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INVITED REVIEW

Stereospecific distribution pattern of fatty acids in triglycerides: A comparative review of human, bovine, bubaline, caprine, and equine milk fat

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Abstract: Major lipid fraction of milk fat is triacylglycerol (TAG) representing about 98% of total lipids. TAG contains a glycerol backbone and three fatty acids. These three fatty acids are positioned at sn-1, sn-2 & sn-3 position in glycerol backbone and the positioning is called as stereospecific positioning. This stereospecific positioning mainly effects the lipid metabolism of dietary lipids. During the digestion of TAG in human beings, lipase preferably attacks the TAG at sn-1 or sn-3 positions resulting in the release of free fatty acids along with sn-2 monoacylglycerol. It has been reported that saturated fatty acids are mainly esterified in sn-2 position whereas unsaturated fatty acids are esterified in sn-1 and 3 positions. The fatty acid distribution among the three sn-positions of the glycerol backbone is non-random. The non-random distribution is a result of the specificity of different enzymes during TAG biosynthesis. The distribution of fatty acids depends upon species, feed, season and lactation period. The stereospecific arrangement of fatty acids in fat also influences some physical properties of fats and oils like crystallization and melting properties. The stereospecific arrangement of milk fat is generally studied by using different lipases along with separation techniques and finally chromatography or spectroscopy. Understanding these aspects can aid in identifying the origin of the fat, detecting potential adulteration of different other species of milk, and predicting nutritional value of different species' milk based on differences in the form of sn-2-MAG and free fatty acids.

Keywords: Digestion; Fatty acids; Positional distribution; Milk fat; Triglycerides; Structure

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Introduction

There are various major dairy animals in different regions of the globe. Cows are the most widely used dairy animals worldwide, accounting for 81.8% of all milk production (FAOSTAT, 2022). On the other hand, India and Pakistan are known for their buffalo milk consumption, these two countries produce more than 90% of the world's buffalo milk (Singh, 2023). In Asia, India and Bangladesh are also the primary producers of goat milk, accounting for 43.4% of the world's output (FAOSTAT, 2022). Central Asian nations such as Kazakhstan and Mongolia have the highest consumption of equine milk compared to other countries (Miraglia et al. 2020). In the high-altitude regions of like Tibet, the Qinghai region of China, and the Ladakh and north Sikkim regions of India yak milk is a popular food (Yang et al. 2018).

The nutritional properties of milk fat have garnered significant public interest. According to McSweeney et al. (2020) human and bovine milk fat comprise approximately 400 fatty acids. The fatty acids are categorized into three groups based on their chain length i.e., short- to medium-chain fatty acids (SMCFA) with a chain length of C4-C12, medium-chain fatty acids (MCFA) with chain length of C13-C16, and long-chain fatty acids (LCFA) with a chain length of C17-C23. The short-chain fatty acids and a portion of the medium-chain fatty acids in milk come from mammary cells' de novo synthesis, whereas long-chain fatty acids and remaining medium chain fatty acids are obtained from dietary sources (Chilliard et al. 2000).

Some saturated fatty acids (SFA) and trans-fatty acids have been linked to long-term health problems (Wales et al. 2009), which makes people more concern about milk fat. On the other hand, it has been observed that a reduced quantity of unsaturated fatty acids (UFA) in milk can positively impact human health. Specifically, the presence of Oleic acid ($C_{18:1c9}$) and linoleic acid ($C_{18:2c9t11}$) fatty acids in low concentrations has been linked to a decrease in plasma lipids and a hindrance in the development of cancer (Calder, 2014). Docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) play a crucial role in facilitating the development of the nervous system in infants (Guesnet & Alessandri, 2011). In addition to fatty acid composition the

arrangement of fatty acids in the triglyceride also plays an important role in fat digestion and absorption (Mehrotra et al. 2019). This arrangement is called stereospecific arrangement or stereospecific distribution of fatty acids. Literature have shown that the distribution of fatty acids among the three sn (stereospecific numberings) positions of milk triacylglycerols (TAG) is non-random (Mugabo et al. 2016). The sn-system is a nomenclature recommended by the IUPAC-IUB Commission on Biochemical Nomenclature (CBN) in 1967 to describe the stereochemistry of glycerol derivatives. It provides information about each fat, that the TAG fraction of each lipid matrix has a unique arrangement of fatty acids among positions of the glycerol backbone (Parodi, 1983). The stereospecific arrangement of fatty acids in fat also influences some physical properties of fats and oils like crystallization and melting properties. Stereospecific arrangement of fats and oils is generally studied by using different lipases along with separation techniques and finally chromatography or spectroscopy. Understanding these aspects can aid in identifying the origin of the fat, detecting potential adulteration of different other species milk, and predicting nutritional value of different milk based on differences in the form of sn-2-MAG and free fatty acids. The main aim of this review article is to compare different stereospecific distribution of fatty acids in triglycerides of bovine, bubaline, caprine and equine milk fat with human milk fat.

Fat digestion mechanism in human body

The digestion and absorption of lipids have some special challenges. Triglycerides are large molecules, but they are not water-soluble, like carbohydrates and proteins. Due to this, they tend to coalesce in huge droplets in a watery environment of the digestive tract. The digestive tract breaks these big fat droplets into smaller droplets and then uses enzymes called lipases to break down lipid molecules (Mu & Høy, 2004). The mouth and stomach play a little role in this process, but the small intestine is responsible for the most of fat digestion. After lipid digestion, the products of fat digestion are absorbed into circulation and spread throughout the body, which again requires for special treatment since lipids are not water-soluble and do not integrate with the watery element of blood (Mu & Høy, 2004).

The stereospecific position of fatty acids also plays an important role in nutrition and digestion of fats. Triacylglycerol breaks down with the help of lipase in the mouth, stomach, and small intestine. To hydrolyse triacylglycerol that has been emulsified by bile acids, pancreatic lipase works in cooperation with colipase. Pancreatic lipase is position specific, while hydrolysing the triglyceride, it attacks at sn-1 and sn-3 position and releases the fatty acids from sn-1 or sn-3 position, but the sn-2 positional fatty acid remains intact as 2-mono acyl glycerol (2-MAG) as depicted in Figure.1 (Akoh, 2017). Fatty acids and monoacylglycerols micelles migrate into enterocytes, and they undergo re-esterification to become triacylglycerols in small intestine (Decker, 1996) . These triacylglycerols are subsequently packed into chylomicrons along with lipoproteins and other components. Although this is how triacylglycerols are usually broken down and absorbed, the fatty acids released from the sn-1 and sn-3 positions often end up in different places in the body's metabolism. Short- and medium-chain fatty acids (5-10 carbons) can be dissolved in the intestine's aqueous content. They are absorbed, attached with albumin, and transported to the liver through the portal vein. Free long-chain fatty acids (palmitic and stearic) have low coefficients of absorption because their melting points are higher than body temperature, and they can form calcium soaps. So, fats with long-chain saturated fatty acids in the sn-1 and sn-3 positions of triacylglycerols may be absorbed differently than fats with palmitic or stearic acids in the sn-2 position (Bracco, 1994; Decker, 1996; Innis, 2011).

