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Evaluation of the lipid composition of the three lactation phases of raw, pasteurized and lyophilized pasteurized human milk

Avaliação da composição lipídica das três fases de lactação do leite humano cru, pasteurizado e pasteurizado liofilizado

Evaluación de la composición lipídica de las tres fases de lactancia de la leche materna pasteurizada cruda, pasteurizada y liofilizada

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Abstract

The aim of this study was to analyze the acidity, fatty acid (FA) composition and the triacylglycerol (TAGs) profile of human milk (HM) from three lactation phases (colostrum, transitional and mature) submitted to different treatments (raw milk, pasteurized, pasteurized in conjunction with lyophilization), in order to verify whether these processes applied to the samples can influence the characteristics of the analyzed components. To carry out the analyzes, the project was approved by the ethics committee and the HM was acquired at the Human Milk Bank (HMB) of the University Hospital of Maringá - HUM (Paraná, Brazil). The acidity analysis was performed using the titratable acidity method in Dornic degrees (ºD), the composition in FAs from Gas Chromatography with Flame Ionization Detector (GC-FID), and the TAGs profile by Mass Spectrometry with Electrospray Ionization source (ESI-MS). From the results obtained, it was possible to observe that the Dornic acidity and the composition in AGs did not undergo significant changes by the Tukey test (p<0.05) and the TAG profile remained similar after the application of the processing, when compared samples of raw HM from their respective phase. Therefore, the pasteurization technique in conjunction with freeze drying can be a promising alternative for HM storage and conservation in HMBs, as it guarantees the preservation of the evaluated components, in addition to reducing the storage volume and facilitating the transport of this HM.

Keywords: Gas chromatography; Mass spectrometry; Fatty acids; Triacylglycerol; Acidity.

Resumo

O objetivo deste estudo foi analisar a acidez, a composição em ácidos graxos (AG) e o perfil de triacilgliceróis (TAGs) do leite humano (LH) das três fases de lactação (colostro, transição e maduro), submetidos a diferentes tratamentos (leite cru, pasteurizado, pasteurizado em conjunto com liofilização), com o intuito de verificar se estes processamentos aplicados nas

amostras podem influenciar nas características dos componentes analisados. Para a realização das análises, o projeto foi aprovado pelo comitê de ética e o LH foi adquirido no Banco de Leite (BLH) do Hospital Universitário de Maringá – HUM (Paraná, Brasil). A análise de acidez foi realizada utilizando-se o método de acidez titulável em graus Dornic (ºD), a composição em AGs a partir de Cromatografia em Fase Gasosa com Detector de Ionização em Chama (CG-DIC), e o perfil de TAGs por Espectrometria de Massas com fonte de Ionização Eletrospray (ESI-MS). A partir dos resultados obtidos, foi possível observar que a acidez Dornic e a composição em AGs não sofreram alterações significativas pelo teste de Tukey (p<0,05) e o perfil de TAG manteve-se similar após a aplicação dos processamentos, quando comparados as amostras de LH cru de sua respectiva fase. Portanto, a técnica de pasteurização em conjunto com a liofilização pode ser uma alternativa promissora de armazenamento e conservação de LH em BLHs, já que garante a preservação dos componentes avaliados, além de favorecer a redução do volume de estocagem e facilitar o transporte deste LH.

Palavras-chave: Cromatografia em fase gasosa; Espectrometria de massas; Ácidos Graxos; Triacilglicerol; Acidez.

Resumen

El objetivo de este estudio fue analizar la acidez, composición de ácidos grasos (AG) y el perfil de triacilglicerol (TAGs) de la leche materna (LH) de tres fases de lactancia (calostro, transición y maduración) sometidas a diferentes tratamientos (leche cruda), pasteurizados, pasteurizados en conjunto con liofilización), con el fin de verificar si estos procesos aplicados a las muestras pueden influir en las características de los componentes analizados. Para realizar los análisis, el proyecto fue aprobado por el comité de ética y la LH se adquirió en el Banco de Leite (BLH) del Hospital Universitario de Maringá - HUM (Paraná, Brasil). El análisis de acidez se realizó mediante el método de acidez titulable en grados Dornic (ºD), la composición en AGs de Cromatografía de Gases con Detector de Ionización de Llama (CG-FID), y el perfil de TAGs por Espectrometría de masas con fuente de ionización Eletrospray (ESI-MS). De los resultados obtenidos se pudo observar que la acidez Dornic y la composición en AGs no sufrieron cambios significativos por la prueba de Tukey (p<0.05) y el perfil de TAG permaneció similar luego de la aplicación del procesamiento, al comparar muestras de LH cruda de su respectiva fase. Por tanto, la técnica de pasteurización en conjunto con la liofilización puede ser una alternativa prometedora para el almacenamiento y conservación de LH en HMB, ya que garantiza la conservación de los componentes

evaluados, además de reducir el volumen de almacenamiento y facilitar el transporte de esta LH.

Palabras clave: Cromatografía de gases; Espectrometría de masas; Ácidos grasos; Triacilglicerol; Acidez.

1. Introduction

Human milk (HM) is considered an ideal food for neonates, as it is a dynamic fluid capable of adapting and perfectly meeting the needs of the newborn (Andreas et al., 2015). For this reason, exclusive breastfeeding during the first six months of life is recommended for the child's proper growth and development (Who, 2003).

Lipids are components of HM that play a fundamental role in neurological development and meet the child's high energy needs; besides being sources of several long-chain polyunsaturated fatty acids (LC-PUFA), such as the docosahexaenoic acid (DHA) of the omega family 3 and arachidonic acid (AA) from the omega 6 family, which are related to cognitive development. The amount of neonatal cerebral DHA is related to good cognitive and behavioral performance and AA is important for cell growth and signaling, from the pathway of formation of eicosanoids and lipoxins (McCann & Ames, 2005). Therefore, some lipids present in the HM have important biological roles related to the newborn's nervous and immune functions (Koletzko, 2016).

Studies carried out recently suggest that there is variation in the composition of some fatty acids (FA) throughout the lactation phases (Wang, 2020; Floris et al., 2020). As for the stages of breastfeeding, colostrum is the first fluid released by the mammary glands in small and sufficient volume to meet the needs of the neonate, during the first 30–40 hours after birth, with a high concentration of immunological components (Pang & Hartmann, 2007). From the fifth day until two weeks postpartum the HM is considered to be transitional milk and has higher concentrations of calories to meet the baby's growth needs. After two weeks, human milk is characterized as mature milk (Ballard & Morrow, 2013).

