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Direct Methylation Method for Quantification of Fatty Acids in Lyophilized Human Milk by Gas Chromatography with Flame Ionization Detector

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This study aims to propose a new method for derivatizing fatty acids (FAs) from human milk (HM), eliminating the lipid extraction step, and simplifying the preparation for gas chromatography with a flame ionization detection (GC-FID) analysis to quantify the FAs. The Design Expert software optimized the reaction times, concentrations, and sample amount. The proposed method (PM) was validated for lyophilized HM and results for the figures of merit for precision in relative standard deviation (RSD) ($RSD_{intra\text{-}day}$ 1.34-4.03% and $RSD_{inter\text{-}day}$ 2.08-5.16%), accuracy (99.87-102.16%), and robustness are within a linear range of 3 to 38% lipids in HM samples. The atmospheric solids analysis probe tandem mass spectrometry (ASAP-MS/MS) technique confirmed the efficiency of PM by expressing the molecular composition of triacylglycerol formed by FAs from the GC-FID technique. The PM requires a small sample size and conducts derivatization directly in the sample matrix, minimizing extraction errors.

Keywords: analytical parameters, lipids, atmospheric-pressure solids analysis probe, ultrasound

Introduction

The World Health Organization and pediatricians recommend human milk (HM) as the exclusive source of nutrition during the first six months of the life of neonate.[1] The composition of HM is rich in essential compounds, including carbohydrates, proteins, and lipids, that are important for the development of the immunologic and cognitive systems of the neonate.[2-4]

Lipids have structural and regulatory functions and are the major source of energy in HM,[5] in which fatty acids (FAs) are essential for the development of the central nervous system in the first two years of life, favoring the action of digestive lipases, increasing anandamide levels, and producing analgesic effects.[5-7] Besides, FAs are important for the immune, cognitive, visual, and motor development of newborns.[8]

The HM consumed by neonates differs in composition and volume. When exploring relationships between maternal impact and infant outcomes, it is important to consider the overall profile of the HM FAs and the intake of each HM component.[9] So, quantifying the FAs present in HM is an important aspect of the HM study. The analysis of FAs in HM by gas chromatography with a flame ionization detector (GC-FID) is extensively reported in the literature.[3,10,11] Usually, this analysis requires three steps: lipid extraction; derivatization to methyl esters by derivatization reactions; and quantification by GC-FID.[12]

The standard lipid extraction method used for the analysis of FAs in HM was proposed by Folch *et al.*[13] However, this methodology needs long extraction times to provide acceptable extraction efficiency, which leads to a laborious procedure and requires large volumes of organic solvents (chloroform and methanol in a 2:1 v/v ratio). In addition, extensive sample manipulation, including agitation, phase separation, filtration, and evaporation, increases the probability of experimental errors.[12,13] After extraction, FAs need to be derivatized to fatty acid methyl esters (FAMEs),[7] which are then detected by GC-FID, due to their volatile and thermal stability characteristics.[14]

Direct methylation has been gaining popularity over the last few years as a derivatization methodology for the analysis of FA composition in different samples, such as

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bovine fat, infant formulas, and fermented milk samples.[15-18] The method is simpler and faster than traditional methods, because lipid extraction and derivatization occur simultaneously in a one- or two-steps (base-catalyzed hydrolysis, followed by acid hydrolysis). In addition, direct methylation requires a low amount of sample and solvents and is less susceptible to experimental errors due to decreased sample manipulation.[15-20]

However, to the best of our knowledge, no study has proposed a method of direct methylation for analysis of FA composition in lyophilized human milk (HM_{lyo}). Therefore, this study proposes a novel procedure of derivatization to determine the FA composition in mature HM_{lyo}, without the need for previous lipid extraction, using low quantities of samples and solvents. Moreover, the proposed method was also applied to samples of HM_{lyo} and liquid HM (HM_{liq}) at different lactation stages (colostrum, transitional and mature). The HM_{lyo} was chosen due to its advantages for the human milk bank (HMB) presented in previous studies,[6] including the increase of the shelf-life of the product, stopping the microbial growth, and retarding the lipid oxidation process. The use of the lyophilization process by the HMB can be a good alternative to prolong the shelf-life of human milk and facilitate its transport and storage.

Experimental

Reagents, solvents and standards

Potassium hydroxide (KOH), hydrochloric acid (HCl), chloroform, *n*-heptane, and methanol (MeOH) (all analytical grade) were acquired from Labsynth (Diadema, Brazil). Reference standards of methyl tricosanoate (23:0; ≥ 99%), tritridecanoin (TAG 13:0; ≥ 99%), FAME Mix, C4-C24 unsaturated (≥ 97%) were purchased from Sigma-Aldrich (São Paulo, Brazil).

HM samples

The present study was approved by the Research Ethics Committee local, under number 2.797.476/2018, of the Universidade Estadual de Maringá (UEM, Maringá, Brazil). HM samples in different lactation phases (colostrum, transitional, and mature) were collected from 20 distinct donors, following a specific protocol for HMB of the Hospital Universitário de Maringá (Maringá, Brazil), and stored at 4 °C in a domestic refrigerator. Samples were homogenized, grouped into pools (2 L) according to each lactation phase, and stored at –32 °C in a vertical series MFV/UFV ultra-freezer (Terroni Scientific equipment, Pauliceia, Brazil) until analysis.

Lyophilization

The 960 mL pool of colostrum, transitional, and mature HM were subjected to the lyophilization process. Samples (80 mL) were lyophilized using an SLH-50 lyophilizer (Terroni Scientific Equipments, São Carlos, Brazil) at –55 °C and 0.05 mmHg for 72 h. The operating conditions used were recommended by the manufacturer. Only mature HM_{lyo} ($HM_{lyo}M$) was utilized for the development of the proposed method. The HM_{lyo} from the other lactation phases, colostrum ($HM_{lyo}C$) and transitional ($HM_{lyo}T$), were utilized to verify the applicability of the proposed method (PM, see sub-section "Proposed method (PM) of lipid extraction and derivatization").

Traditional method (TM) of lipid extraction and derivation

Lipid extraction was performed according to Folch *et al.*[13] Approximately 10 g of samples were added into a beaker. Then 200.0 mL of chloroform:methanol mixture (2:1, v/v) was added, and the solution was stirred vigorously for 3 min. The resulting solution was filtered in a Büchner funnel through a quantitative filter paper. The solution obtained was transferred to a separation funnel and left undisturbed for 24 h to allow phase separation. The lower part of the separation containing chloroform and lipids was transferred to a 250 mL pre-weighed flat bottom flask, and the solvent was evaporated in a rotary evaporator (Prismalab, Rio de Janeiro, Brazil).

Derivatization reactions of the FAs were performed according to the International Organization for Standardization ISO 12966:2/2017.[21] Lipid samples (100.0000 mg) were weighed in a tube, followed by the addition of 500 µL standard 23:0 solution (1.0 mg mL^{-1} in *n*-heptane m/v) and 2.0 mL *n*-heptane were added. The solution was stirred in a vortex for 2 min (Forlab Express, Higienópolis, Brazil). Next, 3.0 mL KOH/MeOH (2 mol L^{-1}) was added and stirred in a vortex for 3 min. The resulting solution was left undisturbed for 24 h to allow phase separation at 4 °C in a domestic refrigerator. The superior portion (organic portion) was collected for further GC-FID analysis.

Experimental design

A rotational central composite design was generated by Design Expert® 7 software[22] to model a second-order response surface, it provides error prediction uniformity.[23] For this, five independent variables (concentrations of acid and base, reaction times of acid and base, and sample mass) at five levels were to associate their individual

effects and interactional influences on the dependent variable (sum of FA). The –α and +α levels for acid and base reactions time, acid and base concentration, and sample mass were: 5.00 and 25.00 min, 0.38 and 1.88 mol L^{-1}, and 30.0000 and 310.0000 mg, respectively. The axial points (± α) provided by the rotational system (k < 5) were ± 1.4142 and were applied to calculate the quadratic terms, as presented in Table S1 (in the Supplementary Information (SI) section). The design comprises 32 experiments, with five replications at the center point (Table 1). The temperature of the experiments was set at 25 °C.

