

## Lipid Profile of Human Milk in Different Lactation Stages Submitted to Pasteurization, Lyophilization and Spray-Drying Processes

Vanessa J. C. Neia,<sup>a</sup> Patrícia D. S. Santos,<sup>b</sup> Christyna B. G. Tavares,<sup>c</sup> Meliana G. Paula,<sup>c</sup> Silvio C. Costa,<sup>d</sup> Joana M. V. Zacarias,<sup>e</sup> Josiane B. Alencar,<sup>e</sup> Roberta Silveira,<sup>f</sup> Oscar O. Santos,<sup>b</sup> Jeane E. L. Visentainer<sup>e</sup> and Jesuí V. Visentainer<sup>\*,b</sup>

<sup>a</sup>Programa de Pós-Doutorado em Ciência de Alimentos, Universidade Estadual de Maringá (UEM), 87020-900 Maringá-PR, Brazil

<sup>b</sup>Departamento de Química, Universidade Estadual de Maringá (UEM), 87020-900 Maringá-PR, Brazil

<sup>c</sup>Hospital Universitário de Maringá (HUM), Universidade Estadual de Maringá (UEM), 87083-240 Maringá-PR, Brazil

<sup>d</sup>Departamento de Bioquímica, Centro de Ciências Biológicas, Universidade Estadual de Maringá (UEM), 87020-900 Maringá-PR, Brazil

<sup>e</sup>Laboratório de Imunogenética, Departamento de Ciências da Saúde, Universidade Estadual de Maringá (UEM), 87020-900 Maringá-PR, Brazil

<sup>f</sup>Programa de Ciência de Alimentos, Universidade Estadual de Maringá (UEM), 87020-900 Maringá-PR, Brazil

The objective of this study was to evaluate the effect of pasteurization, lyophilization and spray-drying on the lipid profile of donated human milk (DHM) at different lactation stages. Nine frozen samples of colostrum, transitional and raw mature human milk (HM) were collected from the Human Milk Bank (HMB) of the Hospital Universitário de Maringá (HUM). Samples were thawed and pooled according to lactation stage. Thereafter, it was submitted to pasteurization, lyophilization and spray-drying processes and later the fatty acid (FA) composition and the lipid profile analyses were performed. Gas chromatograph (GC) with flame ionization detector (FID) and electrospray ionization (ESI) mass spectrometer (MS) were used, respectively. In the FA composition analysis, it was observed that the same classes of saturated FA (SFA), monounsaturated FA (MUFA) and polyunsaturated FA (PUFA) were preserved throughout the process and in all lactation stages. The lipid profiles were also preserved after processing. Therefore, the lyophilization and spray-drying processes are promising techniques to preserve the DHM in the HMB, once the components evaluated were preserved, and both techniques facilitate the transport and storage, as the techniques reduce the sample volume.

**Keywords:** mass spectrometry, human milk, gas chromatography, fatty acids, lipid profile, triacylglycerols

## Introduction

Exclusive breastfeeding (BF) is recommended during the first six months of life and continued until the age of two, or older,<sup>1</sup> being well described in the literature,<sup>2,3</sup> due to the numerous short and long health benefits. Human

milk (HM) is the gold standard for newborn (NB) feeding, as it contains all the nutrients, bioactive compounds and immunological factors essential for the NB proper growth and development.<sup>4,5</sup>

The HM lipids provide approximately 50% of the total energy value necessary for the adequate growth and development of the NB.<sup>6</sup> The HM lipid composition is mainly formed by 98% of triacylglycerol (TAG), which confers lipid stability and are sources of saturated fatty

\*e-mail: jesuivv@gmail.com

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acid (SFA), monounsaturated fatty acid (MUFA) and polyunsaturated fatty acid (PUFA).<sup>7</sup>

SFA are associated with biological antimicrobial activities,<sup>8</sup> MUFA are associated with calcium absorption by the intestinal tract of the NB<sup>9</sup> and PUFA are important for neurological and cognitive development of the NB.<sup>10</sup> Conjugated linoleic acid (Cla) is also included, which refers to positional and geometric isomers of linoleic acid, which are important immunomodulators, in addition to having anti-inflammatory properties.<sup>11</sup>

Even with all the HM benefits, some conditions may lead to difficulties in establishing and maintaining exclusive BF, such as prematurity, maternal illness and death.<sup>3</sup> Thus, donated human milk (DHM) has become an efficient alternative and Human Milk Bank (HMB) are specialized services responsible for collecting the DHM, as well as its processing, quality control, storage activities and transport to the final recipient.<sup>10</sup>

The HMB recommends that the DHM transport must be with it frozen in temperature-controlled isothermal boxes from the donor's home to the neonatal intensive care unit (NICU), where the NB will receive the processed DHM.<sup>12</sup> Therefore, new HM processing technologies are necessary to contribute with alternative measures in the DHM distribution and transport routine by HMB, in order to increase the HM shelf-life, as well as decrease storage and transportation costs in the cold chain, allowing HM to be distributed in portioned and/or individually (mother/child), including units that are not part of the HMB distribution routine. So as to ensure the HM lipid profile quality after processing, the aim of the present study was to evaluate the effect of pasteurization, lyophilization and spray-drying on the lipid profile in different lactation stages of DHM.

## Experimental

### Standards, solvents and reagents

Reference standards of fatty acid methyl esters (FAME) and Cla, respectively, FAME Mix, C4-C24 unsaturated ( $\geq 97\%$ ) and Cla ( $\geq 98\%$ ), were purchased from Sigma-Aldrich (Saint Louis, USA). Reagents for Folch method and ISO esterification (methanol, chloroform, *n*-heptane, sodium hydroxide) were used without purification and were purchased from Millipore Sigma (Darmstadt, Germany). Basic sodium hydroxide/methanol solution ( $2 \text{ mol L}^{-1}$ ) was prepared in 100% methanol and stored at  $-18 \text{ }^\circ\text{C}$ . The solvents used for TAG analysis were high performance liquid chromatography (HPLC) grade chloroform and methanol, both purchased from JT Baker (Mexico City,

Mexico) and ammonium formate were purchased from Sigma-Aldrich (Saint Louis, USA).

### HM samples

DHM was collected at the donor's house, frozen ( $-18 \text{ }^\circ\text{C}$ ) in a domestic refrigerator and transported to the HMB in an insulated box with ice packs. DHM samples were obtained from the HMB of the Hospital Universitário de Maringá (HUM, Maringá, Paraná, Brazil). Milk collection was performed following a specific protocol for HMB-HUM donors, and this work was approved by the Research Ethics Committee (CEP in Brazil), number 2.797.476/2018, of the Universidade Estadual de Maringá (UEM, Maringá, Paraná, Brazil).

