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Comparative lipid analysis of colostrum and mature human milk using UHPLC-Q-TOF-MS

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Abstract

Human milk serves as a complete source of nutrients for newborns and is considered an essential food for child development. One of the key nutrients in milk is lipids, which are the primary energy source for infants. This work presents a comparative study of the lipid profiles of colostrum and mature human milk from Brazilian nursing mothers. The analysis utilized the techniques of gas chromatography with a flame ionization detector and ultra-performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry. The predominant fatty acids found in both types of milk were palmitic acid, oleic acid, and linoleic acid. Furthermore, a lipidomic analysis based on the fatty acids profile identified 48 different lipids, which were classified as glycerophospholipids, glycolipids, and non-esterified fatty acids. When comparing the lipid profiles, colostrum was found to be more nutritious than mature milk in terms of lipid quality. Although the comparison of human milk through lipidomic analysis is not extensively explored in Brazil, this study demonstrated its effectiveness and has the potential to enhance our understanding of lipid absorption processes by the human body.

Keywords: lipid profile; human milk; ultra-performance liquid chromatography; gas chromatography with flame ionization detector.

Practical Application: This study reveals the lipid profiles of colostrum and mature milk, highlighting key fatty acids.

1 INTRODUCTION

Human milk (HM) is a complete and essential nutrient source for newborns, considered the gold standard for child growth. It contains 3–5% fat, primarily composed of triacylglycerols (TAG), diacylglycerols (DGs), glycerophospholipids, sphingolipids, free fatty acids (FA), cholesterol, and glycolipids (Wang et al., 2020). TAGs serve as the main energy source for infants, with their digestion influenced by the specific positioning of FA within their structure. Brazilian regulations encourage HM donation through milk banks, particularly supporting mothers unable to breastfeed due to conditions such as HIV, HTLV, medication use, or insufficient milk production. Donated milk can be frozen for up to 15 days at –18°C, with preservation methods such as freeze-drying and spray-drying, developed to extend the shelf life of this perishable food (Manin et al., 2023; Vyssotski et al., 2015; Zhao et al., 2018).

Several studies globally have investigated the levels of FA, total fat, phospholipids, and cholesterol present in HM. Additionally, some pooled data analyses have evaluated lipids in HM, but these analyses have mainly focused on specific FA such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), total fat, or phospholipids. The composition of HM is highly dynamic and changes according to diet, time of day, and lactation period. Furthermore, this composition varies between individual mothers and across different ethnicities, being influenced by the mother's diet. Fat is one of the nutrients most subject to variations in HM (Zhang et al., 2022).

Lipids in HM are primarily found in fat globules, consisting triglycerides surrounded by a membrane of phospholipids, cholesterol, proteins, and glycoproteins. These lipids supply 50–60% of an infant's energy intake, alongside essential FA and fat-soluble vitamins. TAGs, constituting 98–99% of the fat content, derive their properties from the length and saturation of FA, with long-chain polyunsaturated FA (LC-PUFA) being extensively studied. While higher PUFA concentrations in breast milk are associated with better cognitive development, extremely high levels have been associated with negative effects, such as reduced motor and cognitive performance, increased allergic risks, and higher fat mass (Bernard et al., 2015; Guxens et al., 2011; Much et al., 2013; Waidyatillake et al., 2017; Zielinska et al., 2019).

To analyze the lipid profile of HM, new, more convenient, and rapid approaches are essential. Therefore, lipidomics has been continuously employed in various food studies to investigate the lipid profile (Wu et al., 2023). In this context, lipidomic analysis can provide important information about the quality of HM, as it is the branch of science that studies the biochemical and molecular characterization of lipids within a specific biological system, in addition to the lipid changes induced by various factors (Wenk, 2010).