In addition to the lactation stage, other factors can also influence the components of HM, such as the conditions of the lactating mother's diet, her geographical region and other factors (Delplanque et al., 2015; Deng et al., 2018). Because of this, in exceptional situations in which the baby cannot be breastfed, the receipt of expressed breast milk is provided by the human milk banks (HMB) (Who, 2003) and, under these conditions, factors such as: milk

expression, heat treatments (such as pasteurization), freezing and thawing cycles and the duration of storage can also alter the composition of HM (Ballard & Morrow, 2013).

As there is a risk of transmission of pathogens through the donated HM, it is mandatory that this HM be submitted to a quality control protocol in the HMBs, which includes pasteurization of HM, being carried out by Holder pasteurization, a method carried out at 62.5 ºC for 30 minutes, followed by freezing at -18 °C (Ballard & Morrow, 2013). However, new alternatives are being studied in order to preserve the properties of HMB, such as lyophilization, which is a technology that aims to increase the useful life, facilitate transportation and preserve the nutritional characteristics of HM donated to HMB (Lozano et al., 2014; Cortez & Soria, 2016; Martysiak-Żurowska et al., 2020).

Thus, this work aimed to analyze Dornic acidity and lipid composition, using gas chromatography methodologies with flame ionization detector (GC-FID) and electrospray mass spectrometry (ESI-MS), of HM (colostrum, transitional and mature) raw, pasteurized and lyophilized pasteurized, in order to verify if the techniques are capable of conserving these components.

2. Materials and Methods

Samples

The selected design was a study of a quantitative nature (Pereira, Shitsuka, Pereira, & Shitsuka, 2018). HM samples were collected in the HMB of the University Hospital of Maringá (HUM, Maringá, Paraná), separated according to availability, with the approval number of the Human Research Ethics Committee 2.797.476. HM raw of the three phases was used (colostrum, transitional and mature), that have been separated into three pools according to their respective phases, mixed and agitated until total homogenization of all samples, with a final volume of 300 mL for each phase.

Pasteurization

Pasteurization processing was carried out in accordance with the current Brazilian HMBs standard, from heating in a water bath, using a temperature in the middle of the 62.5 °C flask for 30 minutes, with manual stirring every five minutes. Subsequently, the HM samples were cooled to a temperature below 4 °C, and frozen in a domestic refrigerator at -

18 °C, as described by Almeida, Guimarães & Novak (2005). Subsequently, 200 mL of each HM phase was pasteurized, with half the volume of HM frozen at -18 °C and the other half freeze-dried. To carry out the analyzes, the pasteurized HM was thawed at 4 ºC in a water bath.

Liofilization

Half of the volume (100 mL) of pasteurized HM samples from each lactation phase was lyophilized, in aluminum forms for a period of 36 hours in a Lyophilizer (Alpha 1-2 LD Plus, model 101522), at a temperature of approximately -50 °C and pressure of 0.023 mbar. The powdered milk was vacuum-packed, in aluminum bags free from light, frozen at -18 °C, for further analysis. To carry out the analyzes, pasteurized and lyophilized milk was reconstituted with water according to the volume of water extracted.

Table 1 shows the final volume of HM for each processing.

Table 1 - HM volume (colostrum, transitional and mature) for each processing.

Lactation stages	**Raw** (mL)	**Pasteurized** (mL)	**Lyophilized Pasteurized** (mL)
Colostrum	100	100	100
Transitional	100	100	100
Mature	100	100	100

Source: Authors.

Determination of acidity in Dornic degrees

For all samples of raw HM, pasteurized HM and pasteurized lyophilized HM, of the colostrum, transitional and mature lactation phases, acidity analyzes were performed in Dornic degrees (º D), according to the Instituto Adolfo Lutz (2008).

Extraction and esterification of total lipids

The extraction of total lipids was performed according to the method of Folch et al. (1957) and the esterification of fatty acids (FA), according to the methodology proposed by

ISO 5509 (2000). This procedure was carried out with HM samples, in the three lactation phases, raw HM, pasteurized HM and pasteurized lyophilized HM.

Fatty Acid Composition by GC-FID

The FAs of the HM samples were analyzed in the three lactation phases, raw HM, pasteurized HM and pasteurized lyophilized HM. The fatty acid methyl esters (FAME) were separated according to the methodology proposed by Simionato et al. (2010), using a gas chromatograph (GC) TRACE™ Ultra Thermo Scientific™ (Thermo Scientific™, USA), with flame ionization detector (FID) and a fused silica column (100 m x 0.25 mm internal diameter, 0.25 µm cyanopropyl, CP-7420). Gas flows were 1.4 mL min^{-1} for Hydrogen carrier gas (H_2), 30 mL min^{-1} for Nitrogen auxiliary gas (N_2), and 30 and 300 mL min^{-1} for Hydrogen (H_2) and synthetic air gases, respectively. A sample volume of 2 µL was injected in triplicate with a sample division of 1:100. The column temperature was raised to 65 °C for 4 min, followed by a heating ramp from 16 °C min^{-1} to 185 °C, maintained for 12 min. After that, a new ramp of 20 °C min^{-1} was applied up to 235 °C, and maintained for 14 min, totaling a total analysis time of 40 min.

The identification of FAMEs was carried out by comparing the retention times with the relative analytical standards (FAME Mix, C4-C24, Sigma-Aldrich), and the results expressed in relative percentage (%) of the total fatty acids , automatically processed using the Chromquest software™ 5.0.

Determination of triacylglycerols by direct infusion by ESI-MS

The triacylglycerol profiles (TAG) of HM samples were analyzed in the three lactation phases, raw HM, pasteurized HM and lyophilized pasteurized HM. To obtain the TAG profile via direct infusion by Mass Spectrometry (MS), the samples were infused directly into an XEVO TQ-D mass spectrometer (Waters, Massachusetts, United States) with an electrospray ionization source (ESI) operating in positive mode, comprising the range of *m/z* 100-1200, in triplicate.

