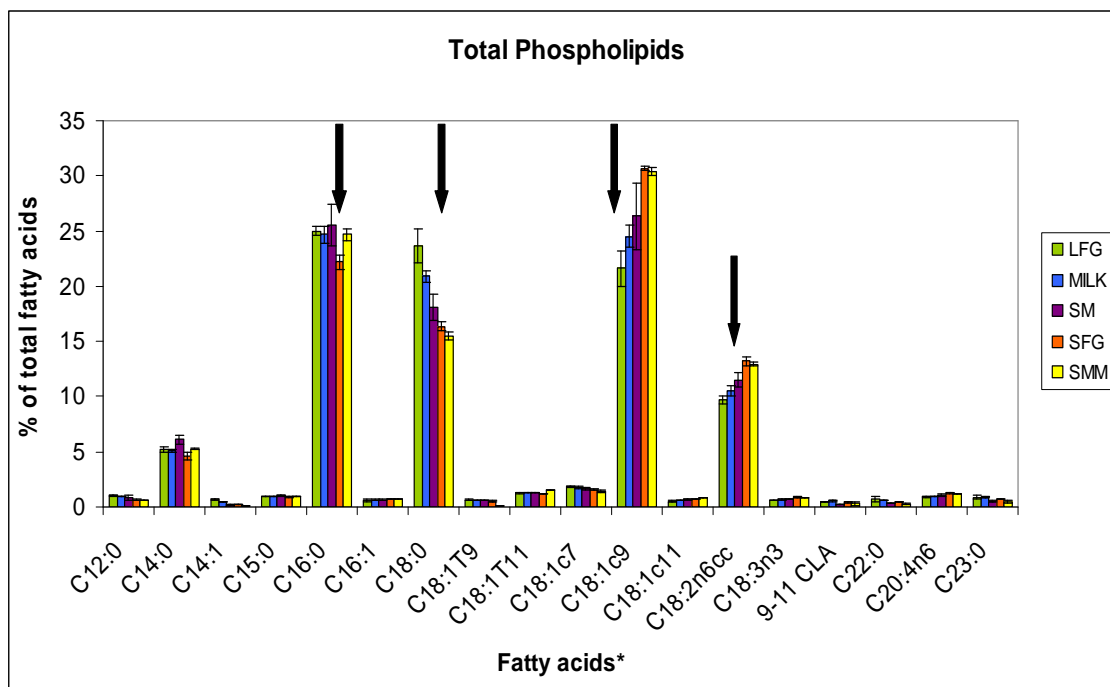
### **Lipid Composition**

After isolation of different lipid aggregates from milk, they were subject to extensive characterization of their constituent lipid components. Fatty acid composition of different lipid classes of each sample is discussed below.

### **Total Phospholipid Composition**

Figure 3 presents the graphical representation of total phospholipid composition of LFG, SFG, SM, SMM, and milk. Significant differences were observed in the C16:0, C18:0, C18:1n9 and C18:2n6cc content of different lipid

aggregates. Data for total phospholipid composition are attached in tabular format in Appendix A.



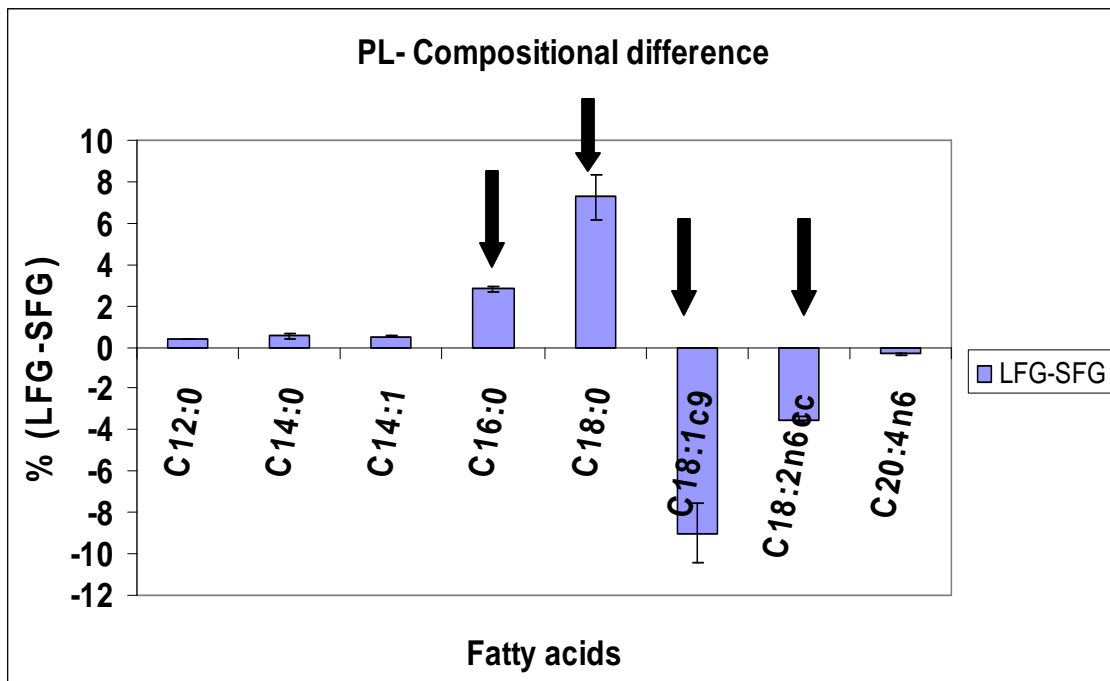
**Figure 3:** Total phospholipid composition of large fat globules (LFG), milk, small fat globules (SFG), skim milk (SM), and skim milk membrane (SMM)

\*Only species contributing >0.5 % of total fatty acids are shown.

(↓) means of fatty acid are significantly different between the samples.

It is clear from the figure 3 that saturated fatty acid C18:0 content decreases as we move from LFG to SMM, whereas unsaturated fatty acids (C18:1n9, C18:2n6cc) content increases as we move from LFG to SMM. Among all the samples, total phospholipid composition of SFG was found to contain significantly less C16:0, whereas LFG were found to contain significantly more C18:0. Unsaturated fatty acids C18:1n9 and C18:2n6cc were found in higher concentration in SMM and SFG.

As a visual tool to directly compare the lipid composition of two samples, data were analyzed by subtracting % content of each fatty acid in large fat globules with small fat globules. Phospholipid compositional difference between LFG and SFG is shown in Figure 4.



**Figure 4:** Total phospholipid compositional difference between large milk fat globules (LFG) and small milk fat globules (SFG). Only fatty acids contributing >0.3 % total fatty acids are shown. (↓) means of fatty acid are significantly different between the samples.

There was a significant difference in C16:0, C18:0, C18:1n9 and C18:2n6cc fatty acid content in large fat globules as compared to small fat globules. LFG were found to contain significantly more C16:0 and C18:0 as compared to SFG, whereas there was significantly more C18:1n9 and C18:2n6cc content in SFG. Comparatively more saturated fatty acids (C12:0, C14:0, C16:0,

C18:0) were found in large fat globules, on the other hand there was more unsaturated long chain fatty acids (C18:1n9, C18:1c11, C18:2n6cc, C18:3n3, and C20:4n6) in small fat globules.

Difference between fatty acid content of skim milk membrane and large or small fat globules were compared to investigate the composition difference between skim milk membrane fraction and large or small fat globules. The rationale behind this comparison is that it can provide an idea of source of skim milk membrane, if it originates from native milk fat globule membrane or from some other sources of membrane.

Results of the compositional difference between LFG vs. SFG and LFG vs. SMM were very similar, except difference in C16:0 content. Unlike in LFG vs. SFG, there is no significant difference was found in C16:0 content in LFG and SMM. Conversely, C16:0 fatty acid content was significantly different between SFG and SMM but no significant difference was observed in content of any other fatty acids. SMM was found to contain significantly more C16:0 as compared to SFG.

Phospholipids account for only 0.8% of milk lipids. About 65% of them are found in the MFGM. The fatty acid composition of the MFGM is rich in unsaturated fatty acids (C18:1 and C18:2) as compared to the lipid core (Christie, 1995; Jensen and Newberg, 1995). As specific surface area in SFG is more than LFG (Table 2), SFG fraction contains proportionally more MFGM and thus phospholipid as compare to LFG fraction. Although there is more phospholipid in SFG proportionally, we have normalized each fatty acid to the total fatty acids in

our analysis. Thus we can compare the bulk differences in membranes by the percent of fatty acids in each fraction.

It is clear from our normalized percent fatty acid results (Figure 4) that SFG phospholipids contain more unsaturated long chain fatty acids (C18:1n9, C18:1c11, C18:2n6cc, C18:3n3, and C20:4n6) but less saturated fatty acids (C12:0, C14:0, C16:0, C18:0) as compared to LFG. As majority of phospholipid is present in the MFGM, higher content of polyunsaturated fatty acids in MFGM of SFG might facilitates the greater curvature.

Similar to the small fat globule fraction, skim milk membrane was also found to contain more unsaturated fatty acids (C18:1 and C18:2) as compared to large fat globules. However, higher content of C16:0 in phospholipid of SMM as compared to SFG suggests that there might be chances these membrane-associated constituents in skim milk originate from some other cellular source than plasma membrane.

### **Composition of Different Phospholipid Species**

Three major phospholipids present in bovine milk are phosphatidylcholine, phosphatidylethanolamine, and sphingomyelin. They are present in similar proportions in the total phospholipids, about 25 to 35 % (MacGibbon and Taylor, 2006). Fatty acid characterization of these three major milk polar lipids was performed for isolated aggregates of lipid samples.

### **Sphingomyelin composition**

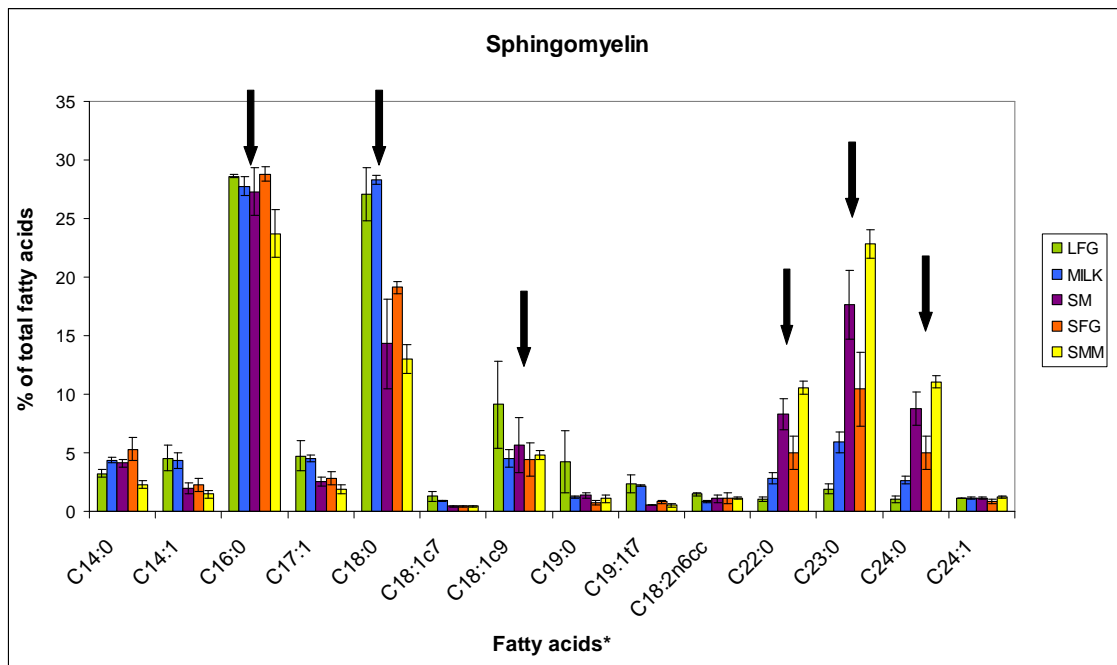
Figure 5 presents the graphical representation of the sphingomyelin composition of LFG, SFG, SM, SMM, and milk. Significant differences were observed in the C16:0, C18:0, C18:1n9, C22:0, C23:0, and C24:0. Data for sphingomyelin composition are attached in tabular format in the appendix A.

Among all the samples, sphingomyelin from LFG contained fewer long chain fatty acids C22:0, C23:0, C24:0, and more C18:0, and C18:1n9. However, some of these results were not significantly different from other samples (SFG, milk, SMM, SM). Content of long chain fatty acids C22:0, C23:0, C24:0 among the samples followed an increasing trend as SMM > SM > SFG > milk > LFG. Where content of these long chain fatty acids were significantly different in SMM vs. SFG and SMM vs. LFG, on the other hand no significant difference was observed between SMM vs. SM and LFG vs. milk. This can be accounted for the fact that LFG was contained most of the fat in milk and SMM was primarily originated from SM.

Content of C18:0 among the samples followed a reverse trend as compared to long chain fatty acids. Increasing trend was observed as LFG > SFG > SMM. Again, no significant difference was observed between LFG vs. milk and SMM vs. SM.

Sphingomyelin compositional difference between LFG and SFG is shown in Figure 6. There was significantly more C18:0 and C18:1n9, but less C23:0 in large fat globules as compared to small fat globules. Other than C23:0, SFG

were also found to contain more long chain fatty acids C22:0 and C24:0, but these results were not statistically significant.

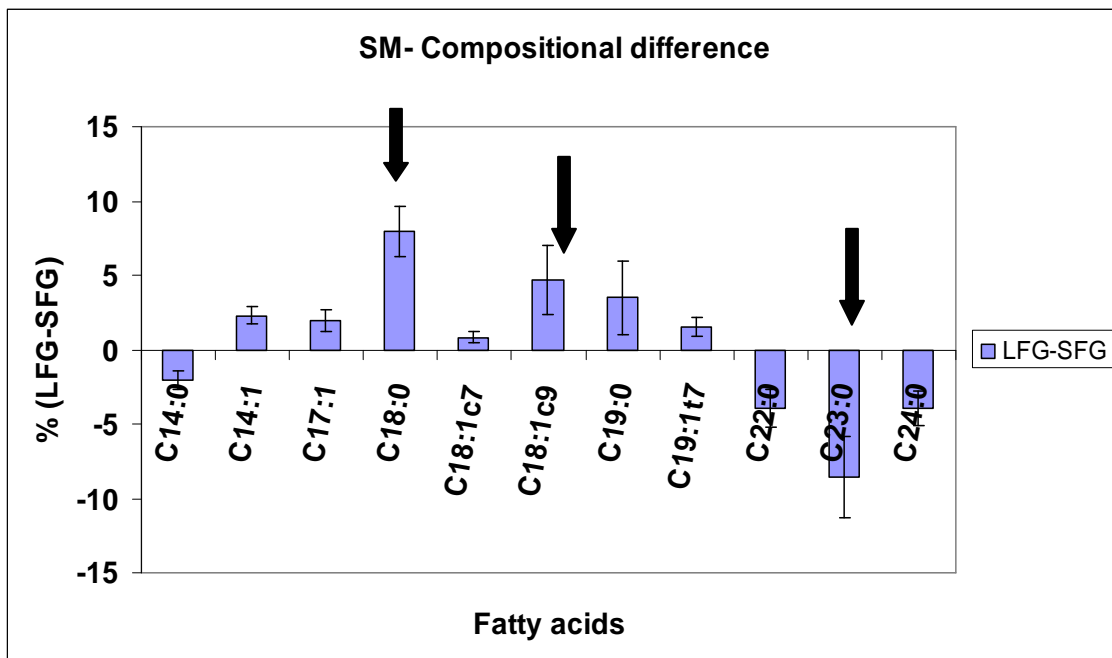


**Figure 5:** Sphingomyelin (SM) composition of large fat globules (LFG), milk, small fat globules (SFG), skim milk (SM), and skim milk membrane (SMM)  
 \*Only species which contribute >0.5 % of total fatty acids are shown.  
 (↓) means of fatty acid are significantly different between the samples.