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There are notable challenges regarding the complete and efficient extraction of the lipidome, including differences in lipid structures and concentration levels. Therefore, careful sample preparation in lipidomics greatly aids in isolating the analytes of interest to achieve high lipidome coverage while avoiding signal suppression. Additionally, lipidomic analysis sample preparation must be reproducible, robust, and efficient, facilitating the extraction of a wide range of analytes with varying polarities, molecular weights, and concentration levels (Bakhytkyzy et al., 2020). Consequently, the study aims to obtain and compare, through chemometric analyses, the lipid profile of lyophilized colostrum and mature HM samples, focusing on lipidomic analysis using UPLC-MS/MS.

1.1 Relevance of the work

The study focuses on analyzing the lipid profiles of colostrum and mature human milk, employing advanced chromatography techniques. Key findings reveal that palmitic acid, oleic acid, and linoleic acid are the major fatty acids present in both colostrum and mature milk. Additionally, through lipidomic analysis, the study identifies 48 different lipids categorized into glycerophospholipids, glycolipids, and non-esterified fatty acids. Interestingly, colostrum demonstrates a higher nutritional quality in terms of its lipid profile compared to mature milk.

This fresh research is not under consideration of publication elsewhere. We believe that such study would have great visibility if published in this estimated journal.

2 MATERIALS AND METHODS

2.1 Materials, solvents, and reagents

Methanol, chloroform, anhydrous sodium sulfate, and sodium hydroxide were purchased from Sigma-Aldrich (Darmstadt, Germany). A standard analytical mixture of fatty acid methyl esters (FAMES 189-19) was purchased from Millipore-Sigma (St. Louis, USA). For chromatographic analysis, all reagents and solvents used were of analytical grade (purity ≥ 99.9%), while mass spectrometry analysis was performed using HPLC-grade solvents.

The research ethics committee of the State University of Maringá approved all procedures with process number 3,430,478. Initially, in the HM bank of the Hospital Universitário de Maringá (Maringá, Paraná, Brazil), samples of HM from the lactation phase, including colostrum and mature HM, were collected while maintaining a temperature of 4°C according to a specific protocol regulated by Agência Nacional de Vigilância Sanitária (ANVISA, 2006), with a total volume per phase of 1,200 mL. Subsequently, a homogenized pool was formed and divided into appropriate containers for further processing of colostrum and mature HM.

A pool volume of colostrum and mature milk was subjected to the freeze-drying process according to Manin et al. (2019), at approximately –54°C and 0.021 mbar for 48 h in the *Enterprise I* freeze-dryer until constant weight was achieved. The powdered milk was vacuum packed in light-blocking aluminum bags, frozen at –18°C, for later analysis. The dried samples were subjected to Folch extraction, followed by lipidomic analysis.

2.2 Extraction of lipids

The method by Folch et al. (1957) was adopted for the extraction of lipids from milk samples, using a sample:solvent ratio of 1:5 (v/v) with chloroform:methanol as the extraction solvent in a 2:1 (v/v) ratio. For each sample, 10 g of freeze-dried milk was mixed with 50 mL of the chloroform:methanol solution and stirred for 15 min. Then, the mixture was centrifuged for 5 min at 6000 rpm and 25°C. Subsequently, 10 mL of 1% Na_2SO_4 solution was added to the sample solution, shaking again for 5 min, and centrifuged under the same conditions. After phase separation, the lower phase was collected into a previously weighed 250 mL flask, and the extracting solvent mixture was evaporated at an appropriate temperature using a rotary evaporator. The lipid fractions were collected with a pipette and a small volume of hexane, transferred to an Eppendorf, and stored at –18°C in a freezer until analysis.

2.3 Lipid derivatization

For the derivatization of FA in the milk sample, the lipid methylation method was applied, as described in International Organization for Standardization 5509 (ISO, 1978). To obtain fatty acid methyl esters, 100 mg of extracted lipids in test tubes were mixed with 2 mL of n-heptane and 500 µL of methyl tricosanoate (23:0; ≥ 99% Sigma-Aldrich, Darmstadt, Germany) as an internal standard. The test tubes were shaken for 2 min using a VX-200 vortex (Labnet International Inc., New Jersey, USA). Then, 2 mL of esterifying reagent (KOH/MeOH 2 mol L^{-1}) was added to the solution, which he stirred again under the same conditions. Post-derivatization, the upper (organic) phase was collected for gas chromatograph coupled with a flame ionization detector (GC-FID) analysis.