### Experimental design

Two hundred mL of raw HM in different lactation stages; colostrum ( $n = 3$ ), transitional ( $n = 3$ ) and mature ( $n = 3$ ) were collected from different donors ( $n = 9$ ), it was frozen, at  $-18 \text{ }^\circ\text{C}$  in the HMB-HUM (Maringá, Paraná, Brazil). The samples were thawed in a pulsating water bath (at  $37 \text{ }^\circ\text{C}$ ) and mixed according to each lactation stage. After thawing, the samples were grouped into pools according to the lactation stage, being: 600 mL pool of raw colostrum ( $n = 3$ ); 600 mL pool of raw transitional HM ( $n = 3$ ); 600 mL pool of raw mature HM ( $n = 3$ ). Each 600 mL pool of colostrum, transitional and raw mature HM was subdivided into 12 groups. Raw colostrum (150 mL), transitional (150 mL) and mature (150 mL) pools were separated and not subjected to processing. Raw colostrum (150 mL), transitional (150 mL) and mature (150 mL) pool were subjected to pasteurization processing. Raw colostrum (150 mL), transitional (150 mL) and mature (150 mL) pool were subjected to lyophilization processing. Raw colostrum (150 mL), transitional (150 mL) and mature (150 mL) pool were subjected to spray-drying processing.

After processing, samples were classified into: donated raw colostrum (Craw), pasteurized colostrum (Cpast), lyophilized colostrum (Clyo), spray-dried colostrum (Cspr), donated raw transitional (Traw), pasteurized transitional (Tpast), lyophilized transitional (Tlyo), spray-dried transitional (Tspr), donated raw mature (Mraw), pasteurized mature (Mpast), lyophilized mature (Mlyo) and spray-dried mature (Mspr). Unprocessed and processed samples were subjected to analysis of FA composition and TAG profile.

### Pasteurization

The 150 mL pool of colostrum, transitional and raw

mature HM were submitted to the pasteurization process according to Brazilian Human Milk Bank Guidelines.<sup>12</sup> The samples were subjected to a water bath (Eco-Sonics Model Q 5.9/25 (São Paulo, Brazil)) at 62.5 °C for 30 min, cooled by immersion in a bath containing water and ice until reach 4 °C and then subjected to analysis.

#### Lyophilization

The 150 mL pool of colostrum, transitional and raw mature HM were subjected to the lyophilization process according to Manin *et al.*<sup>13</sup> The samples were frozen at -18 °C and subsequently lyophilized in a Lyophilizer (Alpha 1-2 LD Plus Model 101522 (Osterode am Harz, Germany)) at approximately -54 °C and 0.021 mbar for 48 h. The lyophilization process was continued until constant weight, but not longer than 48 h. The powdered milk was vacuum-packed in light-free aluminum bags, frozen at -18 °C, for further analysis. The dried samples were weighed, reconstituted in sufficient water to reach the initial mass before the lyophilization process and then subjected to analysis.

#### Spray-drying

The 150 mL pool of colostrum, transitional and raw mature HM were submitted to the mini-Spray-Dryer drying process (Buchi, model B-191 (Flawil, Switzerland)), with inlet temperature of 175 °C and outlet temperature of 103 °C, with vaporized water flow, using 100% compressed air as recommended by Cavazos-Garduño *et al.*<sup>14</sup> The powdered milk was vacuum-packed in light-free aluminum bags, frozen at -18 °C, for further analysis. The dried samples were weighed, reconstituted in sufficient water to reach the initial mass before the spray-drying process and then subjected to analysis.

#### Extraction of total lipids (TL)

Lipid extraction was performed according to Folch *et al.*<sup>15</sup> Approximately 10 mL of samples were weighed into a 250.0 mL beaker, then 200.0 mL of a chloroform/methanol mixture (2:1, v/v) was added while stirring vigorously for 2 min, the solution obtained was filtered through a No. 1 Whatman filter paper in a Buchner funnel coupled to a vacuum pump. Next, 30.0 mL of chloroform and 30.0 mL of distilled water were added into the filtrate and stirred again for 2 min, and subsequently filtered. After filtration, the resulting solution was transferred to a 250.0 mL separation funnel. The extremity containing chloroform and lipids were transferred to a pre-weighed

flat bottom flask, and finally the solvent was rotavaporated. Ten mL of DHM samples from each pool were used to obtain 150 mg of lipids for esterification and subsequent identification of FAMES by gas chromatograph (GC) with flame ionization detector (FID) (Trace Ultra 3300, Waltham, USA) and analysis of lipid profile by direct infusion by electrospray ionization (ESI) mass spectrometer (MS) (Waters, Milford, Massachusetts, USA).

#### Lipid esterification/transesterification

Esterification and transesterification were performed using the ISO<sup>16</sup> methodology; 100.0 mg of sample were weighted, 2.0 mL of *n*-heptane and 2.0 mL of KOH/methanol solution (2 mol L<sup>-1</sup>) were added, the solution was stirred for 2 min and the organic phase was collected for further GC-FID analysis.

#### FA composition by GC-FID

Chromatographic conditions and analyses were performed according to Piccioli *et al.*<sup>17</sup> Chromatographic analyses were performed in a Thermo Scientific GC (Waltham, USA) equipped with FID, split/splitless inlet and CP-7420 fused silica capillary column (Select FAME, 100.0 m long, 0.25 mm internal diameter and 0.25 µm thin film of cyanopropyl as stationary phase). The injector and detector temperatures were 235 °C. The column temperature was raised to 65 °C for 4 min, followed by a 16 °C min<sup>-1</sup> heating ramp to 185 °C, which was maintained for 12 min. Thereafter, a new ramp of 20 °C min<sup>-1</sup> was applied up to 235 °C and maintained for 14 min, totaling an analysis time of 40 min. Gas flows were: 1.2 mL min<sup>-1</sup> for carrier gas (H<sub>2</sub>), 30.0 mL min<sup>-1</sup> for make-up gas (N<sub>2</sub>), and in the FID: 30.0 and 300.0 mL min<sup>-1</sup> of gas (H<sub>2</sub>) and synthetic air, respectively. The samples were injected in split mode, with 1:40 ratio. Injection volume was 1.0 µL. FAMES were identified by comparing the retention time of constituents samples and Sigma FAMES. The Chromquest™ 5.0 software was used to determine peak areas, FA concentrations were expressed as relative percentage of total FA.

#### TAG profile by direct infusion ESI-MS

The TAG profile was obtained by direct infusion into MS using ESI source (Waters, Milford, Massachusetts, USA). Lipid samples were prepared according to Silveira *et al.*<sup>18</sup> and Piccioli *et al.*;<sup>17</sup> approximately 50.0 µL of lipid was added to 950.0 µL of chloroform. 5.0 µL of this solution was transferred to a vial and 1.0 mL of

9:1 methanol/chloroform solution ( $v v^{-1}$ ) was added. In order to obtain the ammonium adducts  $[TAG + NH_4]^+$ , 20.0  $\mu L$  of 0.10 mol  $L^{-1}$  ammonium formate prepared in methanol were added to the final solution. The prepared solutions were infused with a flow of 10.0  $\mu L min^{-1}$  directly into a Xevo TQ-D<sup>TM</sup> triple quadrupole MS (Waters, Massachusetts, USA) equipped with Z spray<sup>TM</sup> ESI, operating in positive mode (ESI+), conditions as follows: desolvation gas flow, 500  $L h^{-1}$ ; source temperature, 150 °C; desolvation temperature, 200 °C; and capillary and cone voltages, 3.00 kV and 20.00 V, respectively. Lipid profiles were evaluated in the mass/charge ( $m/z$ ) range 100-1200 in triplicate. The results obtained were determined using the MassLynx<sup>TM</sup> software.

