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Chapter

Lipids and Fatty Acids in Human Milk: Benefits and Analysis

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Abstract

Human milk is related to the physiological and nutritional welfare of newborns, providing the necessary dietary energy, physiologically active compounds and essential nutrients for breastfed babies. Human milk fat has an important position as energy source, structural and regulatory functions, being one of the most important components of breast milk. It provides approximately 50–60% of the energy of the human milk, and its composition in fatty acids defines its nutritional and physico-chemical properties. Furthermore, human milk contains the long-chain polyunsaturated essential fatty acids (LCPUFA) eicosapentaenoic acid (EPA), arachidonic acid (AA) and docosahexaenoic acid (DHA), which is important for appropriate development of baby's organs, tissues and nervous system. This chapter will address the benefits associated with the consumption of human milk (health, nutritional, immunological and developmental benefits) as well as the analysis applied to determine the lipid quality of this powerful food.

Keywords: lipids, fatty acids, human milk, omega-3, children's health

1. Introduction

Human milk is especially complete and suitable to provide the essential to the infants due to its composition including a variety of nutrients, bioactive compounds and immunological factors, which are indispensable for the newborn growth and optimal development [1]. Moreover, studies have demonstrated the benefits of human milk for newborns concerning gastrointestinal problems, growth, neurological development and immune system [2]. Breastfeeding is recognized as the gold standard for feeding infants and should be the exclusive feed source during the primary 6 months of life, without the demand to supplement with additional food or liquid. Besides, the breastfeeding should be prolonged even after food introduction during the primary 2 years of life [3].

Lipids are allocated in groups according to its solubility in apolar and organic solvents insoluble in water, being classified as neutral lipids: triglycerides (TAG), diglycerides (DAG) and monoglycerides (MAG), polar lipids: phospholipids and glycolipids, and miscellaneous lipids: sterols, carotenoids and vitamins [4].

In human milk, the lipids are present as fat globules form, mainly constituted of TAG surrounded by a structural membrane composed of phospholipids, cholesterol, proteins and glycoproteins [5]. The fat from human milk is its main energy source, consisting 98% (m/m) of neutral lipids (TAG, DAG and MAG) [6]. Hence, the fatty acid composition of these constituents defines the nutritional and physicochemical properties of human milk fat [7].

TAG are molecules of glycerol esterified to three fatty acids (FA), which may be located at the TAG sn-1, sn-2 and sn-3 positions. However, the FA position in TAG is also related to the human milk quality. In TAG from human milk, for example, palmitic acid is positioned normally on sn-2 (the central carbon atom) [8], which facilitates the action of pancreatic lipase. Besides, it leads to improved absorption of fat and calcium by newborns due to the subsequent metabolism of these TAG in the infant's body [9].

Therefore, numerous analytical techniques are employed to attempt the verification of the FA composition as well as TAG structure present in human milk fat. This chapter will address the benefits associated with the consumption of human milk, as well as analytical techniques employed to assess its lipid quality.

2. Benefits and analysis of human milk

2.1 Importance of human milk in newborn health

Breastfeeding provides numerous health benefits, both short and long-term for breastfed newborn [10]. The short-term benefits include immune system development, reduction of gastrointestinal diseases (diarrhea), respiratory diseases (pneumonia), skin diseases (atopic dermatitis), allergies, leukemia, sudden death syndrome, diabetes and ear inflammation during childhood [10, 11]. Long-term evidence has shown various benefits to public health problems such as improved cognitive development [12] and reduction of chronic diseases, for example diabetes (type 1 and 2), obesity, hypertension, cardiovascular diseases, hyperlipidemia and selected categories of cancer in adult life [13].

2.1.1 Importance of different lipid classes of human milk

Milk TAG are formed in the endoplasmic reticulum from circulating FA or newly synthesized in the mammary epithelial cells of glucose. The initial step in the FA synthesis is the conversion of acetyl-CoA to malonyl-CoA, afterwards, the synthase enzyme catalyzes the sequence of fatty acid reactions, then each sequence adds two-carbon unit to the growing chain, resulting in the de novo synthesis of medium-chain and intermediate chain FA as well as explaining the elevated content of these FA in milk [14].

Long-chain TAG are digested by a lingual lipase, while the medium and short chain TAG undergoes the action of a stomach lipase and are absorbed in the stomach as FA and glycerol. In the intestine, TAG non-hydrolyzed, especially long chain triglycerides, undergo the action of bile salts and pancreatic enzyme, being reduced to MAGs, FA and glycerol, which are absorbed, distributed and utilized by the tissues [15].