Graphical representation of sphingomyelin compositional difference between LFG and SMM is shown in Figure 7. Similar to SFG, SMM was also found to contain more long chain fatty acids C22:0, C23:0 and C24:0 as compared to LFG. But unlike the results of compositional difference between LFG and SFG, the compositional difference between LFG and SMM is higher

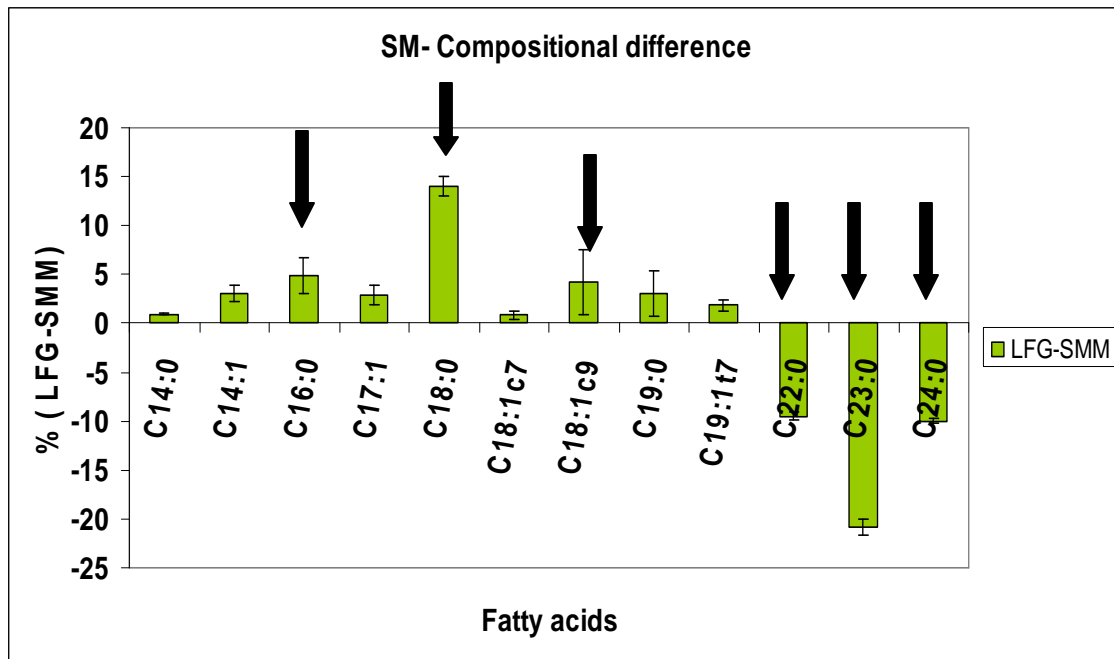
and also significant ( $P < 0.05$ ). There was significantly more C22:0, C23:0, and C24:0 but less C16:0, C18:0, and C18:1n9 content in SMM as compared to LFG.

As mentioned above, SMM shows a similar trend as SFG when compared to LFG, but fatty acid composition of SMM and SFG is not identical. SMM found contains significantly more C22:0, C23:0 and C24:0, but less C16:0, and C18:0 as compared to SFG. These results are shown in Figure 8.



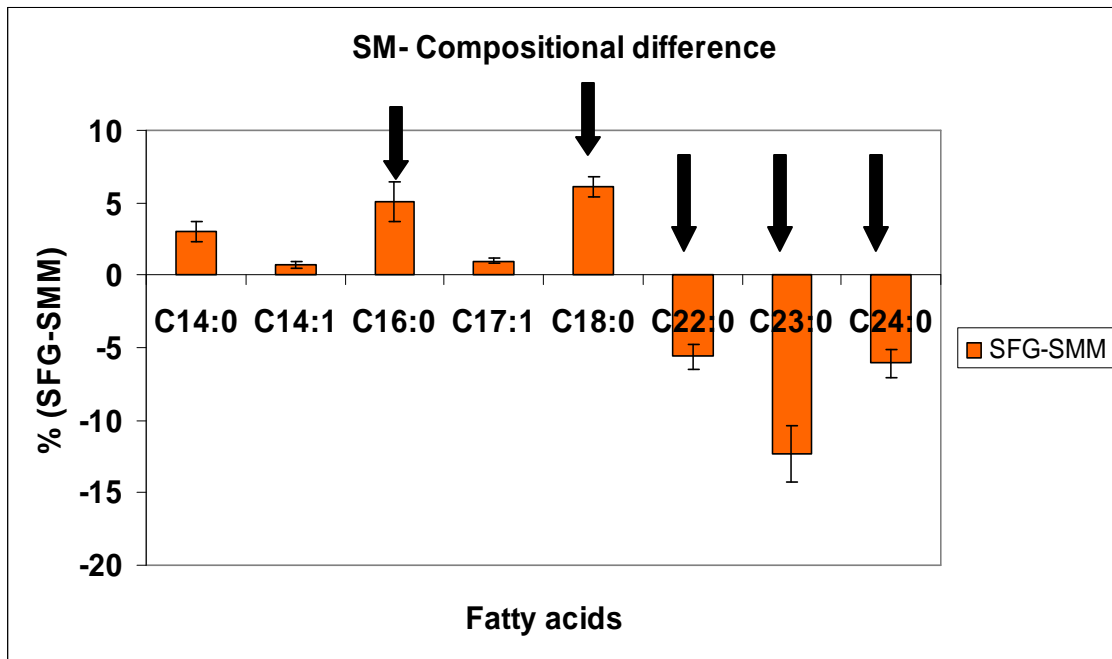
**Figure 6:** Sphingomyelin compositional difference between large milk fat globules (LFG) and small milk fat globules (SFG)  
 Only species which contribute >0.3 % of total fatty acids are shown.  
 (↓) means of fatty acid are significantly different between the samples.





**Figure 7:** Sphingomyelin compositional difference between large milk fat globules (LFG) and skim milk membrane (SMM)  
 Only fatty acids contributing > 0.3 % total fatty acids are shown.  
 (↓) means of fatty acid are significantly different between the samples.

Sphingomyelin contributes almost one-third of the total phospholipids in milk. It is mainly present in the outer layer of bilayer milk fat globule membrane (Christelle et al., 2008). Sphingomyelin has a unique composition of fatty acids compare to the other phospholipids as the fatty acids are mainly long-chain saturated (MacGibbon and Taylor, 2006). As we discussed earlier that SFG contains more MFGM as compare to LFG, content of sphingomyelin is also higher in SFG. Thus, normalization of fatty acid to % of total fatty acid in each fraction allowed us to compare fatty acid composition directly without any biasness of difference in MFGM content.



**Figure 8:** Sphingomyelin compositional difference between small milk fat globules (SFG) and skim milk membrane (SMM)  
 Only fatty acids contributing >0.3 % total fatty acids are shown.  
 (↓) means of fatty acid are significantly different between the samples.

After normalization process, our analysis of results showed that SFG sphingomyelin still contains more long chain saturated fatty acids (C22:0, C23:0, and C24:0) as compared to LFG (Figure 6). As sphingomyelin is mostly present in the outer layer of the membrane bilayer structure, our results suggest that more long chain saturated fatty acids are present in outer layer of small milk fat globule membrane.

Sphingomyelin compositional difference was also observed in SMM vs. SFG and LFG. Similar trend of fatty acid composition was observed in SMM and SFG, but the composition of SMM was not found identical with either SFG or LFG. SMM was found to contain more long chain saturated fatty acids (C22:0,

C23:0, C24:0) and comparatively less short chain fatty acids (C16:0 and C18:0) than SFG and than LFG. Thus, disintegration of the MFGM is not only the possible source of skim milk membrane origin but there may be some other potential sources of this membrane material in skim milk.

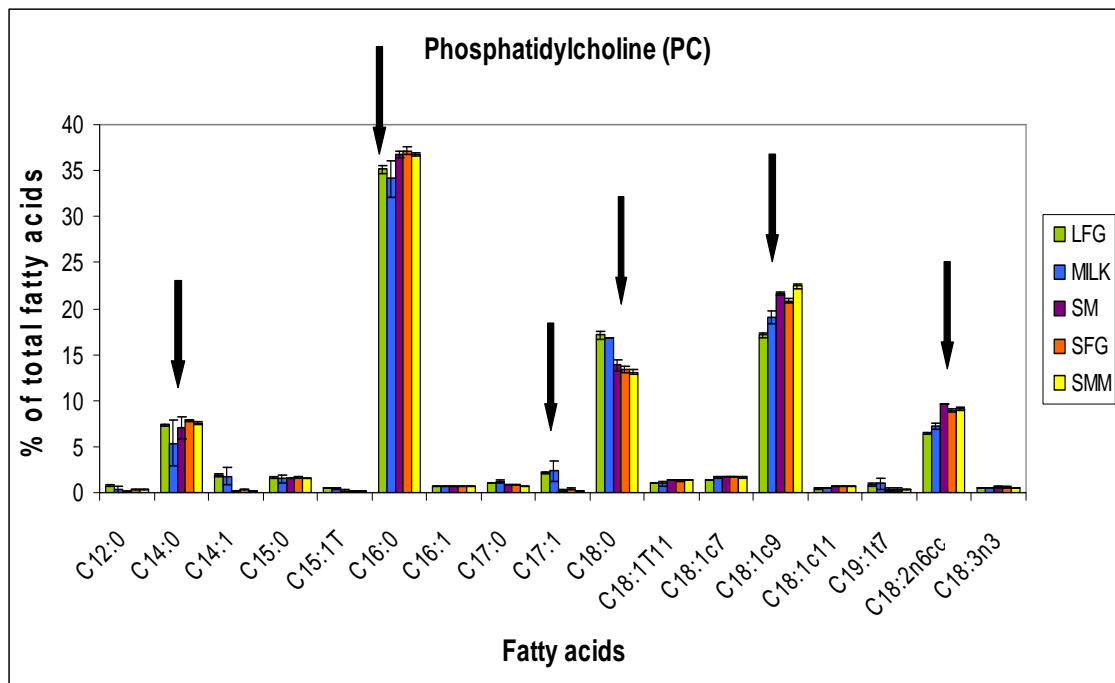
### **Phosphatidylcholine composition**

Figure 9 presents the graphical representation of the phosphatidylcholine composition of LFG, SFG, SM, SMM, and milk. Significant differences were observed in the C14:0, C16:0, C 17:1, C18:0, C18:1n9 and C18:2n6cc content of different lipid aggregates. Data for phosphatidylcholine composition are attached in the tabular format in Appendix A.

Among the samples, milk was found to contain less C16:0 and it was not significantly different from LFG. Content of C18:1n9 and C18:2n6cc was found to increase as we move from LFG < milk < SFG < SM <SMM, whereas a reverse trend was observed for C18:0. Except milk, difference between the content of these fatty acids in LFG and other samples (SM, SMM, and SFG) were found significant. No significant difference was observed between SFG, SM and SMM across all the fatty acids. These insignificant differences between LFG vs. milk and SMM vs. SM can be accounted for the fact that most of the fat in milk was contained by LFG and SM was the primary source of origin of SMM.

Compositional difference between LFG and SFG was found very similar to the results of phospholipid, with the exception of C16:0. Unlike phospholipid, no significant difference was found in C16:0 content of phosphatidylcholine

composition of LFG and SFG. However, similar to the results of total phospholipids, phosphatidylcholine composition of SFG were found to contain significantly more unsaturated fatty acids (C18:1n9 and C18:2n6cc) and less C18:0 as compared to LFG.



**Figure 9:** Phosphatidylcholine composition of large fat globules (LFG), milk, small fat globules (SFG), skim milk (SM), and skim milk membrane (SMM)

\*Only fatty acids contributing >0.5 % total fatty acids are shown.

(↓) means of fatty acid are significantly different between the samples.

Comparison of phosphatidylcholine composition of LFG and SMM also shows the same trend as was observed in total phospholipids. SMM contain significantly more C18:1n9 and C18:2n6cc but less C18:0.

Phosphatidylcholine composition of SFG and SMM was found very close to each other and no significant difference was observed in any fatty acids. This

result again followed the same trend as in total phospholipids composition (except C16:0 fatty acid composition, which was found significantly different in total phospholipids composition of SFG and SMM).

Again, from the results of phosphatidylcholine composition of SFG and LFG, it is clear that there are some compositional differences present in membrane of these two fractions. Thus, it is very likely that these differences are due to some difference in secretion process of milk fat globules of different sizes.

As expected, fatty acid profile of phosphatidylcholine for different aggregates of lipid was found very similar to the total phospholipid. However, unlike phospholipid composition, there was no significant difference was observed in C16:0 content of SFG and SMM and with this phosphatidylcholine fatty acid profile of SFG was found very similar to the SMM. This suggests that skim milk membrane may be originated from common source as that of small fat globules membrane. But difference in PC composition of SMM with LFG may be related to difference in their source of membrane origin in lactating cell.

### **Phosphatidylethanolamine composition**

Figure 10 presents the graphical representation of the phosphatidylethanolamine composition of LFG, SFG, SM, SMM and milk. Significant differences were observed in the C14:0, C16:0, C 17:1, C18:0, and C18:1n9 content of different lipid aggregates. Data for phosphatidylethanolamine composition are attached in the tabular format in Appendix A.

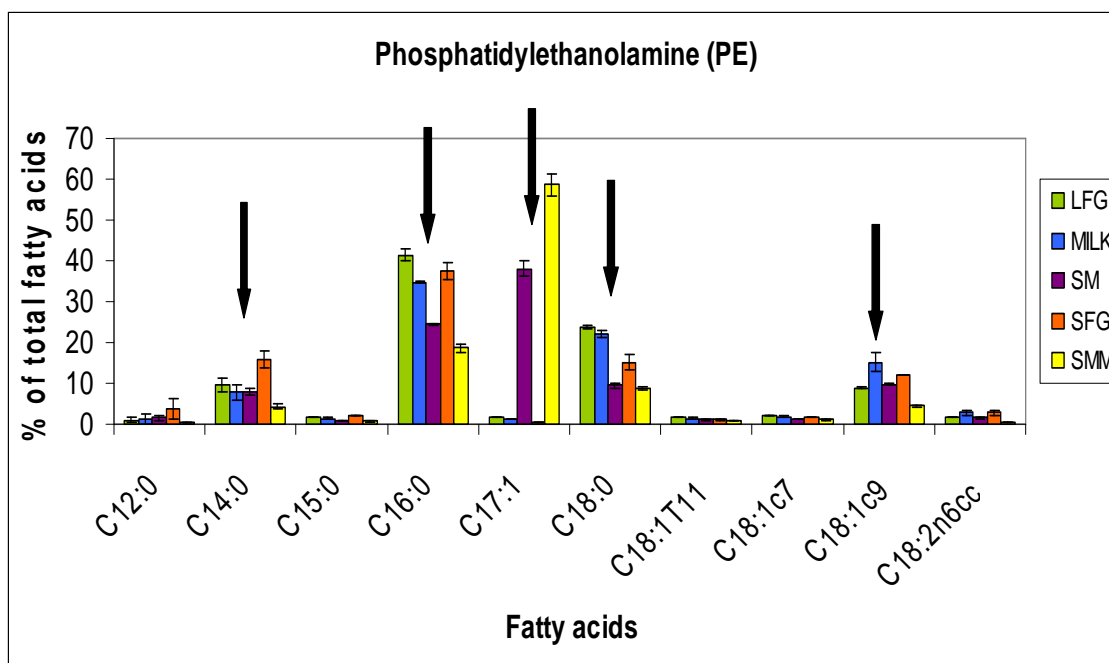
Among the samples, phosphatidylethanolamine composition of SMM was found proportionally lower across all the fatty acids, with the exception of C17:1. SMM and SM were found to contain significantly more C17:1. There was significantly more C16:0 in LFG, whereas SFG contain significantly more C14:0. Other than C16:0, LFG was also found to contain more C18:0, but this result was not significantly different than milk.

Phosphatidylethanolamine compositional difference of LFG and SFG is shown in Figure 11. Phosphatidylethanolamine composition of LFG was found to contain significantly more C16:0 and C18:0, whereas there was significantly more C14:0 content in SFG as compared to LFG. SFG also contains more unsaturated fatty acids C18:1n9 and C18:2n6cc, but these results were not significant. These results follow the same trend as the results seen in phospholipid compositional difference between LFG and SFG, except the result of C14:0 difference and P values (test of significant difference).

Phosphatidylethanolamine composition of SMM contains significantly more C17:1 fatty acid as compared to LFG and SFG, respectively. Except C17:1, all other fatty acids are present in higher concentrations in SFG and LFG than SMM.