This process ensures that the fatty acids in the sn-2 position of the absorbed TAG molecules are retained, similar to those in the dietary TAG. The esterification of fatty acids in the glycerol backbone is significant for physiological and nutritional purposes. The study of intramolecular triacylglycerol (TAG) composition is particularly important due to its influence on various factors (like: melting property, crystallization property, digestion behaviour of fat). There are many studies on compositional differences in fatty acids in different milk species but limited study on positional distribution of milk fatty acids in different milch species are limited.

The fat composition of milk varies between different animal species

Milk of cow, buffalo, goat, and horse, has less saturated fat and more unsaturated fats like monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA). It also has a higher ratio of omega-6 to omega-3 fatty acids (Table. 1). Typically, equine milk, has a reduced concentration of saturated fatty acids and cholesterol and an elevated concentration of polyunsaturated fatty acids in comparison to milks sourced from ruminant animals (bovine, bubaline and caprine). The quantity of conjugated linoleic acid is comparable between human and ruminant milks, although it is comparatively lower in non-ruminant milks, as seen in Table.1.

The fats found in caprine (goat) milk are recognized for their high content of short-chain and medium-chain fatty acids, which contribute to the unique flavor of its milk. Goat milk is also enriched with unsaturated fatty acids, such as oleic, linoleic, and linolenic acids (Ruiz Sala et al.1996). Bubaline or buffalo milk has a higher percentage of medium-chain triglycerides compared to bovine milk. Bovine milk, on the other hand, is characterized by a higher concentration of long chain fatty acids (LCFAs) (Abd El-Salam & El-Shibiny, 2011; Park et al. 2007; Ruiz Sala et al. 1996). It is also reported that bubaline milk has a greater concentration of LCFAs and a comparatively less concentration of short chain fatty acids (SCFAs) when compared to bovine milk (Jilo & Tegegne, 2016).

From the above data, it is evident that among all species equine milk fatty acid composition is nearer to human milk. The variations mentioned above might potentially impact the digestive characteristics of milk fat across various species. This is because lipases are known to be more effectively break down short or medium-chain fatty acids in triglycerides (TAGs) (Lee et al. 2022).

Different techniques for determination of stereospecific arrangements of fatty acids in triglycerides

The study of triglyceride structure has two primary purposes: firstly, to detect the fatty acid present in the sn-2 position, without distinguishing between the sn-1 and sn-3 positions, and secondly, to identify each individual fatty acid on the triacylglycerol molecule by complete stereospecific analysis. Determination of stereospecific arrangement of fatty acids using these methodologies, involving the specificity of lipase enzyme, one can determine the impact of triglyceride composition on the reactions of lipid digestion, absorption, and metabolism.

Fig.1 Fat digestion in human body

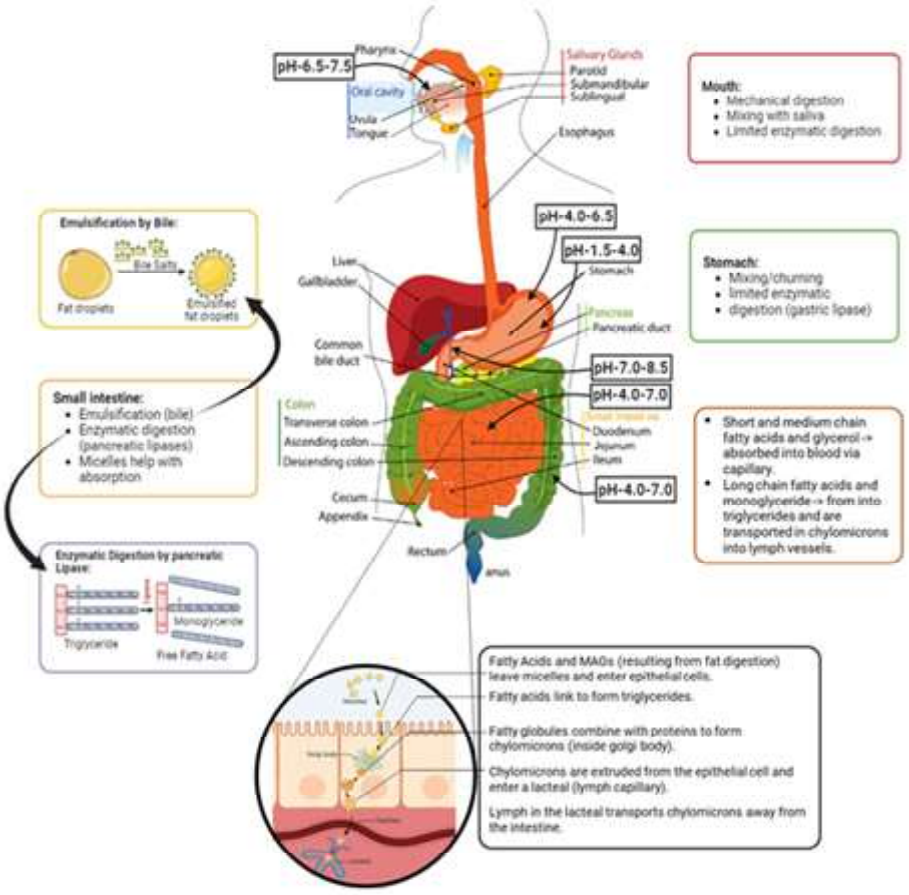

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Table1: Lipid profile of equine, caprine, bubaline, bovine and human milk fat