Proposed method (PM) of lipid extraction and derivatization

In a glass tube containing 100.0000 mg of $HM_{lyo}M$, internal standard solutions 23:0 (500 µL) and TAG 13:0 (2.0 mL) (both prepared at 1.0 mg mL^{-1} in *n*-heptane m/v) were added along with 2.0 mL of KOH/MeOH (using different concentrations according to the experimental design and presented in Table 1).

Then, the solution was stirred in a vortex for 2 min and placed into an ultrasonic bath (Eco-Sonics Q 5.9/25, Unique, São Paulo, Brazil) at different reaction times as determined by experimental design (Table 1), with 165 W

Table 1. Central composite rotational experimental design and sum of FAs obtained for lyophilized mature human milk by GC-FID

Experimental run	Basic reaction time / min	Acid reaction time / min	HCl/MeOH content / (mol L^{-1})	KOH/MeOH content / (mol L^{-1})	Sample mass / mg	Sum of FAs / (mg g^{-1} sample)
1	10 (–1)	10 (–1)	0.75	0.75	240	191.3661
2	20 (+1)	10 (–1)	0.75	0.75	100	251.9260
3	10 (–1)	20 (+1)	0.75	0.75	100	213.8109
4	20 (+1)	20 (+1)	0.75	0.75	240	218.5640
5	10 (–1)	10 (–1)	1.5	0.75	100	409.5600
6	20 (+1)	10 (–1)	1.5	0.75	240	329.0885
7	10 (–1)	20 (+1)	1.5	0.75	240	215.9338
8	20 (+1)	20 (+1)	1.5	0.75	100	249.0849
9	10 (–1)	10 (–1)	0.75	1.5	100	246.1021
10	20 (+1)	10 (–1)	0.75	1.5	240	165.6124
11	10 (–1)	20 (+1)	0.75	1.5	240	160.4444
12	20 (+1)	20 (+1)	0.75	1.5	100	147.1313
13	10 (–1)	10 (–1)	1.5	1.5	240	189.7598
14	20 (+1)	10 (–1)	1.5	1.5	100	195.2520
15	10 (–1)	20 (+1)	1.5	1.5	100	175.7692
16	20 (+1)	20 (+1)	1.5	1.5	240	166.2129
17	5 (–α)	15 (0)	1.125	1.125	170	190.6491
18	25 (+α)	15 (0)	1.125	1.125	170	284.3414
19	15 (0)	5 (–α)	1.125	1.125	170	268.1195
20	15 (0)	25 (+α)	1.125	1.125	170	290.1179
21	15 (0)	15 (0)	0.375	1.125	170	222.9148
22	15 (0)	15 (0)	1.875	1.125	170	221.4920
23	15 (0)	15 (0)	1.125	0.375	170	203.7484
24	15 (0)	15 (0)	1.125	1.875	170	142.2655
25	15 (0)	15 (0)	1.125	1.125	30	211.3690
26	15 (0)	15 (0)	1.125	1.125	310	233.4732
27	15 (0)	15 (0)	1.125	1.125	170	263.1749
28	15 (0)	15 (0)	1.125	1.125	170	212.7515
29	15 (0)	15 (0)	1.125	1.125	170	209.9662
30	15 (0)	15 (0)	1.125	1.125	170	275.0098
31	15 (0)	15 (0)	1.125	1.125	170	225.3410
32	15 (0)	15 (0)	1.125	1.125	170	203.2956

(0): central point; (± 1): factorial points; (± α): axial points.

potency, 25 kHz frequency, and 25 °C temperature. After alkaline reaction time, 2.0 mL of HCl/MeOH (at different concentrations) was added to the mixture. The solution was stirred in a vortex for 2 min and placed into an ultrasonic bath at different reaction times (Table 1) under the same conditions employed for the alkaline reaction. Then, 1.0 mL *n*-heptane was added to the mixture, and the tube was stirred for 30 s in a vortex and centrifuged under 2000 rpm for 1 min. The solution was stored at 4 °C to allow phase separation for 24 h in a domestic refrigerator. The superior portion (organic portion) was collected for further GC-FID and atmospheric pressure solid analysis probe (ASAP) analysis.

Gas chromatography analysis

Chromatographic analyses were carried out in a Shimadzu GC-2010 Plus (Kyoto, Japan) equipped with a flame ionization detector (FID), split/splitless injector, and a fused silica capillary column CP-7420 (Select FAME, 100 m length, 0.25 mm i.d., and 0.25 μm film thickness of cyanopropyl as stationary phase). The gas flows used were 1.4 mL min^{-1} for carrier gas (H_2), 30.0 mL min^{-1} for make-up gas (N_2), and in the FID 30.0 and 300.0 mL min^{-1} for H_2 and synthetic air, respectively. Samples were injected in split mode (1:40) and an injection volume of 2 μL. The column temperature was raised to 65 °C for 4 min, then heated to 185 °C at 16 °C min^{-1}. After 12 min, the temperature was raised to 235 °C at 20 °C min^{-1} and maintained for 9 min, totaling an analysis time of 35 min. The injector and detector temperatures were 230 and 250 °C, respectively. FAMEs were identified by comparing the retention times of the compounds of the samples with those of the analytical standard (FAME Mix, C4-C24). The Lab Solutions software was used to determine peak areas. The analysis was performed in triplicate. Theoretical FID correction factor was used to obtain FA concentration values (mg g^{-1} of sample) carried out by comparing the retention times of the compounds in the samples with those of the analytical standard FAME Mix, C4-C24, and they where quantified using the internal standard 23:0 according to Visentainer *et al.*,[24] as demonstrated by equation 1:

$$Mx = \frac{A_x M_p F_{CT}}{A_p M_A F_{CEA}} \quad (1)$$

where Mx: concentration of the fatty acid (mg g^{-1} of sample); A_X: peak area of the fatty acids; A_p: peak area of the internal standard (23:0); M_p: mass of internal standard (23:0) added to the sample (mg); M_A: mass of sample (g); F_{CT}: theoretical correction factor of the flame ionization detector (FID); F_{CEA}: conversion factor from methyl ester to fatty acid.

In addition, the transesterification performance (%) of the PM was determined on the recovery of the TAG 13:0 internal standard based on the equation 2:[25]

$$\text{Recovery}\ (\%) = \frac{m_{23:0} \times A_{13:0} \times R_{13:0} \times S_{13:0}\,(\text{TAG})}{A_{23:0} \times m_{13:0}} \times 100 \quad (2)$$

where $m_{23:0}$: mass of 23:0 internal standard added to the solution (mg); $A_{13:0}$: peak area of TAG 13:0 internal standard in the chromatogram; $R_{13:0}$: response factor of TAG 13:0 compared to 23:0; $S_{13:0}$ (TAG): stoichiometric conversion factor of 13:0 FAME into 13:0 TAG (0.994); $A_{23:0}$: peak area of 23:0 internal standard in the chromatogram; $m_{13:0}$: mass of TAG 13:0 added to the solution (mg).