The preparation of the samples for analysis by ESI-MS was carried out in accordance Silveira et al. (2017), where 50.0 µL of HM lipid were diluted in 950.0 µL of chloroform. Then, 5.0 µL of this solution was added to 1.0 mL of methanol/chloroform in a 9:1 ratio and also 20.0 µL of 0.10 $mol.L^{-1}$ ammonium formate solution (prepared in methanol). This last

addition was carried out aiming at the formation of ammonium adducts. The operating conditions of the equipment were: capillary voltage 3.00 kV, cone voltage 20.00 V and dessolvation temperature 200 ºC. The samples were injected with a continuous flow of 10.0 µL min^{-1}.

TAG assignment and estimation

TAG ions were assigned and estimated in percentage (%) via LAMES Platform, which is based on the mathematical algorithm that describes the distribution of FAs in TAG molecules (Filho, Mendes, & Lanças, 1995), using the FA percentage determined by GC-FID. With the Lipid maps® database, it was possible to find a molecular formula for TAGs.

Statistical analysis

The results of determination of acidity and composition in FAs obtained were subjected to analysis of variance (ANOVA) with 5% significance (p<0.05) and the means were compared by the Tukey test, with the software Assistat version 7.7 (Silva & Azevedo, 2008).

3. Results and Discussions

Acidity (in Dornic degrees)

Table 2 shows the results of Dornic titratable acidity in samples of raw, pasteurized and lyophilized pasteurized HM, in the colostrum, transitional and mature samples. The acidity values in Dornic degrees (°D) had the following ranges: colostrum, from 3.66 to 4.33 °D; transitional, from 3.66 to 4.33 °D; and mature, from 4.33 to 5.00 °D. The results presented had no significant differences (p<0.05) between them, that is, the acidity was not influenced by the treatments applied.

Table 2 - Dornic acidity of human milk (colostrum, transitional and -mature) raw, pasteurized and pasteurized lyophilized.

Samples	Dornic degrees (º D)		
	Raw	Pasteurized	Pasteurized Lyophilized
Colostrum	3.66 ± 0.57^{A}	4.33 ± 0.57^{A}	4.33 ± 0.57^{A}
Transitional	3.66 ± 0.57^{A}	4.33 ± 0.57^{A}	4.33 ± 0.57^{A}
Mature	4.33 ± 0.57^{A}	4.66 ± 1.15^{A}	5.00 ± 1.00^{A}

Results expressed as mean ± standard deviation of the triplicate. Values with different letters on the same line are significantly different ($p < 0.05$) by the Tukey test.
Source: Authors.

After the immediate milking of the HM, there is no formation of lactic acid and its acidity is considered original, with values ranging between 1 and 4 °D, corroborating with the values found for raw HM in colostrum and in the transitional phases of this work (Anvisa, 2008). The storage time of HM favors elevation conditions of the microbiota, which produces lactic acid and, consequently, the acidity is high (Várquez-Román et al., 2016).

The increase in acidity causes a decrease in nutritional quality, causing the destabilization of serum proteins and casein micelles and, as a result, their coagulation, making the availability of minerals such as calcium and phosphorus difficult. These changes increase osmolarity, in addition to causing sensory changes in odor and taste and decreased immunological properties of HM (Pereira, Dametto, & Oliveira, 2016). According to Anvisa (2008), to be considered appropriated, the acidity of HM for use in HMB must be in the range between 1 to 8 °D. Therefore, the studied samples were appropriate for use, since the different treatments applied, do not interfered in the increase of acidity, in which they varied between 3.66 to 5.00 °D.

Fatty acid composition by GC-FID

Tables 3, 4 and 5 show the results obtained from the FAs compositions in percentage of relative area (%) of HM, in the different lactation phases (colostrum, transitional and mature), and different heat treatments (raw, pasteurized, lyophilized pasteurized), obtained by GC-FID analysis.

Table 3 - Fatty acid composition (%) of colostrum human milk, raw, pasteurized and lyophilized pasteurized.

Fatty acid composition	Colostrum (%)		
	CR	CP	CL
10:0	0.43 ± 0.01^{A}	0.42 ± 0.07^{A}	0.43 ± 0.01^{A}
12:0	3.32 ± 0.06^{A}	3.30 ± 0.16^{A}	3.20 ± 0.12^{A}
14:0	5.89 ± 0.11^{A}	5.97 ± 0.10^{A}	5.79 ± 0.07^{A}
14:1n-9	0.07 ± 0.01^{A}	0.06 ± 0.00^{A}	0.06 ± 0.01^{A}
15:0	0.29 ± 0.01^{A}	0.30 ± 0.00^{A}	0.29 ± 0.00^{A}
15:1n-9	0.07 ± 0.00^{A}	0.07 ± 0.00^{A}	0.07 ± 0.00^{A}
16:0	26.21 ± 0.26^{A}	26.97 ± 0.57^{A}	26.01 ± 0.33^{A}
16:1n-9	1.48 ± 0.06^{A}	1.45 ± 0.07^{A}	1.48 ± 0.05^{A}
17:0	0.41 ± 0.03^{A}	0.45 ± 0.01^{A}	0.43 ± 0.01^{A}
17:1n-9	0.19 ± 0.03^{A}	0.18 ± 0.01^{A}	0.17 ± 0.01^{A}
18:0	7.00 ± 0.07^{A}	6.88 ± 0.20^{A}	7.00 ± 0.07^{A}
18:1n-9	31.69 ± 0.22^{A}	30.62 ± 0.35^{A}	32.14 ± 0.17^{A}
18:2n-6	17.95 ± 0.35^{A}	18.51 ± 0.47^{A}	17.93 ± 0.03^{A}
18:3n-3	1.07 ± 0.10^{A}	1.05 ± 0.05^{A}	1.04 ± 0.03^{A}
20:0	0.20 ± 0.04^{A}	0.19 ± 0.03^{A}	0.20 ± 0.00^{A}
20:1n-9	0.54 ± 0.08^{A}	0.51 ± 0.06^{A}	0.53 ± 0.07^{A}
21:0	0.68 ± 0.10^{A}	0.69 ± 0.03^{A}	0.70 ± 0.03^{A}
20:3n-6	0.81 ± 0.06^{A}	0.84 ± 0.15^{A}	0.85 ± 0.03^{A}
20:3n-3	0.13 ± 0.01^{A}	0.12 ± 0.05^{A}	0.15 ± 0.02^{A}
20:4n-6	0.18 ± 0.02^{A}	0.16 ± 0.00^{A}	0.15 ± 0.04^{A}
22:0	0.50 ± 0.03^{A}	0.46 ± 0.04^{A}	0.48 ± 0.03^{A}
20:5n-3	0.09 ± 0.01^{A}	0.11 ± 0.00^{A}	0.13 ± 0.02^{A}
22:1n-9	0.15 ± 0.03^{A}	0.13 ± 0.02^{A}	0.13 ± 0.02^{A}
24:0	0.14 ± 0.01^{A}	0.12 ± 0.01^{A}	0.15 ± 0.02^{A}
24:1n-9	0.21 ± 0.02^{A}	0.18 ± 0.04^{A}	0.22 ± 0.02^{A}
22:6n-3	0.27 ± 0.03^{A}	0.24 ± 0.00^{A}	0.28 ± 0.02^{A}
ΣSFA	45.09 ± 0.47^{A}	45.76 ± 0.71^{A}	44.68 ± 0.58^{A}
ΣMUFA	34.39 ± 0.50^{A}	33.21 ± 0.95^{A}	34.80 ± 0.74^{A}
ΣPUFA	20.52 ± 0.53^{A}	21.03 ± 0.37^{A}	20.53 ± 0.76^{A}
Σ n-3	1.57 ± 0.13^{A}	1.52 ± 0.09^{A}	1.59 ± 0.05^{A}