Parameters	Species wise different types of milk fat				
	Equine	Caprine	Bubaline	Bovine	Human
Total Fat % (g/100g milk)	8-12	12-16	16-17	12-13	10-13
SFA% (% Total fatty acid)	40.5-67.7	59.9-73.7	62.1-74	55.7-72.8	39.4-45
MUFA% (% Total fatty acid)	15.3-35.0	21.8-35.9	24.0-29.4	22.7-30.3	33.2-45.1
PUFA% (% Total fatty acid)	14.17-30.5	2.6-5.6	2.3-3.9	2.4-6.3	8.1-19.1
ώ-6: ώ-3	0.9-6.1	3-4	2.5-3.0	2.1-3.7	7.4-8.1
CLA% (% Total fatty acid)	0.02-0.1	0.3-1.2	0.4-1	0.2-2.4	0.2-1.1
Cholesterol (mg/100ml milk)	5.8-8.8	10.7-18.1	4-18.0	13.1-31.4	14-20
% of C16:0 at sn-2	30-54	35-36	37-40	38-42	52-74

(Claeys et al. 2014; Gantner et al. 2015; Roy et al. 2020)

Enzyme-linked assays are often used for the analysis of the stereospecific arrangement of fatty acids on milk fat triglycerides. The diacylglycerol acyltransferase (DGAT) test is one example. It can be used to find out the enzyme activity that attaches fatty acids to the glycerol molecule at the sn-1 and sn-3 places (Liu et al. 2012). Although there are several isolation and quantification methods are reported in various studies for determining the positional distributions of fatty acids in triacylglycerol.

Stereospecific Position determining methods of fatty acids in Triglyceride

Stereospecific analysis of TAG can be obtained by the enzymatic reaction catalysed by porcine pancreatic lipase (EC 3.1.1.3) (Christie and Han., 2012) or by Grignard chemical diacylation (Blasi et al. 2008). The latter procedure is the preferred method for the analysis of milk TAG, because it does not show acylic specificity (Turon et al. 2002; Tzompa-Sosa et al. 2014). It allows the direct determination of FA composition of sn-2 position and the differentiation of FA esterified in the primary (sn-1,3) and secondary (sn-2) position of the glycerol backbone. This approach is based on 1,3 random, 2-random distribution theory (RR procedure). In TAG positional analysis, sn-2-monoacylglycerols (sn-2-MAG), obtained by enzymatic hydrolysis or chemical diacylation, are isolated by a preparative TLC and trans esterified as FAME for the subsequent analysis by GC-FID. It allows the determination of the FA composition (%) in the sn-2-position of the native TAG. The FA composition in the sn-1(3) positions can then be estimated from the composition of the sn-2-MAG and TAG, according to the following formula:

$$A_{(1,3)} = \frac{3 \times A_T - A_2}{2}$$

where $A_{1,3}$ = % FA in sn-1 and sn-3 positions; A_T = % FA in total TAG; A_2 = % FA in sn-2 position.

The pancreatic lipase technique does not differentiate between sn-1 and sn-3 positional fatty acids, so, it is mainly applicable to fats and oils with a restricted number of fatty acids that originate from symmetrical triglycerides, such as POP, SOS, and OOO (where P, O, and S, are palmitic, oleic, and stearic acids, respectively). Examples of this include cocoa butter and Borneo tallow.

In order to conduct a stereospecific analysis on a complex triacylglycerol mixture, such as milk fat, a more complex procedure is required. This procedure includes the use of the Grignard reagent to split the triglyceride into diglycerides, derivatization of that diglyceride to phospholipids, splitting with specific phospholipases and finally determination of fatty acids through gas chromatography. Figure.2. provides a concise summary of the analytical procedures that are involved in the stereospecific analysis of complex triglycerides.

These procedures include both chemical and specific enzymatic processes. So, one can specify, for each kind of fat, the fatty acids that are located in the sn-2 position, as well as the overall composition of the triglyceride and the total fatty acid composition. The analytical approaches are based on enzymatic-instrumental and chemical-instrumental procedures. The use of sn-1,2 diglycerol kinase is more suitable for stereospecific analysis of vegetable fat (Han, 2016), whereas the most suitable for milk TAG, containing FA with a short chain, is the phospholipase A2 (PLA_2) procedure.

Another method for determining the positional distribution of fatty acids in triacylglycerols is high-resolution ^{13}C nuclear-magnetic resonance spectroscopy, which is based on the chemical shift of esterified fatty acids in all three locations (Bunga et al. 2023; Edison, 2009; Hamilton, 1998) or by high-performance liquid chromatography (HPLC) of the diastereomeric derivatives (urethane) of partial glycerides (Takagi & Ando, 1991; Takagi & Suzuki, 1992).

a) Thin layer chromatography couples with GLC (Christie,1982)

For a long period, this method has been used in different studies with various modifications. The recently modified version was developed by Chen et al. (2020). They mentioned primary hydrolysis through pancreatic lipase and isolation of MAG through TLC for the determination of sn-2 positional FA. Then Grignard hydrolysis splits the triglyceride into diglycerides, next chemical derivatization of that diglyceride occurs to produce phosphatidylcholine. After that the fatty acids of phosphatidylcholine was splitter with the help of phospholipase A_2(PLA_2). Finally, determination through gas chromatography (GC-FID) of each fraction. The percent FA composition of sn-3 position can be calculated by applying the following formula:

$$A_3 = [3 \times A_T - A_2 - A_1],$$

where A_3 = % FA in sn-3 position; A_T = % FA in total TAG; A_2 = % FA in sn-2 position; A_1 = % FA in sn-1 position.

The most complete characterization of TAG by stereospecific analysis of donkey, cow, ewe, goat, and buffalo milk, carried out using the phospholipase A2 (EC 3.1.1.4) procedure, was obtained by Blasi et al. (2008). The main disadvantage of these methods is that it is time-consuming and not applicable for routine quality control.

b) High-Performance Liquid Chromatography method

One possible method for analysing TAG stereospecifically using chemical-instrumental methods involves the use of chiral HPLC