Reaction efficacy by atmospheric solids analysis probe mass spectrometry

A Xevo TQ-D mass spectrometer (Waters, Massachusetts, USA) with a triple quadrupole mass analyzer, equipped with an ASAP (Waters, Massachusetts, USA) was applied to assess the efficacy of the PM and compare it to the TM of lipid extraction and esterification and transesterification. A capillary tube (100 mm × 1.9 mm) was immersed into a vial containing the sample, placed onto the ASAP probe, and introduced into the ion source chamber. For the 150-500 Da mass scan, a probe temperature ramp of 120-300 °C (gradient of 10 C s^{-1}) was used with a 10-70 V cone ramp (gradient of 0.171 V Da^{-1}). For the 500-1000 Da mass scan, a 10-100 V cone ramp (gradient of 0.171 V Da^{-1}) was used with a probe temperature ramp of 300-600 °C (gradient of 10 °C s^{-1}). ASAP-MS analysis was carried out within 4 min, in positive ion mode, and data was processed using the MassLynx ™ software.[16]

Validation parameters

The validation parameters of the PM were determined following the guidelines of the International Conference on Harmonization.[26] The figures of merit used were: precision (inter- and intra-day), accuracy, linear range, and robustness, obtained with 6 replicates. Relative standard deviation (RSD) was evaluated to estimate the precision of the PM by calculating the repeatability of results on the same day ($RSD_{intra\text{-}day}$) and different days ($RSD_{inter\text{-}day}$). Accuracy was estimated by comparing the results obtained from PM and TM. The linear range was determined by interpolation of the sum of FA obtained by TM concerning the PM. The robustness of the PM was evaluated by varying

the temperature of the ultrasonic bath (28.5-31.5 °C) and sample injection volume (1-3 μL).[14]

Application of the method

The PM was applied to samples of HM_{liq} at different lactation phases, colostrum ($HM_{liq}C$), mature ($HM_{liq}M$), transitional ($HM_{liq}T$), and $HM_{lyo}C$ and $HM_{lyo}T$ to evaluate its applicability using different HM forms and lactation phases.

Statistical analysis

The FA composition of HM samples was acquired in triplicate and expressed as mean values ± standard deviation. The results were subjected to analysis of variance (ANOVA) and values were compared by Tukey's test at a 95% significance level using the Assistat 7.7 software.[27]

Results and Discussion

Experimental design

The PM for the direct methylation of $HM_{lyo}M$ was developed and optimized based on existing methods used for evaluating the FA composition of biological fluids.[17,19,20,25] In the literature,[25] only one study developed and validated a direct method to analyze the FA content in HM_{liq} samples. However, HM_{liq} samples were heated to 100 °C for 60 min. Such high temperatures can easily undergo lipid degradation, including butyric acid (4:0), caproic acid (6:0), caprylic acid (8:0), and capric acid (10:0).[7] In contrast, in our study, the PM is performed at room temperature (25 °C) to maintain the integrity of the lipids, avoiding oxidation reactions. In the present study, two base-catalyzed transesterification methods were explored for methylation: KOH/MeOH and HCl/MeOH. The KOH/MeOH has been used in several studies.[20,28] This method can be performed at room temperature, requiring a short derivatization time, showing good efficiency in the derivatization process for the FA in triacylglycerols (TAGs).[12,28,29] On the other hand, the HCl/MeOH was selected because it gives semi-quantitative yields and is simple to operate.[12,29] It is comparable to other acid catalysts such as sulfuric acid (H_2SO_4) and boron trifluoride (BF_3), with the advantage of low cost and large availability.[12,28,29]

Once the independent variables were defined, a rotational central composite design was generated by Design Expert® 7 software,[22] and the results obtained for each experimental run are presented in Table 1. The highest rate of FA esterification achieved was in experiment 5 with a FAs sum of 409.5600 mg g^{-1} of sample. This result was obtained with the use of KOH/MeOH (0.75 mol L^{-1}) and HCl/MeOH (1.5 mol L^{-1}) using equal reaction times for the alkaline and acid reactions. To evaluate the model obtained and the interactions between the factors, the results were submitted to ANOVA and the response surface was generated by the Design Expert® 7 software. Among the models suggested by the software (linear, two-factor interaction (2FI), quadratic, and cubic), the cubic model was selected as being the most appropriate due to its high order of significance, low lack of adjustment, and reasonable agreement between the correlation coefficients proposed for the model. Table 2 presents the ANOVA parameters.

The statistical significance of the factors was evaluated by employing the *t*-test (Student's *t*-distribution) with *p*-value and *F*-test (Fischer's distribution). Values of Prob. > *F* less than 0.0500 indicate that the terms of the model are significant. In this study, A, D, AE, BC, BD, BE, CD, B^2, D^2, A^2B, A^2C, A^2D, A^2E, and AB^2 are significant model terms. The *F*-value of 24.24 for the model implies the model is significant. There is a 0.04% chance that the *F*-value of the model could occur due to noise. The "lack-of-fit" *F*-value of 4.34 implies that this term is not significant about the pure error. There is a 9.18% chance that the "lack-of-fit" *F*-value could occur due to noise. The coefficient of determination (R^2) defined was $R^2 = 0.9906$ agrees with the adjusted R^2 (0.9494), which signals a good fit between the cubic model and experimental data. The low value of the coefficient of variation (CV: 5.36%), indicates the good reproducibility of the model. "Adequate precision" was applied to measure the signal-to-noise ratio. A ratio greater than 4 is desirable for the PM model. The ratio of 25.36 indicates an adequate signal, which demonstrates that this model can be used to evaluate the PM for $HM_{lyo}M$.[30]

The model was adjusted based on actual values of factor functions studied and presented in equation 3. The relationship between predicted, and actual values is illustrated in Figure S1 (SI section). Besides, the predicted response for the dependent variable (maximum intensity) could be obtained via the second-order polynomial equation (equation 3) by multiple regression analysis on the experimental data.

$$Y = 204.17 + 21.84A - 14.22D + 18.92AE - 11.70BC + 8.13BC + 12.03BE - 18.95CD + 13.69B^2 - 10.93D^2 - 30.91A^2B + 20.15A^2C - 22.49A^2D - 19.75A^2E - 26.56AB^2 \quad (3)$$

Figure S1 shows that the model is well-adjusted because the actual values are close to the straight line, agreeing with

Table 2. ANOVA model parameters of the proposed method of fatty acid derivatization

Source	Sum of squares	DF	Mean square	*F* value	Prob. > *F*	Observation
Model	76871.14	25	3074.85	24.24	0.0004	S
A	3817.63	1	3817.63	30.10	0.0015	S
D	16616.53	1	1616.53	12.74	0.018	S
AE	5724.81	1	5724.81	45.14	0.0005	S
BC	2190.01	1	2190.01	17.27	0.0060	S
BD	1057.71	1	1057.71	8.34	0.0278	S
BE	2317.22	1	2317.22	18.27	0.0052	S
CD	5748.29	1	5748.9	45.32	0.0005	S
B^2	5499.09	1	5499.09	43.36	0.0006	S
D^2	3506.27	1	3506.27	27.64	0.0019	S
A^2B	5095.41	1	5095.41	40.17	0.0007	S
A^2C	2165.59	1	2165.9	17.07	0.0061	S
A^2D	2698.65	1	2698.5	21.28	0.0036	S
A^2E	2080.99	1	2080.99	16.41	0.0067	S
AB^2	3763.20	1	3763.20	29.67	0.0016	S
Residual	761.02	6	126.84	–	–	–
Lack-of-fit	353.47	1	353.47	4.34	0.0918	NS
Pure error	407.55	5	81.51	–	–	–
Total	77632.16	31	–	–	–	–

DF: freedom degree; Prob. > *F*: probability value linked to *F* value; A: KOH/MeOH reaction time; D: KOH/MeOH concentration; AE: interaction of KOH/MeOH reaction time and sample mass; BC: interaction of HCl/MeOH reaction time and HCl/MeOH concentration; BD: interaction of HCl/MeOH reaction time and KOH/MeOH concentration; BE: interaction of HCl/MeOH reaction time and sample mass; CD: interaction of HCl/MeOH reaction time and KOH/MeOH concentration; B^2: HCl/MeOH reaction time; A^2B: interaction of KOH/MeOH reaction time and HCl/MeOH reaction time; A^2C: interaction of KOH/MeOH reaction time and HCl/MeOH concentration; A^2D: interaction of KOH/MeOH reaction time and KOH/MeOH concentration; A^2E: interaction of KOH/MeOH reaction time and sample mass; S: significant; NS: non-significant.