Phospholipids contribute to 1–2% of the total lipids of human milk [16]. The major phospholipids of milk fat globule membrane are phosphatidylcholines, phosphatidylethanolamines and sphingomyelins, and each of it contributes to 20–40% of the total phospholipids [17]. The nutritional importance of these lipids is based on the variety of specific lipids provided, plus it also has particular bioactivities in the gastrointestinal.

The sphingomyelin demonstrates robust anti-tumor activity, may influence the cholesterol metabolism, and exhibits anti-infective activity [4]. The phosphatidylcholine and sphingomyelin contribute to approximately 10% of the total choline intake of infants [18]. Thus, in quantitative terms, water-soluble choline in milk is more significant, although there are good indications that the metabolization of free and esterified dietetic choline is distinctive and it may have specific effects on plasma cholesterol levels and even in the brain development of the baby [19].

Cholesterol content in human milk is low (0.5%), serving as structural component of the milk fat globule membrane, this characteristic is related to the provision of sufficient stabilization and fluidity, it is also essential in lipid metabolism [5]. Breastfed babies present higher plasma cholesterol levels in comparison to babies receiving infant formulas; however, early exposure may favor the metabolic regulation of cholesterol homeostasis in adult life [20].

2.1.2 Importance of fatty acids in human milk

Human milk fat accomplish an important position as energy source, structural and regulatory functions, [21] in which FA are essential for the development of the central nervous system [22] antiprotozoal activity (free fatty acid (FFA) produced during gastric and intestinal digestion of milk fat), increased immune response, anticarcinogenic agents and antidiabetic effects [23].

The principal saturated fatty acid (SFA) in human milk is the palmitic acid (16:0) [24]. It is located in the TAG sn-2 region, simplifying the pancreatic lipase action that specifically hydrolyzes the FA at the sn-1 and sn-3 positions converting the palmitic acid to sn-2 MAG, which is generally well absorbed resulting in improvement of intestinal discomfort, decreasing colic and crying of the newborn. [25] In addition, the palmitic acid position influences the n-acylentanolamides, including levels of anandamide, which presents analgesic effects contributing to the enlightenment of the association between the palmitic acid position and the baby cry behavior [14].

It is also noteworthy that the butyrate SFA (4:0) present functions as modulation of the gene expression regulation and reduction of inflammation processes in the intestine. The SFAs caproic (6:0), caprylic (8:0), capric (10:0) and lauric (12:0) acids are linked to antimicrobial biological activities [26].

In particular, the most significant FA in human milk are the long-chain polyunsaturated fatty acids (LCPUFA) [22]. The homologs of linoleic acid (18:2n-6; LA) from n-6 series are precursors of arachidonic acid (20:4n-6; AA), while homologs of α-linolenic acid (18:3n-3; ALA) from n-3 series are precursors of eicosapentaenoic acid (20:5 n-3, EPA) and docosahexaenoic acid (22:6n-3, DHA). Therefore, breast milk contains the indispensable FA precursors (LA and ALA) to produce AA and DHA, which present crucial function in visual, immune, cognitive and motor development in newborns. Besides, it present important function in allergy protection, asthma, improvement of lung function and reduction of childhood inflammation and obesity rates, plus an additional advantage is the increase of 4.5 IQ points in infants breastfed in comparison to infants that did not received it [24].

2.2 Nomenclature and terminology of main fatty acids in human milk

The IUPAC nomenclature system is technically clear. The fatty acid names are excessively long, principally the long chain acids, therefore, for convenience, common or trivial name and abbreviated notations are often employed in scientific texts. Researchers working in different study areas on fatty acid composition are familiar with the chemical structure and commonly use of the notation C:D to

represent the FA, being C the number of carbon atoms and D the number of double bonds in the carbon chain. Some researches frequently employ the "omega" system (n minus system), Shorthand Designation, as a notation to define the different series, such as n-3, n-6, n-9, n-12. This system is applicable to unsaturated fatty acids from natural sources (cis configuration). Unsaturated fatty acids have double bond in the carbon chain and, commonly, in the PUFA, the double bonds of carbon chain is interrupted by a methylene group (cis, cis-1,4-pentadiene group).

Thus, the term n minus refers to the position of the double bond closest to the methyl end carbon chain. However, in this system, the position of another double bond in PUFA acid carbon chain is not denoted and the configuration (cis or trans) is not specified. So, this system is not employed for FA with trans configuration and PUFA group, although it is widely used by researchers. Therefore, the IUPAC-IUB Commission does not recommend the 'omega' system [27].

2.2.1 Main fatty acids in human milk

Cis fatty acid composition is one of the major components of woman's breast milk, and it is influenced by different factors, that can be grouped as follows: (i) variable: method of feeding, genetic factors, dietary habits, maternal diet composition, hormones, gestational age at birth, parity, seasonality, between lactation daily, caloric content of food and mutual proportions of particular dietary components (carbohydrate and fat contents), (ii) positive modulation: duration of the lactation period, adiposity, stage of lactation and maternal age, (iii) negative modulation: maternal malnutrition, infectious (mastitis), metabolic disorders (diabetes) and medications [28, 29].

