

Title Enzymatic and physical modification of milk
fat: a review
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Citation International Dairy Journal - in press
Date 2010
URL <http://dx.doi.org/10.1016/j.idairyj.2010.05.003>
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Enzymatic and physical modification of milk fat: A review

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Abstract

Bovine milk fat has one of the most complex composition of all natural fats. Being composed of more than 400 different fatty acids present primarily as triacylglycerols gives milk fat highly diverse functional and nutritional properties. The complexity of milk fat provides both opportunities and challenges to modify its composition for different applications. Due to versatility of lipidic compounds, milk fat can be considered as a good source of essential fatty acids and fat-soluble vitamins. Additionally, milk fat has physically and chemically favorable properties, and it also has good sensory quality. However, the negative nutritional image of milk fat, especially certain saturated long-chain fatty acids, and poor spreadability of butter

have inspired researchers to develop technologies to produce milk fat fractions with different physico-chemical or nutritional properties. This article reviews the current literature on enzymatic and physical means to fractionate bovine milk fat into different fractions and highlights their fields of usage.

1. Introduction

The milk of major bovine breeds contains 3.5 – 5 % fat, which is composed of more than 400 different fatty acids, mainly present as triacylglycerols (TAGs), traditionally named triglycerides (Jensen, 2002). Milk fat fractions are widely used in a variety of food products, due to many favorable physical, chemical and nutritional properties of milk fat. People consume most of milk fat in different forms of traditional dairy products, such as liquid milk, cream, butter, ghee, cheese and ice cream. In most industrialized countries consumption of butter and other high-fat dairy products has been on the decline in recent years due to a negative nutritional image of milk fat. This trend is likely to continue in industrialized countries while, in developing countries, milk fat consumption may increase due to increasing milk production (IDF, 2006). In view of above trends, there is an emerging need to expand the scope for usage of milk fat by providing milk fat products with improved melting profile, as well as flavour and nutritional properties.

The negative nutritional image of milk fat is mostly related to the current conception that the intake of milk fat is associated with an increased risk of developing coronary heart disease (CHD) or metabolic syndrome (MacRae, O'Reilly, & Morgan, 2005; Mensink, 2006; Moss & Reed, 2003). This property is claimed to be due primarily to certain saturated medium- and long-chain fatty acids, notably lauric (C12:0), myristic (C14:0) and palmitic (C16:0) acids, which are considered to have atherogenic action through increasing plasma cholesterol and

low-density lipoprotein (LDL) levels (Kris-Etherton & Yu, 1997). These fatty acids comprise about 35 % of total milk fat. Other long-chain fatty acids in milk fat, such as stearic acid (C18:0) and short-chain acids (C4:0 – C10:0) are considered to have neutral effect, whereas linoleic (C18:1) or linolenic (C18:2) acids may reduce cholesterol level (Jensen, 2002). Recently, the putative role of milk fat in the development of CHD and metabolic syndrome has been discussed critically in many review articles (Elwood, Pickering, Fehily, Hughes, & Ness, 2004; Elwood, Pickering, Hughes, Fehily, & Ness, 2004; German, 2008; German & Dillard, 2006; Lock, Destailats, Kraft, & German, 2008; Parodi, 2009; Steijns, 2008; Tholstrup, 2006) and critical views have been supported by case-control studies (Biong et al., 2007; Warensjö et al., 2004). However, some minor components of milk fat, especially conjugated linoleic acid (CLA), sphingomyelin, butyric acid, and ether lipids may have beneficial effects on health (Parodi, 2004). Due to these controversial views, the multi-purpose use of milk fat appears to be an approach warranting more research in the future.

According to current knowledge, there are some possibilities of influencing the amount and composition of milk fat by means of feeding or breeding (AbuGhazaleh & Holmes, 2009; Lock & Bauman, 2004; Palmquist, Stelwagen, & Robinson, 2006; Shingfield et al., 2005). According to Shingfield, Chilliard, Toivonen, Kairenius and Givens (2008) it is possible to significantly reduce the content of saturated fatty acids and enhance the concentration of several bioactive lipids several-fold in milk through changes in the ruminant diet. Most strategies involve supplementing the diet with plant oils or oilseeds, which, due to the metabolism of dietary lipids in the rumen, results in an unavoidable increase of trans fatty acid content in milk. Another possible approach to modify the composition of milk fat is to employ technological means. Over the last thirty years, a great number of studies have been carried out using enzymatic or physico-chemical methods to modify the composition of milk

fat. Modification of milk fat using specific lipolytic enzymes has been tested and applied industrially with varied success (Balcão & Malcata, 1998a). Many technologies are being exploited industrially for chemical modification, e.g. hydrogenation, interesterification, acidolysis and alcoholysis (Balcão & Malcata, 1998b) and physical fractionation of milk fat, e.g., temperature-induced crystallization, short-path distillation and supercritical fluid extraction (Kaylegian, 1999; Rizvi & Bhaskar, 1995).

This literature review compiles the current knowledge on enzymatic and physical methods available for modification of milk fat.

2. Milk fat as a substrate for lipases

2.1. Composition and structure of milk fat

Bovine milk is an oil-in-water emulsion containing ca. 3.5 – 5 % (w/w) milk fat (Jensen, 2002). A major proportion of milk fat exists in small milk lipid globules (average $\phi \sim 4 \mu\text{m}$), which are protected by the surrounding milk fat globule membrane (MFGM) composed of phospholipids, cholesterol, lipoproteins, glycoproteins, and proteins, e.g., xanthine oxidase, butyrophillin, and γ -glutamyl transpeptidase (Dewettinck et al., 2008; Evers, 2004; Jiménez-Flores & Brisson, 2008; Rombaut & Dewettinck, 2006). Hence, prior to extensive enzymatic modification, milk fat should be separated and processed to various forms of anhydrous milk fat, e.g., (anhydrous) butteroil or butterfat.

The lipid fraction of milk is very heterogeneous. The major lipid classes of milk fat are given in Table 1, and the fatty acid composition of milk fat is shown in Table 2. The most abundant lipid class in milk is triacylglycerols (TAG), which are triacylestere of glycerol with fatty acids, constituting ca. 97–98 % (w/w) of all lipids. Figure 1 gives a schematic chemical

structure of a TAG molecule, which is the triacylglycerol of glycerol with oleic (C18:1), palmitic (C16:0), and butyric acid (C4:0). In addition, Figure 1 shows an example of the stereospecific numbering (*sn*) system recommended by the International Union of Pure and Applied Chemistry – International Union of Biochemistry (1967).