the R^2 value. The response surface (Figure S2, SI section) showed a reduction in the FA sum upon decreasing the concentration of HCl/MeOH. The lowest sum of FA was obtained when the lowest concentration of HCl/MeOH was used. A direct correlation between HCl/MeOH concentration and the sum of FAs can be observed on the 3D plot. The positive impact of the acid reaction in the sum of FAs may be because of the broader spectrum of lipid classes (e.g., triacylglycerols (TAGs), diacylglycerols (DAGs), monoacylglycerols (MAGs), and free FAs) that HCl/MeOH enables to derivatize.[31]

Optimization of the model

The optimization of the PM conditions was performed to prepare the experimental conditions for a fast reaction time and low amount of sample. Therefore, both alkaline and acid reaction times parameters were limited to 20 min and the sample amount parameter was restricted to 240 mg. The Design Expert® 7 software theoretically predicted the optimum parameters as the following: 10 min for both alkaline and acid reaction time; concentrations of 1.5 and 0.75 mol L^{-1} for HCl/MeOH and KOH/MeOH, respectively; and 100 mg of sample. These conditions should provide a sum of FA 409.5620 mg g^{-1} of sample.

Assessment of the reaction efficiency by ASAP-MS

The ASAP-MS is an ionization technique for rapid and direct analysis of solids and liquids.[32] In this study, ASAP-MS was used to evaluate the reaction efficiency of the PM compared with the TM. To date, there has been no detailed investigation of the HM lipid profile using ASAP-MS as an analytical technique. However, existing studies on the HM lipid profile,[11,15,33] have shown that ion peaks of DAGs and TAGs appear in the region of *m/z* 500-1000 in the mass spectra. Then, Figures 1 and 2 show the mass spectra of the unesterified compounds (*m/z* 500-1000) and the esterified compounds (*m/z* 150-500), respectively by the PM (HM_{lyo}M-PM) and TM (HM_{lyo}M-TM). In both methods (PM and TM), DAGs and TAGs (*m/z* 500-1000) were found as unesterified products, however, the intensities and quantities of peaks

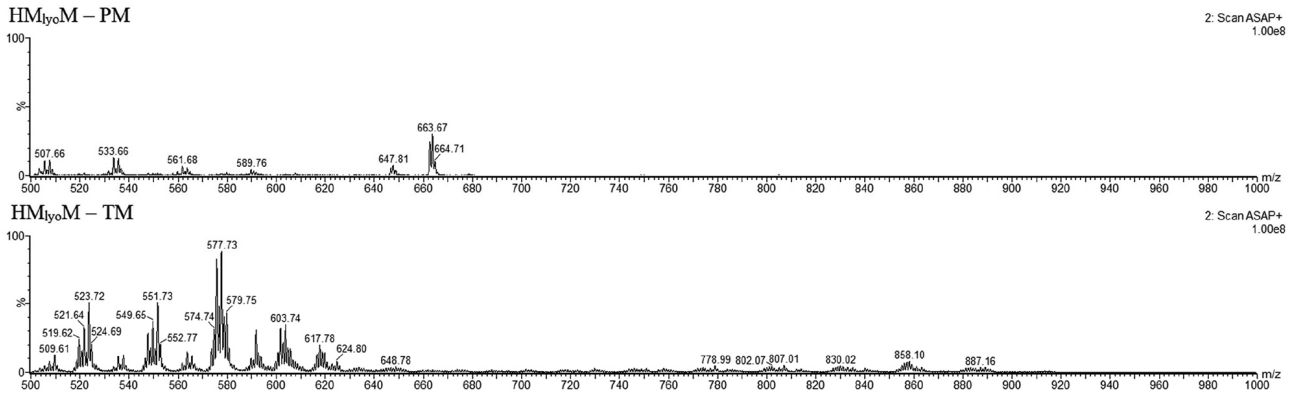

Figure 1. Mass spectra of the unesterified compounds of $HM_{lyo}M$ by the PM ($HM_{lyo}M$-PM) and traditional methodology ($HM_{lyo}M$-TM) in the range of *m/z* 500-1000.

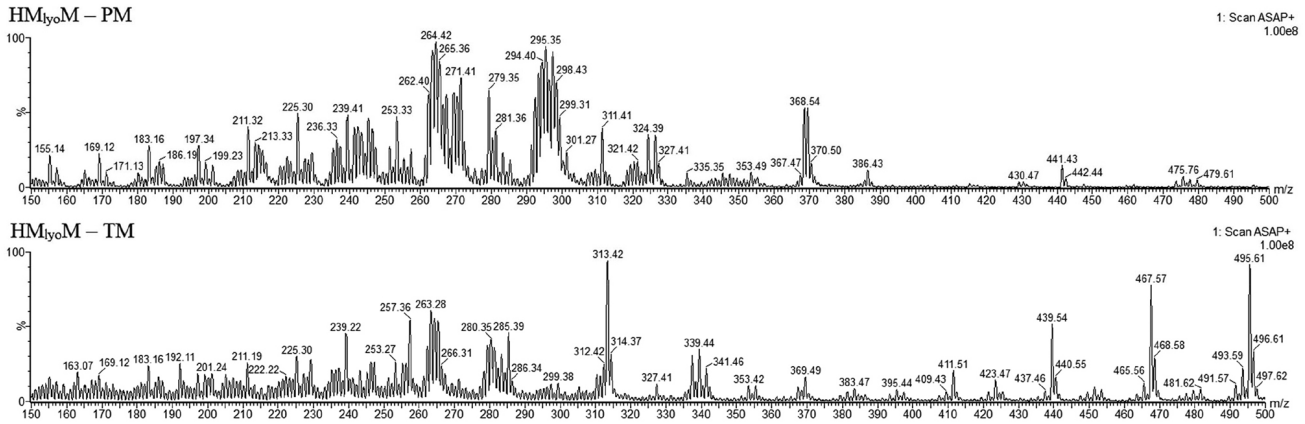

Figure 2. Mass spectra of the esterified compounds of $HM_{lyo}M$ by the PM ($HM_{lyo}M$-PM) and traditional methodology ($HM_{lyo}M$-TM) in the range of *m/z* 150-500.

were smaller using PM than TM (Figure 1), which shows the relevance of PM.

The $[M]^+$, $[M + H]^+$, and $[M + K]^+$ ions were identified in the mass spectra of both derivatization methods. In ASAP ionization, the compounds are volatilized using heated steam of nitrogen and submitted to a proton transfer reaction near the corona discharge needle, which leads to the formation of $[M + H]^+$ and $[M]^+$ ions depending on the content of residual water inside the ion source.[34,35] Therefore, both ionization can occur inside the ion source. Moreover, $[M + K]^+$ ions are also observed in the mass spectra because both PM and TM use a KOH solution in the alkaline catalysis. Thus, during the ASAP process, potassium is ionized, forming potassium adducts.[36]

In both derivatization methods, the $[M]^+$ ions were identified as *m/z* 155, 169, 183, 211, 225, 239, 253, 267, 265, 263, and 293. These ions correspond to the FAME fragments of the respective fatty acids: 10:0, 11:0, 12:0, 14:0, 15:0, 16:0, 17:0, 18:0, 18:1, 18:2, and 20:1. These results are according to the work of Sud *et al.*,[37] which demonstrates that the peaks obtained for ionized FAMEs and FAME fragments previously attached to TAG molecules have distinct *m/z* ratios. In $[M + K]^+$ form, ions were identified with *m/z* 169, 197, 225, 253, 279, and 281 corresponding to the FAME fragments of fatty acids 6:0, 8:0, 10:0, 12:0, 14:1, and 14:0, respectively. The most abundant ion peaks present in the mass spectra, *m/z* 269, 271, 293, 297, and 299 are related to the $[M + H]^+$ form, which corresponds to the FAME fragments of fatty acids 16:1, 16:0, 18:3, 18:1, and 18:0, respectively. These results are according to the literature,[11] which shows that 18:1n-9 and 16:0 are the major fatty acids found in HM and reported similar FAMEs composition for HM.[33]

Assessment of the proposed method by GC-FID

The chromatograms of $HM_{lyo}M$-TM and $HM_{lyo}M$-PM are illustrated in Figure S3 (SI section). Both chromatograms are similar and exhibited a good level of separation, great resolution, and absence of interferences, indicating that PM has good sensibility and detectability.