In particular, trans fatty acids (TFA) in human milk have raised concerns because of the possible adverse effects on infant growth and development. The TFA have been associated with adverse effects on LCPUFA and essential fatty acids (LA, 18:2n-6 and ALA, 18:3n-3) metabolism, oxidative stress and low density lipoprotein cholesterol levels. These negative effects of TFA are predominantly associated with different isomers from hydrogenated vegetable shortening, such as 6/7/8/9/10 (trans) 18:1, and barely with TFA of natural sources such as ruminants fats as 11 (trans) 18:1 [30]. Composition of the TFA in human milk from Canadian and American woman has reduced since the mandatory TFA labeling was introduced in those countries [30, 31].

In this chapter, the group prepared a review concerning the composition of FA commonly encountered in studies of human milk from different countries, including five regions of China, Canada, Spain, Brazil, Poland, Germany, Hungary, Finland, Sweden Slovakia, United Kingdom, Denmark, Egypt, Uganda, and Tanzania. A list of FA is elaborated on **Table 1**, including: saturated FA, monounsaturated FA, polyunsaturated FA, branched chain FA, trans FA and conjugated linoleic [14, 26, 31–34].

2.3 Contribution of omega-3 fatty acids from human milk on immunity

Human milk contains numerous growth and antimicrobial factors, as well as cells and antibodies from mother, which are responsible by innate and acquired immune responses in the newborn [35]. The breastfeeding permits these components to cover the neonatal gastrointestinal tract, the main access of microorganisms in early life, and influences the maturation of immune system. It is assumed that human milk can perform in the induction of specific immune responses in the intestine, favoring a microbiota that competes with pathogenic bacteria [36].

Trivial name	Shorthand designation	Trivial name	Shorthand designation
SFA		**MUFA**	
Butiric	4:0	Lauroleic	12:1n-3
Caproic	6:0	Tsuzuic	14:1n-10
Enantic	7:0	Physeteric	14:1n-9
Caprylic	8:0	None	14:1n-7
Pelargonic	9:0	Myristoleic	14:1n-5
Capric	10:0	None	15:1n-5
Undecylic	11:0	Sapienic	16:1n-10
Lauric	12:0	None	16:1n-9
Tridecylic	13:0	Palmitoleic	16:1n-7
Myristic	14:0	None	17:1n-8
Pentadecylic	15:0	None	17:1n-7
Palmitic	16:0	Petroselinic	18:1n-12
Margaric	17:0	None	18:1n-10
Stearic	18:0	Oleic	18:1n-9
Arachidic	20:0	Vaccenic	18:1n-7
Heneicosylic	21:0	None	18:1n-6
Behenic	22:0	None	18:1n-5
Tricosylic	23:0	None	18:1n-4
Lignoceric	24:0	None	18:1n-3
BCFA		None	18:1n-2
Isotridecylic	13:0 iso	Gadoleic	20:1n-11
Anteisotridecylic	13:0 anteiso	Gondoic	20:1n-9
Isomyristic	14:0 iso	Paullinic	20:1n-7
Isopentadecylic	15:0 iso	Cetoleic	22:1n-11
Anteisopentadecylic	15:0 anteiso	Erucic	22:1n-9
Isopalmitic	16:0 iso	Nervonic	24:1n-9
Isomargaric	17:0 iso		
Anteisomargaric	17:0 anteiso		
PUFA		**TFA**	
Linoleic	18:2n-6 (LA)	Myristelaidic	t-14:1n-5
Gamma-linolenic	18:3n-6	None	t-15:1n-5
Alpha-linolenic	18:3n-3 (ALA)	Isomers	3/4/5/6/7/8/9/10/11/12/13 (trans) 16:1
None	20:2n-6	Isomers	6/7/8/9/10/11/12/13/14 (trans) 18:1
Stearidonic	18:4n-3	Linolelaidic	trans,trans 18:2n-6
Meadacid	20:3n-9	None	cis-9, trans-12 18:2
Dihomo-γ-linolenic	20:3n-6	None	trans-9,cis-12 18:2
Dihomo-ALA	20:3n-3	None	trans-11,cis-15 18:2

Trivial name	Shorthand designation	Trivial name	Shorthand designation
None	20:4n-3		
Aracdonic	20:4n-6 (AA)	**CLA**	
Timnodonic	20:5n-3 (EPA)	Rumenic	cis-9, trans 11 18:2
Adrenic	22:4n-6	None	trans-9, cis-11 18:2
Osbond	22:5n-6	None	trans-11, cis-13 18:2
Clupadonic	22:5n-3	None	trans-11,trans-13 18:2
Cervonic	22:6n-3 (DHA)		

Fatty acids abbreviations: SFA—saturated fatty acid, MUFA—monounsaturated fatty acid, PUFA—polyunsaturated fatty acid, BCFA—branched chain fatty acid, TFA—trans fatty acid, CLA—conjugated linoleic acid.