2.4 Composition in fatty acid

The analysis of the fatty acid composition was carried out through chromatographic separation and identification using a Shimadzu GC-2010 Plus gas chromatograph equipped with a flame ionization detector and fused silica capillary column (Select FAME, 100 m x 0.25 mm i.d. x 0.25 µm cyanopropyl film thickness) along with a split/splitless injection system. A split ratio of 1:100 and an injection volume of 2 µL were used. The injector and detector temperatures were set at 240°C and 250°C, respectively. The flows for carrier, auxiliary, synthetic air, and hydrogen gases were maintained at 1.4 mL. min^{-1}, 30 mL. min^{-1}, 300 mL. min^{-1}, and 30 mL. min^{-1}, respectively. Chromatographic separation followed this heating schedule: the column was heated to 65°C and held stable for 4 min, followed by a heating ramp of 16°C min^{-1} until reaching 185°C. After 12 min, a new heating ramp of 20°C min^{-1} was used so that the column temperature reached 235°C, which was maintained for 9 min, totaling a 35-min analysis duration. Analytical standard methyl tricosanoate (23:0; ≥ 99% Sigma-Aldrich, Darmstadt, Germany) was used as an internal standard for quantification. The results from GC-FID analysis were processed using the Chromquest 5.0 software and expressed as a relative percentage of total FA.

2.5 Lipidomic analysis

In total, 100 mg of the samples were resuspended in 10 mL of isopropanol, 10 µL of each extract was injected, and then the suspension was analyzed using ultra-high-performance liquid chromatography (Shimadzu, Nexera X2, Japan). Separation of individual components was performed using an Acquity UPLC® BEH C18 (Waters, EUA, 1.7 µm, 2.1 × 50 mm) at a flow rate of 0.300 mL min^{-1}. The analysis was carried out in isocratic mode with 1% of solvent A (containing 0.1% ammonium formate in acetonitrile) and 99% of solvent B (containing 0.1% ammonium formate in isopropanol) and the column temperature maintained at 55°C.

MS experiments were performed on an Impact II high-resolution mass spectrometer of Q-TOF geometry (Bruker Daltonics Corporation, Germany) equipped with an electrospray ionization source. The instrument was calibrated using a sodium formate solution (10 mmol L^{-1} NaOH solution in 1:1 (v/v) isopropanol:water solution containing concentrated formic acid). The source was operated in positive ionization mode. Drying gas parameters were set to 450 L h^{-1} at 250°C and mist gas pressure at 4 bar. The source temperature was maintained at 130°C, capillary voltage at 3.00 kV, and cone voltage at 35.0 V. Data were collected in the range of *m/z* 100–1000 with an acquisition rate of 5 Hz, and the most intense ions were used for multiple reaction monitoring (MRM), using a staggered program with collision energies ranging from 15 to 40 eV. The data obtained through an exploratory analysis were attributed using the LAMES Platform, based on the mathematical algorithm that elucidates the distribution of FA in TAG molecules. Furthermore, the LipidMaps® database (2024) was used, which made it possible to find the molecular formula of TAGs in colostrum and mature HM samples for lipidomic analysis.

2.6 Statistical analyses

In this study, triplicate analyses were performed to ensure the precision and reliability of the results. The fatty acid composition results were expressed as mean ± standard deviation. Statistical significance was assessed using analysis of variance, followed by Tukey's test for mean comparisons. For multivariate analyses, Origin software (OriginLab Corporation) was used to create graphs and heatmaps, facilitating the identification of differences among the analyzed samples, variations in the extraction methods, and potential patterns in the data.

3 RESULTS AND DISCUSSION

3.1 Fatty acid composition

A total of 26 FA were identified and quantified by GC-FID in the colostrum and mature milk samples studied, as elucidated in Table 1.