The central carbon of TAG (*sn*-2) shows chirality, resulting in an asymmetrical TAG molecule, if two different fatty acids are in the primary positions (*sn*-1 and *sn*-3) of the molecule. The stereochemical features of TAG molecules have to be taken into account when TAGs are used as a substrate for enzymatic modification, because the distribution of fatty acids in the TAG molecule influences the end products and the action of modifying enzymes. In general, the TAG composition and structure of milk fat are extremely complex, due to a high number (>400) of different fatty acids found in milk fat (Jensen 2002). One to two hundred different TAG species have been analytically determined in bovine milk by RP-LC–GLC (Gresti, Bugaut, Maniongui, & Bezar, 1993) and GLC–EI–MS methods (Kemppinen & Kalo, 2006). In general, even-numbered TAGs with 26–54 acyl carbons dominate, but odd-numbered TAGs are also present in bovine milk fat (Kalo & Kemppinen, 2003). A substantial number of mono-short-chain TAGs are also present in milk fat, which is unique among food fats and oils (Gresti et al., 1993; Kemppinen & Kalo, 2006). An early stereospecific analysis showed that the short-chain fatty acids are mainly located at the *sn*-3-position of TAG molecules (Breckenridge & Kuksis, 1968; Pitas, Sampugna, & Jensen, 1967). Later, the non-random distribution of other fatty acids was confirmed (Kermasha, Kubow, Safari, & Reid, 1993; Parodi, 1979). Figure 2 shows a summary of the most probable location (distribution % > 33.3 %) of the main fatty acids in the *sn*-position of TAGs in milk fat (Jensen, 2002; Parodi, 1979).

2.2. Biosynthesis of TAG in the mammary gland

TAGs of ruminants' milk fat are synthesized in the mammary gland. The principal metabolic route involved is the glycerol-3-phosphate (G-3-P) pathway, but a minor proportion of TAGs is synthesized *via* the dihydroxyacetone phosphate pathway (Hawke & Taylor, 1995). Figure 3 shows the outline of the G-3-P pathway, including the most important enzymes involved.

The fatty acids used in TAG biosynthesis are synthesized *de novo* in the mammary gland or they are uptaken from the TAGs of the circulating lipoproteins (very low-density lipoprotein (VLDL) and chylomicrons) of blood (Hawke & Taylor, 1995). In bovine milk fat, the fatty acids with carbon chain length from C4 to C14 are synthesized *de novo* from acetate and β -hydroxybutyrate, which is a special feature of ruminant lipogenesis (Hawke & Taylor, 1995). The C18 fatty acids are taken up from the blood serum and palmitic acid (C16:0) originates from both sources (Hawke & Taylor, 1995).

Nutritional factors are the main source of the variation of the composition of TAGs, but genetic and lactational factors have also influence on the composition of TAGs in bovine milk fat (Hawke & Taylor, 1995). In addition, the biosynthesis of TAGs *via* the G-3-P pathway has a definite effect on the specific structure of TAGs in bovine milk fat (Figure 2). In general, 1-palmitoyl- and 1-oleoyl-*sn*-G-3-P are considered to be most likely substrates for acyl-CoA:1-acyl-*sn*-G-3-P acyl transferase in TAG synthesis, due to the high concentration of palmitic and oleic acid in bovine mammary gland. 1-palmitoyl- and 1-oleoyl-*sn*-G-3-P are shown to be acylated solely with the acyl-CoA of chain length from C8 to C18 (Marshall & Knudsen, 1977a), resulting in a lack of short-chain acyls in the *sn*-2-position of the glycerol backbone. Furthermore, Marshall and Knudsen (1977b) showed that the acyl-CoA:1,2-DAG acyl transferase catalyzed the synthesis of TAGs in the bovine mammary gland from both

butyroyl-CoA and palmitoyl-CoA with equal efficiency. However, the short-chain acyl groups (C4:0 and C6:0) are known to be more abundant in the *sn*-3-position of TAGs in bovine milk fat than the palmitoyl acyl groups. This is probably due to the higher concentration of free butyroyl-CoA in mammary gland than non-protein-bound palmitoyl-CoA, and a high concentration of bovine serum albumin, which binds efficiently free palmitoyl-CoA but not butyroyl-CoA (Marshall & Knudsen, 1980).

2.3. Digestion and absorption of TAGs

Understanding of digestion, absorption, and further metabolism of dietary TAGs is essential when the effect of composition and structure of fat on human nutrition are evaluated. Various different factors influence the digestion and absorption of TAGs, including several lipases (triacylglycerol lipase EC 3.1.1.3), bile salts, pH of gastrointestinal tract, the presence of other components (salts, calcium, monoacylglycerols (MAGs)), and the composition and structure of metabolized substrate (Bracco, 1994; Kuksis, 2000; Mu & Høy, 2004; Small, 1991). A simplified scheme for the digestion, absorption, transport, and accumulation of TAGs in tissue is shown in Figure 4.

In the aqueous environment of the gastrointestinal tract, dietary fat (mostly non-polar TAGs) forms large fat droplets, the size of which rapidly diminished due to the effective mixing of the food bolus and the emulsifying power of the bile salts released from the liver (Figure 4). Decreasing the droplet size increases the surface area of the fat droplets providing a larger water-oil surface for the action of lipases in the gastrointestinal tract, enabling hydrolysis of TAGs to fatty acids and 2-MAGs. Added to the fat droplet size, the structure and composition of the fat globule membrane has a definite influence on the metabolic rate of dietary TAGs as was reviewed lately by Michalski and Januel (2006). For example, digestion rate of human

milk fat globules with native membrane is faster than that of much smaller homogenized fat globules of infant formula with modified fat globule membrane. As digestion proceeds, small fat droplets shrink further and form small micelles, which are able to enter the microvillous border of the small intestine. After diffusion of fatty acids and 2-MAGs inside the enterocytes, they are reesterified to TAGs (Bracco, 1994; Kuksis, 2000; Mu & Høy, 2004; Small, 1991). The TAG biosynthesis in the intestinal epithelial cells mainly uses the monoacylglycerol pathway. The G-3-P pathway is less frequently utilized, i.e., only during fasting. In the monoacylglycerol pathway, the 2-MAGs and acyl-CoAs are first converted to corresponding *sn*-1,2-DAGs or *sn*-2,3-DAGs using the monoacylglycerol acyltransferase enzyme as catalyst, and then the diacylglycerol (DAG) transferase catalyzes the acylation of *sn*-1,2(2,3)-DAGs to TAGs (Kuksis, 2000). Following the resynthesis, the TAGs are assembled with apolipoproteins to yield chylomicrons and VLDL particles, which are released to the blood stream and the target tissues, i.e., adipose and muscle tissue, *via* the lymph (Kuksis, 2000; Mu & Høy, 2004).