Σ n-6	18.95 ± 0.28^{A}	19.51 ± 0.58^{A}	18.93 ± 0.05^{A}

Results expressed as mean ± standard deviation of the triplicate. Values with different letters on the same line are significantly different (p<0.05) by Tukey test. SFA - saturated fatty acids; MUFA - monounsaturated fatty acids; PUFA - polyunsaturated fatty acids; CR– Raw colostrum milk; CP - Pasteurized colostrum milk; CL - Lyophilized pasteurized colostrum milk.
Source: Authors.

Table 4 - Fatty acid composition (%) of transitional human milk raw, pasteurized and lyophilized pasteurized.

Fatty acid composition	**Transitional (%)**		
	TR	**TP**	**TL**
10:0	1.93 ± 0.05^{A}	1.97 ± 0.04^{A}	1.95 ± 0.05^{A}
12:0	10.42 ± 0.10^{A}	10.34 ± 0.14^{A}	10.06 ± 0.13^{A}
14:0	9.42 ± 0.06^{A}	9.44 ± 0.02^{A}	8.97 ± 0.08^{A}
14:1n-9	0.05 ± 0.00^{A}	0.05 ± 0.00^{A}	0.05 ± 0.00^{A}
15:0	0.16 ± 0.00^{A}	0.16 ± 0.00^{A}	0.16 ± 0.00^{A}
15:1n-9	0.03 ± 0.00^{A}	0.03 ± 0.00^{A}	0.03 ± 0.00^{A}
16:0	23.23 ± 0.14^{A}	23.56 ± 0.20^{A}	23.52 ± 0.03^{A}
16:1n-9	1.67 ± 0.02^{A}	1.72 ± 0.02^{A}	1.72 ± 0.02^{A}
17:0	0.24 ± 0.01^{A}	0.25 ± 0.01^{A}	0.26 ± 0.00^{A}
17:1n-9	0.13 ± 0.01^{A}	0.13 ± 0.00^{A}	0.14 ± 0.00^{A}
18:0	4.80 ± 0.01^{A}	4.80 ± 0.21^{A}	4.87 ± 0.06^{A}
18:1n-9	26.64 ± 0.26^{A}	26.87 ± 0.06^{A}	26.91 ± 0.27^{A}
18:2n-6	17.88 ± 0.09^{A}	17.66 ± 0.07^{A}	18.12 ± 0.22^{A}
18:3n-3	0.93 ± 0.02^{A}	0.97 ± 0.04^{A}	1.07 ± 0.09^{A}
20:0	0.10 ± 0.01^{A}	0.10 ± 0.01^{A}	0.11 ± 0.00^{A}
20:1n-9	0.40 ± 0.01^{A}	0.33 ± 0.05^{A}	0.43 ± 0.06^{A}
21:0	0.54 ± 0.10^{A}	0.51 ± 0.06^{A}	0.52 ± 0.01^{A}
20:3n-6	0.55 ± 0.07^{A}	0.48 ± 0.11^{A}	0.52 ± 0.03^{A}
20:3n-3	0.12 ± 0.01^{A}	0.10 ± 0.02^{A}	0.09 ± 0.04^{A}
20:4n-6	0.09 ± 0.01^{A}	0.08 ± 0.02^{A}	0.10 ± 0.01^{A}
22:0	0.15 ± 0.01^{A}	0.16 ± 0.02^{A}	0.16 ± 0.00^{A}
20:5n-3	0.04 ± 0.00^{A}	0.04 ± 0.01^{A}	0.06 ± 0.01^{A}
22:1n-9	0.06 ± 0.00^{A}	0.05 ± 0.02^{A}	0.07 ± 0.01^{A}
24:0	0.04 ± 0.01^{A}	0.06 ± 0.01^{A}	0.06 ± 0.01^{A}

24:1n-9	0.06 ± 0.00^{A}	0.08 ± 0.01^{A}	0.09 ± 0.00^{A}
22:6n-3	0.16 ± 0.02^{A}	0.13 ± 0.03^{A}	0.17 ± 0.01^{A}
ΣSFA	51.21 ± 0.16^{A}	51.35 ± 0.26^{A}	50.37 ± 0.26^{A}
ΣMUFA	29.28 ± 0.23^{A}	29.26 ± 0.33^{A}	29.78 ± 0.48^{A}
ΣPUFA	19.51 ± 0.18^{A}	19.46 ± 0.18^{A}	19.85 ± 0.32^{A}
Σ n-3	1.27 ± 0.03^{A}	1.24 ± 0.03^{A}	1.30 ± 0.05^{A}
Σ n-6	18.24 ± 0.15^{A}	18.22 ± 0.15^{A}	18.55 ± 0.26^{A}

Results expressed as mean ± standard deviation of the triplicate. Values with different letters on the same line are significantly different ($p < 0.05$) by the Tukey test. SFA - saturated fatty acids; MUFA - monounsaturated fatty acids; PUFA - polyunsaturated fatty acids; TR - Raw transitional milk; TP - Pasteurized transitional milk; TL - Lyophilized pasteurized transitional milk.
Source: Authors.