The colostrum and mature milk samples showed an SFA of 41.09 and 42.20%, respectively. For MUFA, the values were 39.20 and 39.23%, while for PUFA, the results were 19.71 and 18.59%, respectively, as shown in Table 1.

Caprylic acid (8:0) was not identified, and the presence of this FA in colostrum milk was not always identified, representing less than 0.1% of the total set of FAs, as reported in an analysis of combined data from 55 studies carried out globally (Floris et al., 2020). Palmitic acid (16:0), among the SFAs, was the majority, presenting a concentration of 25.04 and 22.65% for colostrum and mature, respectively. Such values are consistent with what was found in other related studies (Demmelmair & Koletzko, 2018; Manin et al., 2023). This fatty acid (16:0), when located in the Sn-2 position of TAG helps with the absorption of calcium in the newborn's intestine, in addition to having an analgesic effect, as it increases the levels of the neurotransmitter anandamide, which has an analgesic effect (Visentainer et al., 2018).

As for MUFAs, oleic acid (18:1n-9) stood out, presenting concentrations of 33.01 and 32.59% for colostrum and mature, respectively. The values are close to those found by Manin et al. (2023). Furthermore, according to the study by Wu et al. (2023), who investigated the lipid composition of donors from Taiwan, a content of approximately 27% oleic acid was observed. Oleic

Table 1. Fatty acid composition of colostrum and mature milk samples.

Fatty acid	Colostrum	Mature
04:00	0.10 ± 0.01^{b}	0.14 ± 0.03^{a}
06:00	0.34 ± 0.01^{b}	0.61 ± 0.08^{a}
10:00	0.37 ± 0.01^{b}	0.56 ± 0.00^{a}
12:00	2.88 ± 0.08^{b}	3.49 ± 0.06^{a}
14:00	4.81 ± 0.09^{a}	4.85 ± 0.10^{a}
14:1	0.20 ± 0.00^{a}	0.20 ± 0.00^{a}
15:00	0.14 ± 0.01^{b}	0.23 ± 0.01^{a}
16:00	25.04 ± 0.41^{a}	22.65 ± 0.45^{a}
16:1n-7	2.31 ± 0.08^{b}	2.64 ± 0.05^{a}
16:1n-9	0.17 ± 0.02^{b}	0.59 ± 0.03^{a}
17:00	0.46 ± 0.01^{a}	0.30 ± 0.04^{b}
18:00	6.13 ± 0.16^{b}	8.95 ± 0.13^{a}
18:1n-9	33.01 ± 0.26^{a}	32.59 ± 0.72^{a}
18:1n-7	1.99 ± 0.02^{a}	2.22 ± 0.30^{a}
18:2n-6	16.76 ± 0.26^{a}	16.42 ± 0.61^{a}
18:3n-3	0.71 ± 0.01^{a}	0.43 ± 0.06^{b}
18:3n-6	0.27 ± 0.05^{b}	0.41 ± 0.06^{a}
20:1n-9	1.52 ± 0.09^{a}	0.99 ± 0.78^{b}
20:3n-3	0.67 ± 0.03^{a}	0.22 ± 0.06^{b}
20:3n-6	0.36 ± 0.02^{a}	0.36 ± 0.00^{a}
20:5n-3 (EPA)	0.11 ± 0.00^{a}	0.09 ± 0.00^{b}
22:00	0.82 ± 0.04^{a}	0.42 ± 0.01^{b}
20:3n-6	0.38 ± 0.03^{a}	0.34 ± 0.01^{b}
20:4n-6 (AA)	0.08 ± 0.01^{a}	0.09 ± 0.00^{a}
22:5n-3	0.14 ± 0.01^{a}	0.10 ± 0.01^{b}
22:6n-3 (DHA)	0.23 ± 0.01^{a}	0.13 ± 0.02^{b}
∑SFA	41.09 ± 0.91^{a}	42.20 ± 0.27^{a}
∑MUFA	39.20 ± 1.88^{a}	39.23 ± 0.73^{a}
∑PUFA	19.71 ± 0.84^{a}	18.59 ± 0.57^{a}
∑n-3	1.89 ± 0.15^{a}	0.97 ± 0.19^{b}
∑n-6	17.85 ± 0.68^{a}	17.62 ± 0.36^{a}
∑n-6/∑n-3	9.60 ± 1.02^{b}	18.16 ± 0.44^{a}