Several lipases have a major role in the digestion of dietary TAGs (Figure 4). The digestion of TAGs begins in the stomach with lipolysis catalyzed by lingual lipase, which is secreted already in mouth from Ebner's glands, or by gastric lipase, which is secreted in stomach. Both enzymes preferentially release fatty acids from the *sn*-3-position of TAGs ca. twice as fast as from the *sn*-1-position (Mu & Høy, 2004). At this early stage of milk fat digestion, a high number of TAG species containing short-chain fatty acids in the *sn*-3-position are effectively hydrolyzed, resulting in the release of mostly short-chain fatty acids and *sn*-1,2-DAGs. At the pH of the stomach, the released short-chain fatty acids are water-soluble and they are absorbed into the portal vein by diffusion in the gastric mucosa (Bracco, 1994). The long-chain fatty acids are diffused back into the fat droplets in the stomach or in the duodenum

(Bracco, 1994). However, the most important enzyme in digestion of fat is pancreatic lipase, which hydrolyzes the fatty acids from the primary positions of TAGs, with a slight preference for the *sn*-1-position resulting in formation of 2-MAGs and 2,3-DAGs and free fatty acids (Kuksis, 2000; Mu & Høy, 2004). In addition to pancreatic lipase, the carboxyl ester hydrolase is active in the small intestine, specially acting on DAGs containing long-chain polyunsaturated fatty acids (Small, 1991).

The structure of TAGs in bovine milk fat has other profound effects on the digestion and absorption of dietary milk fat. For example, human milk fat has been shown to be absorbed more efficiently compared to bovine milk fat (Bracco, 1994; Small, 1991), which is probably due to the differences in the distribution of palmitic acid between secondary and primary positions of TAG molecules. Most of the palmitic acid is in the *sn*-2-position in human milk fat, and is efficiently diffused into the enterocytes from micelles as 2-MAGs (Bracco, 1994; Small, 1991). In bovine milk fat, a high portion of palmitic acid is in the primary position, and is released as a free fatty acid by the pancreatic lipase. At the pH of the intestine, the palmitic acid and corresponding soaps are highly insoluble and could precipitate, especially in the presence of calcium, and pass out in the faeces, impairing total absorption of milk fat (Bracco, 1994; Small, 1991).

3. Lipases as tools for lipid modification

Lipases are ubiquitous esterases that are found in various organisms, including animals, plants, filamentous fungi, yeasts and bacteria. Lipases catalyze particularly the hydrolysis of water-insoluble substrates such as long-chain TAGs at the interface between the substrate and water. Although naturally occurring triglycerides are preferred substrates of lipases, they also

catalyze the hydrolysis or synthesis of a rather broad range of other substrates, such as aliphatic, alicyclic, bicyclic and aromatic esters (Schmid & Verger, 1998). In aqueous environments lipases catalyze the hydrolysis reactions, whereas in organic solvents they catalyze several other types of biotransformations, e.g., esterification and transesterification. Microbial lipases are widely used in different industrial applications due to their broad substrate specificity and outstanding regio- and stereo-selectivity under mild reaction conditions. Moreover, lipases often exhibit a very good stability, remain enzymatically active in a wide range of organic solvents, and do not require cofactors (Jaeger & Eggert, 2002).

3.1. Structure and biochemical properties of lipases

Lipases belong to the family of α/β -hydrolases, which share a characteristic fold of their central catalytic domain (Ollis et al., 1992). The active sites of lipases are composed of serine, aspartate/glutamate and histidine residues, which form a catalytic triad similar in arrangement to those of serine proteases (Brady et al., 1990; Schmid & Verger, 1998). Lipases have a large, hydrophobic scissile fatty-acid-binding site for medium- and long-chain fatty acids. The length of the scissile fatty-acid-binding site varies between 7.8 Å in *Fusarium solani* cutinase and 22 Å in *Candida rugosa* and *Rhizomucor miehei* lipases, and this length was observed to correlate with the activity profile of the lipases on different length of fatty acid chains (Pleiss, Fischer & Schmid, 1998). Common to all lipases is the arrangement of hydrophobic residues around the active site; these residues have been suggested to be crucial for the attachment of the lipase to the aggregated substrate (Fojan, Jonson, Petersen, & Petersen, 2000).

The activity of lipases is low on monomeric (dispersed) substrates but strongly enhanced on aggregated TAGs (e.g., emulsion or micelles) formed above the saturation limit. This enhanced activity, a phenomenon known as interfacial activation (Schmid & Verger, 1998),

distinguishes lipases from esterases acting only on water-soluble carboxylic ester molecules. The three-dimensional structures of *Mucor miehei* and human pancreatic lipases (Brady et al., 1990; Winkler, D'Arcy, & Hunziker, 1990) revealed that a surface loop, called the lid or the flap, covers the active site serine. Binding of the lipase to the lipid-water interface induces a conformational change in the surface loop of the enzyme, rendering the active site accessible to the substrate (Schmid & Verger, 1998). However, not all lipases show interfacial activation, although they have an amphiphilic lid covering the active site (Verger, 1997).

Microbial lipases are typically extracellular enzymes. However, in the case of many microorganisms capable of producing extracellular lipases, a substantial portion of the enzyme apparently remains attached onto hydrophobic surfaces of the cell wall due to the hydrophobic nature of lipases (Liew, Ghazali, Long, Lai, & Yazid, 2001; Long, Ghazali & Ariff, 1996). Production of multiple lipases, which differ in molecular weights or substrate specificities and may have different pH and thermal stability properties, is also characteristic for microorganisms producing lipases. The multiple forms are due to both post-translational processes and to the synthesis of truly different lipases (Sugihara, Shimada, & Tominaga, 1990). It is known that microorganisms, particularly yeasts and filamentous fungi such as *C. rugosa*, *Geotrichum candidum*, *Aspergillus niger* and *Rhizopus niveus*, secrete different lipase isoforms in varying amounts depending on the culture conditions (Chang, Chou, & Shaw, 1994; Höfelmann, Hartmann, Zink, & Schreier, 1985; Kohno, Kugimiya, Hashimoto, & Morita, 1994; Sugihara et al., 1990).

Most microbial lipases exhibit maximum activity in the temperature range of 30–40°C (Malcata, Reyes, Garcia, Hill, & Amundson, 1992) but both lower and higher ranges have been demonstrated (Gupta, Gupta, & Rathi, 2004). Thermophilic lipases retaining activities in

the temperature range of 50–65°C have been isolated, e.g., from filamentous fungi (*Aspergillus niger*, *Thermomyces lanuginosus*) and bacteria (*Pseudomonas* and *Bacillus* sp.). The pH stability of bacterial lipases ranges from 4 to 11; the maximum activity of most microbial lipases is displayed in the pH range of 5.6–8.5, and the maximum stability is in the neutral pH range (Malcata et al., 1992). Some clearly alkaline lipases having pH optima around 9.5 have been found, e.g., from *Bacillus* and *Pseudomonas* species (Rúa, Schmidt-Dannert, Wahl, Sprauer, & Schmid, 1997; Watanabe, Ota, Minoda, & Yamada, 1977).