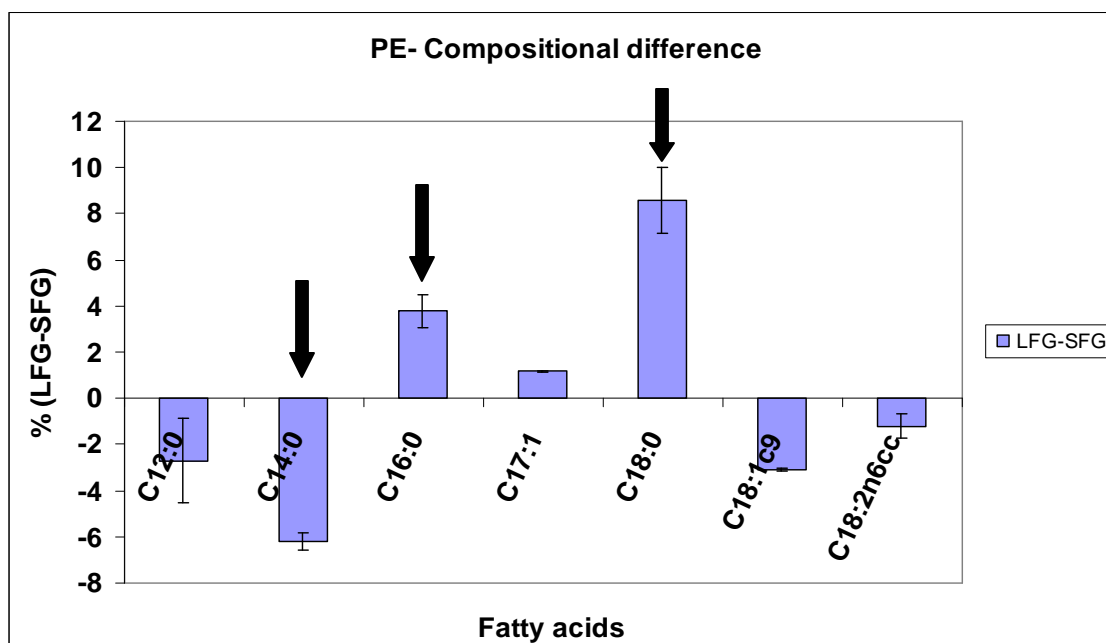
Similar to the phospholipid composition, phosphatidylethanolamine in SFG was found to contain more PUFA and less saturated (C16:0 and C18:0) fatty acids than LFG. Although more PUFA content in PE composition of SFG was not found significant with LFG, significantly more short chain C14:0 and C12:0 (not

significant) was found in SFG and comparatively more long chain saturated fatty acids (C16:0 and C18:0) was found in LFG.



**Figure 10:** Phosphatidylethanolamine composition of large fat globules (LFG), milk, small fat globules (SFG), skim milk (SM), and skim milk membrane (SMM) \*Only species contributing >0.5 % of total fatty acids are shown. (↓) means of fatty acid are significantly different between the samples.

Results obtained from phosphatidylethanolamine fatty acid characterization suggest some difference (especially content of C17:1) in skim milk membrane and membrane of small and large fat globules. These results again suggest that shedding of the MFGM is not only the possible source of skim milk membrane origin but there may be some other potential sources of this membrane material in skim milk, such as Golgi vesicle membranes, membranes from cells which are free in milk, etc.



**Figure 11:** Phosphatidylethanolamine compositional difference between large milk fat globules (LFG) and small milk fat globules (SFG) Only species contributing >0.5 % of total fatty acids are shown. (↓) means of fatty acid are significantly different between the samples.

### Summary of Phospholipid Composition Results

This study demonstrates that there are some compositional differences between native milk fat globule membranes of different sizes (SFG and LFG).

In summarizing the compositional differences of total phospholipids and different classes of phospholipids (PC, PE, SM) between SFG and LFG together, it was observed that SFG contain significantly more C18:1n9 and C18:2n6cc with the exception of sphingomyelin class. Conversely, sphingomyelin composition of SFG contains less C18:1c9 (significant) and C18:2n6cc (not significant).

However, there were more long chain fatty acids C22:0, C23:0, and C24:0 in sphingomyelin composition of SFG with significant difference in C23:0. On the



other hand, LFG were found to contain significantly more C18:0 in the total phospholipids and all the different classes of phospholipids. These differences in MFGM polar lipids might be related to the origin of milk fat globules of different sizes in the lactating cell.

Results of total phospholipid and different classes of phospholipid compositions also demonstrate that there are some compositional differences between skim milk membrane and small and large fat globules. PE composition of SMM was found to contain more C17:1 as compared to SFG and LFG. There was also more long chain fatty acids (C22:0, C23:0, C24:0) content in sphingomyelin composition of SMM as compared to SFG and LFG. SMM phospholipid was found to contain significantly more C16:0 as compared to SFG. Conversely, skim milk membrane SM was found to contain less C16:0 and C18:0 as compared to SFG. Other than these differences SMM is similar to SFG. Thus, these results are consistent with skim milk membrane and milk fat globule membrane not being derived from common source of membrane in lactating cell. But the membrane material observed in skim milk membrane may most likely also have some other possible sources.

### **Triacylglycerol Composition**

Triacylglycerol accounts for around 98% of total milk lipids. The composition of triacylglycerol core of bovine milk lipid is complex due to presence of various fatty acids. Diacylglycerol, free fatty acids and cholesterol ester are other minor classes of neutral lipids present in milk. After isolation of different

lipid aggregates from milk, these lipids were subject to extensive characterization to observe any composition difference.

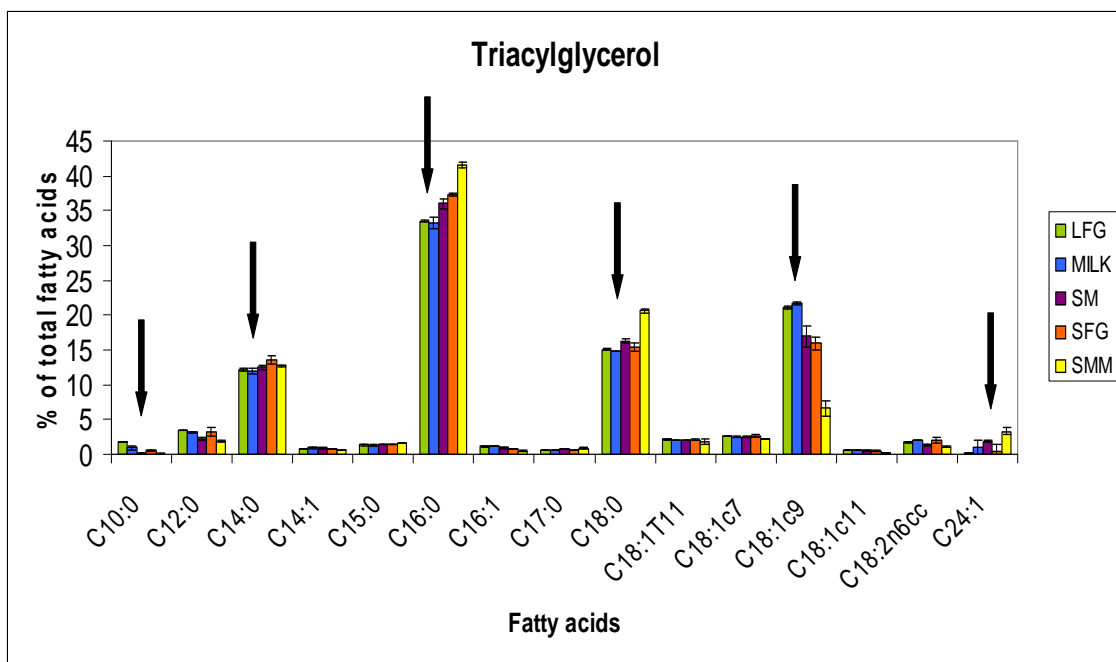
Figure 12 presents the graphical representation of the triacylglycerol composition of LFG, SFG, SM, SMM, and milk. Significant differences were observed in the C10:0, C14:0, C16:0, C18:0, C18:1n9 and C24:1 content of different lipid aggregates. Data for triacylglycerol composition are attached in tabular format in Appendix A.

From Figure 12, it is clear that C16:0 fatty acid content increases as we move from LFG to SMM. However, reverse trend was observed for unsaturated fatty acid C18:1n9. As LFG accounted for a most of the volume of total fat, fatty acid profile of LFG triacylglycerol was found very close to that of milk (total-fat) than the SFG.

To visualize the composition difference between milk fat globules of different sizes and to understand more clearly about the membrane source of skim milk membrane, compositional difference data were analyzed and differences are discussed below.

Triacylglycerol compositional difference between LFG and SFG is shown in Figure 13. LFG were found to contain significantly more C10:0 and C18:1n9, whereas SFG were found to contain significantly more C16:0. Briard et al. (2003) also found more C16:0 and less C18:1n9 fatty acid content in SFG as compared to LFG isolated from winter milk. Other fatty acids did not vary significantly with fat globule size. Unlike with phospholipids, the composition of C18:1n9 in triacylglycerols increased with fat globule size (LFG >SFG), which is consistent

with the results of Tverdokhle (1957). As oleic acid (C18:1n9) content in LFG triacylglycerol is more than SFG, it suggests that the large fat globules cores are richer in unsaturated C18:1n9 fatty acid. However, as discussed earlier that SFG phospholipids contain more C18:1n9 unsaturated fatty acid than LFG, this means that membranes of SFG are richer in C18:1n9 fatty acid.



**Figure 12:** Triacylglycerol composition of lipid extracted from large fat globules (LFG), milk, small fat globules (SFG), skim milk (SM), and skim milk membrane (SMM)

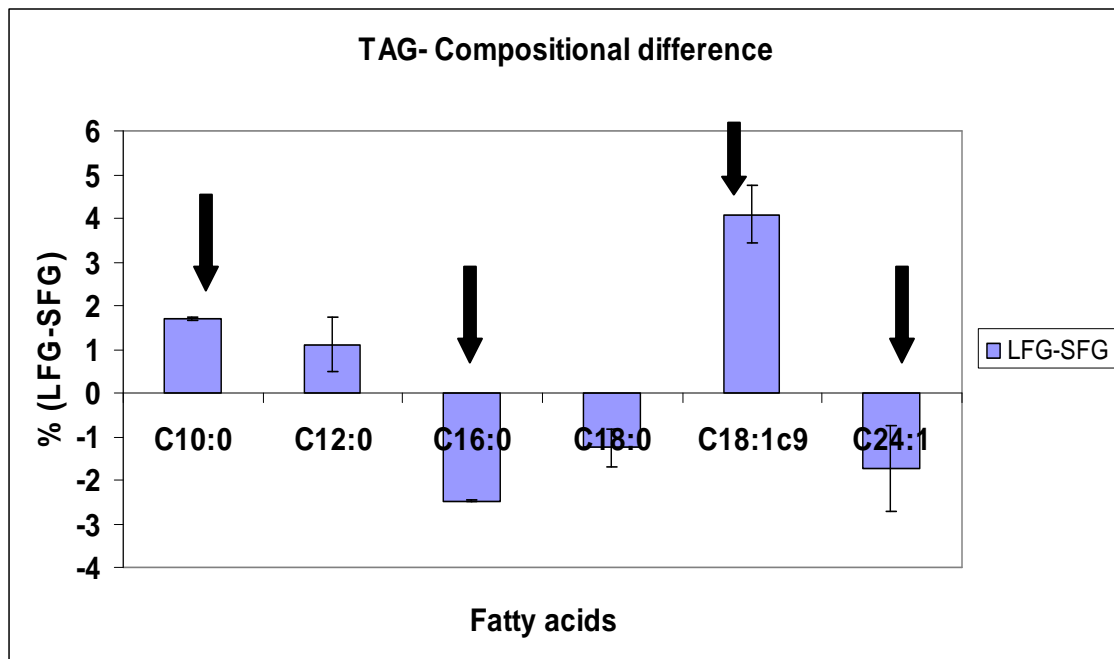
\*Only species contributing >0.5 % of total fatty acids are shown.

(↓) means of fatty acid are significantly different between the samples.

Triacylglycerol compositional difference between LFG and SMM shows very similar fatty acid trend as LFG and SFG. Similar to SFG, triacylglycerol composition of SMM was found to contain more C16:0 and less C10:0 and C18:1n9 as compared to LFG. Other than these fatty acids, triacylglycerol

composition of SMM was found to contain significantly more C 24:1 fatty acid.

The TAG composition difference between LFG and SMM is very similar but not identical to the composition difference between LFG and SFG. Triacylglycerol composition difference between LFG and SMM is more than difference between LFG and SFG. This fact is clearer by seeing the difference between SFG and SMM triacylglycerol composition.



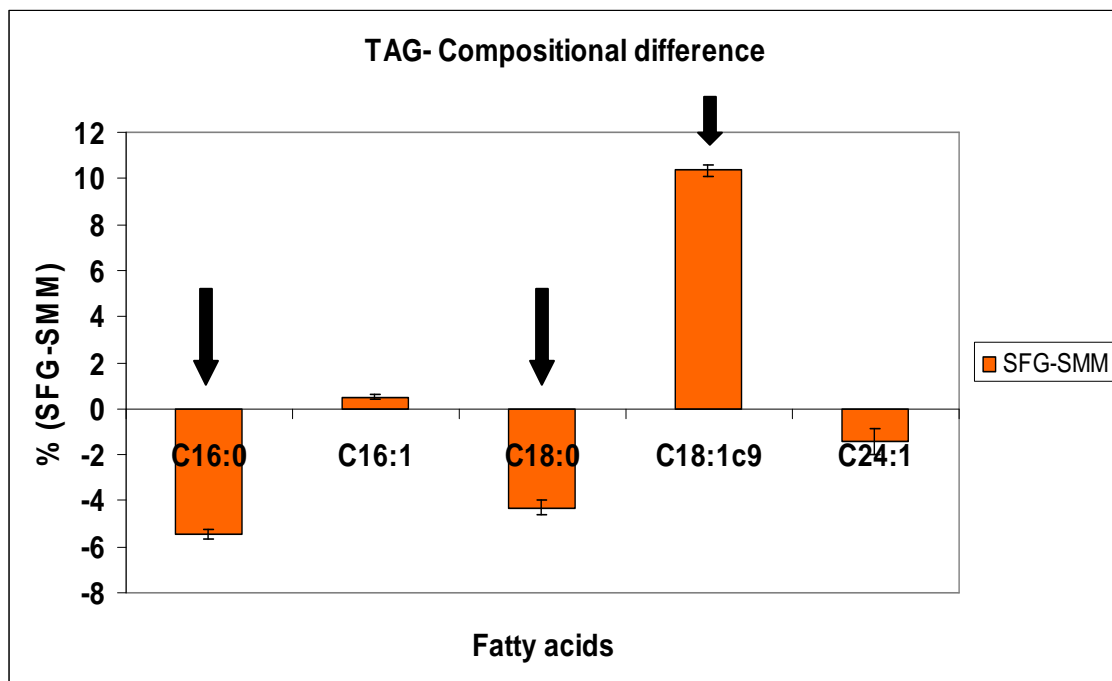
**Figure 13:** Triacylglycerol compositional difference between large milk fat globules (LFG) and small milk fat globules (SFG)  
 Only fatty acids contributing >0.3 % are shown.  
 (↓) means of fatty acid are significantly different between the samples.

Triacylglycerol compositional difference between SFG and SMM is shown in Figure 14. There was significantly more C16:0 and C18:0 fatty acid content in triacylglycerol composition of SMM, whereas SFG were found to contain significantly more C18:1n9 unsaturated fatty acid.

It is clear from these results that there are some differences in the triacylglycerol composition of membrane material originated from skim milk and native small and large fat globules from milk. This means it is possible that there may be other sources of membrane in SMM material other than the MFGM.

### Diacylglycerol Composition

Figure 15 presents the graphical representation of the diacylglycerol composition of LFG, SFG, SM, SMM, and milk. Significant differences were observed in the C14:0, C16:0, C17:0, C18:0, C18:1n9 and C19:0 content of different lipid aggregates. Data for diacylglycerol composition are attached in tabular format in Appendix A.

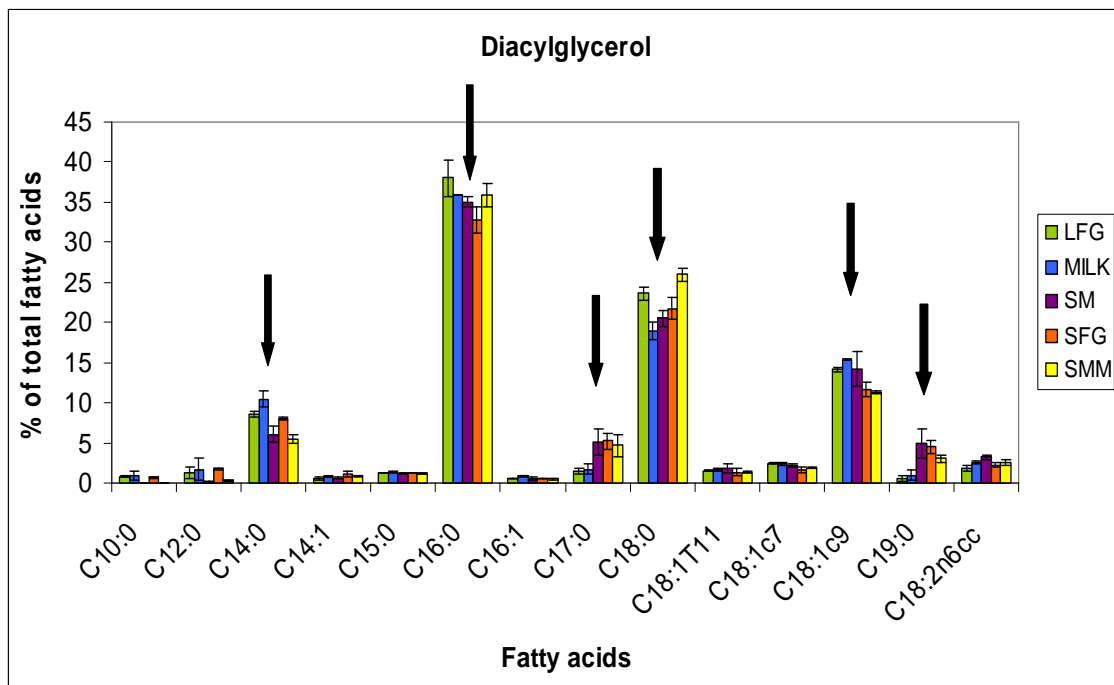


**Figure 14:** Triacylglycerol compositional difference between small milk fat globules (SFG) and skim milk membrane (SMM)

Only fatty acids contributing >0.3 % compositional difference are shown.