Note: Results expressed as mean ± standard deviation. Values with different letters in the same line show significant differences ($p < .05$) using the Tukey's test.

acid plays a crucial role in the development of the baby, being mainly responsible for providing energy to the newborn, helping to absorb fats from the intestine, and acting as a structural component of the brain (Jensen, 1999; Manin et al., 2023).

Regarding PUFAs, the main ones found in the present study were linoleic acids (18:2n-6) and α-linolenic acids (18:3n-3) with concentrations of 16.76 and 0.71% for colostrum milk and 16.42 and 0.43% for mature milk. These nutrients are considered essential, which means that they are not synthesized by the human body; that is, their intake is necessary through food. In addition, they play important roles in the child's immune system and hair growth and maintenance. Furthermore, these nutrients are precursors of LC-PUFA such as arachidonic acid (AA, 20:4n-6), EPA (20:5n-3), and DHA (22:6n-3) (Manin et al., 2023; Silva et al., 2007).

Regarding the AA, DHA, and EPA values found, the results for colostrum milk were 0.08, 0.11, and 0.23%, respectively. For mature milk, the values were 0.09, 0.09, and 0.13%. In a study conducted by Duan et al. (2019) in South Korea, the compositions of FA in HM from lactating mothers in Korea were analyzed, resulting in AA, EPA, and DHA levels of approximately 0.54, 0.22, and 0.77%, respectively. Thus, as the diet of Korean mothers is different from the diet of Brazilian mothers, some acid values found in this study may differ from the literature, given that the diet of lactating mothers directly interferes with the lipid profile of HM.

It is important to highlight that there was no significant difference ($p < .05$) in the levels of SFA, MUFA, and PUFA in both milks analyzed. However, in the case of the major FA, there was a significant decrease in the content of palmitic acid (16:0) in colostrum milk for mature milk, but oleic acids (18:1n-9) and linoleic acids (18:2n-6) showed no significant change. Regarding the content of omega-3 FA, there was a significant decrease, from 1.89% to 0.97%. Thus, for the newborn, colostrum milk, in addition to all the benefits already mentioned in the literature, is more nutritious in terms of lipid quality.

3.2 Lipid composition by LC-MS/MS

Through an exploratory analysis, 48 different lipids were identified, as detailed in Table S1 (see supplementary material). Classified into three lipid categories (GL, GP, and FAA), there were two subclasses of GLs (DG and TAG), four subclasses of GP (phosphatidic acid [PA], choline glycerophospholipid [PC], ethanolamine glycerophospholipid [PE], phosphatidylinositol [PI]), and the free FA (FFA), including 19 PC species (18 for mature milk), two FFA species, two PE species, one PA species, three PI species, two DG species, and 18 TAG species (17 for mature milk). Figure 1 illustrates the quantity of compounds found for each lipid class.

Around 95–98% of the fat present in HM are TAGs. Among the TAGs identified, those that contain palmitic acid (16:0) in the Sn-2 position stand out, given the importance of this TAG in this position to aid calcium absorption in the intestine of newborns. Assistance in the calcium absorption process in the intestine of neonates, along with the ability to induce an analgesic effect, is possible due to increased levels of anandamide, a neurotransmitter with analgesic properties.