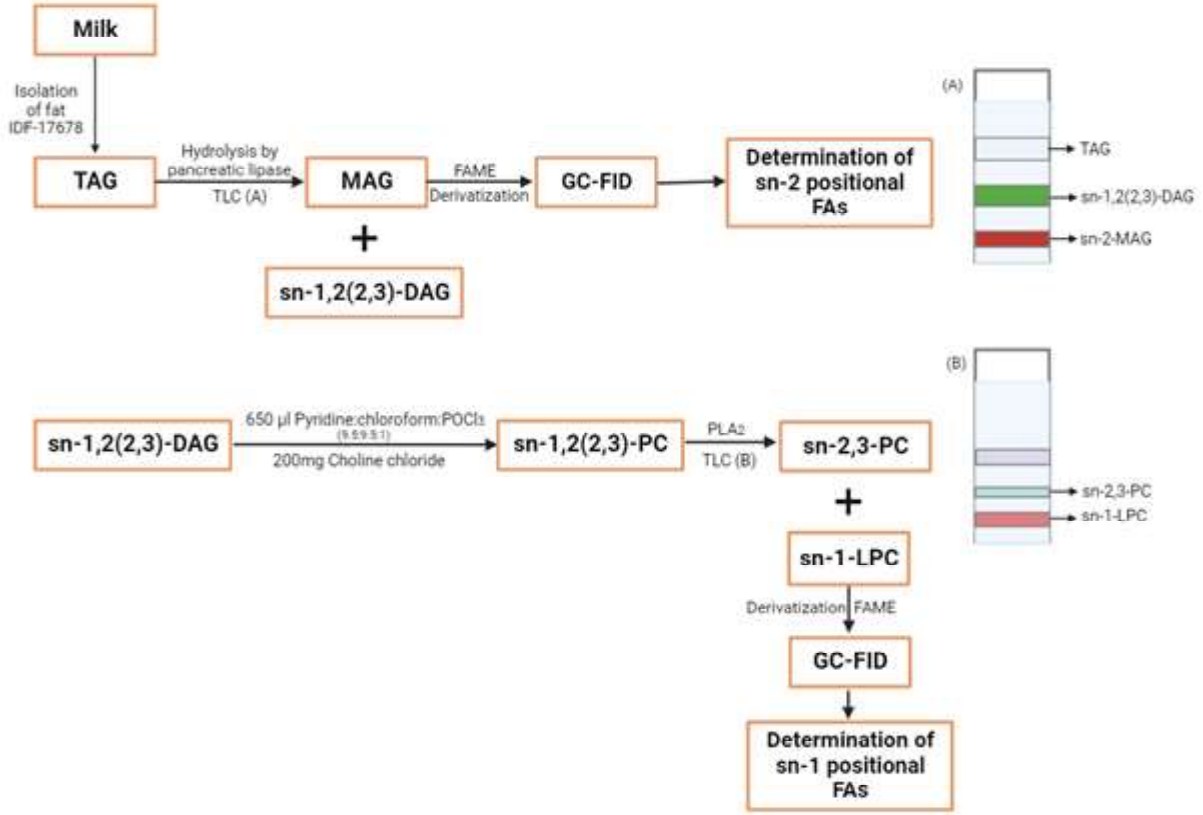

Fig.2 Schematic diagram of stereospecific analysis of fatty acids presents in milk fat TAG

to separate derivatized enantiomers. Unfortunately, only a few articles reported about the application of this approach to milk fat analysis. The first method of this kind to be talked about using the concept of resolution of diastereomeric diacylglycerol products (Figure 3. part a) (Christie, 1996; Laakso & Christie, 1990). The process began with the creation of sn-1,2-, 2,3-, and 1,3-diacylglycerols through a reaction with ethyl magnesium bromide. In the second step, these products were reacted with a chiral derivatizing agent, (S)-(+)-1-(1-naphthyl) ethyl isocyanate and the resulting diacyl-sn-glycerol urethane derivatives were isolated through chromatography on solid-phase extraction columns containing octadecyl silyl phase. The third and most crucial step involved the resolution of the diacylglycerol urethanes through HPLC on columns of silica gel. To achieve the best possible resolution, two columns of silica gel have been used (Hypersil TM 3-m, 250×4.6 mm i.d.) in series. The mobile phase consisted of 0.4 to 0.33% (v/v) 1-propanol (containing 2% water) in isooctane, and the flow rate was set to 1 mL/min. UV rays at 280 nm were used for analysis.

Takagi & Ando. (1991) reported a different but comparable approach (Figure. 3, parts b and c). They used high-performance liquid chromatography (HPLC), on a column that had a stationary phase with chiral moieties chemically bound to a silica gel base. Di- and monoacyl-sn-glycerols made from triacylglycerols, converted to the 3,5-dinitrophenyl urethane (DNPU) derivatives. The 3,5-dinitrophenyl moieties of the urethanes help in charge-transfer with functional groups that have pi-electrons on the stationary phase (Christie, 1996; Takagi & Suzuki, 1992, 1993). After lowering the column temperature and slowing down the flow rate, the method could even be applied to such complex triacyl-sn-glycerols as fish oils. Following isolation of the various fractions, trans-methylation and gas chromatography, the distributions of fatty acids in each of positions sn–1, –2 and –3 can be calculated from the data (Valeille & Martin, 2004).

c) Mass spectrometry

Mass spectrometry is an effective method for examining the stereospecific positioning of fatty acids on triglycerides of milk fat. In 2005, Kuksis and Itabashi developed a method for MS analysis of regio-isomeric triacylglycerols (TAGs). Generally, ammonia negative-ion chemical ionisation (NICI) and collision-induced dissociation (CID) are used for investigating the regio isomerism of fatty acids in TAGs. The molecular ions, $[M\text{-}H]^{É}$, are allowed to hit an inert gas like argon or xenon. The fragment ions, $[M\text{-}H\text{-}RCOOH\text{-}100]^{É}$, $[M\text{-}H\text{-}RCOOH\text{-}74]^{É}$, and $[M\text{-}H\text{-}RCOOH\text{-}56]^{É}$, or $[M\text{-}H]^{É}$ molecular ions are all less abundant for fatty acids in the sn-2 position than for those located in the sn-1 and sn-3 positions. It is the $[M\text{-}H\text{-}RCOOH\text{-}100]^{É}$ ions and the $[RCOO]^{É}$ ions that are used to determine the TAG structures.

Positive $NH4^{É}$ ESI-MS has not been shown to be very useful for identifying the difference between TAG regio isomers. An ion-trap device and ESI-MS in of $[M + NH_4]^+$ ions, on the other hand, have led to better results. It has been shown by Kalo et al. (2003) that successful separation of regio isomers of short-chain TAGs requires the use of positive $NH_4^{É}$ ion ESI-MS in combination with normal phase.