Table 5 - Fatty acid composition (%) of mature human milk raw, pasteurized and lyophilized pasteurized.

Fatty acid composition	**Mature (%)**		
	MR	**MP**	**ML**
10:0	1.03 ± 0.05^{A}	1.02 ± 0.04^{A}	0.99 ± 0.03^{A}
12:0	6.90 ± 0.10^{A}	6.95 ± 0.27^{A}	6.63 ± 0.02^{A}
14:0	7.55 ± 0.10^{A}	7.40 ± 0.29^{A}	7.19 ± 0.06^{A}
14:1	0.08 ± 0.01^{A}	0.08 ± 0.00^{A}	0.09 ± 0.01^{A}
15:0	0.21 ± 0.01^{A}	0.21 ± 0.01^{A}	0.21 ± 0.00^{A}
15:1	0.06 ± 0.00^{A}	0.06 ± 0.00^{A}	0.06 ± 0.00^{A}
16:0	22.47 ± 0.24^{A}	22.23 ± 0.88^{A}	21.78 ± 0.13^{A}
16:1n-9	1.62 ± 0.12^{A}	1.68 ± 0.07^{A}	1.70 ± 0.03^{A}
17:0	0.36 ± 0.01^{A}	0.34 ± 0.01^{A}	0.33 ± 0.03^{A}
17:1	0.20 ± 0.02^{A}	0.20 ± 0.01^{A}	0.19 ± 0.01^{A}
18:0	7.57 ± 0.14^{A}	7.40 ± 0.29^{A}	7.36 ± 0.06^{A}
18:1n-9	31.74 ± 0.59^{A}	32.05 ± 1.26^{A}	32.68 ± 0.44^{A}
18:2n-6	17.29 ± 0.23^{A}	17.91 ± 0.71^{A}	17.64 ± 0.17^{A}
18:3n-3	1.04 ± 0.03^{A}	1.09 ± 0.04^{A}	1.08 ± 0.03^{A}
20:0	0.18 ± 0.03^{A}	0.21 ± 0.01^{A}	0.22 ± 0.01^{A}
20:1n-9	0.33 ± 0.05^{A}	0.35 ± 0.01^{A}	0.39 ± 0.02^{A}
21:0	0.34 ± 0.02^{A}	0.39 ± 0.02^{A}	0.37 ± 0.01^{A}
20:3n-6	0.43 ± 0.07^{A}	0.45 ± 0.02^{A}	0.52 ± 0.03^{A}

20:3n-3	0.11 ± 0.00^{A}	0.11 ± 0.00^{A}	0.09 ± 0.03^{A}
20:4n-6	0.07 ± 0.02^{A}	0.08 ± 0.00^{A}	0.08 ± 0.00^{A}
22:0	0.10 ± 0.01^{A}	0.08 ± 0.00^{A}	0.10 ± 0.01^{A}
20:5n-3	0.04 ± 0.01^{A}	0.05 ± 0.00^{A}	0.04 ± 0.00^{A}
22:1n-9	0.03 ± 0.01^{A}	0.02 ± 0.00^{A}	0.04 ± 0.00^{A}
24:0	0.02 ± 0.00^{A}	0.02 ± 0.00^{A}	0.01 ± 0.00^{A}
24:1n-9	0.11 ± 0.02^{A}	0.08 ± 0.00^{A}	0.11 ± 0.00^{A}
22:6n-3	0.13 ± 0.01^{A}	0.13 ± 0.01^{A}	0.13 ± 0.01^{A}
ΣSFA	46.73 ± 0.35^{A}	46.26 ± 1.82^{A}	45.18 ± 0.21^{A}
ΣMUFA	34.17 ± 0.52^{A}	34.53 ± 1.36^{A}	35.24 ± 0.39^{A}
ΣPUFA	19.10 ± 0.34^{A}	19.83 ± 0.78^{A}	19.58 ± 0.20^{A}
Σ n-3	1.32 ± 0.03^{A}	1.38 ± 0.05^{A}	1.34 ± 0.05^{A}
Σ n-6	17.79 ± 0.30^{A}	18.44 ± 0.73^{A}	18.23 ± 0.16^{A}

Results expressed as mean ± standard deviation of the triplicate. Values with different letters on the same line are significantly different ($p < 0.05$) by the Tukey test. SFA - saturated fatty acids; MUFA - monounsaturated fatty acids; PUFA - polyunsaturated fatty acids; MR - raw mature milk; MP - Pasteurized mature milk; ML - lyophilized pasteurized mature milk.
Source: Authors.

The Tables 3, 4 and 5 show a total of twenty-six FAs were identified in the samples of lyophilized raw, pasteurized and pasteurized HM (colostrum, transitional and mature), when compared to the raw HM of the lactation phases, with pasteurized HM and pasteurized lyophilized HM and did not presented a significant difference at the level of 5% of significance by the Tukey test ($p<0.05$).

According to Tables 3, 4 and 5, the FAs which obtained higher concentrations among the nine samples analyzed were oleic acid (O, 18:1n-9) and palmitic acid (P, 16:0). O (18:1n-9) concentration, considered a monounsaturated fatty acid (MUFA), in colostrum varied from 30.62 to 32.14%; in the transitional milk between 26.64 to 26.91%; and in mature milk 31.74 to 32.68%, being this FA the main energy source of HM. In addition, this FA is strategically positioned at the ends of the TAG, favoring the attack of digestive enzymes, enhancing its absorption, as well as the mineral calcium (Rydlewski et al., 2020). P (16:0) is classified as saturated fatty acid (SFA) and in the colostrum the obtained values between 26.01 to 26.97%; in the transitional milk: 23.23 to 23.56%; and in mature milk: 21.78 to 22.47%. This SFA is important because it has analgesic effects by raising the levels of anandamide, a neurotransmitter, which can produce sedation in neonates (Mayo et al., 2020).