#### Statistical analysis

The percentage values of each FA were calculated in relation to the total percentage and analyses were performed in triplicate. The results were subjected to one-way analysis of variance (ANOVA) at a 5% significance level using the GraphPad Prism<sup>®</sup> v. 5.0 software.<sup>19</sup> The mean values of the samples were compared by Tukey's test.

## Results and Discussion

#### FA composition in HM

Thirty-five FAs were identified and quantified by GC-FID (Table 1). According to the columns, it is possible to observe all FAs and the same classes of SFA, MUFA and PUFA were found in the raw (untreated), pasteurized, lyophilized and spray-dried HM in all lactation stages (colostrum, transitional and mature HM).

Lipids are essential for the NB as energy source, in addition to having structural and regulatory functions, in which FAs<sup>20,21</sup> are critical in the development of the central nervous system,<sup>22</sup> antiprotozoal activities, increased immune response, anticarcinogenic agents and antidiabetic effects.<sup>23</sup>

Palmitic acid is important as it has analgesic effects on the NB.<sup>20</sup> Hence, we observed that 16:0 is the most concentrated FA among the SFAs in all samples and the following results were observed: Craw (24.45%), Cpast (23.09%), Clio (22.58%), Cspr (23.42%), Traw (21.75%), Tpast (21.56%), Tlio (22.22%), Tspr (21.68%), Mraw (23.29%), Mpast (22.78%), Mlio (22.85%) and Mspr (23.29%), similar results were found by Cavazos-Garduño *et al.*,<sup>14</sup> who evaluated the composition of mature HM FAs subjected to pasteurization, lyophilization and spray-drying, and Manin *et al.*,<sup>13</sup> who evaluated lyophilized HM FAs in all lactation stages for 180 days.

Other important SFAs also found in this work are: butyrate acid (4:0) which has functions in the modulation of gene expression and in the reduction of inflammatory processes in the intestine. In addition to caproic (6:0), caprylic (8:0), capric (10:0) and lauric (12:0) acids, all of which related to biological antimicrobial activities.<sup>8,21</sup>

MUFAs are important in the early days of life, as it connects to myelogenesis. Thus, we observed that oleic acid (18:1n-9) is the most concentrated FA among MUFAs in all samples and the following results were observed: Craw (27.85%), Cpast (30.94%), Clio (28.30%), Cspr (29.83%), Traw (27.97%), Tpast (30.15%), Tlio (29.46%), Tspr (28.81%), Mraw (33.25%), Mpast (33.20%), Mlio (33.81%) and Mspr (33.53%), which is in agreement with the results found by Moltó-Puigmartí *et al.*,<sup>24</sup> who evaluated the effects of pasteurization and high pressure in mature HM.

PUFAs are the most important FAs in HM. Table 1 displays that all samples presented concentrations of linoleic (18:2n-6; LA), arachidonic (20:4n-6; AA),  $\alpha$ -linolenic (18:3n-3, ALA), eicosapentaenoic (20:5n-3, EPA) and docosahexaenoic (22:6n-3, DHA) acids and there was no significant difference between processed HM samples and raw DHM in all lactation stages. Similar results were found by Cavazos-Garduño *et al.*,<sup>14</sup> Manin *et al.*<sup>13</sup> and Moltó-Puigmartí *et al.*<sup>25</sup> Therefore, HM contains essential FAs (AL, ALA, AA and DHA); presenting a crucial role in the visual, immunological, cognitive and motor development of the NB, in addition to protection against allergy, asthma, improvement of lung function and reduction of inflammation and obesity rates in childhood.<sup>26,27</sup>

Cla is a group of linoleic acid isomers (18:2n-6) with conjugated double bond<sup>28</sup> and among the most representative isomers, 18:2n-6 C9, t11 and 18:2n-6 t10, C12 showed benefits in immune function and child development.<sup>11,29</sup> In this study, there was no statistically significant difference between processed samples and raw DHM in all lactation stages of Cla. The found values for 18:2n-6 C9, t11 for the Craw (0.18%), Traw (0.32%) and Mraw (0.27%) samples and the values found for 18:2n-6 t10 of C12 Craw (0.24%), Traw (0.20%) and Mraw (0.26%) were in agreement with what was found by Moltó-Puigmartí *et al.*,<sup>24</sup> who evaluated the HM FAs composition according to the gestational phase and Rydlewski *et al.*,<sup>30</sup> who evaluated the FA composition in eutrophic, overweight and obese women.

#### TAG profile in HM

The lipids functional properties are related to the FA composition, as well as its arrangement in the TAG molecule.<sup>31</sup> However, the FA analysis by GC-FID does not

Table 1. Fatty acid composition of raw (untreated), pasteurized, lyophilized and spray-drying human milk