(↓) means of fatty acid are significantly different between the samples.

From Figure 15, it is clear that there are some differences in DG composition of different lipid aggregates. Among the samples, content of C16:0 was found more in LFG. However, diacylglycerol composition of LFG was found very close to that of the milk for most of the fatty acids. Milk and LFG contain more C14:0 and C18:1n9 fatty acids than other samples. On the other hand, SMM was found to contain more C18:0. As SMM originated from SM, composition of SMM was found very close to SM across most of the fatty acids. No significant difference was found between SFG, SMM, and SM in content of C17:0 and C19:0, which is more as compared to LFG and milk.

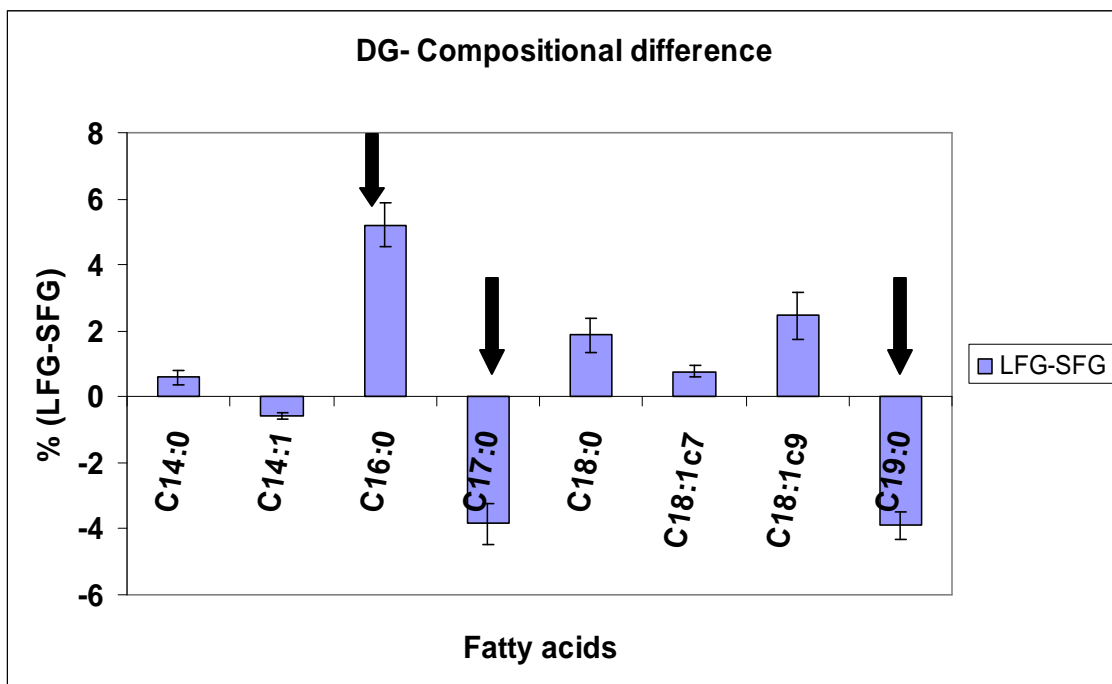


**Figure 15:** Diacylglycerol composition of large fat globules (LFG), milk, small fat globules (SFG), skim milk (SM), and skim milk membrane (SMM)

\*Only fatty acids contributing >0.5 % total fatty acids are shown.

(↓) means of fatty acid are significantly different between the samples.

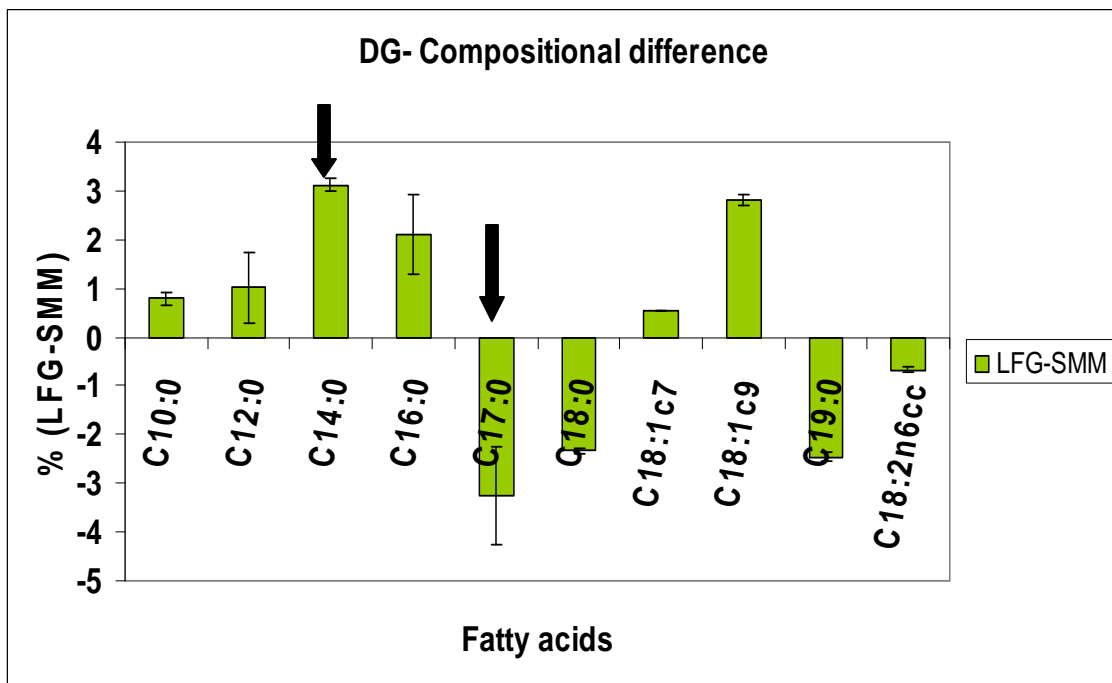
Diacylglycerol compositional difference between LFG and SFG is shown in Figure 16. LFG were found to contain significantly more C16:0 than SFG. There was also more C18:0 and C18:1n9 in LFG but results were not significant. SFG were found to contain significantly more C17:0 and C19:0 fatty acids. Thus, there are some differences in diacylglycerol composition of differently sized native milk fat globules.



**Figure 16:** Diacylglycerol compositional difference between large milk fat globules (LFG) and small milk fat globules (SFG). Only fatty acids contributing >0.3 % total fatty acids are shown. (↓) means of fatty acid are significantly different between the samples.

Data of diacylglycerol compositional difference between LFG and SMM is shown in Figure 17. There was significantly more C14:0 and less C17:0 content

in diacylglycerol composition of LFG as compared to SMM. It was also observed that SMM contains more C18:0 and C19:0 fatty acids, whereas LFG was found to contain more C16:0 and C18:1n9 fatty acids, but these results were not found significant. DG composition of LFG contains comparatively more short chain fatty acids and more unsaturated fatty acids as compared to SMM.



**Figure 17:** Diacylglycerol compositional difference between large milk fat globules (LFG) and skim milk membrane (SMM)

Only fatty acids contributing >0.5 % total fatty acids are shown.

(↓) means of fatty acid are significantly different between the samples.

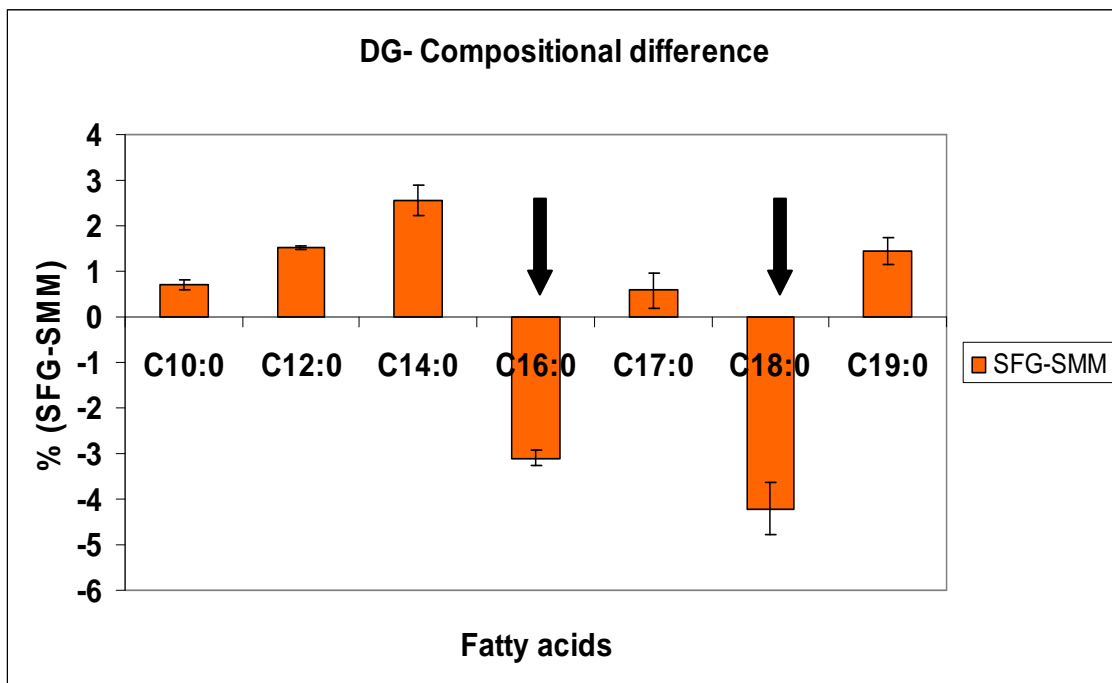
Diacylglycerol compositional difference between SFG and SMM is shown in Figure 18. SMM was found to contain significantly more C16:0 and C18:0.

There was more content of C10:0, C12:0, C14:0, C17:0, and C19:0 in



diacylglycerol composition of SFG as compared to SMM, but these results were not significant.

These results also clearly suggest that there are some composition differences in skim milk membrane and small and large fat globules.



**Figure 18:** Diacylglycerol compositional difference between small milk fat globules (SFG) and skim milk membrane (SMM)

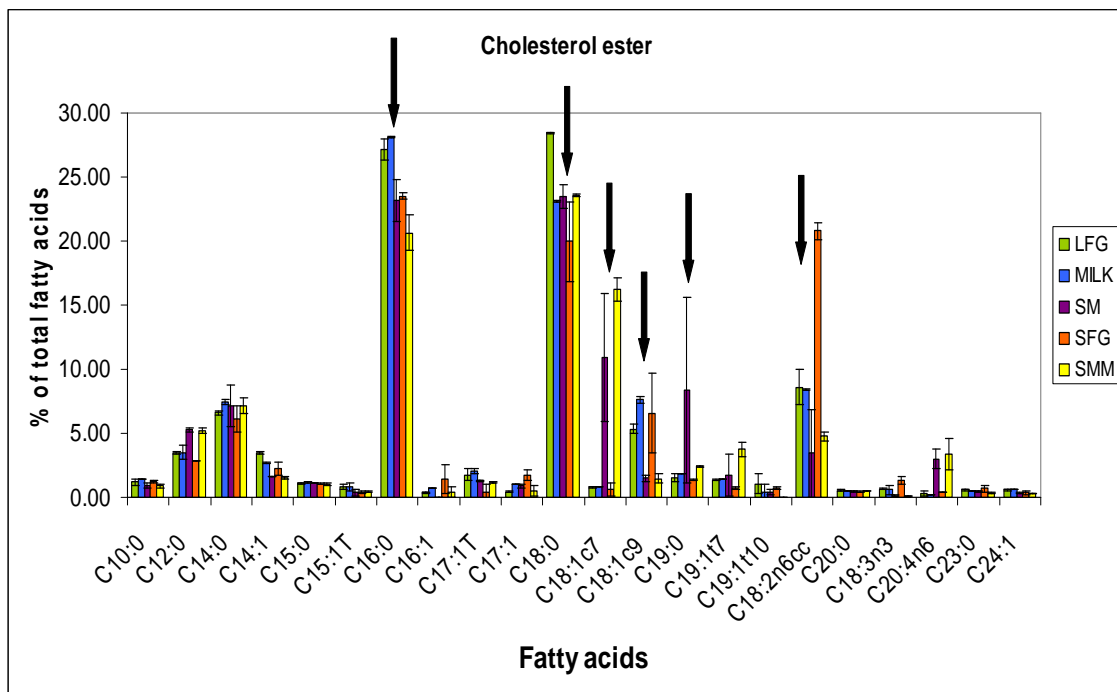
Only fatty acids contributing >0.5 % total fatty acids are shown.

(↓) means of fatty acid are significantly different between the samples.

### Cholesterol Ester Composition

Figure 19 presents the graphical representation of the cholesterol ester composition of LFG, SFG, SM, SMM, and milk. Significant differences were observed in the C16:0, C18:0, C18:1n11, C18:1n9, C19:0, and C18:2n6cc. Data for CE composition are attached in tabular format in Appendix A.

As expected, similar to other lipid classes, cholesterol ester composition of LFG is very close to milk (total-fat), with the exception of C18:0 (Figure 19). Among all the samples, cholesterol ester composition of LFG was found to contain significantly more C18:0, whereas SFG contain significantly more C18:2n6cc. Milk and LFG was found to contain more C16:0 than SFG and than SMM. There was significantly more C18:1n11 in SMM as compared to other samples.



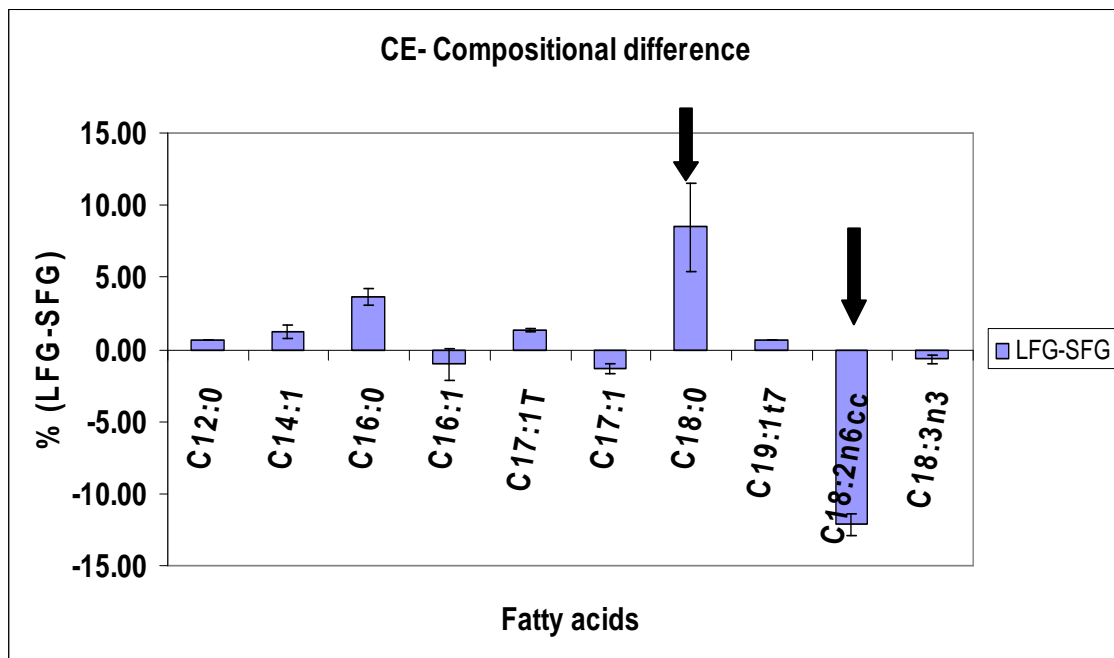
**Figure 19:** Cholesterol ester composition of large fat globules (LFG), milk, small fat globules (SFG), skim milk (SM), and skim milk membrane (SMM)

\*Only fatty acids contributing >0.5 % total fatty acids are shown.