HPLC matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) is also a technique that can precisely measure the fatty acid composition and distribution of milk fat triglycerides. MALDI-TOF MS is an extremely sensitive and precise technique capable of detecting even trace amounts of triglycerides and fatty acids (Christie, 1996; Cossignani et al. 2019).

d) Nuclear Magnetic Resonance (NMR) Spectroscopy

NMR spectroscopy is another technique that is utilized to analyse the stereochemistry of milk fat triglycerides. The locations of fatty acids on the glycerol backbone and other structural and kinetic characteristics of molecules may be thoroughly studied using NMR spectroscopy (Lopes et al. 2018). Tengku & Birch, (2014) reported in their study, fat sample of 180 mg was mixed with 700 ml of deuterated chloroform and put into a 5mm NMR tube using a syringe. NMR spectra was obtained on a Varian 500MHz VNMRS device with a 5mm OneprobeTM working at 125.705 MHz. Peak recognition was done according to the work of Aursand et al. (1995) and Suárez et al. (2010). The integrator response of the NMR spectra, which was done with Varian VnmrJ version 3.0 software, showed the percentage of fatty acids in the carbonyl region.

Mass spectrometry, nuclear magnetic resonance, and enzyme-linked assays are highly effective methods for analysing the stereospecific positioning of fatty acids on milk fat triglycerides. These techniques are used to determine the distribution of fatty acids on the glycerol backbone, which can be used to better understand and regulate the physical properties and nutritional quality of milk and dairy products.

Merits and demerits of different techniques used for positional analysis of fatty acid in triglyceride in milk fat

Multiple methods are employed to analyse the stereospecific positioning of fatty acids in milk fat triglycerides. Table.2 presents a compilation of various techniques utilized by different researchers, along with their respective findings and associated costs.

Mass spectrometry, nuclear magnetic resonance, and enzyme-linked assays are all powerful techniques for analysing the stereospecific positioning of fatty acids on milk fat triglycerides. These methods can provide detailed information about the positional distribution of fatty acids on the glycerol backbone, which can be used to understand and control the milk and dairy products' physical properties and nutritional quality.

Table 2: Merits and demerits of different techniques used for Stereospecific positioning of fatty acids in milk fat TAG

Author	Milk Species	Methodology	Findings	Cost involved
Parodi, 1979	Bovine milk	Pancreatic lipase diacylation and isolation through GLC	There is a change in medium chain fatty acids in sn-1(3), than sn-2 with season.	Low cost
Parodi et al.1983	Prepartum and Postpartum milk of Friesian cow	Pancreatic lipase diacylation and GLC	Prepartum mammary gland secretion has twice the amount of 16:0, than normal milk, and the amount of 14:0 is also higher.	Low cost
Andreotti et al. 2002	Cow, Sheep, goat	NMR-nuclear magnetic resonance	The short-chain fatty acids, 4:0 and 6:0, almost exclusively at the sn-3 position.	High cost
Blasi et al.2008	Donkey, cow, ewe, goat and buffalo milk	Phospholipase A2 hydrolysis, TLC and HRGC.	The FA distribution in glycerol backbone is non-random, SFA is esterified at sn-3 position, while MUFA are in sn-2. EFA as linoleic acid present in sn-2 at donkey milk.	High cost
Haddad et al. 2010	Camel milk	Pancreatic lipase + Grignard degradation + TLC + phosphor lipase A2 + GC	The sn-2 position is esterified with C16:0 (40.8% mol), C14:0(18.1% mol), C18:1(14% mol). LCFA are esterified at outer position (50.7% and 42.6% respectively in sn-1 & sn-3).	High cost

Haddad et al. 2012	Human colostum, transitonal and mature milk	Grignard degradation, TLC and GLC.	Significant differences in fatty acid composition occurred between lactation times, quantities in each position can be changed.	Costly
Gotoh et al. 2012	Huma, Rat, Cow, buffalo, goat and sheep milk	HPLC-UV-APCI-MS/MS	Palmitic acid in cow or buffalo milk cheese fat TAG is mainly present at sn-1, (3) position.	High cost
Tzompa-Sosa et al. 2014	HF cows	Grignard degradation +preparative TLC +GC-FID	GPAT indirectly controls the amount of FA that are esterified at the sn-2 position by selectively esterifying C 16:0 at sn-1.	High cost
Sun et al. 2018	Human milk fat and human milk formula	Pancreatic lipase hydrolysis, TLC and GLC.	Formula fat contains lower level of PA, SFA, LC-PUFA and higher level of Oleic, linoleic and α-linoleic Acid at sn-2 position.	Costly
Chen et al.2020	Human milk	Pancreatic lipase and phospholipase A2 hydrolysis, TLC and GLC	MUFA are sn-1, sn-3> sn-2, PUFA are sn-3> sn-1> sn-2. SCFA and MCFA are in sn-3.	Costly
Yener et al. 2021	Bovine milk	Candida antarctica lipase B Hydrolysis + SPE + GC-FID	Reported seasonal variation in the sn-2 and sn-1(3) FA compositions between summer and winter months.	Moderately Cost
Karrar et al. 2022	Camel, cow, donkey, goat, and yak milk	Pancreatic lipase hydrolysis + TLC + GC-FID	sn-2 position was mainly esterified by C18:1 n-9 and C16:0. Goat milk fat had a lower C18:1n-9/C16:0 ratio at the sn-2 position compared with milk fat from other species. Donkey and camel milk fat have more UFAs and less SFAs at the sn-2 and sn-1,3 positions than milk fat from other species.	Low cost
Pacheco-	Cow milk	Candida antarctica	Changes in FA positioning in different	Moderately

Stereospecific distribution of fatty acids in triglycerides of human, bovine, bubaline, caprine and equine milk fat

The positional distribution of fatty acids in milk fat has received considerable attention in recent years due to its large importance in understanding fat digestion, absorption, and metabolism in humans (Bakry et al. 2020; Cossignani et al. 2019; Karrar et al. 2022; L. Yang et al. 2022). Literature suggested that in milk fat of different species, the short-chain fatty acids are esterified in sn-1 or sn-3 position, medium-chain fatty acids like lauric acid ($C_{12:0}$), myristic acid ($C_{14:0}$) is esterified in sn-2 and sn-3 position. The long-chain fatty acids are mainly esterified at the sn-1 or sn-2 position.