Fatty acids <sup>a</sup> / %	Colostrum						Sample					
	Colostrum			Transitional HM			Mature HM			Mature HM		
	CrAw	CpAst	Clyo	CrAw	TrAw	TpAst	Tlyo	Tspr	MrAw	MpAst	Mlyo	Mspr
04:0 (B) <sup>b</sup>	0.06 ± 0.01	0.03 ± 0.01	0.05 ± 0.01	0.05 ± 0.01	0.05 ± 0.01	0.04 ± 0.01	0.03 ± 0.01	0.03 ± 0.01	0.05 ± 0.01	0.06 ± 0.01	0.05 ± 0.01	0.05 ± 0.01
06:0 (Cp) <sup>b</sup>	0.02 ± 0.01	0.03 ± 0.01	n.d.	0.03 ± 0.01	0.03 ± 0.01	0.02 ± 0.01	n.d.	0.02 ± 0.01	0.03 ± 0.01	0.03 ± 0.01	n.d.	0.03 ± 0.01
08:0 (Ca) <sup>b</sup>	0.13 ± 0.01	0.23 ± 0.01	n.d.	0.13 ± 0.01	0.13 ± 0.01	0.12 ± 0.01	n.d.	0.17 ± 0.04	0.17 ± 0.04	0.22 ± 0.03	n.d.	0.12 ± 0.02
10:0 (C) <sup>b</sup>	0.35 ± 0.01 <sup>b</sup>	0.13 ± 0.01 <sup>b</sup>	0.56 ± 0.01 <sup>a</sup>	0.21 ± 0.01 <sup>c</sup>	1.02 ± 0.10 <sup>c</sup>	0.64 ± 0.07 <sup>f</sup>	0.81 ± 0.04 <sup>f</sup>	0.74 ± 0.01 <sup>f</sup>	0.09 ± 0.01 <sup>h</sup>	0.22 ± 0.01 <sup>i</sup>	0.12 ± 0.01 <sup>k</sup>	0.34 ± 0.02 <sup>j</sup>
12:0 (La) <sup>b</sup>	3.75 ± 0.03 <sup>b</sup>	2.53 ± 0.04 <sup>c</sup>	4.16 ± 0.08 <sup>c</sup>	2.56 ± 0.05 <sup>c</sup>	5.76 ± 0.18 <sup>c</sup>	4.73 ± 0.08	6.04 ± 0.06	4.68 ± 0.03	2.74 ± 0.11	2.72 ± 0.04	2.77 ± 0.02	2.58 ± 0.08
14:0 (M) <sup>b</sup>	5.60 ± 0.05 <sup>b</sup>	4.69 ± 0.01 <sup>b</sup>	5.75 ± 0.04 <sup>b</sup>	4.84 ± 0.02 <sup>c</sup>	6.48 ± 0.06	6.16 ± 0.19	6.04 ± 0.04	6.11 ± 0.08	4.41 ± 0.09 <sup>h</sup>	4.20 ± 0.06 <sup>h</sup>	4.27 ± 0.04 <sup>h</sup>	4.65 ± 0.07 <sup>h</sup>
15:0	0.20 ± 0.01 <sup>b</sup>	0.04 ± 0.01	0.04 ± 0.01	0.04 ± 0.01	0.06 ± 0.01	0.05 ± 0.01	0.05 ± 0.01	0.06 ± 0.01	0.07 ± 0.01	0.06 ± 0.01	0.07 ± 0.01	0.07 ± 0.01
16:0 (P) <sup>b</sup>	24.45 ± 0.02 <sup>b</sup>	23.09 ± 0.03 <sup>b</sup>	22.58 ± 0.13 <sup>c</sup>	23.42 ± 0.01 <sup>b</sup>	21.75 ± 0.12	21.56 ± 0.21	22.22 ± 0.23 <sup>c</sup>	21.68 ± 0.08	23.29 ± 0.13 <sup>h</sup>	22.78 ± 0.07 <sup>i</sup>	22.85 ± 0.17 <sup>j</sup>	23.29 ± 0.05 <sup>j</sup>
17:0 (Mg) <sup>b</sup>	0.36 ± 0.01	0.34 ± 0.01	0.35 ± 0.03	0.10 ± 0.01 <sup>a</sup>	0.37 ± 0.01	0.38 ± 0.02	0.42 ± 0.02	0.34 ± 0.08	0.36 ± 0.02	0.33 ± 0.01	0.35 ± 0.02	0.40 ± 0.02
18:0 (S) <sup>b</sup>	6.83 ± 0.04 <sup>b</sup>	7.29 ± 0.12 <sup>b</sup>	6.45 ± 0.04 <sup>b</sup>	7.22 ± 0.01 <sup>a</sup>	6.91 ± 0.11 <sup>b</sup>	7.40 ± 0.07 <sup>g</sup>	7.63 ± 0.15 <sup>f</sup>	8.26 ± 0.09 <sup>g</sup>	7.93 ± 0.21 <sup>h</sup>	7.86 ± 0.03 <sup>h</sup>	7.12 ± 0.2 <sup>i</sup>	8.10 ± 0.10 <sup>i</sup>
20:0 (A) <sup>b</sup>	0.08 ± 0.02	0.10 ± 0.01	0.10 ± 0.01	0.12 ± 0.01	0.06 ± 0.01	0.07 ± 0.01	0.07 ± 0.01	0.09 ± 0.01	0.03 ± 0.01	0.04 ± 0.01	0.06 ± 0.03	0.03 ± 0.01
22:0 (Be) <sup>b</sup>	0.91 ± 0.02	0.88 ± 0.01	0.91 ± 0.01	0.86 ± 0.01	0.61 ± 0.02	0.61 ± 0.02	0.57 ± 0.02	0.59 ± 0.01	0.41 ± 0.01	0.41 ± 0.01	0.36 ± 0.02	0.34 ± 0.05
24:0 (Lg) <sup>b</sup>	0.54 ± 0.02	0.56 ± 0.01	0.55 ± 0.01	0.56 ± 0.01	0.19 ± 0.01	0.20 ± 0.01	0.21 ± 0.01	0.04 ± 0.01 <sup>c</sup>	0.05 ± 0.01	0.04 ± 0.01	0.08 ± 0.01 <sup>i</sup>	0.03 ± 0.01
ΣSFA	43.28 ± 0.66 <sup>a</sup>	39.97 ± 0.33 <sup>b</sup>	41.50 ± 0.34 <sup>b</sup>	40.15 ± 0.33 <sup>c</sup>	43.42 ± 1.03 <sup>c</sup>	41.98 ± 0.94 <sup>b</sup>	42.69 ± 0.75 <sup>i</sup>	42.81 ± 1.20 <sup>g</sup>	39.63 ± 0.85 <sup>j</sup>	38.97 ± 0.28 <sup>k</sup>	38.10 ± 0.78 <sup>k</sup>	40.03 ± 0.62 <sup>k</sup>
14:1	0.02 ± 0.01 <sup>a</sup>	0.17 ± 0.01	0.19 ± 0.01	0.18 ± 0.01	0.24 ± 0.01	0.22 ± 0.02	0.21 ± 0.01	0.23 ± 0.01	0.25 ± 0.01	0.21 ± 0.01 <sup>i</sup>	0.25 ± 0.02	0.25 ± 0.01
16:1n-9 (Pa) <sup>b</sup>	0.39 ± 0.01	0.37 ± 0.01 <sup>b</sup>	0.43 ± 0.04 <sup>a</sup>	0.38 ± 0.01	0.36 ± 0.01	0.35 ± 0.01	0.34 ± 0.01	0.34 ± 0.01	0.27 ± 0.01	0.25 ± 0.01	0.26 ± 0.01	0.26 ± 0.01
16:1n-7 (Po) <sup>b</sup>	1.38 ± 0.03 <sup>b</sup>	1.22 ± 0.03 <sup>b</sup>	1.48 ± 0.08 <sup>b</sup>	1.31 ± 0.02 <sup>c</sup>	1.76 ± 0.04	1.73 ± 0.04	1.70 ± 0.01	1.66 ± 0.05 <sup>c</sup>	1.50 ± 0.01	1.57 ± 0.03	1.48 ± 0.06	1.56 ± 0.01
17:1	0.12 ± 0.01	n.d.	0.12 ± 0.01	0.35 ± 0.01 <sup>a</sup>	0.18 ± 0.01	0.18 ± 0.01	0.17 ± 0.01	0.18 ± 0.04	0.10 ± 0.01	n.d.	0.12 ± 0.01	0.13 ± 0.02