Table 3 presents the quantification of FAs for $HM_{lyo}M$-TM and $HM_{lyo}M$-PM obtained by GC-FID. A total of 35 FAs were identified and quantified in $HM_{lyo}M$ for both methods.

Table 3. Fatty acid quantification for lyophilized human milk in the mature lactation phase by the traditional method of extraction and derivatization compared to the proposed method by gas chromatography with flame ionization detector

Fatty acid / (mg g^{-1} of sample)	Lyophilized human milk	
	Traditional method	Proposed method
4:0	0.3519[a] ± 0.0696	0.4654[a] ± 0.0152
6:0	0.1821[b] ± 0.0090	0.3372[a] ± 0.0100
8:0	0.1013[b] ± 0.0270	0.3994[a] ± 0.0139
10:0	2.2768[b] ± 1.8480	3.8474[a] ± 0.3923
11:0	0.0526[b] ± 0.0667	0.1435[a] ± 0.0116
12:0	15.3170[b] ± 11.6290	22.7664[a] ± 2.4655
14:0	25.8887[a] ± 0.4093	27.5870[a] ± 3.0020
14:1n-5	0.2510[a] ± 0.0048	0.2626[a] ± 0.0328
15:0	0.6927[a] ± 0.1145	0.8975[a] ± 0.0447
15:1n-7	0.2612[a] ± 0.0349	0.2292[a] ± 0.0393
16:0	44.2994[b] ± 1.4295	107.3300[a] ± 1.9761
16:1n-7	0.5116[a] ± 0.2408	0.8274[a] ± 0.1671
16:1n-9	6.4244[b] ± 0.4488	8.6474[a] ± 0.2680
17:0	0.5712[b] ± 0.1573	1.0819[a] ± 0.0694
17:1n-9	0.4363[a] ± 0.0887	0.5198[a] ± 0.0324
18:0	12.7646[b] ± 2.3104	25.8741[a] ± 0.9993
18:1n-9	109.7036[b] ± 6.8992	125.2718[a] ± 2.3014
18-:1n-7	3.6595[b] ± 0.3195	5.4783[a] ± 0.2751
18:2n-6	55.2697[b] ± 0.5374	65.6860[a] ± 0.8970
18:2n-6 *cis*9,*trans*11	0.2326[b] ± 0.0428	0.4756[a] ± 0.0828
18:2n-6 *trans*10,*cis*12	0.6764[a] ± 0.4217	0.8875[a] ± 0.1314
18:3n-3	2.9131[b] ± 0.2148	4.0041[a] ± 0.5542
18:3n-6	0.9176[a] ± 0.5212	1.1031[a] ± 0.1616
20:0	0.2599[a] ± 0.1446	0.3440[a] ± 0.0587
20:1n-9	1.1128[a] ± 0.1359	1.3347[a] ± 0.1928
21:0	0.0007[b] ± 0.0003	0.0016[a] ± 0.0002
20:4n-6	1.8467[a] ± 0.3160	2.1332[a] ± 0.2585
20:3n-3	0.0698[b] ± 0.0089	0.2332[a] ± 0.0246
22:0	0.3509[a] ± 0.1206	0.4313[a] ± 0.0215
20:3n-6	0.0976[b] ± 0.0028	0.1415[b] ± 0.0175
20:5n-3	0.3221[a] ± 0.1104	0.2696[a] ± 0.0277
22:1n-9	0.4115[b] ± 0.1242	0.6644[b] ± 0.0817
24:0	0.0001[a] ± 0.0001	0.0002[a] ± 0.0001
24:1n-9	0.0004[a] ± 0.0001	0.0005[a] ± 0.0001
22:6n-3	0.4100[b] ± 0.0980	0.6270[b] ± 0.0770
∑SFA	102.4488[b] ± 14.5898	190.6323[a] ± 6.6892
∑MUFA	122.0839[b] ± 6.6189	142.2581[a] ± 1.7087
∑PUFA	61.6823[b] ± 1.6723	75.8363[a] ± 1.1489
∑n-6	57.6581[b] ± 1.1580	68.8301[a] ± 0.6558
∑n-3	3.7054[b] ± 0.1953	5.1340[a] ± 0.6743
∑(n-6)/(n-3)	15.6238[b] ± 0.7712	13.6176[a] ± 1.9865
Total sum of fatty acids	277.5035[b] ± 8.9380	409.5620[a] ± 5.8429

Results expressed as mean ± standard deviation for analysis in three replicates. Means followed by distinct letters in the same line are significantly different by *t*-test ($p < 0.05$). Description of the chemical name of fatty acids: butyric acid (4:0); caproic acid (6:0); caprylic acid (8:0); capric acid (10:0); undecylic acid (11:0); lauric acid (12:0); myristic acid (14:0); myristoleic acid (14:1n-5); pentadecylic acid (15:0); 8-pentadecenoic acid (15:1n-7); palmitic acid (16:0); palmitoleic acid (16:1n-7); 7-hexadecanoic acid (16:1n-9); margaric acid (17:0); heptadecenoic acid (17:1n-9); stearic acid (18:0); oleic acid (18:1n-9); vaccenic acid (18:1n-7); linoleic acid (18:2n-6); linoleic conjugated acid (18:2n-6 *cis*9,*trans*11); linoleic conjugated acid (18:2n-6 *trans*10,*cis*12); α-linolenic acid (18:3n-3); γ-linoleic acid (18:3n-6); arachidic acid (20:0); eicosenoic acid (20:1n-9); heneicosylic acid (21:0); arachidonic acid (20:4n-6); eicosatrienoic acid (20:3n-3); behenic acid (22:0); dihomo-gamma-linolenic acid (20:3n-6); eicosapentaenoic acid (20:5n-3); erucic acid (22:1n-9); lignoceric acid (24:0); nervonic acid (24:1n-9); docosahexaenoic acid (22:6n-3); saturated fatty acids (SFA); monounsaturated fatty acids (MUFA); polyunsaturated fatty acids (PUFA); omega-6 fatty acids series (n-6); omega-3 fatty acids series (n-3); omega-6 to omega-3 ratio ((n-6)/(n-3)).

The use of GC-MS could aid in identification, as the MS detector provides information about the molecular weight and structure of the analyte. However, the FID was chosen due to its high detectability and selectivity for compounds containing carbon in their composition, such as methyl esters of fatty acids.

However, the sum of FA was higher for PM (409.5620 ± 5.8429 mg g^{-1} of sample) than TM (277.5035 ± 8.9380 mg g^{-1} of sample). The results obtained are justified because TM requires an extraction step of the lipid matrix before derivatization, which may lead to errors related to sample manipulation, resulting in a decrease in the amount of lipid. In addition, non-lipid compounds can also be extracted due to the interactions of the solvents. Therefore, non-lipid compounds are not esterified/transesterified and are not considered. This error is minimized in the PM, as the derivatization is performed directly on the sample matrix and quantification involves the mass of the sample. Then, the results obtained using PM are not influenced by extraction errors.

Validation of the method

The accuracy values of PM ranged from 99.87 to 102.16%, below the values defined in the guidelines.[26] The $RSD_{intra\text{-}day}$ (1.34-4.03%) and $RSD_{inter\text{-}day}$ (2.08-5.16%) showed that the PM presents good precision. The correlation between the sum of FA obtained by PM and TM allowed the evaluation of linearity. The coefficient of determination was $R^2 = 0.9996$, indicating that PM is well

correlated with TM, within the linear range of 3 to 38% of the total lipids in the sample.