(↓) means of fatty acid are significantly different between the samples.

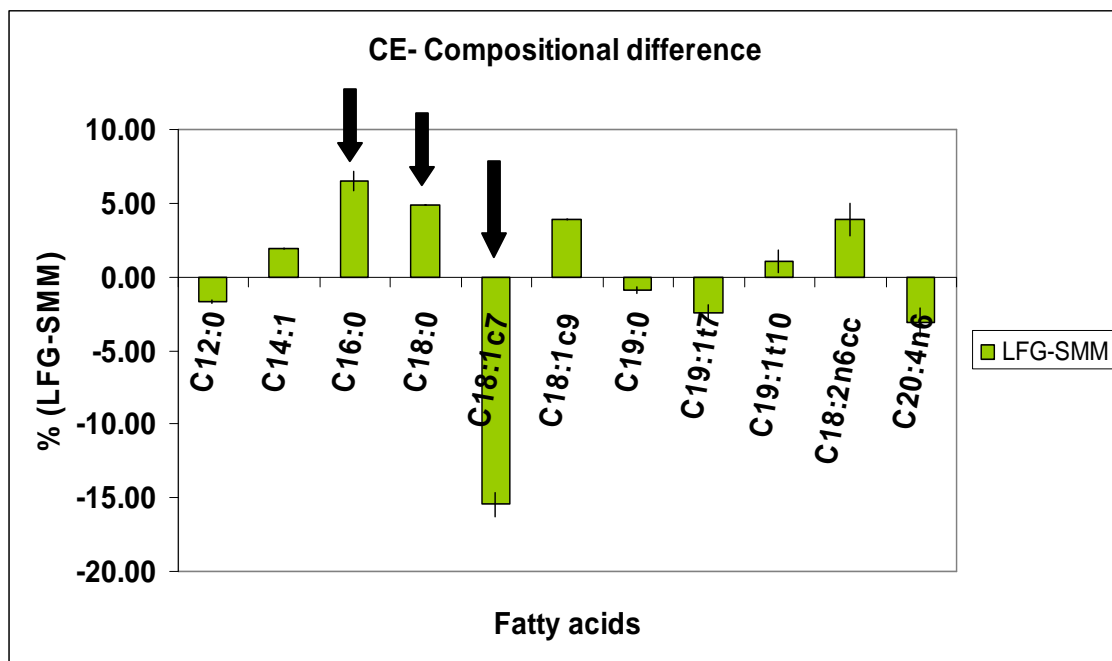
Cholesterol ester compositional difference between LFG and SFG is shown in Figure 20. SFG was found to contain significantly more unsaturated

C18:2n6cc fatty acid as compared to LFG, whereas there was significantly more saturated C18:0 fatty acid content in LFG as compared to SFG. Palmitic acid (C16:0), which is another saturated fatty acid was also observed to be in higher amount in LFG, whereas other unsaturated fatty acids C16:1 and C17:1 were found to be higher in SFG. However, these results were not found significant. Similar to the results seen in phospholipid composition, cholesterol ester composition of SFG contain more unsaturated fatty acids and less saturated fatty acids than LFG. These results suggest that there is clear composition differences exist between large and small fat globules isolated from same native milk.



**Figure 20:** Cholesterol ester compositional difference between large milk fat globules (LFG) and small milk fat globules (SFG). Only fatty acids contributing >0.3 % compositional difference are shown. (↓) means of fatty acid are significantly different between the samples.

Cholesterol ester compositional difference between LFG and SMM is shown in Figure 21. Cholesterol ester composition of LFG was found to contain significantly more saturated C16:0 and C18:0 fatty acids, and significantly less unsaturated C18:1n11 as compared to SMM. It was observed that LFG contain more C18:1n9, C18:2n6cc and less C19:1t7, C20:4n6 fatty acids as compared to SMM, but these results were not found significant.

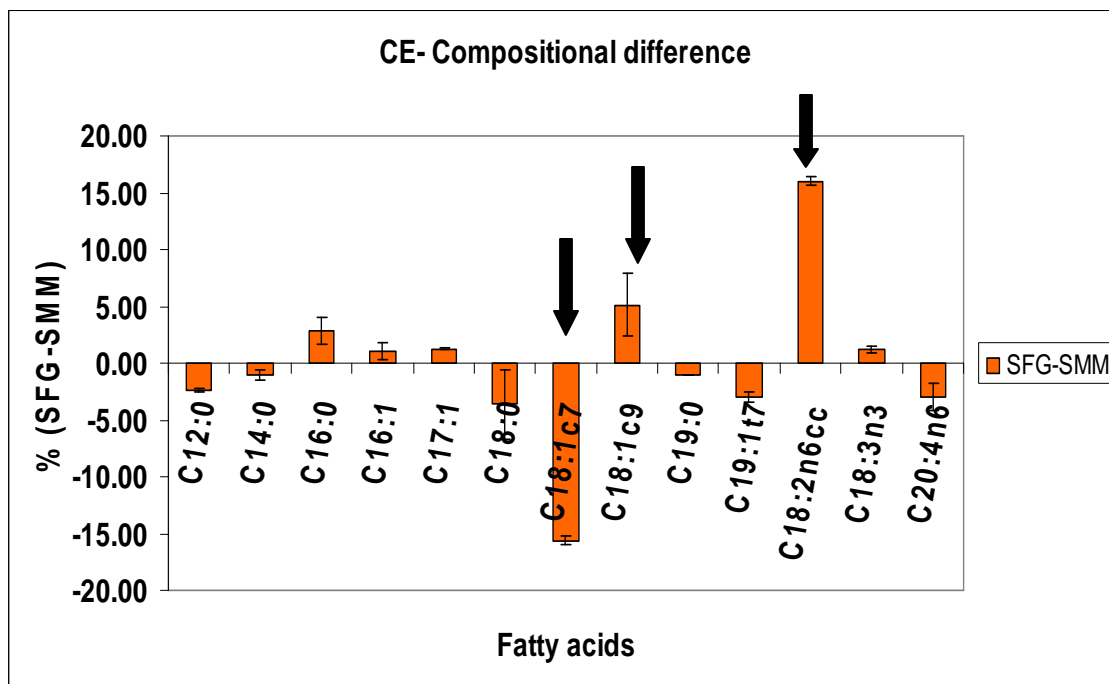


**Figure 21:** Cholesterol ester compositional difference between large milk fat globules (LFG) and skim milk membrane (SMM)  
 Only fatty acids contributing >0.3 % compositional difference are shown.  
 (↓) means of fatty acid are significantly different between the samples.

Figure 22 presents the cholesterol compositional difference between SFG and SMM. Cholesterol ester composition of SFG was found to contain

significantly more C18:2n6cc and C18:1 n9 fatty acids as compared to SMM.

However, there was significantly more C18:1n11 content in SMM as compared to SFG. There was more content of C18:0, C19:1t7, C20:4n6 and less content of C16:0 found in SMM as compared to SFG, but these results were not found significant. These results suggest that there are definitely some composition differences exist between membrane material originated from skim milk and small or large fat globules.



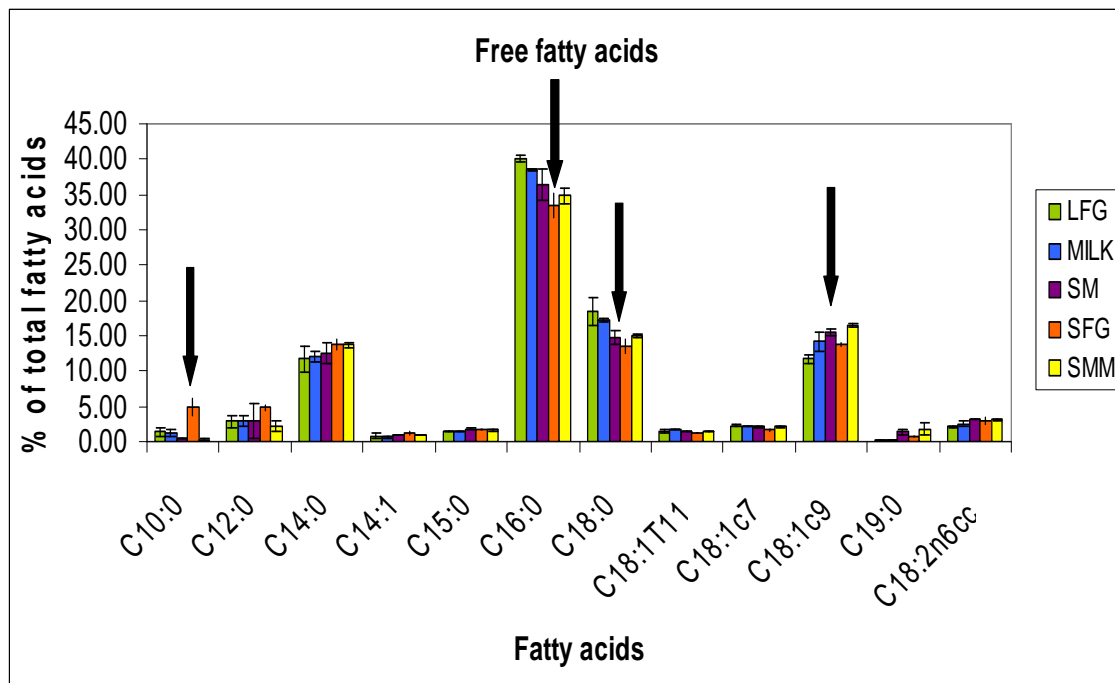
**Figure 22:** Cholesterol ester compositional difference between small milk fat globules (SFG) and skim milk membrane (SMM)

Only fatty acids contributing >0.3 % compositional difference are shown.

(↓) means of fatty acid are significantly different between the samples.

## Free Fatty Acids Composition

Figure 23 presents the graphical representation of the free fatty acids composition of LFG, SFG, SM, SMM, and milk. There are significant differences in the content of the C10:0, C16:0, C18:0, and C18:1n9 of different lipid aggregates. Data for FFA composition are attached in tabular format in Appendix A.



**Figure 23:** Free fatty acids composition of large fat globules (LFG), milk, small fat globules (SFG), skim milk (SM), and skim milk membrane (SMM)

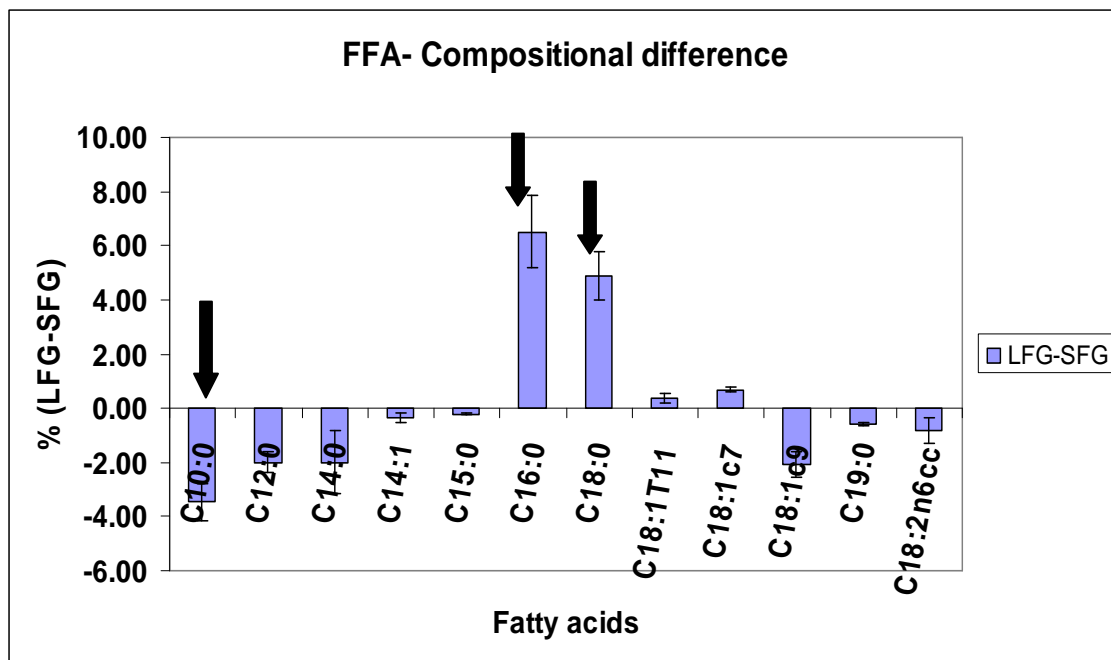
\*Only fatty acids contributing >0.5 % of total fatty acids are shown.

(↓) means of fatty acid are significantly different between the samples.

Among all the samples, free fatty acids composition of SFG was found to contain significantly more C10:0. LFG was found to contain more C16:0 and C18:0 but it was not significantly different from milk composition. There was more

C18:1n9 unsaturated fatty acid in SMM but difference was only found significant with LFG. Across all the fatty acid, free fatty acids composition for LFG is very close to milk and SMM composition is very close to SM. These results are expected as LFG originated from milk and SMM originated from SM.

Free fatty acid compositional difference between LFG and SFG is shown in Figure 24. Clear difference between FFA composition of SFG and LFG are observed Free fatty acid composition of SFG was found to contain significantly more C10:0 and less C16:0 and C18:0 as compared to LFG. SFG was also found to contain more C12:0, C14:0, C18:1n9, and C18:2n6 but these results were not found significant.

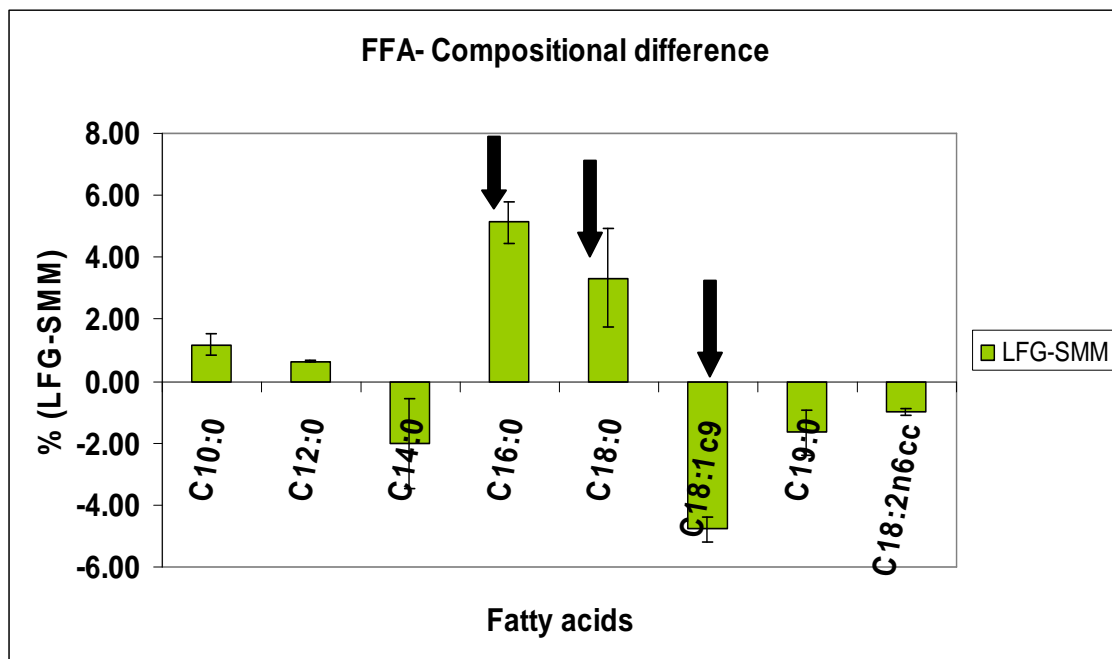


**Figure 24:** Free fatty acids compositional difference between large milk fat globules (LFG) and small milk fat globules (SFG).

Only fatty acids contributing >0.3 % compositional difference are shown.

(↓) means of fatty acid are significantly different between the samples.