18:1n-9 (O) <sup>b</sup>	27.85 ± 0.05 <sup>d</sup>	30.94 ± 0.02 <sup>a</sup>	28.30 ± 0.03 <sup>c</sup>	29.83 ± 0.02 <sup>b</sup>	27.97 ± 0.08 <sup>b</sup>	30.15 ± 0.10 <sup>b</sup>	29.46 ± 0.44 <sup>f</sup>	28.81 ± 0.09 <sup>g</sup>	33.25 ± 0.14 <sup>h</sup>	33.20 ± 0.09 <sup>i</sup>	33.81 ± 0.14 <sup>i</sup>	33.53 ± 0.16 <sup>j</sup>
18:1n-7 (V) <sup>b</sup>	2.28 ± 0.06 <sup>d</sup>	2.47 ± 0.06 <sup>c</sup>	2.84 ± 0.03 <sup>b</sup>	3.05 ± 0.10 <sup>a</sup>	1.84 ± 0.15	1.80 ± 0.18	2.29 ± 0.39	2.98 ± 0.02 <sup>c</sup>	1.57 ± 0.03 <sup>h</sup>	2.06 ± 0.01 <sup>i</sup>	2.51 ± 0.34 <sup>i</sup>	1.70 ± 0.09 <sup>k</sup>
20:1n-9	0.17 ± 0.01	0.17 ± 0.01	0.15 ± 0.01	0.17 ± 0.01	0.15 ± 0.01	0.15 ± 0.01	0.16 ± 0.01	0.16 ± 0.01	0.15 ± 0.01	0.16 ± 0.01	0.16 ± 0.01	0.15 ± 0.01
22:1n-9 (Er) <sup>b</sup>	0.25 ± 0.01	0.26 ± 0.02	0.23 ± 0.02	0.27 ± 0.02	0.13 ± 0.01	0.14 ± 0.01	0.15 ± 0.01	0.15 ± 0.01	0.05 ± 0.01	0.04 ± 0.01	0.04 ± 0.01	0.04 ± 0.01
24:1n-9	0.26 ± 0.01	0.23 ± 0.01	0.22 ± 0.01	0.22 ± 0.0	0.13 ± 0.01	0.13 ± 0.01	0.09 ± 0.02	0.13 ± 0.02	0.17 ± 0.02 <sup>i</sup>	0.11 ± 0.01 <sup>j</sup>	0.14 ± 0.01 <sup>i</sup>	0.11 ± 0.01 <sup>j</sup>
ΣMUFA	32.72 ± 0.40 <sup>c</sup>	35.83 ± 0.36 <sup>c</sup>	33.96 ± 0.25 <sup>b</sup>	35.76 ± 0.32 <sup>a</sup>	32.76 ± 0.29 <sup>b</sup>	34.85 ± 0.46	34.57 ± 0.89	34.64 ± 0.35	37.31 ± 0.22	37.60 ± 0.11	38.77 ± 0.48 <sup>h</sup>	37.73 ± 0.21
18:2n-6 LA (L) <sup>b</sup>	18.80 ± 0.07	18.86 ± 0.07	19.14 ± 0.20 <sup>a</sup>	18.67 ± 0.10	19.53 ± 0.17 <sup>c</sup>	18.88 ± 0.13	18.80 ± 0.16	18.68 ± 0.04	19.32 ± 0.23	19.72 ± 0.03	19.68 ± 0.13	18.50 ± 0.07
18:3n-6 (Ln) <sup>b</sup>	1.02 ± 0.01 <sup>b</sup>	0.94 ± 0.03	1.12 ± 0.01 <sup>a</sup>	0.99 ± 0.05	1.20 ± 0.02	1.21 ± 0.04	1.15 ± 0.05	1.07 ± 0.05 <sup>c</sup>	1.25 ± 0.04	1.23 ± 0.04	1.17 ± 0.06	1.18 ± 0.08
18:3n-3 ALA (Li) <sup>b</sup>	0.67 ± 0.01 <sup>b</sup>	0.73 ± 0.01 <sup>a</sup>	0.65 ± 0.01 <sup>b</sup>	0.75 ± 0.01 <sup>a</sup>	0.45 ± 0.01	0.46 ± 0.01	0.48 ± 0.02	0.47 ± 0.03	0.49 ± 0.01 <sup>i</sup>	0.48 ± 0.01 <sup>i</sup>	0.44 ± 0.01 <sup>i</sup>	0.44 ± 0.01 <sup>i</sup>
20:3n-3	1.22 ± 0.07 <sup>b</sup>	1.35 ± 0.03 <sup>a</sup>	1.25 ± 0.01 <sup>b</sup>	1.33 ± 0.01 <sup>a</sup>	0.70 ± 0.01	0.72 ± 0.01	0.73 ± 0.01	0.72 ± 0.03	0.35 ± 0.01 <sup>i</sup>	0.40 ± 0.01	0.39 ± 0.02	0.41 ± 0.01
20:3n-6	0.77 ± 0.01 <sup>c</sup>	0.79 ± 0.01 <sup>b</sup>	0.81 ± 0.01 <sup>a</sup>	0.81 ± 0.01 <sup>a</sup>	0.55 ± 0.01	0.57 ± 0.01	0.55 ± 0.01	0.49 ± 0.02 <sup>c</sup>	0.36 ± 0.02	0.34 ± 0.01 <sup>i</sup>	0.34 ± 0.01	0.37 ± 0.02
20:4n-6 (AA) <sup>b</sup>	0.16 ± 0.02	0.19 ± 0.01	0.19 ± 0.01	0.18 ± 0.01	0.11 ± 0.01	0.12 ± 0.01	0.12 ± 0.01	0.12 ± 0.01	0.06 ± 0.01	0.07 ± 0.01	0.06 ± 0.01	0.08 ± 0.01
20:5n-3 EPA (Ep) <sup>b</sup>	0.19 ± 0.02	0.21 ± 0.01	0.20 ± 0.01	0.21 ± 0.01	0.14 ± 0.01	0.14 ± 0.01	0.14 ± 0.01	0.14 ± 0.01	0.15 ± 0.01	0.13 ± 0.01	0.12 ± 0.01	0.15 ± 0.01
22:4n-6 (Ad) <sup>b</sup>	0.23 ± 0.01 <sup>a</sup>	0.28 ± 0.01	0.25 ± 0.01 <sup>a</sup>	0.28 ± 0.01	0.16 ± 0.01	0.16 ± 0.02	0.17 ± 0.01	0.19 ± 0.02	0.11 ± 0.01	0.09 ± 0.01	0.11 ± 0.01	0.09 ± 0.01
22:5n-6	0.30 ± 0.01 <sup>a</sup>	0.24 ± 0.01	0.24 ± 0.01	0.26 ± 0.01	0.39 ± 0.07	0.34 ± 0.01	n.d.	0.11 ± 0.01 <sup>c</sup>	0.14 ± 0.02	0.15 ± 0.01	n.d.	0.16 ± 0.01
22:5n-3 DPA (Dp) <sup>b</sup>	0.21 ± 0.01 <sup>b</sup>	0.15 ± 0.01 <sup>c</sup>	0.30 ± 0.01 <sup>a</sup>	0.14 ± 0.01 <sup>c</sup>	0.04 ± 0.01	0.04 ± 0.02	0.04 ± 0.02	0.05 ± 0.01	0.29 ± 0.06	0.30 ± 0.01	0.29 ± 0.03	0.32 ± 0.01
22:6n-3 DHA (D) <sup>b</sup>	0.04 ± 0.01	0.04 ± 0.01	0.03 ± 0.01	0.04 ± 0.03	0.03 ± 0.01	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.01	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.01
18:2n-6 CLA, C9, t11	0.18 ± 0.01	0.19 ± 0.01	0.15 ± 0.01	0.18 ± 0.01	0.32 ± 0.01	0.30 ± 0.01	0.32 ± 0.01	0.28 ± 0.02	0.27 ± 0.01	0.27 ± 0.01	0.26 ± 0.01	0.27 ± 0.01
18:2n-6 CLA, t10, C12	0.24 ± 0.01	0.23 ± 0.01	0.21 ± 0.01	0.25 ± 0.01	0.20 ± 0.01	0.21 ± 0.01	0.22 ± 0.02	0.21 ± 0.02	0.26 ± 0.01	0.24 ± 0.01	0.26 ± 0.01	0.26 ± 0.01
ΣPUFA	24.00 ± 0.41	24.20 ± 0.33	24.54 ± 0.23	24.09 ± 0.20	23.82 ± 0.45	23.17 ± 0.34	22.74 ± 0.38 <sup>c</sup>	22.55 ± 0.28 <sup>c</sup>	23.06 ± 0.47	23.43 ± 0.17	23.13 ± 0.47	22.24 ± 0.26
ΣTotal	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00