Distribution of short chain saturated fatty acids and medium chain saturated fatty acids in human, bovine, bubaline, caprine and equine milk fat

Short chain fatty acids ($C_{4:0}$, $C_{6:0}$, $C_{8:0}$ and $C_{10:0}$) are primarily esterified at either the sn-1 or sn-3 position, with a preference for the sn-3 position. sn-3 interpositional level (almost 90%) is far higher than sn-1 position. In human milk concentration of $C_{4:0}$, $C_{6:0}$, and $C_{8:0}$ can't be detected at sn-1 and sn-2 position, demonstrating that SCFAs are all distributed only at sn-3 position. In case of short chain fatty acids there are significant variation in between different species. Butyric acid ($C_{4:0)}$ represents only 0.09-0.2% of total sn-3 fatty acids in the human milk, but almost 20% in buffalo milk (Blasi et al. 2008). Equine milk has a lower percentage (2.5-5%) of sn-3 position than bovine, bubaline, and caprine milk (Figure.3).

Human milk and equine milk possessed lowest sn-3 levels of caproic acid (0.03-0.08% and 0.9-1% respectively) and bovine milk possessed highest sn-3 concentration caproic acid (almost 8%). According to Blasi et al. (2008), amount of $C_{6:0}$ at sn-1/sn-2 position in bubaline milk was not detectable, so $C_{6:0}$ is mainly esterified at sn-3 position.

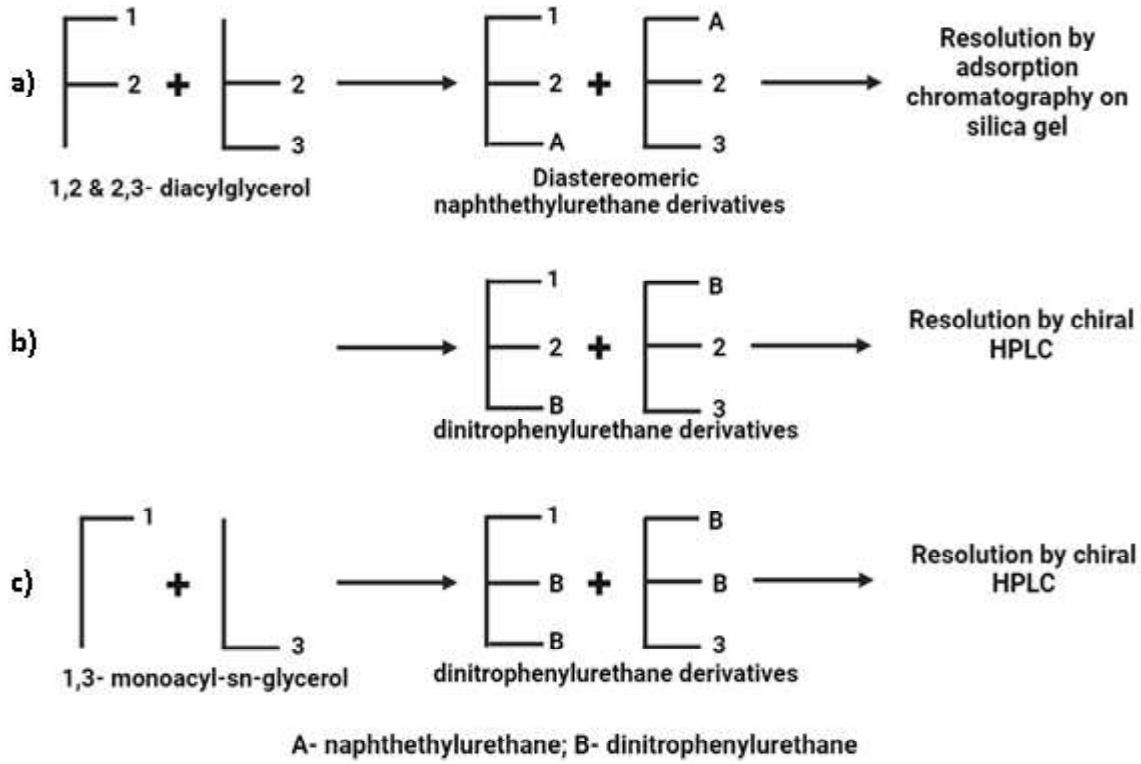

Fig 3. Stereospecific analysis of triacylglycerols via di- and monoacylglycerol for chiral chromatography. a) Preparation of diastereomeric naphthethyl urethane derivatives of diglycerol and resolution by HPLC in the adsorption mode. b) Preparation of dinitrophenyl urethane (DNPU) derivatives of diglycerol and resolution by chiral HPLC. c) Preparation of DNPU derivatives of monoglycerols and resolution by chiral HPLC

Equine milk has higher amount of caprylic acid ($C_{8:0}$) at sn-3 position, 9-10% of total sn-3 fatty acids, while in human milk its just 0.07-0.1% of total sn-3 fatty acids. At sn-1 and sn-3 positions concentration of $C_{8:0}$ is very minimal and almost similar in all species (Blasi et al. 2008; Karrar et al. 2022).

Capric acid ($C_{10:0}$) represented almost 18-28% in equine and 13-30% in caprine milk fat, where in human milk its only 1-2% of total sn-3 fatty acids. In bovine and bubaline milk, the percentage is 9% and 6% of total sn-3 fatty acids respectively. $C_{10:0}$ is also consists almost 8% of total sn-2 fatty acids in caprine milk fat but only 1% in bovine milk fat (Karrar et al. 2022).

Lauric acid ($C_{12:0}$) in human milk first preferentially esterified at sn-3 position and second preferentially esterified at sn-2 position. Concentration of lauric acid in different species varies in sn-2 position. It represents almost 13% of total sn-2 FAs in Equine milk fat, where in bovine and bubaline milk it is only 3-4%.

Myristic acid ($C_{14:0}$) considerably higher in bovine milk (10-22%) but lower in human milk (7-9%) at sn-2 position (Blasi et al. 2008). At sn-3 position its percentage is too less in human milk, while in bubaline milk its almost 18% of total sn-3 fatty acids.