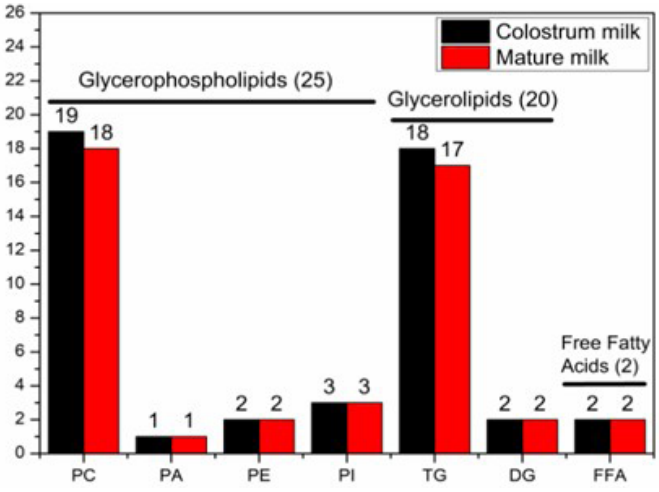
TAGs in HM have a unique structure associated with the efficient absorption and utilization of FAs. In the present study, TAGs in HM were identified by UPLC-Q-TOF-MS.

As shown in Figure 1, through exploratory analysis, the most abundant TAGs in colostrum milk were L-P-L (18:2/16:0/18:2), followed by O-L-L (18:1/18:2/18:2), represented by one of its isomeric forms with 16.93 and 9.76% of the total TAGs, respectively. For mature milk, the TAGs highlighted were TAG O-32:8 with 18.81% and L-P-L (18:2/16:0/18:2) with 15.82% of the total TAGs.

It is worth highlighting the presence of palmitic acid, one of the main TAGs found in both HM samples, in the Sn-2 position, mainly influencing endogenous hormonal functions. Therefore, it is inferred that the neonate may have good digestibility, as palmitic acid in the Sn-2 position is more easily digested by the body compared to other FA in the Sn-1 or Sn-3 position. The presence of palmitic acid in the Sn-2 position helps to increase the efficiency of digestion and absorption of TAGs. Our findings similarly agree with the study by Castro et al. (2023) through research on breast milk.

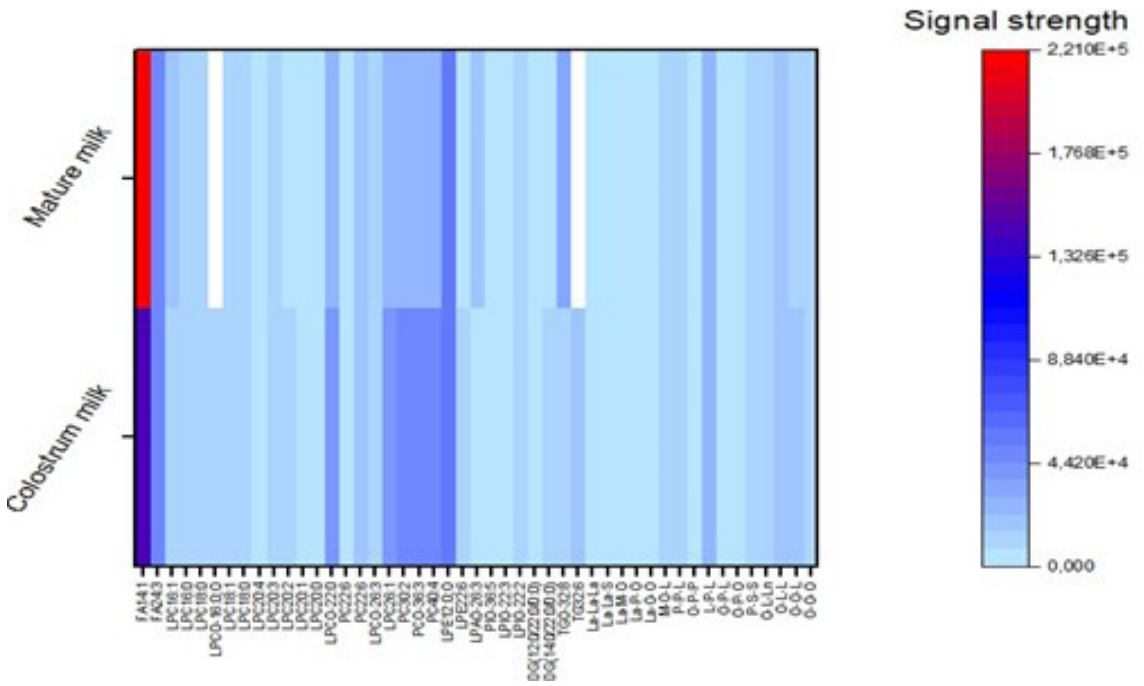
Furthermore, there is also the presence of linoleic acid in the same position, and there are many benefits when a PUFA is located in the Sn-2 position, given that there is a low activity of pancreatic lipase in relation to these FA when present in the Sn-1 and Sn-3 positions. Thus, due to the action of lipases, the fatty acid in the Sn-2 position has greater bioavailability, being more easily absorbed by the newborn's body.