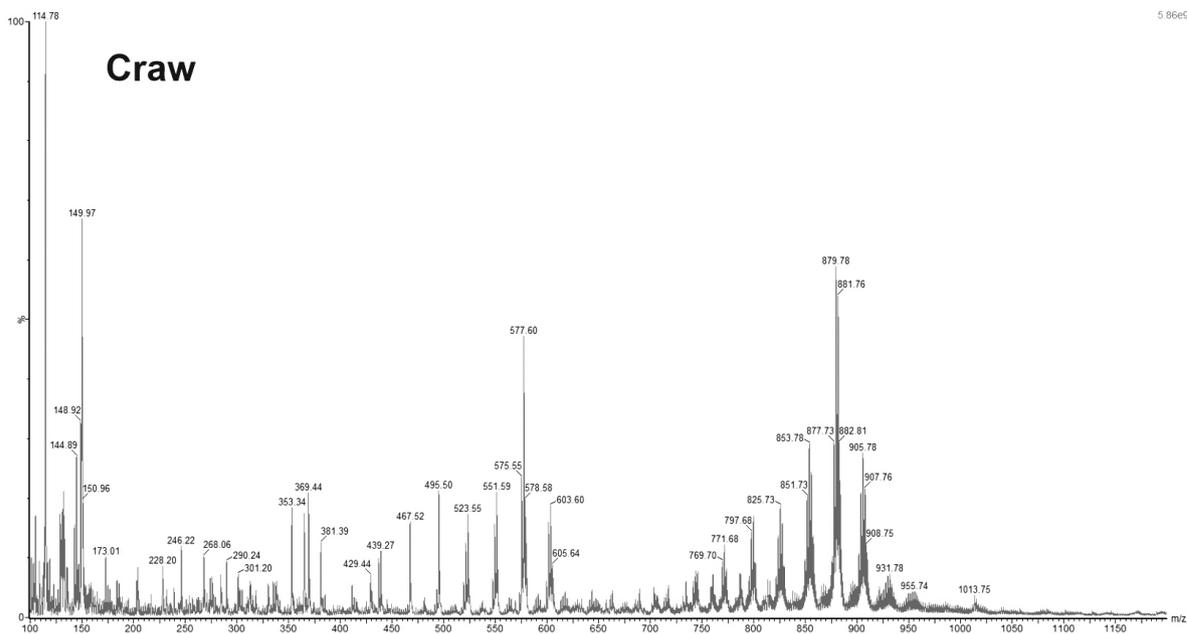
<sup>a</sup>Results are expressed as mean ± SD (standard deviation) in triplicate; <sup>b</sup>abbreviation of each FA used in TAG combinations B: butyric acid (4:0); Cp: caproic acid (6:0); Ca: caprylic acid (8:0); C: capric acid (10:0); La: lauric acid (12:0); M: myristic acid (14:0); P: palmitic acid (16:0); Pa: 7-hexadecenoic acid (16:1n-9); Po: palmitoleic acid (16:1n-7); Mg: heptadecanoic acid (17:0); S: stearic acid (18:0); O: oleic acid (18:1n-9); V: vaccenic acid (18:1n-7); L: linoleic acid (LA, 18:2n-6); Ln: γ-linolenic acid (18:3n-6); Li: α-linolenic acid (ALA, 18:3n-3); A: arachidic acid (20:0); AA: arachidonic acid (20:4n-6); Be: behenic acid (22:0); AA: arachidonic acid (20:4n-6); Er: erucic acid (22:1n-9); Ad: docosahexaenoic acid (22:6n-3); Ep: eicosapentaenoic acid (EPA, 20:5n-3); Ig: tetraenoic acid (24:0); Ad: adrenic acid (22:4n-6); Dp: adrenic acid (DPA, 22:6n-3); D: docosahexaenoic acid (DHA, 22:6n-3). <sup>c</sup>Values with different lowercase letters (<sup>b</sup> to <sup>k</sup>) in the same row for each HM of colostrum are significantly different between the groups (raw, pasteurized, lyophilized and spray-dried) ( $P < 0.05$ ) by ANOVA and Tukey's test; <sup>e</sup>values with different lowercase letters (<sup>a</sup> to <sup>h</sup>) in the same row for each HM of transitional are significantly different between the groups (raw, pasteurized, lyophilized and spray-dried) ( $P < 0.05$ ), whereas values without a superscript did not ( $P > 0.05$ ) by ANOVA and Tukey's test; <sup>h</sup>values with different lowercase letters (<sup>a</sup> to <sup>h</sup>) in the same row for each HM of mature are significantly different between the groups (raw, pasteurized, lyophilized and spray-dried) ( $P < 0.05$ ), whereas values without a superscript did not ( $P > 0.05$ ) by ANOVA and Tukey's test; <sup>i</sup>values with different lowercase letters (<sup>a</sup> to <sup>h</sup>) in the same row for each HM of mature are significantly different between the groups (raw, pasteurized, lyophilized and spray-dried) ( $P < 0.05$ ), whereas values without a superscript did not ( $P > 0.05$ ) by ANOVA and Tukey's test; CrAw: raw colostrum; CpAst: pasteurized colostrum; Clyo: lyophilized colostrum; TrAw: raw transitional; TpAst: pasteurized transitional; Tlyo: lyophilized transitional; Tspr: spray-drying transitional; MrAw: raw mature; MpAst: pasteurized mature; Mlyo: lyophilized mature; Mspr: spray-drying mature; n.d.: not detected; LA: linoleic acid; ALA: α-linolenic acid; EPA: eicosapentaenoic acid; DHA: docosahexaenoic acid; CLA: conjugated linoleic acid; SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids; ΣTotal: SFA + MUFA + PUFA.