Table 1.
Fatty acids commonly encountered in human milk.

Other components constantly present in human milk are the LCPUFA DHA and AA [37], essentials as cell membranes components and also as immunomodulators, by production and regulation of inflammatory cytokines, leukotrienes, prostaglandins, and thromboxanes, recognized as eicosanoids [38].

LCPUFA in human milk can modulate immunological responses, affecting the balance between T-helper cell type-1 (Th1) and Th2 [39], and regulatory T and T helper 17 cells from the acquired immune response [40].

These subsets of CD4+ T cells, Th1, Th2 [41], Th17 [42], and regulatory T (Treg) cells [43] participle producing cytokines with the most diverse functions. Interferon-γ (IFN-γ), tumor necrosis factor-alpha (TNF-α) and interleukin-2 (IL-2) are the products of Th1, and IL-4, IL-5, IL-9, IL-10, IL-13, and IL-25 are of Th2 cells. Th17 cells produce IL-17A, IL-17F, IL-21 and IL-22; while Treg cells produce IL-10 and TGF-β1.

Th1 cells present important functions in cellular immunity against intracellular bacteria and protozoa, while Th2 cells mediate the response against extracellular parasites, as helminths, and participate in allergies [44]. Th17 cells apparently perform against different classes of pathogens and autoimmune conditions [45], and Treg cells perform regulating the inflammation, autoimmunity, allergy, infection, and tumors.

In general, preterm infants have an immature immunoregulatory system, with potential for chronic inflammation [46], but an increase in Tregs and their function in early neonates has been observed [47], suggesting a transient increase of activated Treg in mature and full-term infants.

Although, generally, AA is considered proinflammatory and DHA immunoregulatory, the addition of it to infant formula has been indicated to increase the immunoregulatory system and to reduce inflammatory cytokines in infants, indicating an effect of LCPUFA on immune maturation [48].

However, according to [49], diets rich in DHA can reduce suppressive and migratory functions of regulatory T-cells [48]. Thereby, we must carefully examine the influence of FA during breastfeeding, since the knowledge from the DHA data on immune response in preterm infants, and the generation and maintenance of Tregs are still not well comprehended. Finally, the addition of AA and DHA in infant formulas should consider balancing its amounts, as DHA in excess may suppress the benefits provided by AA [50].

2.4 Atopic disease and polyunsaturated fatty acids (PUFA)

Atopic disease is defined as a set of disease such as atopic dermatitis, asthma and rhinitis, but it may differ according to the authors. The prevalence of childhood atopic

disease has been increasing and studies suggested the possibility that breastfeeding may reduce allergic manifestations in high-risk individuals [51, 52]. This association is possible linked with the breast milk PUFA, which are essential for adequate growth and development. It is also recognized that an early ingestion of it may affect the growth as well as the neurological and immune functions in later life [53].

Part of the PUFA breast milk composition depends on the mother dietary, and it has provided support to the hypothesis that omega-3 (n-3) PUFA in breast milk possibly protect against atopic diseases. The ratio between n-3 and omega-6 (n-6) PUFA levels seems to influence the development of atopic disorders [54, 55].

The n-3 FA supplementation, during pregnancy, and lactation have been extensively studied. Pregnant mothers consuming n-3 FA may enhance levels of IgA (adaptative immune response) and soluble CD14 (innate immune response) in breast milk. This theory reinforces the importance of the PUFA immune modulation and the idea that it can improve the immune system directly and indirectly [56].

The composition of breast milk has been shown to reflect in the infant's serum, by increase of immunomodulatory cytokines, such as TGF-1 and TGF-β2, associated to the protection against atopic diseases [57]. Moreover, EPA and DHA levels in colostrum and early mature milk were related to the protective effect in the development of IgE-associated with allergic disease in infancy [58].

An Asian study revealed that n-3 FA supplementation during pregnancy could reduce the chance of preterm birth. According to this article the intake recommendation to pregnant women is minimum of 200 mg (DHA) per day over and above the intake level recommended for adult general health, resulting in a total DHA intake of at least 300 mg/day. Likewise, PUFA supply and fish ingestion may positively influence the development of immune responses involved in allergic reactions, and reduce the risk of allergic diseases (asthma and eczema). It is recommended to women who breastfeed to achieve a minimum average daily supply of 200 mg DHA, to result in a milk with DHA content of 0.3% of FA [53].