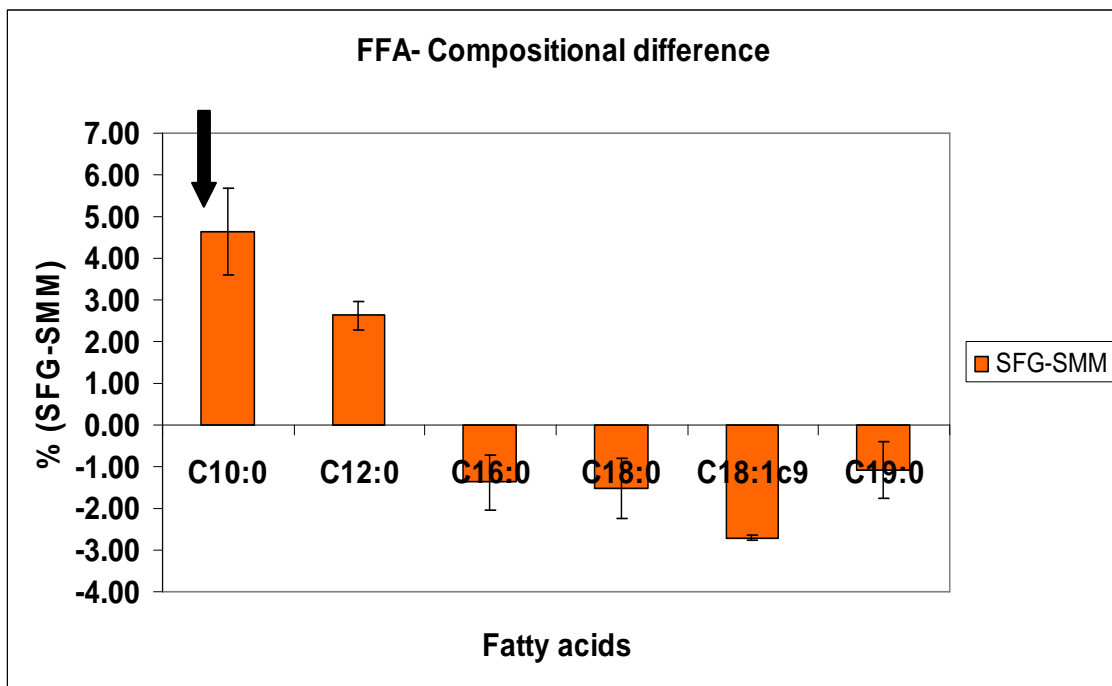
Free fatty acid compositional difference between LFG and SMM is shown in Figure 25. There was significantly more C16:0 and C18:0 content in LFG as compared to SMM, respectively. However, SMM was found to contain significantly more unsaturated C18:1n9 fatty acid as compared to LFG. SMM was also found to contain more long chain fatty acids C19:0 and C18:2n6cc, whereas LFG was found to contain more short chain fatty acids C10:0 and C12:0. However, these results were not significant.



**Figure 25:** Free fatty acids compositional difference between large milk fat globules (LFG) and skim milk membrane (SMM). Only fatty acids contributing >0.3 % compositional difference are shown. (↓) means of fatty acid are significantly different between the samples.



In Figure 26, difference in FFA composition of SMM and SFG can be easily seen. SMM was found to contain more long chain fatty acids C16:0, C18:0, C18:1n9, and C19:0, whereas there was more content of small chain fatty acids C10:0 and C12:0 in SFG as compared to SMM. However, significant difference was found only in C10:0. Again, these differences suggest that the skim milk membrane and milk fat globule membrane may not be arise from common source in lactating cell, but skim milk membrane may have some different potential sources of membrane.



**Figure 26:** Free fatty acids compositional difference between small milk fat globules (SFG) and skim milk membrane (SMM). Only fatty acids contributing >0.3 % compositional difference are shown. (↓) means of fatty acid are significantly different between the samples.

## CONCLUSION

This study demonstrates that there are some compositional differences between native milk fat globules of different sizes.

- Total Phospholipid composition of SFG contains significantly more unsaturated C18:1n9 and C18:2n6cc but less saturated C16:0 and C18:0 as compared to LFG.
- Phosphatidylcholine composition of SFG contains significantly more C18:1n9 and C18:2n6cc and less C18:0 as compared to LFG.
- Phosphatidylethanolamine composition of SFG contains significantly more C14:0 but less C16:0 and C18:0 as compared to LFG.
- Sphingomyelin composition of SFG contains significantly more C23:0 and less C18:0 and C18:1n9 as compared to LFG.
- Triacylglycerol composition of SFG contains significantly more C16:0 but less C10:0 and C18:1n9 as compared to LFG.
- Diacylglycerol composition of SFG contains significantly more odd chain fatty acids C17:0 and C19:0 but less C16:0 as compared to LFG.
- Cholesterol ester composition of SFG contains significantly more C18:2n6cc but less C18:0 as compared to LFG.
- Free fatty acids composition of SFG contains significantly more C10:0 but less C16:0 and C18:0 as compared to LFG.

Composition differences between skim milk membrane and native milk fat globules of different sizes suggest that origin of this membrane material in skim

milk might have some different source than that of milk fat globule membrane.

Data in this study do not support the concept that this skim milk membrane material arises by disintegration of the milk fat globule membrane. Summary of the lipid composition differences between SMM and native SFG and LFG is listed below:

- Total phospholipid composition of SMM contains significantly more C18:1n9, C18:2n6cc, and less C18:0 as compared to LFG.
- Phosphatidylcholine composition of SMM contains significantly more C18:1n9 and C18:2n6cc but less C17:1 and C18:0 as compared to LFG.
- Phosphatidylethanolamine composition of SMM contains significantly more C17:1 but less C14:0, C16:0, C18:0, and C18:1n9 as compared to both SFG and LFG.
- Sphingomyelin composition of SMM contains more C22:0, C23:0, and C24:0 but less C16:0 and C18:0 as compared to both SFG and LFG.
- Triacylglycerol composition of SMM contains significantly more C16:0 and C18:0 but less C18:1n9 as compared to SFG.
- Triacylglycerol composition of SMM contains significantly more C16:0 and C24:1 but less C10:0 and C18:1n9 as compared to LFG.
- Diacylglycerol composition of SMM contains significantly more C16:0 and C18:0 as compared to SFG.
- Diacylglycerol composition of SMM contains significantly more C17:0 but less C14:0 as compared to LFG.

- Cholesterol ester composition of SMM contains significantly more C18:1n11 but less C18:1n9 and C18:2n6cc as compared to SFG.
- Cholesterol ester composition of SMM contains significantly more C18:1n11 but less C16:0 and C18:0 as compared to LFG.
- Free fatty acids composition of SMM contains significantly less C10:0 as compared to SFG.
- Free fatty acids composition of SMM contains significantly more C18:1n9 but less C16:0 and C18:0 as compared to LFG.

As this study suggests some differences in lipid composition of native milk fat globules of different sizes, more studies are needed to identify other constituents compositional differences between these two distinct distributions of fat globules in milk. These distributions should be study to see any difference in protein composition by running SDS PAGE electrophoresis. To collect more information about the source of membrane material in skim milk and to confirm the results of this study that skim milk membrane may not have common source as that of milk fat globule membrane, more facts should be collected by performing protein analysis of these isolated fractions.

As there is some difference in fatty acid profile of small and large native milk fat globules, it might be interesting to study the interaction of rumen micro-organism with these different fractions of fat globule size in milk. This can give more information with nutritional significance of these fat globules, which can lead to the development of new applications of these fractions in food industry.

Development of quantitative technique to quantify the amount of each fatty acid in milk fat globules of different sizes will help to determine the fortification amount of these fractions in food to have nutritional and functional significance. In further research, fortification of these different globule size fractions at different amount can be performed in dairy/ food products to develop new products with improved functional, nutritional and sensory characteristics.

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APPENDICES

APPENDIX A.

TABLES

**Table A1.** Total phospholipid composition of large fat globules (LFG), milk, small fat globules (SFG), skim milk (SM), and skim milk membrane (SMM)

Fatty acids*	LFG (% of total fatty acids)	Milk	SFG	SM	SMM
C12:0	1.05 ± 0.06	0.97 ± 0.03	0.62 ± 0.07	0.85 ± 0.26	0.56 ± 0.03
C14:0	5.15 ± 0.24	5.07 ± 0.09	4.60 ± 0.34	6.09 ± 0.39	5.24 ± 0.04
C14:1	0.70 ± 0.06	0.49 ± 0.01	0.19 ± 0.01	0.18 ± 0.05	0.09 ± 0.02
C15:0	0.94 ± 0.02	0.96 ± 0.00	0.90 ± 0.03	1.03 ± 0.07	0.96 ± 0.01
<b>C16:0</b>	<b>25.00 ± 0.46<sup>a</sup></b>	<b>24.67 ± 0.73<sup>a</sup></b>	<b>22.17 ± 0.59<sup>b</sup></b>	<b>25.51 ± 1.90<sup>a</sup></b>	<b>24.67 ± 0.54<sup>a</sup></b>
C16:1	0.60 ± 0.08	0.64 ± 0.03	0.73 ± 0.02	0.64 ± 0.07	0.72 ± 0.02
<b>C18:0</b>	<b>23.61 ± 1.55<sup>a</sup></b>	<b>20.87 ± 0.55<sup>b</sup></b>	<b>16.36 ± 0.45<sup>cd</sup></b>	<b>18.04 ± 1.18<sup>c</sup></b>	<b>15.54 ± 0.35<sup>d</sup></b>
C18:1T9	0.61 ± 0.04	0.56 ± 0.02	0.53 ± 0.04	0.60 ± 0.02	0.15 ± 0.01
C18:1T11	1.28 ± 0.07	1.27 ± 0.03	1.21 ± 0.01	1.27 ± 0.02	1.52 ± 0.01
C18:1c7	1.85 ± 0.05	1.78 ± 0.07	1.58 ± 0.06	1.68 ± 0.12	1.39 ± 0.10
<b>C18:1n9</b>	<b>21.61 ± 1.61<sup>a</sup></b>	<b>24.49 ± 0.99<sup>b</sup></b>	<b>30.63 ± 0.17<sup>c</sup></b>	<b>26.32 ± 3.05<sup>b</sup></b>	<b>30.41 ± 0.34<sup>c</sup></b>
C18:1c11	0.53 ± 0.05	0.61 ± 0.03	0.76 ± 0.00	0.68 ± 0.08	0.78 ± 0.01
<b>C18:2n6cc</b>	<b>9.70 ± 0.36<sup>a</sup></b>	<b>10.49 ± 0.45<sup>a</sup></b>	<b>13.18 ± 0.47<sup>b</sup></b>	<b>11.50 ± 0.67<sup>ab</sup></b>	<b>12.94 ± 0.14<sup>b</sup></b>
C18:3n3	0.60 ± 0.01	0.65 ± 0.01	0.87 ± 0.08	0.72 ± 0.02	0.82 ± 0.03
9-11 CLA	0.51 ± 0.00	0.56 ± 0.05	0.43 ± 0.02	0.23 ± 0.02	0.29 ± 0.13
C22:0	0.71 ± 0.25	0.62 ± 0.01	0.44 ± 0.01	0.35 ± 0.03	0.27 ± 0.07
C20:4n6	0.92 ± 0.04	0.97 ± 0.03	1.25 ± 0.08	1.04 ± 0.08	1.18 ± 0.02
C23:0	0.85 ± 0.18	0.88 ± 0.06	0.70 ± 0.00	0.50 ± 0.04	0.43 ± 0.12

<sup>a,b,c,d</sup> Means in a row with different superscripts are significantly different ( $P < 0.05$ ).

Highlighted rows: fatty acids are significantly different between the samples. For example, composition of C16:0 content in LFG and SFG, SFG and milk, SFG and SM, SFG and SMM is significantly different.

\* Only species contributing >0.5 % of total fatty acids are shown.

**Table A2.** Sphingomyelin (SM) composition of large fat globules (LFG), milk, small fat globules (SFG), skim milk (SM), and skim milk membrane (SMM)

Fatty acids*	LFG (% of total fatty acids)	Milk	SFG	SM	SMM
C12:0	0.12±0.04	0.49±0.03	0.64±0.02	0.17±0.02	0.09±0.08
C14:0	3.25±0.36	4.36±0.23	5.32±1.02	4.11±0.36	2.31±0.29
C14:1	4.55±1.10	4.38±0.66	2.25±0.54	1.98±0.50	1.48±0.31
C15:0	1.03±0.07	1.09±0.09	2.07±1.47	0.89±0.00	0.63±0.02
C15:1T	0.92±0.3	0.76±0.10	0.43±0.06	0.38±0.09	0.27±0.11
<b>C16:0</b>	<b>28.62±0.13<sup>a</sup></b>	<b>27.75±0.81<sup>ab</sup></b>	<b>28.79±0.61<sup>a</sup></b>	<b>27.30±2.05<sup>ab</sup></b>	<b>23.71±2.02<sup>b</sup></b>
C16:1	0.40±0.04	1.11±1.00	1.41±0.01	0.18±0.02	0.17±0.01
C17:1T	0.31±0.38	0.25±0.29	0.76±0.84	0.08±0.04	0.03±0.01
C17:1	4.76±1.31	4.51±0.28	2.84±0.56	2.55±0.39	1.86±0.38
<b>C18:0</b>	<b>27.07±2.23<sup>a</sup></b>	<b>28.31±0.34<sup>a</sup></b>	<b>19.11±0.53<sup>b</sup></b>	<b>14.31±3.85<sup>c</sup></b>	<b>13.01±1.22<sup>c</sup></b>
C18:1T7	0.45±0.18	0.58±0.11	0.31±0.08	0.32±0.10	0.17±0.08
C18:1T11	0.57±0.47	0.37±0.06	0.30±0.19	0.24±0.14	0.20±0.02
C18:1c7	1.28±0.41	0.90±0.01	0.45±0.03	0.43±0.01	0.45±0.03
<b>C18:1n9</b>	<b>9.11±3.71<sup>a</sup></b>	<b>4.52±0.72<sup>b</sup></b>	<b>4.41±1.42<sup>b</sup></b>	<b>5.68±2.34<sup>ab</sup></b>	<b>4.83±0.40<sup>b</sup></b>
C19:0	4.24±2.66	1.22±0.11	0.72±0.18	1.39±0.24	1.11±0.32
C19:1t7	2.36±0.76	2.21±0.02	0.84±0.14	0.58±0.02	0.53±0.14
C18:2n6cc	1.48±0.15	0.86±0.06	1.14±0.47	1.12±0.32	1.15±0.11
C20:0	0.47±0.05	0.60±0.02	0.55±0.06	0.65±0.01	0.77±0.03
<b>C22:0</b>	<b>1.06±0.17<sup>a</sup></b>	<b>2.83±0.47<sup>a</sup></b>	<b>4.98±1.43<sup>ab</sup></b>	<b>8.32±1.32<sup>bc</sup></b>	<b>10.58±0.58<sup>c</sup></b>
<b>C23:0</b>	<b>1.91±0.41<sup>a</sup></b>	<b>5.90±0.89<sup>a</sup></b>	<b>10.47±3.16<sup>b</sup></b>	<b>17.63±2.93<sup>c</sup></b>	<b>22.80±1.23<sup>d</sup></b>
<b>C24:0</b>	<b>1.06±0.25<sup>a</sup></b>	<b>2.67±0.32<sup>a</sup></b>	<b>4.98±1.44<sup>ab</sup></b>	<b>8.75±1.43<sup>bc</sup></b>	<b>11.07±0.50<sup>c</sup></b>
C24:1	1.15±0.01	1.13±0.13	0.84±0.19	1.14±0.06	1.24±0.08

<sup>a,b,c,d</sup> Means in a row with different superscripts are significantly different ( $P < 0.05$ ).

Highlighted rows: fatty acids are significantly different between the samples. For example, composition of C16:0 fatty acid in LFG and SMM, and SFG and SMM is significantly different.

\* Only fatty acids contributing >0.5 % total fatty acids are shown.