provide information on how FAs are arranged in order to form the TAG molecules.

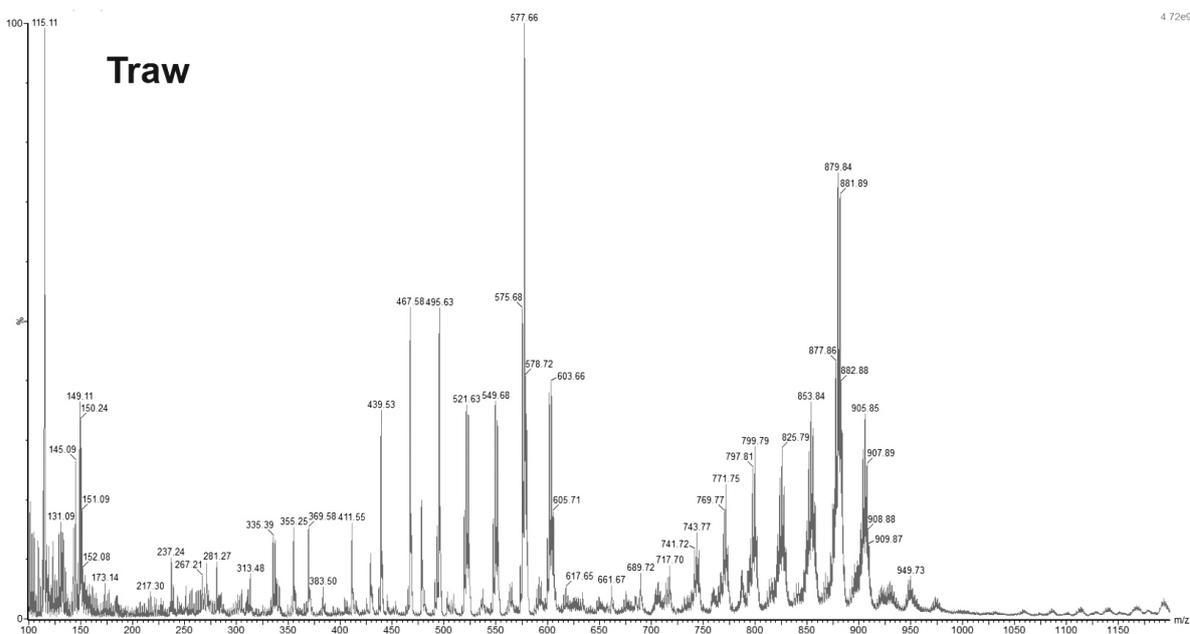
Direct ESI-MS infusion is an accurate technique that has been used in the TAG analysis. It is a fast, simple and a sensitive method.<sup>18</sup> The TAGs were identified using the Lipid Maps platform<sup>®</sup> database with LAMES platform,<sup>32</sup> through this platform it is possible to obtain an estimated concentration (in percentage) of each TAG, that is, the most intense ionic peak in each mass spectrum was designated as 100% and the others were designated as relative intensity in relation to the most intense peak. The peaks identified in the

mass/charge ( $m/z$ ) range from 100 to 1200 in the spectra of samples without processing of the colostrum, transitional and mature HM (Craw, Traw and Mraw) were obtained and the results are displayed in Figures 1-3.

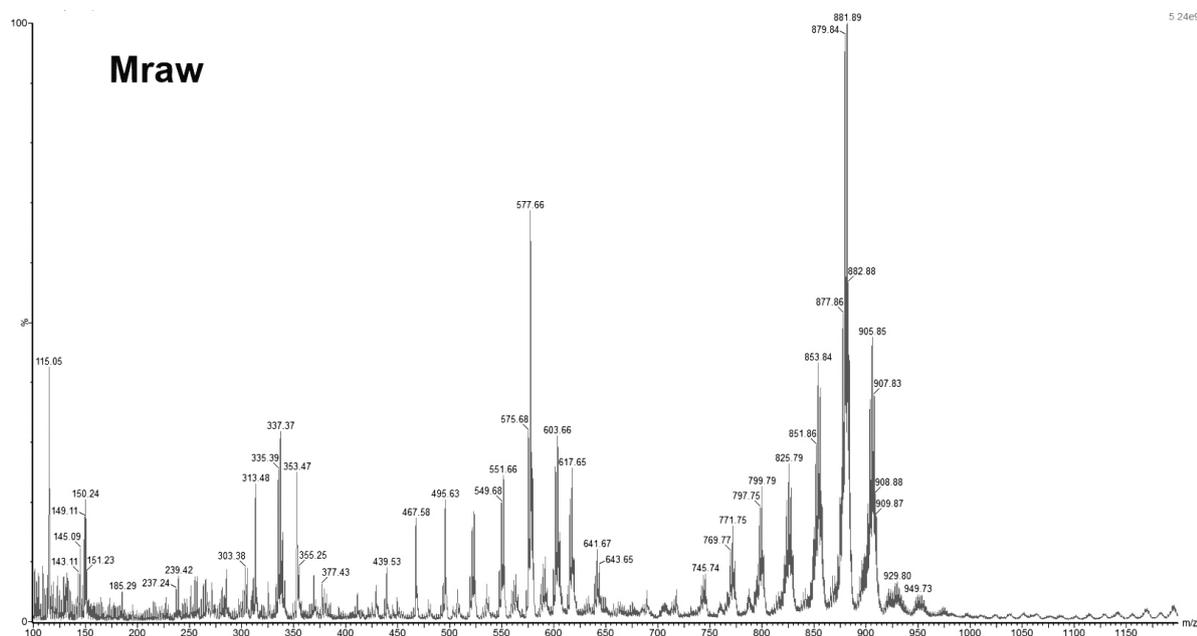
Figures S1-S9 (Supplementary Information section) show the results of the peaks identified in the  $m/z$  range from 100 to 1200 in the spectra of colostrum, transitional and mature HM samples submitted to pasteurization (Cpast, Tpast and Mpast), lyophilization (Clyo, Tlyo and Mlyo) and spray-drying (Cspr, Tspr and Mspr), respectively. In addition to determining the estimated concentration,



**Figure 1.** Ion spectrum of [TAG + NH<sub>4</sub>]<sup>+</sup> raw colostrum (Craw) from ESI(+)-MS.



**Figure 2.** Ion spectrum of [TAG + NH<sub>4</sub>]<sup>+</sup> raw transitional HM (Traw) from ESI(+)-MS.



**Figure 3.** Ion spectrum of  $[\text{TAG} + \text{NH}_4]^+$  raw mature HM (Mraw) from ESI(+)-MS.

it is possible to obtain the FAs distribution for the TAG molecule formation in a sample through the FAs relative area obtained from the GC-FID analysis.