Distribution of long-chain saturated fatty acids in human, bovine, bubaline, caprine, and equine milk fat

Palmitic acid ($C_{16:0}$) is the main saturated fatty acid esterified at the sn-2 positions (Table.3). For $C_{16:0}$ at the sn-2 position, notable differences were observed between different species, ranging from 52-74% of total sn-2 fatty acids in human milk fat to 12.1-13.4% in equine milk fat. In human milk fat palmitic acid at the sn-1 position is only 9.7-10.2% where in case of bovine and bubaline milk fat palmitic acid is higher, 45.7-46.8% and 51.5% respectively, of total sn-1 fatty acids. In human body, when pancreatic lipase hydrolyses the milk fat, this sn-1 or sn-3 positional palmitic acid is easily separated, due to lipase's positional specificity. This separated palmitic acid exhibits poor absorption in its free form. May be because of their melting point is higher than the human body temperature. This palmitic acid reacts with the available calcium or magnesium in the intestine, forming insoluble soaps. These calcium or magnesium soaps cannot be absorbed by the human body and are excreted through the stool. This phenomenon leads to stool hardening or constipation and calcium excretion and increased stool fat, which can pose as a significant health challenge in infants (Linderborg & Kallio, 2005; Mehrotra

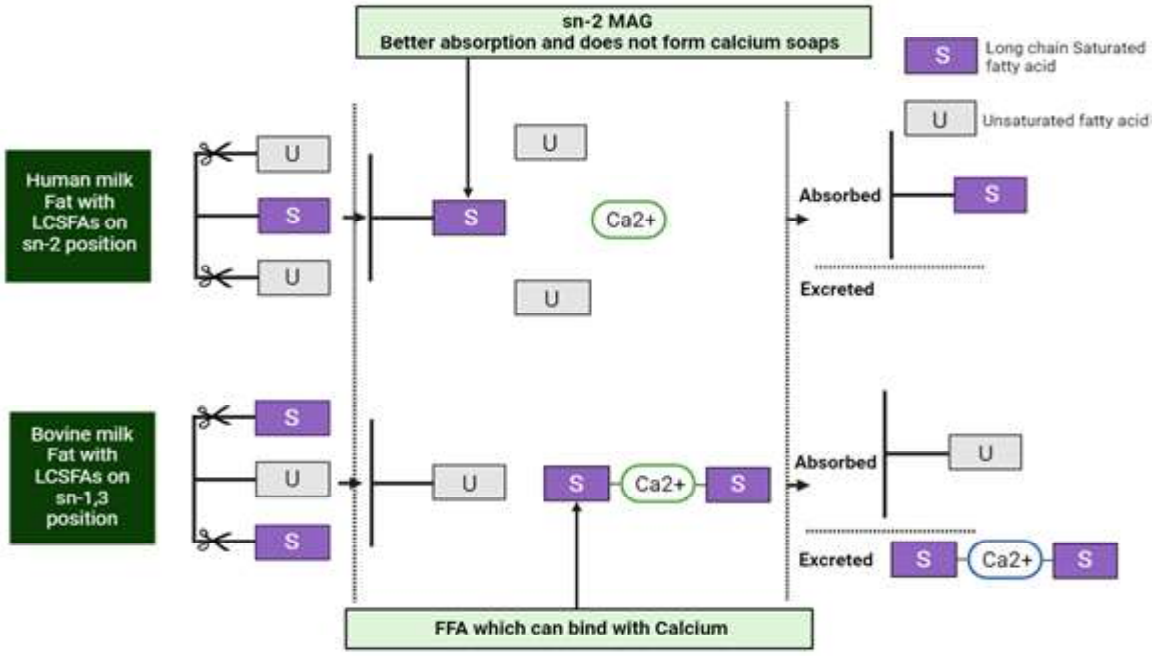

Fig. 4 The sn-2 positioning of fatty acids in TAG of human milk and bovine milk

et al. 2019). The effect of sn-2 positioning of fatty acids in human milk and bovine milk has been pictorially summarised in Figure.4. In a crossover trial in 12 preterm infants conducted by Carnielli et al. (1995), they compared two formulas with similar fatty acid composition and mineral content, but differing in the positional distribution of palmitic acid (in sn-1/3 or sn-2 positions). The study found that infants fed the sn-1(3) palmitic acid formula had higher levels of myristic, palmitic, and stearic acids and calcium, while exhibiting lower levels of oleic and linoleic acids in their feces compared to infants fed the sn-2 palmitic acid formula.

Human milk contains almost 70% of its palmitic acid in the sn-2 position. Pancreatic lipase mainly hydrolyses the fatty acids in the sn-1 and sn-3 positions, leaving palmitic acid as a sn-2 monoglyceride, which is easily absorbed in the body. The sn-2 MAGs are more soluble and polar, which allows for passive diffusion and prevents the formation of insoluble soaps with calcium and magnesium. As a result, human milk causes less constipation and improves the absorption of calcium and magnesium (Lasekan et al. 2017; Mehrotra et al. 2019).

It has been found that an infant's PUFA metabolism is influenced by the distribution pattern of saturated fatty acids. According to studies, linoleic acid increased, long-chain PUFAs in chylomicron phospholipids reduced, and the percentage of sn-2 palmitic acid increased (Innis et al. 1997). In another trial, the formula fat containing sn-2 palmitic acid caused lower proportions of arachidonic and docosahexaenoic acid in chylomicron TAGs, LDL phospholipids, and cholesteryl esters compared with the formula containing palmitic acid in the sn-1 and sn-3 positions (Innis & Dyer, 1997).

Stearic acid ($C_{18:0}$) is mainly found at the sn-1 position (13-16%) in human milk, other species also exhibits a similar percentage as human milk, among them caprine milk has the highest percentage of $C_{18:0}$ at the sn-1 position (>17%) and equine milk has the lower percentage of $C_{18:0}$ at sn-1 position (2-3%) (Karrar et al. 2022).

Distribution of Monounsaturated fatty acids (MUFA) in human, bovine, bubaline, caprine, and equine milk fat

As per different studies, in mammalian milk palmitoleic acid ($C_{16:1}$) and oleic acid ($C_{18:1\ n-9}$) are major MUFA. In equine milk, the percentage of $C_{16:1}$ is higher in sn-1 position, where in human milk it is only ~1% at sn-1 position, comparatively higher in sn-2 position (1.6-2.5%). The most abundant MUFA is oleic acid ($C_{18:1\ n-9}$), which is abundant in sn-1 or sn-3 position. In human milk it ranges in between 40-45% at sn-1 and sn-3 position and 8-9% in sn-2 position. Bovine and bubaline milk has very similar type of distribution, while in caprine milk its ranges 19.2-43.4% at sn-3 position (Table.3.).