Among the studied HM samples, polyunsaturated fatty acids (PUFA) ranged from 19.10 to 21.03%; MUFAs from 29.26 to 35.24%; and SFAs, from 44.68 to 46.73%. Our results were approximate results to the work of Silva et al. (2005) and Lubetzky et al. (2016), who studied the composition in HM FAs of Brazilian lactating mothers and obtained concentrations of PUFAs, MUFAs and SFAs of 23.04, 27.60 and 39.70%, and 20.6, 38.0 and 43.0%, respectively.

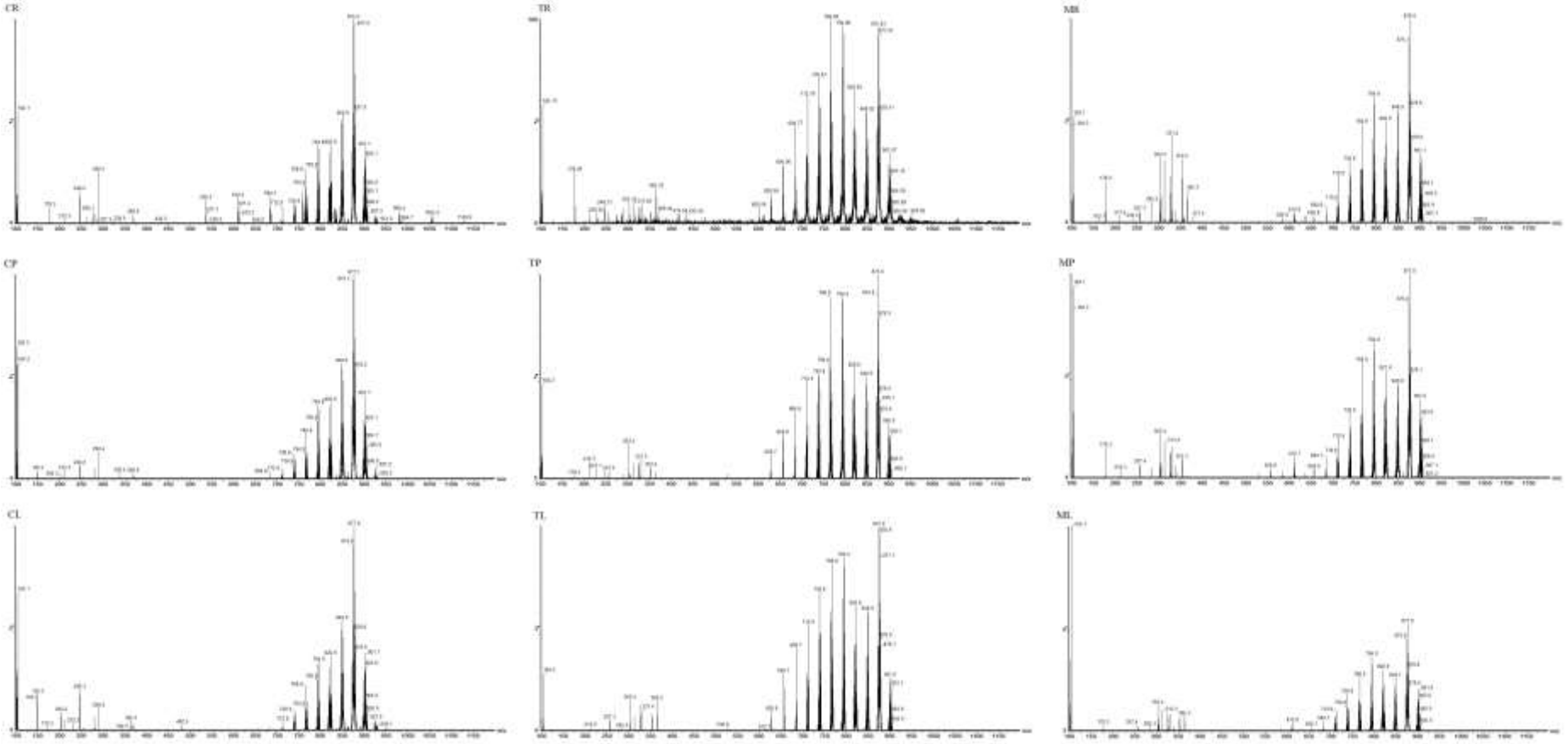
Regarding MUFAs, linoleic acid (18:2n-6) from the omega-6 family and α-linolenic acid (18:3n-3) from the omega-3 family are considered essential FAs, that is, they need to be purchased from the feeding, the evaluated HMs presented values between 17.29 to 18.51%, and 0.93 to 1.09%, respectively, values similar to other studies found in the literature (Kuipers et al., 2012; Manin et al., 2019). These MUFAs have important functions, especially in early life, where growth is intense, in addition to helping to form the immune system and the central nervous system. Consequently, the neonate will benefit in terms of preventing the development of diseases in adulthood (Koletzko, 2017).

Nessel et al. (2019) wrote a review of the effects of storage conditions on donated HM FAs, noting that Holder pasteurization, currently applied to HMBs, did not significantly change the content of FAs in HM, showing that the technique is effective for the conservation of this nutrient. Cavazos-Garduño et al., (2016) studied the effect of pasteurization and lyophilization applied on HM and these processes did not change the profile of FAs. Martysiak-Żurowska et al. (2020) and Manin et al. (2019) evaluated the effect of lyophilization on the FA profile of lyophilized HM, and when compared to raw HM, the results did not presented significant difference. Thus, it was possible to conclude that lyophilization can be a promising method, because it allows a longer storage time of HM, being a viable alternative to freezing, currently used in HMBs.

Determination of Triacylglycerol by ESI(+)-MS

Figure 1 presented the results of the determination of TAGs carried out by direct infusion in ESI-MS, which shows the spectra of HM in the different phases (colostrum, transitional and mature) in different heat treatments (raw, pasteurized, pasteurized lyophilized), obtained by this analysis, in the range of *m/z* from 110 to 1200.

Figure 1 - TAG ion spectra of human milk from different lactation phases (colostrum, transitional, mature) submitted to different treatments (raw, pasteurized, pasteurized and lyophilized pasteurized) defined by direct infusion in ESI(+)-MS.



CR - Raw colostrum milk; CP - Pasteurized colostrum milk; CL - Lyophilized Pasteurized colostrum milk; TR - Raw transitional milk; TP - Pasteurized transitional milk; TL - Lyophilized Pasteurized transitional milk; MR - Raw mature milk; MP - Pasteurized mature milk; ML - Lyophilized Pasteurized mature milk.
Source: Authors.