**Table A3.** Phosphatidylcholine (PC) composition of large fat globules (LFG), milk, small fat globules (SFG), skim milk (SM), and skim milk membrane (SMM)

Fatty acids*	LFG (% of total fatty acids)		Milk		SFG		SM		SMM
C12:0	0.74±0.07		0.31±0.35		0.40±0.00		0.12±0.06		0.34±0.05
<b>C14:0</b>	<b>7.31±0.02<sup>ab</sup></b>		<b>5.39±2.53<sup>b</sup></b>		<b>7.82±0.11<sup>a</sup></b>		<b>7.00±1.24<sup>ab</sup></b>		<b>7.54±0.13<sup>a</sup></b>
C14:1	1.91±0.13		1.78±0.88		0.31±0.04		0.18±0.03		0.15±0.01
C15:0	1.62±0.04		1.49±0.42		1.64±0.02		1.56±0.05		1.60±0.00
C15:1T	0.59±0.00		0.45±0.13		0.25±0.00		0.14±0.12		0.23±0.00
<b>C16:0</b>	<b>35.19±0.43<sup>ab</sup></b>		<b>34.08±2.01<sup>b</sup></b>		<b>37.12±0.43<sup>a</sup></b>		<b>36.70±0.36<sup>a</sup></b>		<b>36.73±0.20<sup>a</sup></b>
C16:1	0.65±0.04		0.70±0.02		0.71±0.00		0.71±0.02		0.72±0.01
C17:0	1.04±0.01		1.21±0.10		0.85±0.04		0.84±0.01		0.70±0.01
<b>C17:1</b>	<b>2.18±0.09<sup>a</sup></b>		<b>2.33±1.08<sup>ab</sup></b>		<b>0.42±0.06<sup>abc</sup></b>		<b>0.21±0.11<sup>bc</sup></b>		<b>0.19±0.00<sup>c</sup></b>
<b>C18:0</b>	<b>17.12±0.43<sup>a</sup></b>		<b>16.84±0.01<sup>a</sup></b>		<b>13.36±0.30<sup>b</sup></b>		<b>13.86±0.57<sup>b</sup></b>		<b>13.12±0.28<sup>b</sup></b>
C18:1T11	0.98±0.03		0.94±0.27		1.29±0.01		1.36±0.00		1.35±0.01
C18:1c7	1.35±0.01		1.67±0.07		1.65±0.01		1.74±0.00		1.66±0.08
<b>C18:1n9</b>	<b>17.09±0.22<sup>a</sup></b>		<b>19.01±0.71<sup>ab</sup></b>		<b>20.86±0.21<sup>bc</sup></b>		<b>21.64±0.23<sup>c</sup></b>		<b>22.42±0.3<sup>c</sup></b>
C18:1c11	0.44±0.02		0.56±0.03		0.62±0.01		0.65±0.00		0.68±0.00
C19:1t7	0.90±0.16		0.97±0.58		0.37±0.14		0.33±0.16		0.36±0.01
<b>C18:2n6cc</b>	<b>6.40±0.08<sup>a</sup></b>		<b>7.20±0.34<sup>ab</sup></b>		<b>8.87±0.15<sup>bc</sup></b>		<b>9.65±0.01<sup>c</sup></b>		<b>9.12±0.20<sup>c</sup></b>
C18:3n3	0.45±0.02		0.52±0.01		0.59±0.01		0.61±0.02		0.55±0.02

<sup>a,b,c</sup> Means in a row with different superscripts are significantly different ( $P < 0.05$ ).

Highlighted rows: fatty acids are significantly different between the samples. For example, composition of C16:0 content in SFG and milk, SM and milk, and SMM and milk is significantly different.

\*Only fatty acids contributing >0.5 % total fatty acids are shown.

**Table A4.** Phosphatidylethanolamine (PE) composition of large fat globules (LFG), milk, small fat globules (SFG), skim milk (SM), and skim milk membrane (SMM)

Fatty acids*	LFG (% of total fatty acids)	Milk	SFG	SM	SMM
C12:0	1.04±0.67	1.24±1.28	3.75±2.49	1.47±0.43	0.28±0.24
<b>C14:0</b>	<b>9.63±1.62<sup>ac</sup></b>	<b>7.76±1.99<sup>acd</sup></b>	<b>15.83±1.97<sup>b</sup></b>	<b>8.02±0.91<sup>cd</sup></b>	<b>4.33±0.53<sup>d</sup></b>
C14:1	1.16±0.11	1.14±0.07	0.94±0.20	0.42±0.03	0.20±0.01
C15:0	1.54±0.07	1.42±0.12	1.97±0.02	0.92±0.04	0.64±0.05
<b>C16:0</b>	<b>41.34±1.41<sup>a</sup></b>	<b>34.66±0.15<sup>b</sup></b>	<b>37.56±2.12<sup>b</sup></b>	<b>24.38±0.35<sup>c</sup></b>	<b>18.60±1.10<sup>d</sup></b>
C16:1	0.48±0.00	0.72±0.01	0.92±0.09	0.58±0.04	0.22±0.01
<b>C17:1</b>	<b>1.53±0.01<sup>a</sup></b>	<b>1.38±0.01<sup>a</sup></b>	<b>0.36±0.04<sup>a</sup></b>	<b>38.08±1.83<sup>b</sup></b>	<b>58.56±2.77<sup>c</sup></b>
<b>C18:0</b>	<b>23.80±0.37<sup>a</sup></b>	<b>22.00±0.79<sup>a</sup></b>	<b>15.20±1.80<sup>b</sup></b>	<b>9.39±0.45<sup>c</sup></b>	<b>8.74±0.40<sup>c</sup></b>
C18:1T11	1.56±0.06	1.39±0.31	1.16±0.22	1.14±0.21	0.68±0.03
C18:1c7	2.07±0.12	1.84±0.04	1.60±0.12	1.37±0.02	1.05±0.04
<b>C18:1n9</b>	<b>8.95±0.03<sup>a</sup></b>	<b>15.19±2.18<sup>b</sup></b>	<b>12.03±0.09<sup>ab</sup></b>	<b>9.74±0.15<sup>a</sup></b>	<b>4.42±0.18<sup>c</sup></b>
C19:0	0.60±0.09	2.74±1.54	0.35±0.15	0.34±0.04	0.31±0.15
C18:2n6cc	1.58±0.03	2.83±0.68	2.79±0.57	1.47±0.05	0.59±0.01
C18:3n3	0.24±0.00	0.47±0.09	0.68±0.05	0.28±0.03	0.11±0.00

<sup>a,b,c,d</sup> Means in a row with different superscripts are significantly different ( $P < 0.05$ ).

Highlighted rows: fatty acids are significantly different between the samples. For example, composition of C14:0 content in LFG and SFG, LFG and SMM, SFG and SM, SFG and SMM, SFG and milk is significantly different.

\* Only fatty acids contributing >0.5 % total fatty acids are shown.



**Table A5.** Triacylglycerol (TAG) composition of large fat globules (LFG), milk, small fat globules (SFG), skim milk (SM), and skim milk membrane (SMM)

Fatty acids*	LFG (% of total fatty acids)	Milk	SFG	SM	SMM
C10:0	1.80±0.06 <sup>a</sup>	0.93±0.34 <sup>ab</sup>	0.12±0.10 <sup>b</sup>	0.58±0.12 <sup>ab</sup>	0.10±0.01 <sup>b</sup>
C12:0	3.41±0.04	3.16±0.04	2.30±0.65	3.25±0.20	1.92±0.07
C14:0	12.17±0.11 <sup>ab</sup>	11.99±0.39 <sup>a</sup>	12.47±0.66 <sup>ab</sup>	13.62±0.37 <sup>b</sup>	12.68±0.12 <sup>ab</sup>
C14:1	0.89±0.01	0.93±0.03	0.90±0.02	0.76±0.02	0.53±0.02
C15:0	1.32±0.01	1.31±0.04	1.45±0.02	1.50±0.04	1.66±0.01
C16:0	33.55±0.20 <sup>a</sup>	33.19±0.81 <sup>a</sup>	36.02±0.17 <sup>b</sup>	37.29±0.70 <sup>b</sup>	41.47±0.40 <sup>c</sup>
C16:1	1.12±0.01	1.18±0.03	0.96±0.01	0.90±0.06	0.46±0.11
C17:0	0.64±0.00	0.64±0.01	0.76±0.00	0.69±0.01	0.84±0.08
C18:0	15.03±0.13 <sup>ab</sup>	14.83±0.05 <sup>a</sup>	16.29±0.56 <sup>a</sup>	15.42±0.27 <sup>a</sup>	20.59±0.25 <sup>b</sup>
C18:1T11	2.13±0.01	2.10±0.00	2.02±0.13	2.09±0.04	1.80±0.38
C18:1c7	2.57±0.00	2.51±0.04	2.51±0.15	2.62±0.04	2.18±0.04
C18:1n9	21.02±0.23 <sup>a</sup>	21.67±0.17 <sup>a</sup>	16.94±0.89 <sup>b</sup>	15.97±1.53 <sup>b</sup>	6.60±1.10 <sup>c</sup>
C18:1c11	0.64±0.00	0.66±0.01	0.51±0.05	0.49±0.04	0.27±0.00
C18:2n6cc	1.76±0.04	1.98±0.05	1.37±0.38	2.04±0.12	1.05±0.12
C24:1	0.16±0.04 <sup>a</sup>	0.93±1.00 <sup>ab</sup>	1.88±1.02 <sup>bc</sup>	0.47±0.09 <sup>ab</sup>	3.31±0.49 <sup>c</sup>

<sup>a,b,c</sup> Means in a row with different superscripts are significantly different ( $P < 0.05$ ).

Highlighted rows: fatty acids are significantly different between the samples. For example, composition of C10:0 content in LFG and SFG, LFG and SMM is significantly different.

\*Only species contributing >0.5 % of total fatty acids are shown.

**Table A6.** Diacylglycerol (DG) composition of large fat globules (LFG), milk, small fat globules (SFG), skim milk (SM), and skim milk membrane (SMM)

Fatty acids*	LFG (% of total fatty acids)	Milk	SFG	SM	SMM
C10:0	0.79±0.12	0.89±0.48	0.71±0.1	0.01±0.00	0.01±0.00
C12:0	1.29±0.78	1.73±1.37	1.80±0.1	0.16±0.1	0.28±0.05
<b>C14:0</b>	<b>8.63±0.37<sup>ab</sup></b>	<b>10.43±0.97<sup>a</sup></b>	<b>8.05±0.17<sup>abc</sup></b>	<b>6.09±1.02<sup>bc</sup></b>	<b>5.51±0.50<sup>c</sup></b>
C14:1	0.53±0.2	0.82±0.16	1.12±0.3	0.63±0.04	0.85±0.05
C15:0	1.20±0.01	1.36±0.01	1.27±0.07	1.15±0.08	1.13±0.06
<b>C16:0</b>	<b>38.04±2.25<sup>a</sup></b>	<b>35.87±0.07<sup>ab</sup></b>	<b>32.83±1.59<sup>b</sup></b>	<b>35.02±0.61<sup>ab</sup></b>	<b>35.93±1.42<sup>a</sup></b>
C16:1	0.55±0.09	0.82±0.06	0.51±0.03	0.56±0.15	0.47±0.07
<b>C17:0</b>	<b>1.39±0.38<sup>a</sup></b>	<b>1.73±0.69<sup>ac</sup></b>	<b>5.23±0.99<sup>b</sup></b>	<b>5.18±1.63<sup>bc</sup></b>	<b>4.65±1.38<sup>bc</sup></b>
C17:1T	0.07±0.03	0.27±0.04	0.61±0.36	0.41±0.1	0.93±0.25
<b>C18:0</b>	<b>23.63±0.78<sup>ac</sup></b>	<b>18.97±1.10<sup>b</sup></b>	<b>21.76±1.31<sup>ab</sup></b>	<b>20.50±0.99<sup>b</sup></b>	<b>25.97±0.74<sup>c</sup></b>
C18:1T9	0.53±0.01	0.24±0.12	0.25±0.14	0.31±0.19	0.35±0.11
C18:1T11	1.51±0.1	1.61±0.21	1.32±0.49	1.83±0.57	1.28±0.09
C18:1c7	2.41±0.1	2.36±0.18	1.64±0.28	2.11±0.20	1.86±0.1
<b>C18:1n9</b>	<b>14.13±0.24<sup>ab</sup></b>	<b>15.39±0.00<sup>a</sup></b>	<b>11.66±0.95<sup>b</sup></b>	<b>14.19±2.18<sup>ab</sup></b>	<b>11.33±0.13<sup>b</sup></b>
<b>C19:0</b>	<b>0.56±0.38<sup>a</sup></b>	<b>0.98±0.59<sup>a</sup></b>	<b>4.47±0.79<sup>b</sup></b>	<b>4.94±1.85<sup>b</sup></b>	<b>3.02±0.47<sup>ab</sup></b>
C18:2n6cc	1.87±0.35	2.58±0.12	2.23±0.28	0.01±0.00	2.55±0.29

<sup>a,b,c</sup> Means in a row with different superscripts are significantly different ( $P < 0.05$ ).

Highlighted rows: fatty acids are significantly different between the samples. For example, composition of C14:0 fatty acid in LFG and SMM, SM and milk, SMM and milk is significantly different.

\* Only fatty acids contributing >0.5 % total fatty acids are shown.

**Table A7.** Cholesterol ester (CE) composition of large fat globules (LFG), milk, small fat globules (SFG), skim milk (SM), and skim milk membrane (SMM)

Fatty acids*	LFG (% of total fatty acids)	Milk	SFG	SM	SMM
C10:0	1.20±0.27	1.44±0.02	1.22±0.06	0.92±0.22	0.88±0.14
C12:0	3.49±0.08	3.49±0.58	2.86±0.05	5.27±0.12	5.20±0.19
C14:0	6.58±0.13	7.47±0.22	6.09±1.03	7.17±1.65	7.14±0.64
C14:1	3.44±0.10	2.70±0.02	2.24±0.52	1.62±0.03	1.51±0.13
C15:0	1.08±0.04	1.16±0.02	1.07±0.02	1.08±0.00	1.05±0.11
C15:1T	0.79±0.22	0.85±0.31	0.39±0.11	0.37±0.22	0.45±0.02
C16:0	27.15±0.79 <sup>ac</sup>	28.11±0.02 <sup>a</sup>	23.51±0.25 <sup>abc</sup>	23.16±1.66 <sup>bc</sup>	20.65±1.41 <sup>b</sup>
C16:1	0.39±0.05	0.72±0.02	1.42±1.14	0.02±0.00	0.38±0.40
C17:1T	1.76±0.46	2.04±0.17	0.45±0.57	1.28±0.06	1.17±0.03
C17:1	0.43±0.05	1.02±0.02	1.75±0.39	0.88±0.18	0.49±0.40
C18:0	28.42±0.06 <sup>a</sup>	23.10±0.02 <sup>b</sup>	19.96±3.10 <sup>b</sup>	23.46±0.94 <sup>b</sup>	23.58±0.07 <sup>b</sup>
C18:1T7	0.33±0.15	0.40±0.02	0.85±0.55	0.41±0.08	0.41±0.11
C18:1T11	0.42±0.26	0.51±0.23	0.47±0.36	0.52±0.08	0.37±0.44
C18:1c7	0.77±0.08 <sup>a</sup>	0.83±0.00 <sup>a</sup>	0.62±0.54 <sup>a</sup>	10.92±4.97 <sup>b</sup>	16.23±0.91 <sup>c</sup>
C18:1c9	5.33±0.35 <sup>ab</sup>	7.62±0.04 <sup>a</sup>	6.57±3.14 <sup>a</sup>	1.52±0.27 <sup>b</sup>	1.46±0.37 <sup>b</sup>
C19:0	1.51±0.29 <sup>a</sup>	1.81±0.67 <sup>a</sup>	1.37±0.09 <sup>a</sup>	8.37±7.28 <sup>b</sup>	2.42±0.06 <sup>a</sup>
C19:1t7	1.34±0.07	1.43±0.01	0.72±0.07	1.75±1.65	3.73±0.52
C19:1t10	1.05±0.79	0.37±0.01	0.70±0.11	0.39±0.20	0.00±0.00
C18:2n6cc	8.61±1.41 <sup>a</sup>	8.41±0.34 <sup>a</sup>	20.77±0.67 <sup>b</sup>	3.42±3.38 <sup>c</sup>	4.75±0.33 <sup>ac</sup>
C20:0	0.55±0.04	0.49±0.01	0.45±0.03	0.49±0.05	0.52±0.00
C18:3n3	0.63±0.05	0.58±0.01	1.32±0.32	0.11±0.04	0.09±0.04
C20:4n6	0.27±0.21	0.21±0.00	0.40±0.03	3.00±0.79	3.35±1.23
C23:0	0.57±0.07	0.54±0.01	0.69±0.24	0.47±0.07	0.36±0.08
C24:1	0.57±0.03	0.63±0.02	0.37±0.16	0.34±0.05	0.32±0.04

<sup>a,b,c</sup> Means in a row with different superscripts are significantly different ( $P < 0.05$ ).

Highlighted rows: fatty acids are significantly different between the samples. For example, composition of C16:0 content in LFG and SMM, milk and SMM, SM and milk is significantly different.

\* Only fatty acids contributing >0.5 % total fatty acids are shown.