The robustness of the PM was achieved by changing the temperature of the ultrasonic bath (29 to 32 °C) and the injection volume (1 to 3 µL). Small variations in injection volume did not significantly change the results, remaining within the coefficient of variation range (5.36%). However, the variation in the temperature of the ultrasonic bath significantly affected the results, leading to decreased accuracy. The recovery experiments were performed by adding TAG 13:0 into $HM_{lyo}M$ samples. Recovery values for PM ranged between 99 to 100%, which is considered acceptable, demonstrating that the PM is adequate.

Application of the method

Table 4 shows the results of the FA quantification of $HM_{liq}C$, $HM_{liq}T$, $HM_{liq}M$, $HM_{lyo}C$, and $HM_{lyo}T$ by PM. The sum of FA obtained by PM was 373.4417 ± 0.0001 mg g^{-1} ($HM_{lyo}C$), 376.1192 ± 0.0001 mg g^{-1} ($HM_{lyo}T$), 35.4857 ± 0.0001 mg g^{-1} ($HM_{liq}C$), 38.8711 ± 0.0001 mg g^{-1} ($HM_{liq}T$), and 42.9602 ± 0.0001 mg g^{-1} ($HM_{liq}M$). The results have been shown in absolute quantity (mg g^{-1} of sample) because it provides much more information about the absolute FA content in HM. However, very few studies have presented absolute FA concentrations in HM, then it is difficult to compare the results from this study.

Regarding HM_{liq} and HM_{lyo}, an 8-fold decrease in the sum of FA was observed in HM_{liq}. For example, $HM_{lyo}C$ and $HM_{liq}C$ presented the sum of FA of 373.4417 ± 0.0001 and 35.485 ± 0.0001, respectively. This high sum of FA in HM_{lyo} is related to the lyophilization process, which is used to remove the water from the sample, so concentrating its compounds, such as FAs.[38,39] Another fact is that the HM_{liq} is composed of 85% water;[40] thus, the water content HM_{liq} may affect the performance of the derivatization, producing undesirable reactions, such as saponification,[28] which may decrease the detected quantity of FAs.

Table 4. Fatty acid quantification of lyophilized human milk in the colostrum and transitional lactation phases, and liquid human milk in all lactation phases (colostrum, transitional, and mature) by the proposed method

Fatty acid / (mg g^{-1} of sample)	Lyophilized human milk		Liquid human milk		
	Colostrum	Transition	Colostrum	Transition	Mature
4:0	0.3994^{a} ± 0.3520	0.4248^{a} ± 0.1001	0.6846^{a} ± 0.2485	0.3898^{a} ± 0.1846	0.7855^{a} ± 0.2029
6:0	0.2574^{a} ± 0.0849	0.3498^{a} ± 0.0885	0.7864^{a} ± 0.2501	0.5115^{a} ± 0.1200	0.9515^{a} ± 0.2653
8:0	0.3571^{a} ± 0.0496	0.3845^{a} ± 0.3675	0.2487^{a} ± 0.0646	0.1643^{a} ± 0.0510	0.3019^{a} ± 0.0748
10:0	3.7091^{a} ± 0.9514	3.9049^{a} ± 1.3495	0.3114^{a} ± 0.0573	0.4478^{a} ± 0.0734	0.4294^{a} ± 0.0948
11:0	1.3423^{a} ± 0.8468	1.5283^{a} ± 0.3790	0.3660^{a} ± 0.1162	0.0251^{a} ± 0.0015	0.5740^{a} ± 0.2098
12:0	22.3313^{a} ± 0.4814	25.2226^{a} ± 0.7840	1.6199^{a} ± 0.4859	2.3601^{a} ± 0.3592	2.5481^{a} ± 0.4278
14:0	23.0962^{a} ± 0.9281	24.1404^{a} ± 1.4160	2.4629^{a} ± 0.4864	3.1146^{a} ± 0.4166	3.1562^{a} ± 0.3620
14:1n-5	0.1622^{b} ± 0.0098	0.1783^{b} ± 0.0072	0.0158^{a} ± 0.0044	0.0153^{b} ± 0.0017	0.0299^{a} ± 0.0032
15:0	0.7004^{a} ± 0.0619	0.7485^{a} ± 0.0322	0.0727^{a} ± 0.0147	0.0773^{b} ± 0.0104	0.1130^{a} ± 0.0111
15:1n-7	0.2505^{a} ± 0.0072	0.1796^{a} ± 0.0228	0.0200^{a} ± 0.0099	0.0217^{a} ± 0.0027	0.0239^{a} ± 0.0017
16:0	97.5610^{a} ± 0.5897	106.0671^{a} ± 0.7631	8.7901^{a} ± 1.3885	9.9996^{a} ± 0.9856	10.3677^{a} ± 0.6950
16:1n-7	0.8224^{a} ± 0.0998	0.7195^{b} ± 0.0955	0.0869^{a} ± 0.0125	0.0798^{a} ± 0.0070	0.0768^{a} ± 0.0034
16:1n-9	6.2970^{a} ± 0.3752	6.0391^{a} ± 0.5331	0.6246^{a} ± 0.0957	0.5645^{a} ± 0.0640	0.7994^{a} ± 0.0786
17:0	1.0445^{a} ± 0.0520	1.0474^{a} ± 0.0350	0.0981^{a} ± 0.0143	0.1037^{a} ± 0.0123	0.1175^{a} ± 0.0168
17:1n-9	0.4543^{a} ± 0.0623	0.4606^{a} ± 0.0647	0.0537^{a} ± 0.0078	0.0472^{a} ± 0.0073	0.0668^{a} ± 0.0004
18:0	20.7420^{a} ± 0.4862	22.0405^{a} ± 0.7210	1.7990^{a} ± 0.2375	2.2289^{a} ± 0.1763	2.1879^{a} ± 0.1716
18:1n-9	120.4042^{a} ± 0.3378	121.7219^{a} ± 0.3666	11.077^{a} ± 1.5887	11.8376^{a} ± 1.2541	13.1275^{a} ± 0.9043
18:1n-7	0.1838^{b} ± 0.0167	0.1067^{b} ± 0.0267	0.0221^{b} ± 0.0051	0.0239^{b} ± 0.0104	0.0209^{b} ± 0.0021
18:2n-6	60.2799^{a} ± 1.3397	47.0999^{b} ± 0.9966	4.9607^{a} ± 0.6307	5.5808^{a} ± 0.2962	5.9306^{a} ± 0.2220
18:2n-6 *cis*9,*trans*11	0.3594^{b} ± 0.2106	0.3888^{a} ± 0.1839	0.0485^{a} ± 0.0069	0.0568^{a} ± 0.0141	0.0682^{b} ± 0.0023
18:2n-6 *trans*10,*cis*12	0.5605^{a} ± 0.0273	0.6679^{a} ± 0.0644	0.0427^{a} ± 0.0099	0.0423^{a} ± 0.0014	0.0410^{b} ± 0.0008
18:3n-3	2.8701^{a} ± 0.1773	3.4857^{a} ± 0.4541	0.2863^{a} ± 0.0178	0.2807^{a} ± 0.0335	0.3102^{a} ± 0.0099
18:3n-6	1.4748^{a} ± 0.3384	1.3324^{a} ± 0.1046	0.1845^{a} ± 0.0076	0.1797^{a} ± 0.0211	0.1344^{a} ± 0.0155
20:0	0.2071^{a} ± 0.0669	0.2139^{a} ± 0.0212	0.0314^{a} ± 0.0065	0.0180^{a} ± 0.0034	0.0190^{a} ± 0.0067
20:1n-9	2.5517^{b} ± 0.1510	2.4825^{a} ± 0.4150	0.2167^{a} ± 0.0334	0.2184^{a} ± 0.0287	0.2484^{a} ± 0.0778
21:0	0.0017^{b} ± 0.0001	0.0020^{a} ± 0.0004	0.0001^{a} ± 0.0001	0.0002^{a} ± 0.0001	0.0001^{a} ± 0.0001

Table 4. Fatty acid quantification of lyophilized human milk in the colostrum and transitional lactation phases, and liquid human milk in all lactation phases (colostrum, transitional, and mature) by the proposed method (cont.)