A systematic review concluded that there is heterogeneity among studies in terms of presenting the association between PUFA and allergy, which could influence the results [59]. Some studies observed associations between n-3 and n-6 PUFAs and allergic disease [60], and the magnitude of this effect varied greatly. Otherwise it is known that breast milk contains different composition of PUFA, which could explain the variability of the results [61].

A cohort study has shown the ratio of n-6: n-3 FA in milk is associated with the risk of non-atopic eczema at 6 months, and perhaps the high level of n-6 may increases the risk of rhinitis [62]. Other authors hypothesized that variations in the lipid composition of milk could, in part, explain some of the controversies regarding the protective effects of breastfeeding against allergy, and concluded that the fatty acid composition of human milk is disturbed in atopic mothers having an effect on atopic sensitization in the primary 12 months of life [58].

2.5 Processing, composition in antioxidants and lipid stability in human milk

The methods of processing human milk employed in milk banks aim the preservation or inhibition of microbial growth, the prevention or delay of decomposition caused by the presence of enzymes, chemical reactions, and the preservation by grime, such as insects, hair, animals, etc. [63]. Generally, pasteurization and freezing techniques are employed. In addition, recent studies combine both processes with lyophilization in order to preserve the original characteristics of milk for an extended period [64].

However, food processing can cause nutritional loss and structural modifications. In milk banks, pasteurization is followed by freezing storage at −18°C for

up to 6 months. But, the thermal process causes modifications in the milk due to the inadequate intensity of the set time and temperature that has been applied. Consequently, proteins can be denatured, enzymes become inactive, lipids suffer oxidation and vitamins and minerals are unstructured [65].

The Holder pasteurization (30 minutes of heat at 62.5°C and frozen at −20°C) imposed by the global guideline was evaluated by studies demonstrating that the lipolytic activity increased, doubling the concentration of free fatty acids (FFA), while the low temperature reduced the lipolysis rate, even if it had been increased by the storage time under freezing [66].

The lyophilization process removes the water from food by sublimation, allowing its preservation at room temperature, with the addition of water the product returns to its original form without nutritional losses. This technique, applied in human milk, demonstrated to be effective, as it inhibits microbial contamination, preserves nutrients and oxidative markers, as well as ensures a prolonged conservation period in comparison to pasteurized human milk [67].

Lipids are the most compromised macronutrients present in milk during processing due to the autoxidation of the fatty acid. This degradation reaction can occur with or without oxygen, as well as be catalyzed by light, heat, irradiation and free radicals, forming toxic compounds, such as peroxides. According to the thermal processes, fatty acid may undergo structural isomers by generating trans molecules or losing their total or partially insaturations, damaging the product and causing nutritional loss. However, studies indicate that the PUFA is stable during pasteurization and it may be justified due to the high antioxidant activity of the human milk [68].

The milk naturally presents antioxidant compounds that delaying or preventing molecules from being affected by the oxidative processes [69]. These compounds operate according to diverse action mechanisms for cell protection, such as: (i) eliminate substances that initiate peroxidation, (ii) chelate metallic ions, turning it incapable of decomposing peroxides or forming free radicals, (iii) block the action of reactive species, (iv) interrupt the auto oxidizing chain reaction, and/or (v) reduce the local concentrations of O_2 [70].

Regarding the antioxidant category, it can be classified chemically by enzymatic and non-enzymatic, both perform synergistic actions in free radicals elimination [71]. Among the antioxidant enzymes, the milk is composed by the superoxide dismutase and the catalase, and the glutathione peroxidase that contains selenium. There are also other enzymes that catalyze the synthesis or regeneration of non-enzymatic antioxidants, named support enzymes, among which are glucose-6-phosphate dehydrogenase and glutathione reductase [72].

Non-enzymatic antioxidants present in breast milk are glutathione, amino acids arginine, citrulline and taurine, creatine, metallic ions selenium and zinc, ascorbic acid (vitamin C), carotenoids, flavonoids, coenzyme Q10, vitamins E and lactoferrin. Among these antioxidants that are three distinct classes: (i) antioxidants that performs as free radical abductor in the lipid milk portion, such as vitamin E and A, carotenoids and coenzyme Q10, (ii) antioxidants that performs in the aqueous phase, such as ascorbic acid, and (iii) antioxidants that performs in both cases, such as flavonoids [73].