Furthermore, some compounds may present different interactions in different samples. Therefore, to highlight the identification of the lipid profile of colostrum and mature HM samples, and to analyze patterns and relationships between samples and variables, heat map analysis was performed. Figure 2 shows a heat map of the 48 lipids found in HM. Heat maps are useful tools in chemical analysis and interpretation of multivariate data. They are two-dimensional graphs that present color information to represent the magnitude of a given value in a data array. In analytical chemistry, heat maps are often used to visualize patterns in spectral data, such as mass spectra



Source: The author.

Figure 1. Lipid subclasses identified in colostrum and mature milk.



Bu, 4:0; Co, 6:0; Cy, 8:0; Ca, 10:0; La, 12:0; M, 14:0; P, 16:0; Po, 16:1; O, 18:1; S, 18:0; L, 18:2; Ln, 18:3; Eo, 20:1; Ed, 20:2; Et, 20:3; ARA, 20:4; EPA, 20:5; DPA, 22:5; DHA, 22:6. Source: The author.

Figure 2. Screening results (heat map) of lipids present in human milk from the colostrum and mature lactation phases.

obtained in the present analysis, helping to compare data from different samples, experiments, or treatments in an analysis (Kumar et al., 2014).

The results in Figure 2 reveal that HM varies in lipid composition during different phases of lactation. However, they also demonstrate some similarities to lipid levels when examining individual components. The columns in the heat map represented, through colors, the amounts of each lipid component in the samples, allowing us to infer which components are present in greater abundance; this is represented by darker tones.

A main difference is in the 14:1 fatty acid, which was found to be higher in mature milk. However, TAG 32:6 and LPC O-16:0;O were found only in colostrum milk, showing an obvious difference in the heat map. Other compounds showed significant differences between milk samples, such as PC 40:4, PC O-36:3, PC 30:2, LPC 26:1, and LPC O-22:0, which were present in greater quantities in milk colostrum. From the glycerolipid class, the same TAGs found in the present study were also found in the research by Yuan et al. (2021).

In the study conducted by Song et al. (2021), a phospholipidomic analysis was performed on breast milk samples collected from Chinese mothers, identifying 258 different phospholipids. The current research identified 25 compounds from this class that are consistent with their findings, with the exception of PC30:2 (12:0/18:2) and LPE12:0. This discrepancy may be attributed to the high concentration of AG 18:2n-6 present in the analyzed milk, which could influence the presence of this AG in the phospholipid structure.

It is worth mentioning that depending on the region and location, mothers' diets can be completely different from each other, causing changes in the lipid profile of breast milk. Lipidomic analysis to differentiate the phases of breast milk has been little explored, with no reports found in the literature of such analysis in Brazil, for example. Therefore, the present research can be a pioneer in this sense.

4 CONCLUSION

This study, using MRM via UPLC-Q-TOF-MS with electrospray ionization and lipidomic analysis, revealed differences in the lipid profiles between HM colostrum and mature milk. Colostrum had a higher proportion of phospholipids and omega-3 FA, offering superior nutritional quality compared to mature milk. These findings are significant for understanding the nutritional needs of newborns in their early days. While lipidomic analysis of breast milk is widely studied globally, it is less explored in Brazil. This research contributes to a deeper understanding of the lipid composition and dynamic changes in HM from colostrum to mature phases in the country.

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