This analysis is important to identify the profile of possible TAGs present in milk samples in different stages of lactation (colostrum C, transitional T and mature M) raw (R), pasteurized (P) and lyophilized pasteurized (L), and compare their correlation given the composition of TAG present. The most intense ion spectral peak of C, T and M in HM was present between *m/z* 875 $[TAG+NH_4]^+$ PLO and 877 $[TAG+NH_4]^+$ POO. These results corroborate with Manin et al. (2019) and Rydlewski et al. (2019), in which, analyzed the lipid profile of TAG in HM in the different stages of lactation, in addition to being in accordance with Table 3, 4 and 5, the FAs. O: oleic acid (18:1n-9); P: palmitic acid (16:0) and L: linoleic acid (18:2n-6) showed higher concentrations in the samples analyzed by GC-FID and the TAGs found through the *m/z* ratio by ESI(+)-MS were composed mainly of these three FAs.

Estimation of identification of the Triacylglycerols (TAG) by the LAMES platform

Table 6 shows the estimates of 24 *m/z*, with their respective HM TAGs from different stages of lactation, submitted to different heat treatments, found in the region between 740 to 904 *m/z*. This analysis was performed using the LAMES Platform, developed for the random configuration of TAGs for vegetable oils (Filho et al., 1995).

Table 6 - Estimation of TAG ions (%) determined by ESI(+)-MS of human milk from different lactation phases submitted to different heat treatments defined by the LAMES software.

					Estimate (%)								
Molecular				**TAG**	**Colostrum**			**Transitional**			**Mature**		
formula	**CN/DB**	**Ionização**	***m/z***	**assigment**	**CR**	**CP**	**CL**	**TR**	**TP**	**TL**	**MR**	**MP**	**ML**
$C_{45}H_{86}O_6$	42:0	$[M+NH_4]^+$	740	PMLa	0.310	0.321	0.292	2.067	2.095	2.005	0.699	0.690	0.622
$C_{47}H_{86}O_6$	44:2	$[M+NH_4]^+$	764	LaLM	0.213	0.220	0.199	1.595	1.580	0.537	0.537	0.556	0.502
$C_{47}H_{86}O_6$	44:1	$[M+NH_4]^+$	766	LaOM	0.366	0.364	0.360	2.396	2.352	2.261	0.994	0.979	0.932
$C_{47}H_{90}O_6$	44:0	$[M+NH_4]^+$	768	PLaP	0.695	0.722	0.659	2.550	2.630	2.617	1.483	1.509	0.941
$C_{49}H_{90}O_6$	46:2	$[M+NH_4]^+$	792	PLLa	0.955	0.989	0.900	3.936	3.967	4.032	2.281	2.433	2.230
$C_{49}H_{92}O_6$	46:1	$[M+NH_4]^+$	794	POLa	2.446	2.431	2.357	5.914	5.906	5.903	4.219	4.281	4.143
$C_{49}H_{94}O_6$	46:0	$[M+NH_4]^+$	796	PMP	1.851	1.950	1.733	2.305	2.400	2.332	1.612	1.595	1.507
$C_{51}H_{92}O_6$	48:3	$[M+NH_4]^+$	818	LaLO	1.680	1.666	1.610	4.563	4.455	4.546	3.244	3.452	3.345
$C_{51}H_{94}O_6$	48:2	$[M+NH_4]^+$	820	LaOO	0.970	0.927	1.453	3.429	3.316	3.328	3.000	3.038	3.108
$C_{51}H_{94}O_6$	48:2	$[M+NH_4]^+$	820	PLM	2.543	2.673	2.377	3.557	3.620	3.592	2.479	2.572	2.433
$C_{51}H_{96}O_6$	48:1	$[M+NH_4]^+$	822	POM	4.374	4.421	4.272	5.346	5.390	5.260	4.586	4.526	4.520
$C_{51}H_{96}O_6$	48:1	$[M+NH_4]^+$	822	SOLa	0.434	0.418	0.434	0.806	0.786	1.007	1.425	0.979	0.958
$C_{51}H_{98}O_6$	48:0	$[M+NH_4]^+$	824	PPP	2.772	2.925	2.609	1.896	2.009	2.030	1.612	1.595	1.521
$C_{53}H_{96}O_6$	50:3	$[M+NH_4]^+$	846	MLO	3.004	3.029	2.918	4.124	4.065	4.051	3.526	3.649	3.649
$C_{53}H_{98}O_6$	50:2	$[M+NH_4]^+$	848	PLP	5.711	6.013	5.347	4.390	4.545	4.690	3.719	3.858	3.683
$C_{53}H_{98}O_6$	50:2	$[M+NH_4]^+$	848	MOO	2.583	2.505	2.633	3.099	3.026	2.966	3.261	3.211	3.390
$C_{53}H_{100}O_6$	50:1	$[M+NH_4]^+$	850	POP	9.822	9.946	9.648	6.597	6.766	6.867	6.879	6.789	6.843
$C_{53}H_{100}O_6$	50:1	$[M+NH_4]^+$	850	SOM	0.776	0.760	0.787	0.728	0.717	1.094	1.549	1.509	1.534
$C_{53}H_{102}O_6$	50:0	$[M+NH_4]^+$	852	SPP	2.197	2.243	2.091	0.775	0.802	1.154	1.634	1.595	1.549

$C_{55}H_{98}O_6$	52:4	$[M+NH_4]^+$	872	PLL	3.922	2.772	3.653	3.387	3.428	3.612	2.860	3.111	2.973
$C_{55}H_{101}O_6$	52:3	$[M+NH_4]^+$	875	PLO	13.491	13.630	13.184	10.180	10.207	10.578	10.579	10.948	11.049
$C_{55}H_{103}O_6$	52:2	$[M+NH_4]^+$	876	SLP	3.017	3.074	2.857	1.816	1.849	1.956	2.512	2.572	2.500
$C_{55}H_{104}O_6$	52:2	$[M+NH_4]^+$	877	POO	11.601	11.273	11.895	7.649	7.598	7.743	9.784	9.633	10.264
$C_{55}H_{104}O_6$	52:1	$[M+NH_4]^+$	878	SOP	5.189	5.084	5.156	2.730	2.752	2.864	4.647	4.526	4.646
$C_{57}H_{100}O_6$	54:5	$[M+NH_4]^+$	898	OLL	4.633	4.670	4.504	3.927	3.849	4.074	4.067	4.414	4.460
$C_{57}H_{103}O_6$	54:4	$[M+NH_4]^+$	900	OLO	7.968	7.724	8.126	5.902	5.730	5.964	7.523	7.767	8.287
$C_{57}H_{104}O_6$	54:3	$[M+NH_4]^+$	902	OOO	4.567	4.259	4.888	2.956	2.844	2.911	4.638	4.556	5.132
$C_{57}H_{104}O_6$	54:3	$[M+NH_4]^+$	902	SLO	3.564	3.483	3.522	2.106	2.076	2.206	3.573	3.649	3.751
$C_{57}H_{106}O_6$	54:2	$[M+NH_4]^+$	904	SOO	3.064	2.881	3.178	1.583	1.545	1.615	3.305	3.211	3.484