Among the lipophilic antioxidants, the principals are: carotenoids, vitamin A and α-tocopherol. A vitamin E constituent is present in greater amount in colostrum, first phase of breast milk, providing it a yellowish color due to the intense presence of the pigment carotenoid β-carotene, and decrease from the beginning of lactation, despite the increase of total lipids [74]. The average level of vitamin A

on the third day of lactation comes to be three times superior than in mature milk. Similarly, the amount of vitamin E in colostrum can be the triple of that found in the mature milk and the carotenoids can present a level up to 10 times higher [75]. In this way, the precise balance of various antioxidants in breast milk, instead of any isolated factor, determines its oxidative stability [76].

2.6 Analytical methods for determination of fatty acids in human milk by gas chromatography: flame ionization detector (GC-FID)

2.6.1 Different extraction methods for total lipids (TL)

The fatty acid composition of human milk has been extensively studied over the last 25 years and almost all of the studies are obtained after lipid extraction.

Different methods of fat extraction have been proposed to determine the fat content in human milk by traditional method: crematocrit [77], esterified FA [78], Gerber method (butyrometer) [79], and gravimetric [80].

Recent methods have been proposed in the direct quantification of FA in human milk by gas chromatography [81]. However, the gravimetric method is considered the gold standard for extracting the total lipid (TL) content in human milk, and Folch et al. [80] is one of the most recommended methods for it. This methodology extracts non polar, polar and neutral lipid using mixture of cold solvents for extraction and not compromising the chemical structure of the lipids.

2.6.2 Quantification of fatty acids in human milk by GC-FID

Prior to chromatographic analysis, a derivation step of the TL is required, converting the different lipid classes in fatty acids methyl esters (FAMEs). This step is necessary to enable the volatilization of the compounds of interest, and to allow the determination by GC; there are several methods for this purpose [82].

Normally, the quantitative determination of FA in the human milk is data generally normalized as g of FA per 100 g of FA or expressed as percentage of weight (area normalization) FA relative to all FA exposed in a chromatogram. In the normalization methods, all the FA of the sample must be considered and, in the case of omission of a component, the other components are affected. On the other hand, the results presented by the normalization present difficulties of interpretation and, therefore, in nutritional values of the human milk [81]. The main drawback about normalization methods is that the data set does give information on the amount of FA (in mass) per volume or mass in human milk.

Recent studies express the composition of FA in mass concentrations of FA by mass of liquid human milk, and direct quantification of FA in human milk by gas chromatography has been proposed [81].

The determination qualitative and quantitative of FAMEs by GC-FID is among the most commonplace analyses in lipid matrices. Quantification of FAMEs by GC-FID has been effectively performed whereas detection with GC tandem mass spectrometry (CG-MS) has been employed mainly for qualitative analysis of FA. Both detectors FID and MS, for chromatographic analysis, the derivation step of the lipids classes is required to conversion of TAG, DAG, MAG, phospholipids (transesterification process), FFA (esterification process) in FAMEs [83].

The American Oil Chemists Society (AOCS) and the Association of Analytical Chemists (AOAC) recommend parameters for accurate quantification of FA. Both sources indicate the use of internal standard (IS, methyl tricosanoate - 23:0) and

capillary columns. The IS are used to minimize the experimental errors, control extraction, transesterification and esterification, undesired saponification. The IS cannot be part of the composition of lipid sample or whole sample [83].

2.6.2.1 Relative response factor in the FID and methodology

The FID has become one of the most popular measuring devices employed in GC and it is the most sensitive detector for hydrocarbons, being the FA merely carboxylic acids with long chains of hydrocarbons.

As the FAMEs respond differentially in FID because of the combustion of carbon compounds that produces ions due to the chain size, presence of FA-substituted functional groups (carboxylics group, double bond) in a hydrocarbon, it reduces the combustion efficiency, and therefore, relative response factors in the FID depends on the effective carbons number. Thus, it is necessary to use correction factors for the FAMEs in relation to the IS. The applied factors are the experimental (empirical correction factor) and the theoretical correction factor (F_{CT}), theoretically determined from the number of effective carbons. It is also important the conversion factor from FAME to FA (F_{CEA}) [83, 84]. In this chapter, methodologies employing IS (23:0) and correction factors FCT, and FCEA for the FID response are described below [83].