3.2. Substrate specificity of lipases

Lipases can be classified according to their substrate specificity (Jensen, Galluzzo, & Bush, 1990). Lipid class (substrate) specificity is defined as the ability of lipases to preferentially hydrolyze a particular glycerol ester, not only the ester bonds of TAGs but also DAGs and MAGs as well as phospholipids (Villeneuve & Foglia, 1997). Animal, plant and microbial lipases generally have a preference for TAG and DAG rather than MAG (Svendsen, 2000). Fatty acid specificity describes the specificity of lipases for a particular fatty acid or, more generally, for a class of fatty acids regardless of their position on the glycerol backbone (Villeneuve & Foglia, 1997). Regiospecificity (positional specificity) is defined as the ability of lipases to distinguish between the two external positions (primary positions) and the internal position (secondary position) of the TAG backbone (Villeneuve & Foglia, 1997). Lipases can also be stereospecific, i.e., they have an ability to distinguish *sn*-1 and *sn*-3 position of TAGs or non-specific being able to hydrolyze all ester bonds in TAG, regardless of positions or types of fatty acids (Villeneuve & Foglia, 1997).

4. Modification of milk fat

4.1. Lipase-catalyzed modification

Lipase-catalyzed modification of milk fat is used commercially to improve physical, chemical, nutritional, and sensory properties of milk fat, and to make it more suitable for manufacture of other products with desired properties (Balcão & Malcata, 1998b; Kalo & Kemppinen, 2003). Enzymatic modification enables production of highly specific structured lipids and tailor-made fats. Most commonly used methods of enzymatic modification are hydrolysis and lipase-catalyzed interesterification reactions, including ester-ester exchange (transesterification), acidolysis, and alcoholysis (Balcão & Malcata, 1998b; Kalo & Kemppinen, 2003). Ester-ester exchange is a reaction between two or more esters, e.g., acylglycerols. In acidolysis, the reaction takes place between an acylglycerol and a free fatty acid, whereas in alcoholysis an acyl group is changed between an acylglycerol and an alcohol. Table 3 gives an overview of recent studies concerning lipase-catalyzed modification of milk fat.

Spontaneous and extensive hydrolysis of milk fat generally results an unacceptable rancid flavour in milk fat products, whereas controlled hydrolysis of milk fat could be used in the production of cheese-like flavour products and additives (Balcão & Malcata, 1998b), which could be used in several branches of the food industry, e.g., in dairy products, bakery products, candies, and snack foods (Regado et al., 2007). In addition to controlled hydrolysis, the synthetic nature of lipases utilized in interesterification reactions of milk fat will result in changed flavour and melting properties and a distinguishing solid fat content, fatty acid composition, and TAG structure compared with those of untreated milk fat (Lee & Swaisgood, 1997). Industrial-scale applications for lipase-catalyzed interesterification of milk fat have been rare, but several potential applications have been reported. Lipase-catalyzed

modifications have been used to improve limited spreadability of butter-based spreads (Marangoni & Rousseau, 1998), to prepare human milk fat substitutes (Christensen & Holmer, 1993), and to enhance nutritional properties of milk fat by increasing the proportion of unsaturated fatty acids and/or decreasing that of C12:0–C16:0 fatty acids by incorporation of healthy unsaturated vegetable oils (Marangoni & Rousseau, 1998; Pal, Bhattacharyya, & Ghosh, 2001; Ronne, Yang, Mu, Jacobsen, & Xu, 2005) or free unsaturated fatty acids (α -linolenic, oleic or conjugated linoleic acid) with milk fat (Balcão, Kemppinen, Malcata, & Kalo, 1998a; Garcia, Storkson, Pariza, & Hill, 1998; Garcia, Arcos, Keough, & Hill, 2000, 2001; Kim et al., 2002; Lee & Swaisgood, 1997; Sehanputri & Hill, 2003).

The early studies with nonspecific lipases (Kalo, Huotari, & Antila, 1990; Kalo, Parviainen, Vaara, Ali-Yrkkö, & Antila, 1986) showed that enzymatic interesterification of milk fat will result in similar end-products as the use of chemical interesterification, i.e., randomization of the TAG structure, and thus milk fat with unchanged fatty acid composition but higher softening temperature due to a changed TAG structure. However, use of 1,3-specific lipases for interesterification could improve the spreadability of modified butterfat compared to untreated butterfat by decreasing (by 2–5°C) the softening temperature of the fat (Marangoni & Rousseau, 1998). However, the loss of buttery flavour due to the necessary refining procedures following interesterification (Marangoni & Rousseau, 1998) is a serious drawback of enzymatic modification of butter-based spreads. Christensen and Holmer (1993) used interesterification of butter oil with polyunsaturated fatty acid concentrates by an immobilized 1,3-specific lipase of *Rhizomucor miehei* for production of a human milk fat substitute having a fatty acid profile and TAG structure resembling those of human milk fat. Recently, Maduko, Akoh, & Park (2007) have applied similar techniques to production of caprine milk infant formula analogs from vegetable oil blends.

In recent years, the most intensively studied application of lipase-catalyzed modification of milk fat has been the incorporation of different unsaturated fatty acids by both nonspecific and 1,3-specific lipases. Garcia et al. (1998, 2000, 2001) and Sehanputri and Hill (2003) have shown the feasibility of lipase-catalyzed acidolysis to produce CLA-enriched butter fat. By combining selective hydrolysis and transesterification reactions in milk fat modification, profound and nutritionally advantageous changes could be achieved, which cannot be obtained with any other processing method,. For example, Balcão & Malcata (1998a) and Balcão, Kemppinen, Malcata, & Kalo (1998b) have shown simultaneous and significant increase in the proportion of oleic acid and decrease in the proportion of saturated medium- and long-chain fatty acids (C12:0–C16:0) by combining different lipase-modification reactions.

4.2. Fractionation of milk fat

The unique physical characteristics of milk fat, especially its wide melting range, make milk fat, as such, unsuitable for a number of food applications. Fractionation of milk fat into oil, plastic and solid fat fractions with different chemical and physical characteristics has provided more specified milk fat products for a broad range of food applications. Because fractionation of milk fat has been frequently reviewed in literature (Dewettinck, Vanhoutte, Foubert, & Huyghebaert, 2004; German & Dillard, 1998; Hamm, 1995; Kaylegian, 1999, Rombaut & Dewettinck, 2006), and an excellent and extensive handbook on milk fat fractionation is available (Kaylegian & Lindsay, 1995), the present survey will discuss this subject only briefly focusing mostly to the very recent findings.