Distribution of Polyunsaturated fatty acids (PUFA) within human, bovine, bubaline, caprine and equine milk fat

Human milk has highest amount of PUFA within following species. Equine milk is nearer to it. In human milk, PUFA (Linoleic and linolenic acid) are abundant in sn-3 position. In human milk at sn-3 position, linoleic acid is almost 30-39%, while bovine, bubaline, and caprine milk fat its ranges between 2-4%, equine milk is little higher, 10-10.5% at sn-3 position (Table.3) (Bakry et al. 2020; Blasi et al. 2008; Karrar et al. 2022).

Table 3: Stereospecific distribution of fatty acids in milk fat triglycerides of different milch species

Species	Human			Bovine (cow)		Bubaline (buffalo)			Caprine (goat)			Equine (donkey)		
	sn-1	sn-2	Sn-3	sn-1	sn-2	Sn-3	Sn-1	Sn-2	Sn-3	sn-1	sn-2	Sn-3	sn-1	sn-2	Sn-3
C4:0	ND	ND	0.09-0.2	1.3-1.6	0.3-0.4	17.9-18.3	1.2±0.4	1.4±0.5	20.5±1.0	1.4-1.5	0.2-0.3	13.0-15.9	1.0-1.1	0.6	2.5-5.1
C6:0	ND	ND	0.03-0.08	0.3-0.35	0.9-1.0	7.6-8.2	ND	ND	2.1±0.5	0.4-0.5	0.7-0.8	6.2-8.6	0.3-0.5	0.1-0.4	0.9-1.0
C8:0	ND	ND	0.07-0.16	0.3-0.32	0.2-0.3	4.5-4.7	ND	ND	3.4±0.1	1.2-1.7	0.1-0.3	1.1-10.6	0.9-1.1	0.9-1.5	9.2-10.6
C10:0	0.4-0.6	0.2-0.38	1.43-2.38	1.1-1.4	0.98-1.4	8.0-8.7	0.7±0.1	0.9±0.5	5.6±0.7	2.0-4.9	3-7.9	13.6-30.2	3.6-4.4	7.2-8.2	18.7-28.5
C12:0	2.8-3.5	3.7-6.9	4.1-7.08	2.3-2.5	2.02-4.8	4.7-5.1	1.6±0.1	3.1±0.7	4.3±0.7	2.2-3.3	5.9-6.35	2.2-3.4	6.1-7.2	3.19-12.7	8.3-8.6
C14:0	2.5-3.7	7.3-9.8	0.6-1.15	11.2-11.7	10.25-22.8	8.0-8.2	9.8±0.6	16.7±0.9	10.9±1.1	7.5-9.0	17.2-18.6	1.2-4.9	7.8-8.0	12.1-13.4	3.7-6.2
C16:0	9.7-10.2	52-74	0.8-1.6	45.7-46.8	38-42	11.0-12.0	51.5±2.0	37-40	17.8±1.8	38.4-45.4	31.2-36.0	1.4-3.2	23.2-28.6	30-54	2.1-7.9
C16:1	0.9-1.3	1.6-2.5	1.1-1.8	1.4-1.5	1.2-2.5	1.8-1.9	1.6±0.1	2.8±0.1	2.0±0.1	0.5-1.0	0.5-0.8	0.2-2.8	5.5-10.71	0.6-5.6	3.9-6.1
C18:0	13.0-16.0	2.2-4.9	0.8-1.06	11.1-11.6	5.3-6.12	5.2-6.1	13.8±0.6	10.2±1.0	9.9±1.5	17.6-17.9	7.9-12.0	7.1-13.1	2.0-3.0	1.7-2.3	0.9-1.6
C18:1	41.2-43.0	8.1-9.8	38.7-45.2	21.5-22.8	15.7-17.6	25.1-27.3	18.1±2.1	21.0±0.9	21.2±1.3	17.5-21.7	18.8-23.0	19.2-43.4	19.2-26.4	14.9-19.2	13.2-21.4
C18:2	21-22	14.5-16.9	30.9-39.0	1.5-1.7	1.9-4.35	2.5-2.7	1.6±0.2	2.7±0.1	2.0±0.1	0.7-0.9	2.2-4.2	1.6-6.8	9.0-13.7	9.1-9.7	10.0-10.4
C18:3	0.73-0.83	1.1-1.4	2.5-3.5	0.1-0.3	0.2-0.3	0.2-0.3	0.2±0.0	0.3±0.0	0.2±0.1	0.3-0.5	0.3-0.4	0.1-0.6	6.3-6.8	4.2-4.3	4.1-5.7

Source: (Blasi et al. 2008; Chen et al. 2020; Cossignani et al. 2011; Haddad et al. 2012; Karrar et al. 2022; Marai et al. 1969; Myher et al. 1986; Roy et al. 2020; Straarup et al. 2006; Sun et al. 2018; Zhang et al. 2020).

MUFA and some PUFA can help to prevent cardiovascular and inflammatory diseases (Blasi et al. 2008; Ulbricht & Southgate, 1991). Thus, equine milk fat provides have better health benefits than other milks.

Overall, there were significant ($p < 0.05$) variations in stereospecific positioning of fatty acids in TAG among milk fat from different species. The variations in positional distribution of fatty acids in TAG between species could be attributed to the differences in genetic background, animal species, feeding, lactation stage, and season (Karrar et al. 2022).

Conclusion

There is difference between the genetic structure, feed habit, season, region, lactation periods, infant requirement in different mammalian species. These differences affect the composition and distribution of fatty acids in different stereospecific position of fatty acids in milk fat triglycerides. This review also highlights the effect of stereospecific arrangements of fatty acids in triglyceride on nutrition and digestion of milk fat. In addition, various methods used to identify the position of fatty acids in triglyceride molecules are briefly described. Where, the most commonly reported Thin-Layer Chromatography (TLC) coupled with Gas-Liquid Chromatography (GLC) technique is maximum time-consuming, but the less explored technique using Nuclear Magnetic Resonance (NMR) required very minimal time. The bovine, bubaline and caprine milk have LCSFAs at sn-1 position at a major concentration, which can cause stool hardening or constipation problem in infant body. In equine milk long chain saturated fatty acids are mainly esterified at sn-2 position which is similar to the human milk. Equine milk's MUFA and PUFA distribution (especially linolenic acid) among the three position of triglycerides is also nearer to human milk. In spite of its low-fat percentage, equine milk is beneficial for human consumption due to its higher essential fatty acid (EFA) percentage and lower SFA percentage at sn-1/3 position than other milks; it has a higher EFA% in sn-2 position. Depending upon fat globule size also caprine and equine milk has the better digestion capability than other species.

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