The follow column and chromatography condition has been used with efficiency in separation process of the methyl esters (FAMEs) in total lipid of milk. The FAMEs are prepared by transesterification and esterification of total lipids. It is injected and separated into a CG-FID. The column Select FAME (part number CP-7420) fused silica capillary column 100 × 0.25 mm, and 0.25 μm of 100% cyanopropylpolysiloxane (high polarity) was employed. The carrier gas (H_2) flow rates are 1.2 mL min^{-1}; auxiliary gas (N_2) 30 mL min^{-1}; H_2 and synthetic air 35 and 350 mL min^{-1}, respectively. The volumes of the sample injection are 1.0 μL, split of 1:80. The injection temperature: 200°C, detector temperature: 240°C. The column temperature-programmed: 165°C for 7.00 min, the heating ramp of 4°C min^{-1} until 185°C (4.70 min.) after that another programming heating of 6°C min^{-1} until 235°C (5.00 min.). The FAMEs are identified by comparison of their retention times, determined by computer software analysis, with those of individual purified standards or secondary standards. The quantifications of FAMEs are performed with internal standard (IS 23:0) and the corrections factors for the FID response are utilized for the determination of concentrations [84]. The composition of fatty acids (FA) in the total lipids of samples is calculated in mg g^{-1} of total lipids (TL) using the Eq. (1) [83, 84].

$$Mx = \frac{A_X M_p F_{CT}}{A_p M_A F_{CEA}} \tag{1}$$

M_x is the concentration of FA "x" in mg g^{-1} of TL, A_x is the FA "x" peak area, A_p is the IS (FAME 23:0) peak area, M_p is the IS mass added to the sample in mg, M_A is the sample mass in grams, F_{CT} is the theoretical correction factor of the FID and F_{CEA} is the conversion factor from FAMEs to FA.

It is concluded that to determine the FA composition by GC-FID with high accuracy, and to express the composition of fatty acids in mass of FA per sample (by volume or mass) it is necessary to apply the correct derivation technique, internal standard and flame ionization detector relative response factors using the effective carbon number and conversion from FAMEs to FA.

2.7 Analytical techniques for analysis of lipids by electrospray ionization (ESI) and other techniques

2.7.1 Lipid extraction for analysis by liquid chromatography-mass spectrometry (LC-MS)

In order to determine TAG in food, initially, is essential to extract the lipids contained on it. Folch [80] and Bligh and Dyer [85] methods are extensively employed for the extraction of milk lipids. The addition of antioxidant, such as BHT is recommended prior to extraction to avoid lipid oxidation [86]. The internal standards for each lipid class are added to the matrix prior to extraction [87].

Preceding the LC–MS analysis, the extraction solvents (chloroform/methanol) must to be removed by evaporation, and the lipids are reconstituted with solvent compatible with the mobile phase of LC. Moreover, a pre-sample of SPE or TLC columns previous to LC–MS analysis may facilitate the lipid species identification due to improved resolution.

2.7.2 Direct infusion analysis in MS

Sample direct infusion into the mass spectrometer was one of the first techniques employed in TAG analysis [88]. Its main advantage is the rapidity. However, despite the progresses in the last 15 years, there are three main problems associated with the technique: (1) ion suppression, (2) isotopic interference and (3) differentiation of isomers. Consequently, a chromatographic separation is crucial to avoid ions suppression and to differentiate the isomeric species, being the direct infusion technique rarely employed for characterize lipid in milk sample.

2.7.3 TAG determination by LC-MS

Nowadays, milk TAG are characterized in three levels: (1) carbon chain size, (2) level of composition in FA and (3) level of FA position; providing information, respectively, on the composition of milk fat TAG, FA composition of a lipid species and regiospecific distribution of FA on TAG molecules.

2.7.4 Identification of the carbon chain size

TAG is defined in this chapter as a series of species with the equal total number of acylated carbons (CN) and the equal number of double bonds (DB), regardless of its constitution in FA. So each TAG group has a unique chemical formula and a precise mass. Ammonium salt is added to the mobile phase so the TAG are detected as ammoniated adducts (to become more stable and avoid the formation of different adducts with the same molecule) in the positive ionization mode ESI+. The most abundant TAG groups contain 26–54 acyl CN and 1–8 DB, with molecular mass ranging from 500 to 1000.

2.7.5 Composition of FA contained in the TAG

Aiming the determination of the FA contained in the TAG, the diacylglycerols (DAG+) are formed after neutral loss of one of the three FA chains. For each TAG molecule, three DAG+ ions correspond to the loss of each of the three FA, so the FA composition of any TAG molecule can easily be deduced by the mass difference (for example, neutral loss of 245, 273 and 301 corresponding to the loss of FA 14:0, 16:0 and 18:0, respectively). As expected, each TAG group may contain different

FA compositions (referred as isomeric species), being the liquid chromatography with reverse phase (RP-LC) combined to a C18 column, non-aqueous mobile phase (generally acetonitrile and isopropanol) and a surface gradient (up to 150 min) the greatest recognized method to separate species of TAG isomers from the same group or even ECN [89].