Solvent-free temperature crystallization (dry melt fractionation) is thus far the most widely used fractionation method for milk fat. In conventional dry melt fractionation, a suspension of liquid (olein) and solid (stearin) fat is produced and the phases are then typically separated by membrane filtration. Although milk fat can be crystallized into fractions with differences in functionalities and physical characteristics (i.e. melting behaviour), there are usually rather small differences between the fatty acid compositions of the fractions. In fact, in commercially available milk fat fractions the fatty acid compositions fall within the range of fatty acid compositions typically found in natural milk fats (German & Dillard, 1998). However, some evident changes in the milk fat composition occur during the fractionation process. Unsaturated fatty acids and short saturated fatty acids are concentrated in liquid olein fractions and long saturated fatty acids are enriched in solid stearin fractions. Flavouring compounds, pigments, cholesterol, and vitamin A are enriched in olein fractions (Boudreau & Arul, 1991). Several differences can also be seen in composition of TAGs between the fractions. The average molecular size of TAGs is higher in stearin fractions and lower in olein fractions. Full saturated TAGs are highly concentrated in hard stearin fractions. However, in complex lipid systems, such as milk fat, it is in any case impossible to obtain complete separation between all different TAGs by fractional crystallization, because of the tendency of different TAG molecules to create mixed crystals (solid solutions) during the process (Timms, 1994).

Layer crystallization is a filtration-free alternative where the crystal layers are produced on a heat-transferring surface and the residual melt is separated by gravity. Belkacemi, Angers, Fischer, & Arul (2003) optimized process parameters for falling-film layer crystallization of milk fat. Crystallization temperature and flow rate of the circulated melt had a significant influence on the separation efficiency and yields of the fractions. It was also necessary to add

a sweating step in the process, where the crystallized fraction was slightly heated to remove low-melting triglycerides that were adhered to crystals. An optimized one-step process provided satisfactory yields of stearin and olein fractions with different physiochemical properties (Belkacemi et al., 2003).

In suspension crystallization, process parameters have an obvious influence on the physical and chemical properties of the fractions. Vanhoutte, Dewettinck, Vanlerberghe & Huyghebaert (2003) observed a negative correlation between fractionation temperature and stearin yield, as well as between residence time and oil entrapment. In contrast, a positive correlation was found between oil entrapment and agitation rate. However, the influence of these process parameters on the chemical composition of the fractions was almost negligible (Vanhoutte et al., 2003).

The functional properties of milk fat fractions are related to the amount and type of fat crystals formed at the temperature of the application and thus, the crystallization behaviour of milk fat fractions has been under exhaustive research. Cisneros, Mazzanti, Campos, & Marangoni (2006) showed that a very high-melting stearin fraction of milk fat crystallized initially in the exceptionally stable α form. It was concluded that the lack of liquid phase hindered the mobility of crystals and impeded the formation of the more stable polymorphs. In stearin fraction, enriched with saturated long-chain fatty acids, two polymorphic forms ($\alpha + \beta'$) were dominant (Lopez, Bourgaux, Lesieur, Riaublanc, & Ollivon 2006). Cisneros et al. (2006) noted that the mixture of high- and low-melting milk fat fractions crystallized originally in the α polymorph but, due to the presence of liquid phase, the transformation into β' polymorph occurred rapidly. Lopez et al. (2006) reported that, in a low-melting olein fraction, the crystals corresponded mainly to the α form. Furthermore, in the olein fraction,

the formation of liquid crystals was observed, which was considered to favour the entrapment of aromatic molecules in the fraction (Lopez et al., 2006).

Since the chemical composition of the fractions differs from the intact milk fat, the chemical reactivity and stability of the fractions is also different. Fatouh, Singh, Koehler, Mahran, & Metwally (2004) reported that both the oxidative and the hydrolytic stability of fractionated buffalo butter oil were highest in the high melting fractions and lowest in the low melting fractions. Abd El-Aziz (2008) showed that the higher content of unsaturated fatty acids in the low-melting milk fat fractions was related directly to the oxidative instability.

De et al. (2007) reported that, in solvent crystallization, the crystallization temperature has to be much lower when non-polar solvents (e.g., hexane) are used as opposed to polar ones (e.g., acetone, isopropanol). It was shown that, in all solvent systems used, the milk fat was readily fractionated into three fractions, with distinctive differences in their melting points (De et al., 2007). However, it should be noted that, while solvent crystallization is common practice in vegetable oil fractionation, the International Dairy Federation (IDF) does not support applying any kind of solvents for fractionation processes of milk fat (Illingfort, 2002).

Milk fat components can also be fractionated on the basis of their molecular size by molecular (short-path) distillation. First distillates are typically enriched in volatile and low molecular weight milk fat components, i.e. free fatty acids, lactones, cholesterol, mono- and diglycerides and short chain TAGs (Stark, Urbach, Hamilton, & Forss, 1973; Boudreau & Arul, 1991; Craven & Lencki, 2007). Gradual increase in the evaporation of high molecular weight TAGs exhibits during the distillation process. Molecular distillation requires high vacuum and relative high temperatures, and this may result in thermal damage to the milk fat and loss in

flavour. This separation technique has high energy costs and is best applied to specialty fat products (Jiménez-Flores, 1997). Campos, Litwinwnko, & Marangoni (2003) short-path-distillated milk fat at different temperatures; as expected, the distillates were enriched with short-chain fatty acids and low molecular weight acylglycerols, while the retentates were enriched with long-chain fatty acids (both saturated and unsaturated) and high molecular weight acylglycerols. Increasing the distillation temperature increased distillate yield, as well as the dropping point of distillate, which is related directly to the melting point (Campos et al., 2003).

Fractionation by supercritical fluid extraction (SFE) offers another opportunity for size-based separation of TAGs, even the separation according to molecular weight is not as distinct as it is with molecular distillation (Boudreau and Arul, 1991). Fatouh, Mahran, El-Ghandour, & Singh (2007) used a multi-step supercritical carbon dioxide extraction procedure for buffalo butter oil fractionation. Four fractions with different chemical and physical properties were obtained. Cholesterol, short-chain fatty acids and saturated fatty acids were enriched in the first fractions obtained at lower pressure, while long-chain fatty acids and unsaturated fatty acids were enriched in the later fractions. The melting of the last fractions occurred at higher temperatures than the melting of the first ones, as could be expected (Fatouh et al., 2007). The influence of temperature and pressure on the supercritical carbon dioxide fractionation of sheep milk fat was studied by Spano, Salis, Mele, Madau, & Mosduzzi (2004); the most promising extraction conditions for single-step fractionation comprised a temperature of 40°C and pressure of 250 bar, at which two fractions, extract and residue, with distinctively different properties were obtained with almost equal yields (Spano et al., 2004).