The FAs distribution along the glycerol structure directly influences its availability; if glycerol is drawn with the first and third hydroxyl groups on the right and the second on the left, the first carbon is called (sn-1); if glycerol is drawn with the second, the first carbon is called (sn-2), and if glycerol is drawn with the third, the first carbon is called (sn-3), therefore, the stereospecific numbering (sn) determines where the FAs are located in the TAG, in addition to this influence on digestion, absorption, metabolism and tissues distribution.<sup>9,33,34</sup>

The FAs in the sn-1 and sn-3 positions are preferentially lost compared to the FAs in the sn-2 position. Steric and electronic energies favor the loss of FAs in the sn-1 and sn-3 positions, where both will form a 6-membered intermediate ring; however, the FA loss from the sn-2 position results in a 5-membered intermediate ring, which is less stable. If the two FAs at the sn-1 and sn-3 position are different, the loss of FA in the sn-1 position generates a relatively more abundant diacylglycerol ion than that produced from the loss of FA at the sn-3 position.<sup>35,36</sup>

Palmitic acid (16:0) is an SFA that represents approximately 22% of the total FAs determined in HM, similar results were found by Manios *et al.*<sup>37</sup> This FA in HM is predominantly esterified at the sn-2 position of TAGs, this positioning is of fundamental importance for the absorption of calcium and magnesium, greater availability of fat, improvement of bone strength and intestinal flora in the NB.<sup>38,39</sup>

Unsaturated FAs, such as oleic (18:1n-9) and linoleic (18:2n-6) acids, are generally esterified at the sn-1 and sn-3 positions of the glycerol structure. Gastric lipase acts directly on stereoselectivity in sn-3, releasing medium and long-chain FAs in the stomach that are important in triggering pancreatic lipase activity and are essential for total digestion.<sup>38,40</sup>

This study is a pioneer in comparing the TAG composition of raw, pasteurized, lyophilized and spray-dried in different HM lactation stages. From the results obtained from the HM samples in the different processing, it was observed that the relative area of the main TAGs was preserved after processing. In Table S1 (Supplementary Information section) it is possible to observe the TAGs with the highest intensities in all HM samples considering its lactation stage and its different processing, in the range of  $m/z$  878-904.

The possibilities of TAG's are: BeNeCp, BeOLa, BeVLa, BePaM, BePoM, COLg, CVLg, CNeS, LgNeB, LgPaLa, LgPoLa, CaNeA, SPaS, SPoS, SOP, SVP, SHpMg, AOM, AVM, APaP, APoP, LaNeP, MNeM, MgOMg, MgVMg; BeCaBe, BeAC, BeCpLg, BeLaS, BePM, CSLg, LgBLg, LgACa, LgPLa, LgMM, SPS, SMA, SMgMg, ALaA, APP; BeDoC, BeEiLa, BeLM, BePaPa, BePoPa, BePoPo, CEiLg, LgDoCa, LgLLa, CpNeNe, SLS, SDoM, SEiP, SOO, SVO, SVV, ADoLa, AEiM, ALP, APaO, APoO, AHpHp, AVPa, AVPo, LaNeO, LaVNe; being: B: butyric acid (4:0); Cp: caproic acid (6:0); Ca: caprylic acid (8:0); C: capric acid (10:0); La: lauric acid (12:0); M: myristic acid (14:0); P: palmitic acid (16:0); Pa: 7-hexadecenoic acid (16:1n-9); Po: palmitoleic acid (16:1n-7); Mg: heptadecanoic acid (17:0); S: stearic acid (18:0); O: oleic acid (18:1n-9);

V: vacenic acid (18:1n-7); L: linoleic acid (LA, 18:2n-6); Ln:  $\gamma$ -linolenic acid (18:3n-6); Li:  $\alpha$ -linolenic acid (ALA, 18:3n-3); A: arachidic acid (20:0); E1: eicosadienoic acid (20:2n-6); Be: behenic acid (22:0); AA: arachidonic acid (20:4n-6); Er: erucic acid (22:1n-9); Ad: docosatetraenoic acid (22:4n-6); Ep: eicosapentaenoic acid (EPA, 20:5n-3); Ig: tetracosanoic acid (24:0); Ad: adrenic acid (22:4n-6); Dp: adrenic acid (DPA, 22:5n-3); D: docosahexaenoic acid (DHA, 22:6n-3); docosadienoic acid (22:2n-6); Hp: heptadecenoic acid (17:1n-9); Ne: tetracosenoic acid (24:1n-9); palmitic acid being predominant among the TAG combinations.

These results are in agreement with the results obtained by the GC-FID analysis, as the 16:0, 18:1n-9, 18:2n-6, 20:0 and 22:0 FAs showed higher concentrations in all HM samples considering all lactation stages and different processing. GC-FID, and the possible lipids found by *m/z* by ESI(+)-MS were mainly composed by these FAs. Furthermore, these findings are in agreement with those found by Rydlewski *et al.*,<sup>7</sup> who evaluated the FA and TAG composition of all HM lactation stages by different lipid extraction methods and Manin *et al.*,<sup>13</sup> who evaluated FA and TAG in all lactation stages of lyophilized FA for 180 days.

## Conclusions

The results of this study indicate that pasteurization, lyophilization and spray-drying processes in DHM samples did not result in changes in the lipid quality of DHM samples after processing, since the FA composition and the TAG profile remained preserved after processing. Therefore, considering these results presented, lyophilization and spray-drying techniques are promising alternatives to improve the quality of lipid preservation processes in DHM samples in HMB, in addition to reducing the storage volume and facilitating the HM transport.

## Supplementary Information

Supplementary data are available free of charge at <http://jbcbs.s bq.org.br> as PDF file.

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## Author Contributions

Vanessa J. C. Neia was responsible for the conceptualization, methodology, formal analysis, investigation, writing - review and editing, project administration; Patrícia D. S. Santos for the formal analysis, investigation; Christyna B. G. Tavares, Meliana G. Paula and Silvio C. Costa for the methodology; Joana M. V. Zacarias for the formal analysis, investigation; Josiane B. Alencar for the writing - review and editing; Roberta Silveira for the writing - review and editing; Oscar O. Santos for the writing - review and editing; Jeane E. L. Visentainer for the writing - review and editing; Jesu V. Visentainer for the conceptualization, methodology, resources, writing - review and editing, supervision, project administration and funding acquisition.

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