Fatty acid / (mg g^{-1} of sample)	Lyophilized human milk		Liquid human milk		
	Colostrum	Transition	Colostrum	Transition	Mature
20:4n-6	2.1988[a] ± 0.1483	2.3886[a] ± 0.3436	0.2432[a] ± 0.0190	0.2206[a] ± 0.0284	0.2514[a] ± 0.0338
20:3n-3	0.2804[a] ± 0.0337	0.3111[a] ± 0.0158	0.0563[a] ± 0.0175	0.0309[a] ± 0.0030	0.0474[a] ± 0.0127
22:0	0.3833[a] ± 0.0293	0.3872[a] ± 0.0376	0.0411[a] ± 0.0050	0.0490[a] ± 0.0057	0.0440[a] ± 0.0041
20:3n-6	0.3845[a] ± 0.0239	0.3670[a] ± 0.0812	0.0449[a] ± 0.0096	0.0425[a] ± 0.0018	0.0394[a] ± 0.0018
20:5n-3	0.1353[b] ± 0.0564	0.2424[a] ± 0.0764	0.0400[a] ± 0.0043	0.0047[a] ± 0.0208	0.0386[a] ± 0.0092
22:1n-9	0.8481[a] ± 0.1204	0.7298[a] ± 0.0709	0.0832[a] ± 0.0089	0.0644[a] ± 0.0060	0.0499[a] ± 0.0072
24:0	0.0002[a] ± 0.0001	0.0002[a] ± 0.0001	ND	ND	ND
24:1n-9	0.0004[a] ± 0.0001	0.0005[a] ± 0.0001	ND	ND	ND
22:6n-3	0.7903[a] ± 0.0622	0.7547[a] ± 0.0787	0.0661[a] ± 0.0127	0.0695[a] ± 0.0063	0.0598[a] ± 0.0065
∑SFA	170.8478[a] ± 2.9443	186.1292[a] ± 0.6537	16.7614[a] ± 3.1116	19.3865[a] ± 2.0080	21.7209[a] ± 2.0993
∑MUFA	131.989[a] ± 0.8499	132.3168[a] ± 1.0545	12.1937[a] ± 1.7500	12.8728[a] ± 1.3780	14.4507[a] ± 1.0615
∑PUFA	69.6408[a] ± 1.1149	57.6168[a] ± 1.4711	5.9011[a] ± 0.7095	6.4752[a] ± 0.2251	6.9735[a] ± 0.2749
∑(n-6)	62.2775[a] ± 1.5096	49.0150[b] ± 0.9725	5.1853[a] ± 0.6474	5.8052[a] ± 0.2744	6.1179[a] ± 0.2370
∑(n-3)	3.9014[a] ± 0.2682	4.6226[a] ± 0.4335	0.4087[a] ± 0.0465	0.3811[a] ± 0.0256	0.4175[a] ± 0.0092
∑(n-6)/(n-3)	16.0437[a] ± 1.2836	10.6033[a] ± 0.8428	14.3790[a] ± 1.2955	15.4895[a] ± 1.0889	14.3864[b] ± 0.3352
Total sum of fatty acids	373.4417[a] ± 0.0001	376.1192[a] ± 0.0001	35.4857[a] ± 0.0001	38.8711[a] ± 0.0001	42.9602[a] ± 0.0001

Results expressed as mean ± standard deviation for analysis in three replicates. Means followed by distinct letters in the same line are significantly different by *t*-test ($p < 0.05$). ND: not detectable. Fatty acids composition: butyric acid (4:0); caproic acid (6:0); caprylic acid (8:0); capric acid (10:0); undecylic acid (11:0); lauric acid (12:0); myristic acid (14:0); myristoleic acid (14:1n-5); pentadecylic acid (15:0); 8-pentadecenoic acid (15:1n-7); palmitic acid (16:0); palmitoleic acid (16:1n-7); 7-hexadecanoic acid (16:1n-9); margaric acid (17:0); heptadecenoic acid (17:1n-9); stearic acid (18:0); oleic acid (18:1n-9); vaccenic acid (18:1n-7); linoleic acid (18:2n-6); linoleic conjugated acid (18:2n-6 *cis*9,*trans*11); linoleic conjugated acid (18:2n-6 *trans*10,*cis*12); α-linolenic acid (18:3n-3); γ-linoleic acid (18:3n-6); arachidic acid (20:0); eicosenoic acid (20:1n-9); heneicosylic acid (21:0); arachidonic acid (20:4n-6); eicosatrienoic acid (20:3n-3); behenic acid (22:0); dihomo-gamma-linolenic acid (20:3n-6); eicosapentaenoic acid (20:5n-3); erucic acid (22:1n-9); lignoceric acid (24:0); nervonic acid (24:1n-9); docosahexaenoic acid (22:6n-3); saturated fatty acids (SFA); monounsaturated fatty acids (MUFA); polyunsaturated fatty acids (PUFA); omega-6 fatty acids series (n-6); omega-3 fatty acids series (n-3); omega-6 to omega-3 ratio ((n-6)/(n-3)).

In addition, note that there is a variation of FA concentration between HM at different lactation stages, which is common according to previous research.[2,41,42]

Conclusions

In the present study, the method developed, optimized, and validated can be used to quantify the FA in HM_{lyo}. The proposed method requires a low volume of solvents and a low amount of sample, it is less susceptible to experimental errors due to decreased sample manipulation and experimental steps and provides a fast sample preparation procedure. The proposed method is robust, efficient, and has good accuracy compared to traditional methodologies. The application of the method demonstrated that there is a variation of FA concentration between HM at different lactation stages and between HM_{liq} and HM_{lyo}. This type of information is important to evaluate the intake and needs of newborns, and the proposed method can assess it in a very short time compared with traditional methods.

Supplementary Information

Supplementary data with the experimental conditions generated by experimental design, linear regression graph, 3D plot of the response surface, and chromatograms are available free of charge at http://jbcs.sbq.org.br as PDF file.

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Author Contributions

Patrícia M. Souza was responsible for the data curation, formal analysis, investigation, validation, visualization, writing (original draft, review and editing); Patrícia D. S. Santos for formal analysis;

Isadora B. Ponhozi for formal analysis; Victor H. M. Cruz for writing (review and editing); Jéssica S. Pizzo for formal analysis; writing (review and editing); Eloize S. Alves for writing (review and editing); Oscar O. Santos for data curation, project administration, supervision, visualization; Jesuí V. Visentainer for writing (review and editing), conceptualization, funding acquisition, resources.