4.3 Applications of milk fat fractions

Milk fat fractions with different physical characteristics and specific properties are valuable for several food applications, and they have been produced commercially for over three decades. Today, several companies offer milk fat fractions as functional food ingredients for bakery, confectionery, chocolate and dairy industries. High-melting milk fat fractions are used in laminated pastries to promote layering and in chocolate industry to inhibit fat blooming. Low-melting milk fat fractions can be used, e.g., for creaming applications in the biscuit and cheese industries (Gibon, 2006).

The blending of selected milk fat fractions to achieve a solid fat content similar to that of margarine has been a popular approach for the production of spreadable butters. Depending on the markets, different kinds of spreadable butters ('hard' and 'soft') can be produced. De et al. (2007) fractionated milk fat by solvent crystallization. The fractions were blended and interesterified with different plant oils and fats; plastic fats with different consistencies and excellent spreadability features were obtained.

Modified milk fat differs functionally from natural milk fat and may influence the physical and chemical attributes in emulsified food systems, such as creams. Scott, Duncan, Sumner, & Waterman (2003) showed that 20% milk fat creams formulated with a low-melting milk fat fraction (dropping point of 18°C) had lower viscosity than natural cream, while creams formulated with a middle-melting milk fat fraction (dropping point of 26°C) had a higher viscosity. It was postulated that higher solid fat content (higher saturation level of fatty acids) in the middle-melting fraction cream produced higher apparent viscosity to the product (Scott et al., 2003). By contrast Abd El-Aziz (2008) reported that the viscosity of creams was highest when low-melting milk fat fractions were used and lowest with high-melting milk fat

fractions. The result was related to the number of fat globules, which was highest in the cream with low melting milk fat fraction (Abd El-Aziz, 2008). Since the fat globules are surrounded by a film of protein, the increase in the number of fat globules increases the protein amount held in the film and, as a consequence, there is a higher susceptibility for interactions between the globules which, accordingly, increases the viscosity of the cream. It appears that in the study of Scott et al. (2003) the total amount of solid constituents (i.e. solid fat content) was the primary factor altering the viscosity of different cream formulations, while in the study of Abd El-Azis (2008) the viscosity was mainly decided by the state of dispersion of the solid constituents, particularly proteins. It should be noted that Abd El-Azis (2008) used buffalo milk and Scott et al. (2003) cow milk in their cream formulations, and the behaviour of these milks during cream processing is certainly dissimilar, e.g., the size distribution of the fat globules after homogenization is significantly different (as a result of different protein profiles) as was already shown by Ismail & El Deeb (1973). While Scott et al. (2003) could not perceive any differences in creaming rates between natural and reformulated creams, Abd El-Aziz (2008) reported that cream with low-melting milk fat fraction had higher creaming stability than natural cream.

Creams formulated with high-melting milk fat fractions had a slightly higher surface tension, foam capacity and foaming stability than creams with intact milk fat (Abd El-Aziz, 2008). It is possible that the increase in surface tension was at least partly originating from the decrease in the free fatty acid (FFA) content (compared to intact milk fat) since FFAs are known to decrease the surface tension in aqueous systems. Another possibility is that the whey proteins in the cream formulations behaved differently during processing and less denatured and hence, less surface active protein forms were produced into the high-melting milk fat cream. Creams formulated with low-melting and middle-melting milk fat fractions were found to be slightly

more susceptible to feathering (determined in pH range from 4.70 to 5.60) than natural cream (Scott et al., 2003). It was explained that the harsher processing conditions (e.g. homogenization pressures) needed for preparation of cream formulates compared to processing of natural cream made the protein membranes around the fat globules more sensitive to denaturation and flocculation and, consequently, the cream formulates were less resistant to feathering (Scott et al., 2003). Creams with low-melting milk fat fractions were also found to be more prone to oxidative deterioration and oxidative off-flavours resulting from the higher levels of unsaturated fatty acids than natural cream (Abd El-Aziz, 2008; Scott et al., 2003).

Ice creams with modified structural features may be obtained when fractionated milk fat is used. Bazmi, Launay, Cuvelier, & Relkin (2008) showed that the shear stability and structure recovery of ice cream emulsions is related to the rate of development of fat crystals in the milk fat droplets, which in turn is depended on milk fat composition; replacing a part of the anhydrous milk fat with high-melting fractions made the initial gel network more stable to shearing. However, during 24 h storage at 4°C, the solid fat content increased and the degree of structural recovery decreased in the emulsion with high-melting milk fat fraction, while these characteristics remained unchanged in the emulsion with low-melting milk fat fraction (Bazmi et al., 2008). On the other hand, during quiescent freezing, the ice cream emulsion formulated with low-melting milk fat fraction was less stable and much more susceptible to fat droplet aggregation-coalescence than the emulsions formulated with high-melting milk fat fraction or intact milk fat (Bazmi & Relkin, 2006). In another study of Bazmi & Relkin (2009) it was shown that the whipping ability of ice cream emulsion can be improved by replacing part of the milk fat with olein-rich fraction. Lower crystalline fat content assisted higher air incorporation into the emulsion and consequently, higher overrun (Bazmi & Relkin,

2009). Abd El-Aziz, Seleet, & Hammad (2006) studied the effects of different milk fat olein fractions obtained at different crystallization temperatures on the properties of low-fat ice cream ('ice milk'). The whipping ability and overrun increased when milk fat was replaced with the olein fractions of crystallization temperature at 35°C and 25°C but decreased with olein fraction made at 15°C. Milk fat aroma compounds are enriched in the low-melting fractions, together with unsaturated fatty acids, and the flavour score of ice milk was improved when milk fat was replaced with olein fractions of 35°C and 25°C but oxidative off-flavour was noticed when olein fraction of 15°C was used (Abd El-Aziz et al., 2006).

Milk fat, and especially its high-melting fractions, has long been known to have anti-bloom effects in chocolate. Addition of milk fat fractions affects the crystallization characteristics of lipids in chocolate and it has been suggested that the mechanism of bloom inhibition is related to the inhibition of polymorphic transition of cocoa butter (Lonchamp & Hartel, 2004). The ability of a high-melting milk fat fraction to increase the fat bloom stability in chocolate was recently confirmed by Pajin & Jovanovic (2005).

Currently, the fractionation of milk fat is more a method for improving technical functionality of milk fat than for improving its nutritional properties. However, some applications with nutritional benefits have been suggested. The use of milk fat fractions with decreased concentrations of saturated fatty acids may improve the nutritional properties of dairy products, such as yoghurt, mayonnaise, formulated creams and butter sauce. A high-melting fraction of milk fat obtained by supercritical fluid extraction had high concentrations of CLA, β -carotene and unsaturated fatty acids and was proposed for application in nutritionally improved dairy products (Romero, Rizvi, Kelly, & Bauman, 2000). CLA is also enriched in the soft fractions during temperature crystallization, but the increase has been considered to

be too minor and the fractionation process too costly for industrial CLA enrichment (Rehberger, Bütikofer, Bisig, & Collomb, 2007).