2.7.6 Determination of FA position in the TAG

Determining the position of FA in the TAG (sn-1, sn-2 or sn-3) is more challenging. Two protocols are commonly employed to achieve it, both based on the partial hydrolysis of TAG to cleave FA at sn-1 and sn-3 with Grignard reagent [90] or pancreatic lipase [91], being the last one, the method approved by the AOCS [92]. It is followed by MAG TLC isolation and lastly the GC determination of the FA that was at the sn-2 position after transesterification of the MAG-sn-2-FA. Both methods may reveal the percentage distribution of the different FA at sn-2 position and also for a particular FA, the percentage at sn-2 position compared to sn-1/sn-3 position. In other words, this approach generates information on the overall percentage of FA in sn-2, the FA composition of a mixture and not for each individual TAG species.

It is essential to mention that the precision of these two methods depends on the complete conversion of TAG and DAG into MAG, which needs to be carefully monitored. This is a rather time-consuming procedure, so a simplified protocol combining lipase digestion with direct LC–MS and quantification of MAG is urgently needed.

2.8 Determination of TAG, phospholipids and sterols from human milk by HPLC analysis

The first approaches for the analysis of TAG in lipid-rich matrices have been based on GC coupled with FID and MS. However, in recent years, to simplify sample preparation, *i.e.*, to avoid time-consuming preliminary treatments, TAG analysis have been carried out by high performance liquid chromatography (HPLC) [93, 94]. TAG analysis by HPLC can be performed by normal phase (NP) and reversed phase (RP). Among it, RP with non-aqueous (NA) mobile phases is the most extensively used mode. In NARP-HPLC the separation is based on the equivalent carbon numbers (ECNs); TAG with the same ECN can be separated based on the position, configuration of the double bounds, the length and unsaturation of FA. In NP separation mode it has been used silver ions impregnated columns, and this mode is named as silver ion (Ag)-HPLC. In (Ag)-HPLC the separation is based on specific silver ion/double bond interactions and the retention times depends on the position, on the cis/trans double bonds configuration and increases with the unsaturation number of the chains [93, 95].

Several detection systems have been used for TAG analysis, such as ultra-violet (UV) detector, evaporative light scattering detector (ELSD) and refractive index detector (RID) which are suitable for quantitative analysis by means of reference, but it does not permit structural information. Thus, HPLC coupled to MS is the currently preferred TAG analysis technique, being electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) the favorite ionization sources [93, 96].

For phospholipids analysis, HPLC is the most commonly used chromatographic technique. PLs are primary determined by NP-HPLC, but RP-HPLC and, in the last years, hydrophilic interaction liquid chromatography (HILIC) has also been

used. In NP and HILIC modes the separation is based on the different polarity of the "headgroups" and in RP mode the separation is based on the features of the chain lengths, number of unsaturation and geometry of acyl chains [97, 98]. Among the detection systems used for phospholipids determination, low-wavelength UV detectors, ELSD, RID and most recently, charged aerosol detector (CAD), ELSD is probably the most extensively reported for phospholipids class analysis in the food matrices. The determination of phospholipids by HPLC coupled to MS has been also increased in the last years, being ESI and matrix-assisted laser desorption ionization (MALDI) the preferred ionization sources. Despite the advantages of MALDI, ESI is more used due to the difficulty in coupling HPLC to MALDI-MS [99–101].

Finally, for sterols analysis the most conventional used chromatographic techniques are GC coupled to FID or MS detection systems and HPLC coupled to UV detection systems. However, the high temperatures achieved during GC methods can cause degrade some sterols and HPLC-UV methods have relatively poor sensitivity and selectivity towards sterol molecules [102–104]. Thus, during the last decade, based on the accurate identifications and good selectivity and sensitivity of MS detectors, the use of HPLC coupled to MS for sterols analysis gained ground. Because sterols are highly lipophilic and have few polar groups, APCI is the most widely used ionization technique, although conventional ESI methods have also been applied. In the same way, RP-HPLC is the preferred analysis mode, and the analyte interactions with the stationary phase increase with increasing molecular sizes and decreasing number of double bonds in sterol molecules [104, 105].

3. Conclusions

The human milk fat contains 98% of neutral lipids (TAG, DAG and MAG) and the fatty acid composition of these constituents is directly related to the nutritional and physico-chemical properties of human milk fat. Especially, the most significant fatty acids in human milk are LCPUFA, including EPA, DHA and AA, essential for proper growth and development. Analytical techniques such as gas chromatography with flame ionization detector (GC-FID) can be employed to evaluate the fatty acid composition of human milk fat. Prior the chromatographic analysis, the lipids derivatization is essential to allow volatilization of the interest compounds. Moreover, TAG analysis can be carried out by mass spectrometry and high performance liquid chromatography (HPLC), in order to determine the FA contained in the TAG, as well as TAG's FA position. Conclusively, breastfeeding is an incomparable ideal food for the healthy growth and development of infants and offers numerous short- and long-term health benefits for breastfed newborns.

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Conflict of interest

None.

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