**Table A8.** Free fatty acids (FFA) composition of large fat globules (LFG), milk, small fat globules (SFG), skim milk (SM), and skim milk membrane (SMM)

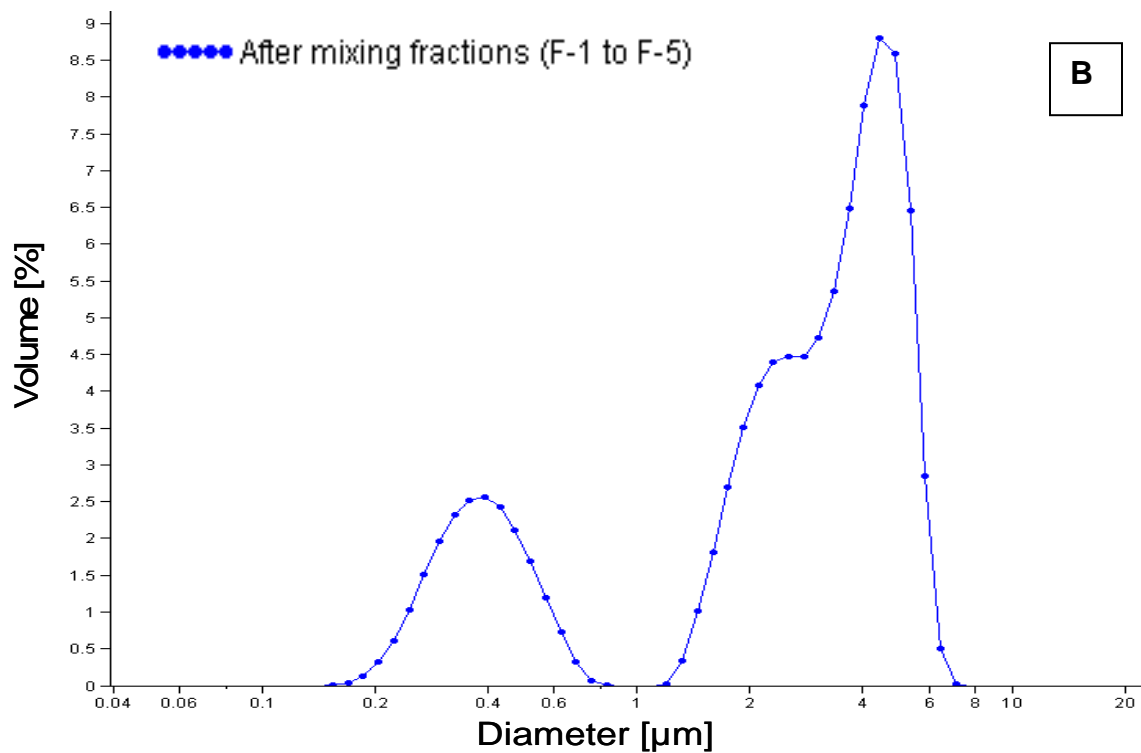
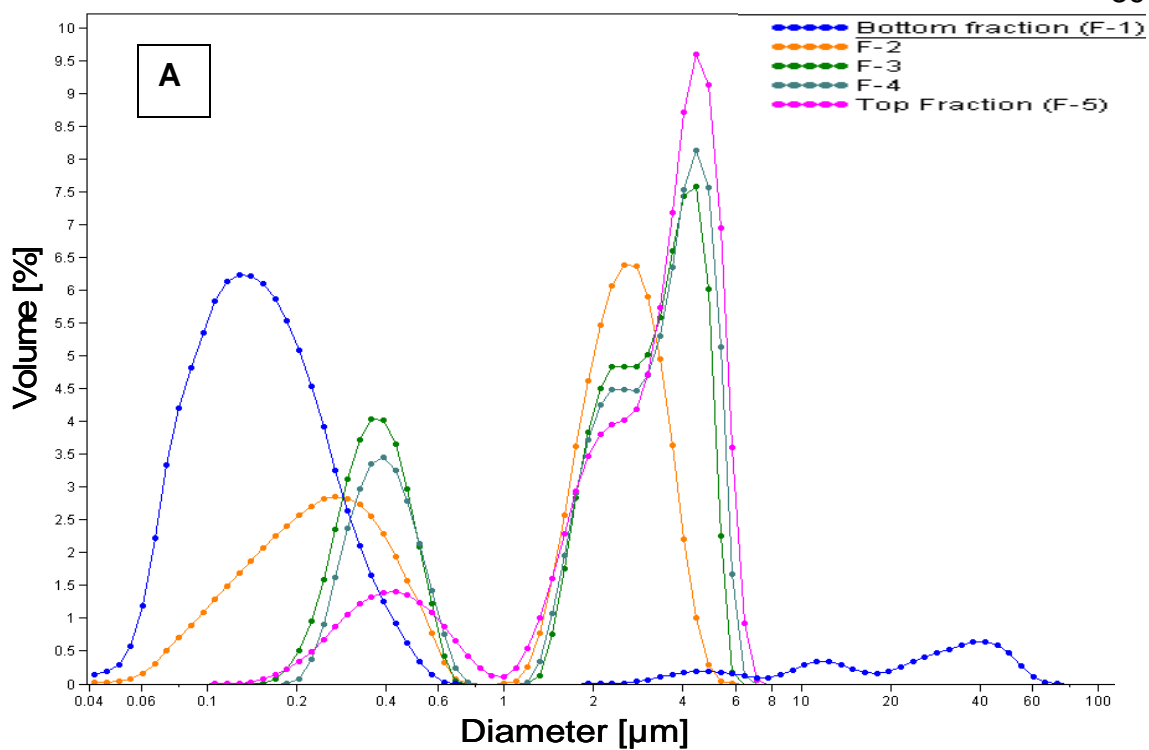
Fatty acids*	LFG (% of total fatty acids)	Milk	SFG	SM	SMM
C10:0	1.43±0.58 <sup>a</sup>	1.22±0.60 <sup>a</sup>	4.90±1.27 <sup>b</sup>	0.54±0.07 <sup>a</sup>	0.25±0.22 <sup>a</sup>
C12:0	2.87±0.80	2.91±0.70	4.86±0.42	2.86±2.44	2.23±0.77
C14:0	11.72±1.84	12.04±0.75	13.73±0.69	12.58±1.39	13.74±0.37
C14:1	0.80±0.39	0.62±0.04	1.16±0.2	0.93±0.07	1.02±0.01
C15:0	1.56±0.03	1.50±0.04	1.78±0.01	1.76±0.09	1.62±0.11
C16:0	40.00±0.46 <sup>a</sup>	38.50±0.17 <sup>ac</sup>	33.48±1.80 <sup>b</sup>	36.31±2.19 <sup>cd</sup>	34.86±1.13 <sup>bd</sup>
C16:1	0.66±0.03	0.85±0.08	1.14±0.05	1.07±0.03	1.21±0.04
C17:0	0.88±0.07	0.97±0.02	1.04±0.08	1.70±0.39	1.18±0.05
C17:1	0.52±0.26	0.28±0.03	0.27±0.08	0.33±0.10	0.23±0.03
C18:0	18.43±1.86 <sup>a</sup>	17.15±0.22 <sup>ac</sup>	13.57±0.97 <sup>b</sup>	14.69±0.93 <sup>bc</sup>	15.09±0.26 <sup>bc</sup>
C18:1T11	1.55±0.24	1.72±0.03	1.20±0.08	1.55±0.04	1.54±0.03
C18:1n11	2.32±0.05	2.27±0.02	1.65±0.13	2.16±0.10	2.10±0.08
C18:1n9	11.70±0.70 <sup>a</sup>	14.14±1.46 <sup>ab</sup>	13.77±0.24 <sup>ab</sup>	15.47±0.42 <sup>b</sup>	16.48±0.31 <sup>b</sup>
C18:1n7	0.35±0.01	0.41±0.05	0.38±0.02	0.43±0.01	0.45±0.01
C19:0	0.15±0.05	0.32±0.05	0.73±0.10	1.41±0.36	1.81±0.79
C18:2n6cc	2.09±0.02	2.55±0.34	2.91±0.50	3.26±0.01	3.09±0.11
C18:3n3	0.42±0.05	0.54±0.07	0.72±0.14	0.72±0.04	0.75±0.06

<sup>a,b,c,d</sup> Means in a row with different superscripts are significantly different ( $p < 0.05$ ).

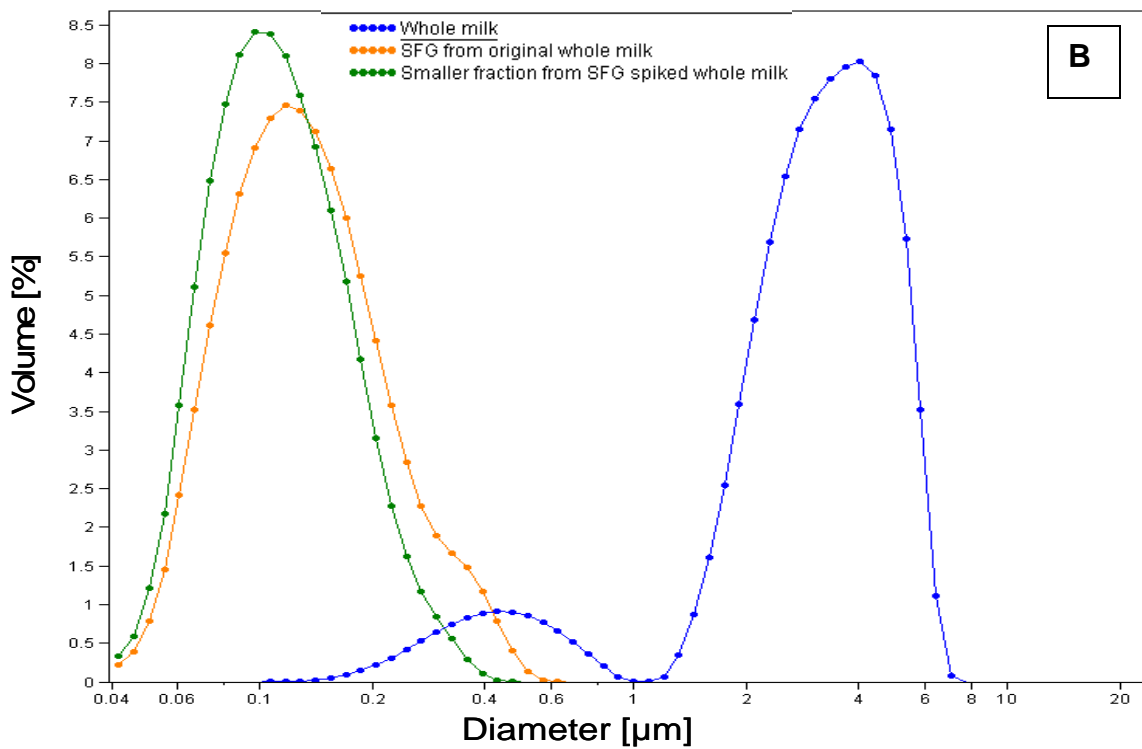
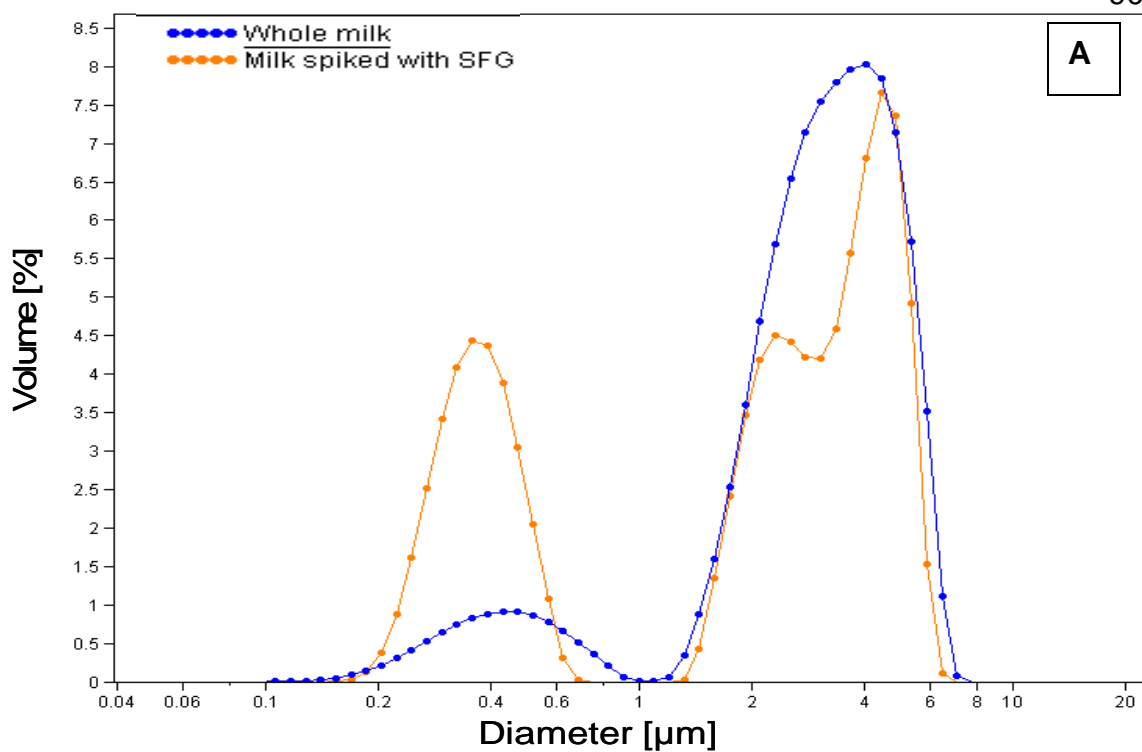
Highlighted rows: fatty acids are significantly different between the samples. For example, composition of C14:0 fatty acid in LFG and SMM, SM and milk, SMM and milk is significantly different.

\* Only fatty acids contributing > 0.5% of total fatty acids are shown.

APPENDIX B.  
FIGURES

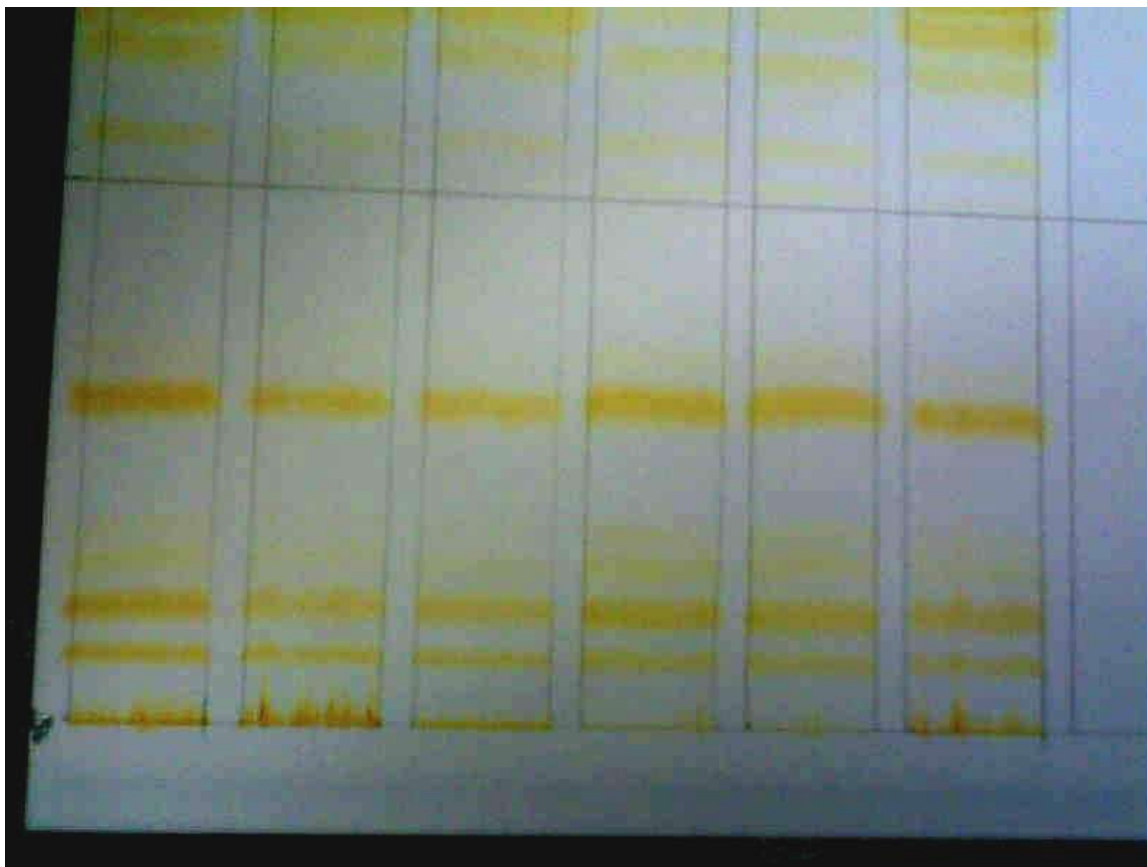


**Figure B1:** Milk fat globule size distribution of the different fractions (F 1 – F5) collected after separation of milk by gravity at 7 °C for 24 h. A) Individual fractions B) after mixing all fractions together.



**Figure B2:** Size distribution of fat globules A) after spiking milk with SFG  
B) isolation of small fat globules from SFG spiked milk.

### Example of Thin Layer Chromatography



**Figure B3:** Separation of total phospholipid into different classes by Thin Layer Chromatography. Iodine vapor was used to visualize different lipid spots on developed TLC plate. These lipid bands were scrapped out from the plate for further analysis.