5. Conclusions

Milk fat possesses favorable properties, both physically and chemically, and it also has good nutritional properties, as a source of essential fatty acids and fat-soluble vitamins. The efforts to exploit milk fat and its different fractions have resulted in many physico-chemical and physical fractionation and enzymatic modification techniques for production of fat fractions with different characteristics. The unique physical characteristics of milk fat, especially its wide melting point range, make milk fat as such unsuitable for several food applications. Therefore, the fractionation of milk fat mostly aims for improving technical functionality of milk fat.

The ability of lipases to catalyze the hydrolysis, esterification and transesterification can be exploited in modification of milk fat. The specificity of lipases enables production of highly specific structured lipids and tailor-made fats for broad range of food applications. However, the negative changes in sensory quality due to the release of short-chain fatty acids as result of enzymatic action or necessary refining procedures are serious drawbacks in enzymatic modification of milk fat.

Very few attempts have been made to modify milk fat using combinations of enzymatic and physical techniques. Such an approach may result in new strategies for expanding the use of milk fat in the form of fractions with tailored properties. These fractions could find applications in many fields, for example as foodstuffs, feeds and in non-food applications.

6. References

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Captions to Figures

Figure 1

Schematic Fischer projection formula of 1-oleoyl-2-palmitoyl-3-butyroyl-*sn*-glycerol. *O*, *P*, and *B* are esterified fatty acids: *O* = oleic acid, *P* = palmitic acid, and *B* = butyric acid.

Figure 2

The most probable *sn*-position (distribution % > 33.3 %) of the main fatty acids in milk fat (data adapted from Parodi, 1979 and Jensen, 2002).

Figure 3

Biosynthesis of TAGs in the mammary gland *via* the glycerol-3-phosphate pathway.
G = glycerol; G-3-P = glycerol-3-phosphate; acyl-CoA = acyl coenzyme A; DAG = diacylglycerol; TAG = triacylglycerol (Data compiled from Hawke & Taylor, 1995).

Figure 4

Digestion, absorption, and metabolism of dietary TAGs.

G-3-P = glycerol-3-phosphate; VLDL = very low density lipoprotein; FA = fatty acid;
MG/MAG = monoacylglycerol; DAG = diacylglycerol; TAG = triacylglycerol (Data compiled from Bracco, 1994; Kuksis, 2000; Mu & Høy, 2004; Small, 1991).

Tables

Table 1
Major lipid classes of milk fat (data adapted from Jensen, 2002).

Lipid class	Average composition, % (w/w)
Triacylglycerols	97–98
Diacylglycerols	0.28–0.59
Monoacylglycerols	0.16–0.38
Free fatty acids	0.10–0.44
Phospholipids	0.20–1.00
Cholesterol	0.42
Cholesterol esters	traces

Table 2
Major fatty acids of milk fat (data adapted from Jensen, 2002).

Fatty acid	CN:DB ^a	wt% ^b	wt% ₁₀₀ ^c	mol% ^d
Butyric acid	4:0	3.79	4.19	10.93
Caproic acid	6:0	2.10	2.32	4.59
Caprylic acid	8:0	1.19	1.31	2.10
Capric acid	10:0	2.44	2.70	3.60
Lauric acid	12:0	2.98	3.29	3.78
Myristic acid	14:0	9.75	10.77	10.85
Myristoleic acid	14:1	1.08	1.19	1.21
Pentadecanoic acid	15:0	1.35	1.49	1.41
Palmitic acid	16:0	23.46	25.92	23.24
Palmitoleic acid	16:1	2.00	2.21	2.00
Margaric acid	17:0	0.72	0.80	0.68
Stearic acid	18:0	10.58	11.69	9.45
Oleic acid	18:1	19.44	21.48	17.48
Other C18:1 acids	18:1	6.85	7.57	6.16
Linoleic acid	18:2	2.17	2.40	1.97
Linolenic acid	18:3	0.61	0.67	0.56
Total		90.51	100.00	100.00

^a CN:DB = the number of carbons: the number of double bonds

^b Data adapted from Jensen (2002)

^c wt% normalized to 100 per cent

^d mol% is calculated on the basis of wt%₁₀₀

Table 3
Studies (1997–2007) on the lipase-catalyzed modifications of milk fat.

Type of modification	Source of lipase	Substrate	Properties of modified milk fat	Reference
Acidolysis + Interesterification/es ter-ester exchange	<i>Pseudomonas fluorescens</i>	Butterfat + FFA	Interesterification resulted in lower SFC; Acidolysis with PUFA and SAFA decreased and increased SFC of BF, respectively.	Lee and Swaisgood, 1997
Acidolysis	<i>Pseudozyma antarctica</i> (prev. <i>Candida antarctica</i>), <i>Pseudomonas</i> spp., <i>C. rugosa</i> , <i>Mucor miehei</i>	Anhydrous butteroil + CLA	CLA enriched BO was synthesized. CLA content increased from 0.6% (w/w) to max 15 % (w/w) by <i>P. antarctica</i> .	Garcia et al., 1998
Acidolysis	<i>Mucor circinelloides</i> (prev. <i>M. javanicus</i>)	Anhydrous butterfat + oleic acid	Acidolysis resulted in 27% increase of C18:1 and 6–8% decrease of C12:0, C14:0, and C16:0 residues of the modified TAGs. The proportion of low-melting TAGs increased by 19% and that of high-melting TAGs decreased by 83%.	Balcão et al., 1998a
Acidolysis	<i>C. rugosa</i> , <i>Pseudomonas</i> spp., <i>P. antarctica</i> , <i>M. miehei</i>	Butteroil + CLA	CLA was incorporated up to ca. 17% (w/w) in BO. The proportion of TAGs with ACN34–42 and ACN46–52 decreased and increased, respectively.	Garcia et al., 2000, 2001
Acidolysis	<i>Rhizopus arrhizus</i>	Butterfat + α -linolenic acid	α -linolenic acid was incorporated up to ca. 24% (w/w) in BO. Spreadability of modified BF was better than untreated BF.	Kim et al., 2002
Acidolysis	<i>P. antarctica</i>	Butteroil + CLA	The extent of incorporation of original free CLA in BO was max 85%.	Sehanputri and Hill, 2003
Interesterification/es ter-ester exchange	<i>R. arrhizus</i>	Butterfat; butterfat + canola oil	Interesterification decreased SFC and dropping point and altered crystallization behaviour of the blends.	Rousseau and Marangoni, 1998a,b, 1999
Interesterification/es ter-ester exchange	<i>M. miehei</i>	Stearin fractions from AMF + sunflower oil/soybean oil	Modification allowed control over the slip points of the blends resulting in spread fats with increased proportion of essential FAs and almost zero <i>trans</i> FA content.	Pal et al., 2001
Interesterification/es ter-ester exchange	<i>Thermomyces lanuginosus</i> (prev. <i>Humicola</i>)	Butteroil + rapeseed oil	At long reaction times, the proportion of C12:0–C16:0 decreased, and that of C18:0 and C18:1 increased in the <i>sn</i> -2 position (BO/RO, 70/30).	Ronne et al., 2005