La: lauric acid (12:0); M: myristic acid (14:0); P: palmitic acid (16:0); S: stearic acid (18:0); O: oleic acid (18:1n-9); L: linoleic acid (18:2n-6); CN: total number of acyl carbons; DB: total number of double bonds; CR– Raw colostrum milk; CP - Pasteurized colostrum milk; CL - Lyophilized Pasteurized colostrum milk; TR - Raw transitional milk; TP - Pasteurized transitional milk; TL - Lyophilized Pasteurized transitional milk; MR - Raw mature milk; MP - Pasteurized mature milk; ML - Lyophilized Pasteurized mature milk.
Source: Authors.

Comparing the results obtained by the composition in FAs (Tables 3, 4 and 5) with the estimates of TAGs presented in Table 6, it was possible to observe the high frequency of combinations of oleic acid (O, 18:1n-9), palmitic acid (P, 16:0) and linoleic acid (L, 18:2n-6) in these TAGs. These results were similar to the work by Manin et al. (2019), who evaluated the possible TAGs in HM from different stages of lactation analyzed after being subjected to pasteurization and pasteurization + lyophilization processes.

The highest estimates in percentage of TAGs in the colostrum lactation phase are present in the *m/z* 875 $[TAG+NH_4]^+$ PLO with estimates in CR (13.491), CP (13.630) and CL (13.184); *m/z* 877 $[TAG+NH_4]^+$ POO in CR (11.601), CP (11.273) and CL (11.895); *m/z* 850 $[TAG+NH_4]^+$ POP in CR (9.822), CP (9.946) and CL (9.648); *m/z* 900 $[TAG+NH_4]^+$ OLO in CR (7.968), CP (7.724) and CL (8.126).

The highest estimates in percentage of TAGs in HM of the transitional lactation phase are present in the *m/z* 875$[TAG+NH_4]^+$ PLO with values in TR (10.180), TP (10.207) and TL (10.578); *m/z* 877$[TAG+NH_4]^+$ POO in TR (7.649), TP (7.598) and TL (7.743); *m/z* 850 $[TAG+NH_4]^+$ POP in TR (6.597), TP (7.598) and TL (7.743); *m/z* 900 $[TAG+NH_4]^+$ OLO in TR (5.902), TP (5.730) and TL (5.964).

The highest estimates in percentage of TAGs in HM of the mature lactation phase are present in the *m/z* 875$[TAG+NH_4]^+$ PLO with values in MR (10.579), MP (10.948) and ML (11.049); *m/z* 877$[TAG+NH_4]^+$ POO in MR (9.784), MP (9.633) and ML (10.264); *m/z* 900 $[TAG+NH_4]^+$ OLO in MR (7.7523), MP (7.767) and ML (8.287); *m/z* 850 $[TAG+NH_4]^+$ POP in MR (6.879), MP (6.789) and ML (6.843).

In a study by Haddad et al. (2011), in which the composition of molecular TAG species in mature milk was analyzed, the POO species (13.587%), PLO (3.045%), POP (9.790%) also stood out. Despite the intensity found in this work for PLO to be lower and for POP to be higher than those found in our study, it is noted that these species of TAGs were predominant, in addition to LaPO, MPO and PLL, which represented approximately 50% of the total of TAGs.

Manin et al. (2019) analyzed pasteurized and lyophilized HM in the three different stages of lactation for a period of six months of storage. The authors observed that the possible combinations of TAGs in the samples remained similar after the lyophilization process, confirming that the lipid profile did not change with the application of the processes or with the passage of time.

In a study by Kallio et al. (2017) the authors noted that P is one of the main constituents in the composition of possible HM TAGs and that P may have its concentrations

altered by several factors, among them, maternal feeding. However, in this work, the authors conclude that the location of P in the TAG skeleton (sn-1, sn-2, sn-3) is not influenced by this factor. Koletzko (2017) reveals that P (16:0) is mostly in the sn-2 position, but the positioning of P was not evaluated in our study.

This study is limited to the evaluation of some lipid components from different phases of HM, which has more than 150 different components. Although our results have not shown a significant difference, the evaluation of components not studied in our study is necessary in future research, to elucidate whether there is maintenance of other compounds important for the development and health of the newborn.

4. Conclusions

The results of this study indicate that the pasteurization and lyophilization processes do not induce significant negative changes in the lipid quality of HM, due to the fact that the Dornic acidity, composition in FAs and the TAG profile remain preserved after the treatments. The results of the FA composition, in the different lactation phases (colostrum, transitional and mature), raw, pasteurized and lyophilized pasteurized revealed that among the FAs found, oleic acid, palmitic acid and linoleic acid were the ones with the highest concentrations. In addition, none FA showed a significant difference for any of the processes applied to the analyzed samples. In relation to the TAG profile, the TAG with the highest estimate in all samples was *m/z* 875 $[TAG+NH_4]^+$ PLO and 877 $[TAG+NH_4]^+$ POO.

Therefore, for these results presented, pasteurization techniques in conjunction with lyophilization, can be a promising alternative, aiming to improve the processes of quality and conservation of HM lipids in HMBs, in addition to reducing the storage volume and facilitating transport of this food.

For future work, we suggest the evaluation of the factors that influence the composition and production of HM, and relate these to the composition in FAs and the TAG profile present in the HM.

Conflicts of interest

The authors declare that there are no conflicts of interest regarding the publication of this article.

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