References

1. World Health Organization (WHO); *Global Strategy for Infant and Young Child Feeding*; WHO: Geneva, 2003. [Link] accessed April 2024
2. Floris, L. M.; Stahl, B.; Berkeveld, M. A.; Teller, I. C.; *Prostaglandins, Leukotrienes Essent. Fatty Acids* **2020**, *156*, 102023. [Crossref]
3. Jagodic, M.; Snoj Tratnik, J.; Potočnik, D.; Mazej, D.; Ogrinc, N.; Horvat, M.; *Food Chem. Toxicol.* **2020**, *141*, 111299. [Crossref]
4. Roche, M. L.; Creed-Kanashiro, H. M.; Tuesta, I.; Kuhnlein, H. V.; *Matern. Child Nutr.* **2011**, *7*, 284. [Crossref]
5. Wei, W.; Jin, Q.; Wang, X.; *Prog. Lipid Res.* **2019**, *74*, 69. [Crossref]
6. Manin, L.; Rydlewski, A.; Galuch, M.; Pizzo, J.; Zappielo, C.; Senes, C.; Santos, O. O.; Visentainer, J. V.; *J. Braz. Chem. Soc.* **2019**, *30*, 1579. [Crossref]
7. Rydlewski, A. A.; Pizzo, J. S.; Manin, L. P.; Zappielo, C. D.; Galuch, M. B.; Santos, O. O.; Visentainer, J. V.; *Rev. Virtual Quimica* **2020**, *12*, 155. [Crossref]
8. Lauritzen, L.; Fewtrell, M.; Agostoni, C.; *Pediatr. Res.* **2015**, *77*, 263. [Crossref]
9. George, A. D.; Gay, M. C. L.; Wlodek, M. E.; Murray, K.; Geddes, D. T.; *Nutrients* **2021**, *13*, 4183. [Crossref]
10. Chen, Y. J.; Zhou, X. H.; Han, B.; Li, S. M.; Xu, T.; Yi, H. X.; Liu, P.; Zhang, L. W.; Li, Y. Y.; Jiang, S. L.; Pan, J. C.; Ma, C. H.; Wang, B. C.; *Food Res. Int.* **2020**, *133*, 109196. [Crossref]
11. Rydlewski, A.; Silva, P.; Manin, L.; Tavares, C.; Paula, M.; Figueiredo, I.; Neia, V.; Santos, O. O.; Visentainer, J. V.; *J. Braz. Chem. Soc.* **2019**, *30*, 1063. [Crossref]
12. Hewavitharana, G. G.; Perera, D. N.; Navaratne, S. B.; Wickramasinghe, I.; *Arabian J. Chem.* **2020**, *13*, 6865. [Crossref]
13. Folch, J.; Lees, M.; Stanley, G. H. S.; *J. Biol. Chem.* **1957**, *226*, 497. [Crossref]
14. Collins, C. H.; Braga, C. G. L.; Bonato, P. S.; *Fundamentos de Cromatografia*, 1st ed.; Editora da Unicamp: Campinas, 2006.
15. Dai, X.; Yuan, T.; Zhang, X.; Zhou, Q.; Bi, H.; Yu, R.; Wei, W.; Wang, X.; *Food Funct.* **2020**, *11*, 1869. [Crossref]
16. Figueiredo, I. L.; Claus, T.; Santos Jr., O. O.; Almeida, V. C.; Magon, T.; Visentainer, J. V.; *J. Chromatogr. A* **2016**, *1456*, 235. [Crossref]
17. Piccioli, A.; Santos, P.; da Silveira, R.; Bonafe, E.; Visentainer, J. V.; Santos, O. O.; *J. Braz. Chem. Soc.* **2019**, *30*, 1350. [Crossref]
18. Park, P.; Goins, R. E.; *J. Food Sci.* **1994**, *59*, 1262. [Crossref]
19. Liu, Z.; Ezernieks, V.; Rochfort, S.; Cocks, B.; *Food Chem.* **2018**, *261*, 210. [Crossref]
20. Liu, Z.; Wang, J.; Li, C.; Rochfort, S.; *Food Chem.* **2020**, *315*, 126281. [Crossref]
21. International Organization for Standardization (ISO); *ISO 12966: Animal and Vegetable Fats and Oils - Gas Chromatography of Fatty Acid Methyl Esters Part 2: Preparation of Methyl Esters of Fatty Acids*, ISO: Geneva, 2017. [Link] accessed in April 2024
22. *Design Expert*, version 7.0; Stat-Ease, Minneapolis, MN, USA, 2007.
23. Leonzio, G.; Zondervan, E.; *Comput.-Aided Chem. Eng.* **2019**, *46*, 151. [Crossref]
24. Visentainer, J. V.; Franco, M. R. B.; *Ácidos Graxos em Óleos e Gorduras: Identificação e Quantificação*, 2nd ed.; Eduem: Maringá, 2012.
25. Cruz-Hernandez, C.; Goeuriot, S.; Giuffrida, F.; Thakkar, S. K.; Destaillats, F.; *J. Chromatogr. A* **2013**, *1284*, 174. [Crossref]
26. International Conference on Harmonization (ICH); *Guideline Q2(R1) Validation of Analytical Procedures: Text and Methodology*, ICH: Stanford, 2005. [Link] accessed April 2024
27. Silva, F. A. S.; *The ASSISTAT Software: Statistical Assistance*; Universidade Federal de Campina Grande, Brazil, 1996.
28. Milinsk, M. C.; Matsushita, M.; Visentainer, J. V.; Oliveira, C. C.; Souza, N. E.; *J. Braz. Chem. Soc.* **2008**, *19*, 1475. [Crossref]
29. Chiu, H. H.; Kuo, C. H.; *J. Food Drug Anal.* **2020**, *28*, 60. [Crossref]
30. Chiavelli, L. U. R.; Godoy, A. C.; da Silveira, R.; Santos, P. D. S.; Lopes, T. A. M.; Santos, O. O.; Visentainer, J. V.; *Food Anal. Methods* **2020**, *13*, 166. [Crossref]
31. Cruz-Hernandez, C.; Destaillats, F. In *Gas Chromatography*; Poole, C. F., ed.; Elsevier: Amsterdam, 2021, ch. 27. [Crossref]
32. Smith, M. J. P.; Cameron, N. R.; Mosely, J. A.; *Analyst* **2012**, *137*, 4524. [Crossref]
33. Rydlewski, A. A.; Manin, L. P.; Pizzo, J. S.; Silva, P. D.; da Silveira, R.; Tavares, C. B. G.; de Paula, M.; Pereira, O.; Santos, O. O.; Visentainer, J. V.; *J. Food Compos. Anal.* **2021**, *100*, 103797. [Crossref]
34. Petucci, C.; Diffendal, J.; *J. Mass Spectrom.* **2008**, *43*, 1565. [Crossref]
35. Tose, L. V.; Murgu, M.; Vaz, B. G.; Romão, W.; *J. Am. Soc. Mass Spectrom.* **2017**, *28*, 2401. [Crossref]
36. Krejčí, P.; Cechová, M. Z.; Nádvorníková, J.; Barták, P.; Kobrlová, L.; Balarynová, J.; Smýkal, P.; Bednář, P.; *Talanta* **2022**, *242*, 123303. [Crossref]
37. Sud, M.; Fahy, E.; Cotter, D.; Brown, A.; Dennis, E. A.; Glass, C. K.; Merrill, A. H.; Murphy, R. C.; Raetz, C. R. H.; Russell, D. W.; Subramaniam, S.; *Nucleic Acids Res.* **2007**, *35*, D527. [Crossref]

38. Pisano, R.; Arsiccio, A.; Capozzi, L. C.; Trout, B. L.; *Eur. J. Pharm. Biopharm.* **2019**, *142*, 265. [Crossref]
39. Morais, A. R. V.; Alencar, E. N.; Xavier Júnior, F. H.; de Oliveira, C. M.; Marcelino, H. R.; Barratt, G.; Fessi, H.; do Egito, E. S. T.; Elaissari, A.; *Int. J. Pharm.* **2016**, *503*, 102. [Crossref]
40. Meng, F.; Uniacke-Lowe, T.; Ryan, A. C.; Kelly, A. L.; *Trends Food Sci. Technol.* **2021**, *112*, 608. [Crossref]
41. Jiang, J.; Wu, K.; Yu, Z.; Ren, Y.; Zhao, Y.; Jiang, Y.; Xu, X.; Li, W.; Jin, Y.; Yuan, J.; Li, D.; *Food Funct.* **2016**, *7*, 3154. [Crossref]
42. van de Heijning, B. J. M.; Stahl, B.; Schaart, M. W.; van der Beek, E. M.; Rings, E. H. H. M.; Mearin, M. L.; *Adv. Pediatr. Res.* **2017**, *4*, 16. [Link] accessed April 2024

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