	<i>lanuginosa</i>), <i>Rhizomucor miehei</i> , <i>P. antarctica</i> , <i>Burkholderia cepacia</i>			
Hydrolysis (selective) + Interesterification/ester-ester exchange	<i>M. circinelloides</i>	Butterfat	Modification induced widening of the melting temperature range and <i>ca.</i> 11–13% decreasing in the proportion of C12:0–C16:0 residues in TAGs as compared to untreated BF.	Balcão et al., 1998b
Hydrolysis	<i>Penicillium roquefortii</i>	Short-chain TAG fraction of butter fat / butter fat	The ratio of released aromatic short-chain FFA to medium-chain FFA was higher (1.80) when short-chain fraction of BF was used as a substrate as compared to BF (0.75).	Lencki, Smink, Snelting, & Arul, 1998
Hydrolysis	<i>C. rugosa</i> , <i>C. lipolytica</i> , <i>Rhizopus delemar</i> , <i>R. niveus</i> , <i>P. roquefortii</i> , <i>P. camembertii</i> , <i>P. fluorescens</i> , <i>H. lanuginosa</i> , <i>Geotrichum candidum</i> , <i>M. circinelloides</i>	Anhydrous MFs from cows, ewes, and goats	All combination of 3 different MFs and 10 different lipases was shown to be an alternative way to produce cheesy flavour by lipase modification. The net hydrolysis was in all cases below 10%.	Regado et al., 2007

FFA = free fatty acids

SFC = solid fat content

CLA = conjugated linoleic acid

PUFA/SAFA = polyunsaturated/saturated fatty acids

BF/BO = butter fat/butter oil

TAG = triacylglycerol

ACN = the number of acyl carbons in TAGs

RO = rapeseed oil

Figures

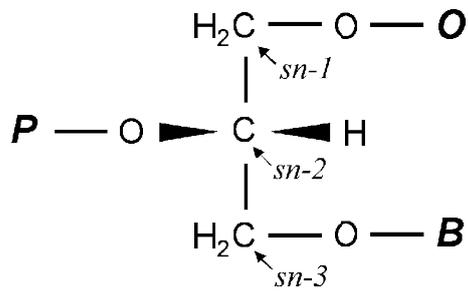


Figure 1.

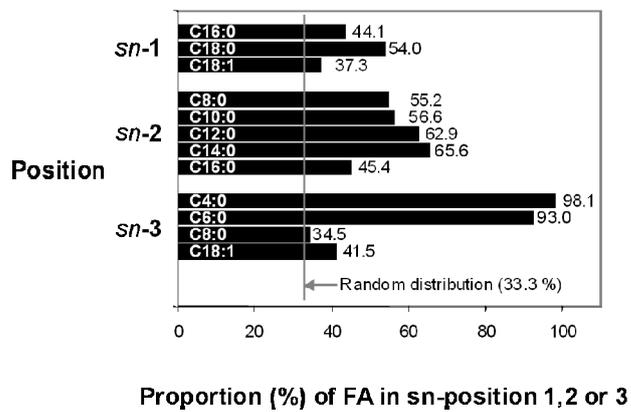


Figure 2.

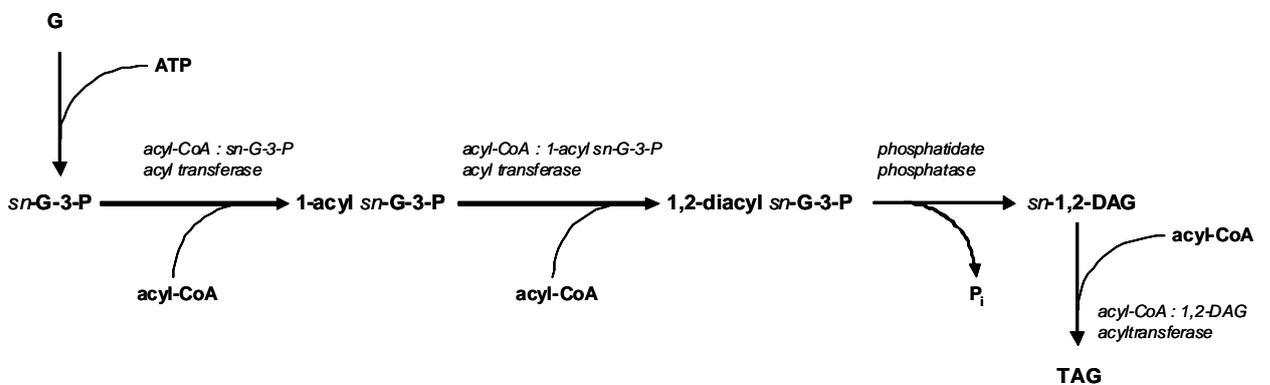


Figure 3.

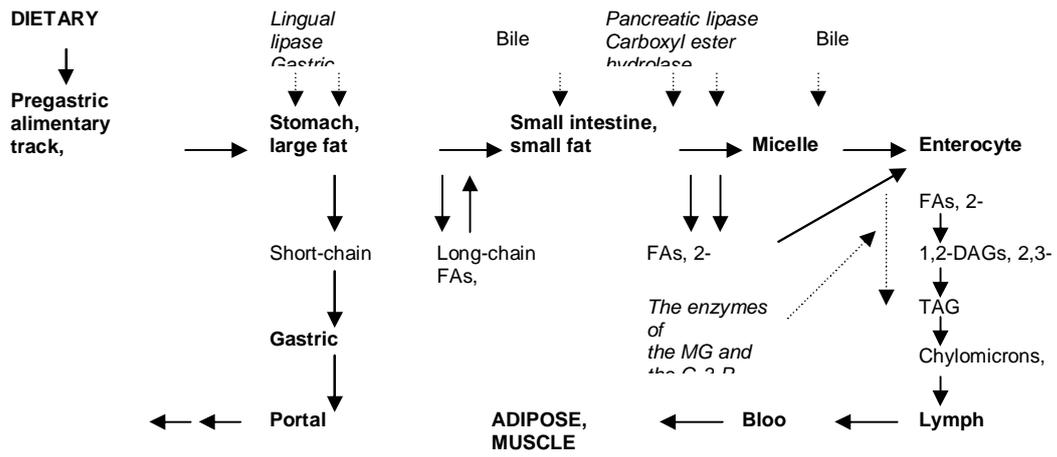


Figure 4.