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RESEARCH PAPER

Leveraging the use of ionic liquid capillary columns and GC×GC-MS for fatty acid profiling in human colostrum samples

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Abstract

Lipids in human colostrum provide the majority of energy intake and essential fatty acids for developing infants. The fatty acid composition of human colostrum is highly variable and influenced by multiple factors. Human colostrum is a complex sample bringing challenges to fatty acid profiling. This work aimed to optimize the use of ionic liquid (IL) columns and flow-modulated comprehensive two-dimensional gas chromatography coupled to mass spectrometry (FM-GC×GC-MS) for fatty acid profiling in human colostrum. Derivatization strategies were optimized and the elution behavior of fatty acid methyl esters (FAME) on various ^{1}D column phases (Solgel-WAX, SLB-IL60i, SLB-IL76i, and SLB-IL111i). Derivatization with sodium methoxide yielded a satisfactory recovery rate (90%) at milder conditions and reduced time. The use of IL60 as the ^{1}D column provided superior separation, good peak shape, and better utilization of elution space. As a proof of concept, the developed method was applied to access the effects of the mode of neonatal delivery (vaginal vs. C-section) on the fatty acid profile of human colostrum samples. The integrated multidimensional gas chromatography strategy improved FAME detection and separation and can be a useful tool for accessing the effects of different factors on the fatty acid profiling of complex samples.

Keywords Fatty acid methyl esters · Comprehensive two-dimensional gas chromatography · Human colostrum · Lipidomics

Introduction

Early life is a critical period for the growth and development of an infant's organs and tissues [1]. Nutrition plays a pivotal role during this development phase, as it influences long-term health and the individual's resilience against diseases later in life [2]. Human milk is considered an optimal nutritional source to supply the nutritional needs of infants during the first six months of life [3, 4].

Human milk is a complex fluid, containing lipids, proteins, carbohydrates, vitamins, minerals, and immunocompetent substances (IgA, enzymes, interferon), in addition to growth modulators [5, 6]. The lipid fraction in milk is the primary source of energy for developing infants, contributing to nearly 50% of the total energy intake [6, 7]. Human colostrum and milk provide all the dietary essential fatty acids, such as linoleic acid (LA; 18:2n-6) and α-linolenic acid (ALA, 18:3n-3), as well as their longer-chain-unsaturated metabolites, including arachidonic acid (ARA, 20:4n-6) and docosahexaenoic acid (DHA, 22:6n-3) to support the growth and development of the breastfed infant [7]. However, lipid content and the fatty acid composition in human milk can vary considerably as they can be modulated by many factors, including lifestyle, nutritional status, ethnicity, stage of lactation, maternal diet, gestational age, and mode of neonatal delivery [1, 8]. Very few studies have reported the effect of mode of delivery on the composition of human milk and the influence of mode of

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delivery on the fatty acid composition of human milk remains unclear [8]. The great complexity of fatty acid structures in human milk and its tremendous importance on infant development highlights the importance of accurate separation, identification, and quantification of those compounds.

Fatty acid accurate identification and separation in complex samples is challenged by several factors including derivatization efficiency [9], and the high complexity of their structures in terms of chain length, degree of unsaturation, branching, and functional groups with different positions, geometries, and structures [10, 11]. Fatty acids are most commonly measured by gas chromatography (GC) after their extraction and conversion into their fatty acid methyl esters (FAME) derivatives by utilizing polar and long capillary columns [12] in a time-consuming manner [13]. The commercial introduction of capillary columns coated with polar ionic liquids (IL) provided significantly different separation characteristics for FAME [14, 15]. These differences are due to their higher polarity, their selective interaction with the double bonds of FAME, and the unique chemical structures of these novel dicationic and tricationic stationary phases [11–13]. However, challenges regarding proper separation and identification of the FAME in complex samples still warrant further investigation.

In this context, comprehensive gas chromatography (GC×GC) emerges as a technique capable of making FAME profiling in complex samples possible [16]. The great advantage of GC×GC over conventional gas chromatography is greater resolving power, enhanced peak capacity, improved sensitivity, and unique structured patterns aiding in compound identification [17]. Specifically, flow-modulated comprehensive two-dimensional gas chromatography coupled to mass spectrometry (FM-GC×GC-MS) enables proper sample separation and characterization of complex samples due to the enhanced peak capacity, being an attractive alternative for FAME characterization and quantification. The simplicity of flow modulators combined with their low cost and robustness makes them a good choice for routine analysis [16].

Importantly, the analysis of fatty acid profiling holds significant importance in biological samples, such as plasma, blood, human milk, and colostrum among others due to its potential relevance to a wide array of diseases like hypertension, diabetes, coronary heart disease, inflammatory disorders, autoimmune disorders, and cancer, and it is noteworthy that relatively few studies have been published in this area [18].

This work aims to demonstrate the use of an integrated approach that combines IL and FM-GC×GC for FAME profiling in complex samples such as human colostrum. Derivatization conditions were optimized and the elution behavior of FAME on ^{1}D column phases (Solgel-WAX and ionic liquid columns: SLB-IL60i, SLB-IL76i, and SLB-IL111i). As a proof of concept, the developed method was also applied to access the effects of the mode of neonatal delivery (vaginal vs. C-section) on the fatty acid profile of human colostrum samples. This paper demonstrates the application of inverse phase orthogonal sets to improve FAME separation and identification in complex samples. The use of FM-GC×GC is a useful tool for accessing the effects of different factors on fatty acid profiling.

It is important to highlight that our study is distinctly oriented toward clinical practitioners who work with human colostrum, a field that faces notable challenges in method development despite the extensive literature available on FAME in various matrices. To the best of our knowledge, the exploration of the three ionic liquid (IL) column sets, namely SLB-IL60i × HP-5, SLB-IL76i × HP-5, and SLB-IL111i × HP-5, has not been previously documented in the scientific literature. Additionally, our application of these column sets to investigate different delivery modes in human colostrum represents a novel and unexplored avenue in research. These unique aspects of our study distinguish it from previous work and contribute to the advancement of knowledge in the field of FAME analysis in human colostrum.

Materials and methods

Samples

Colostrum samples were collected from 27 mothers (coded *s1* to *s27*) at the Hospital "Estadual Infantil e Maternidade Alzir Bernardino Alves (HEIMABA)," located in Vila Velha City, ES, Brazil. Information on gestational age, weight of newborn, and mother's age can be found in Supplementary material—Table 1S. Only patients over 18 years old who agreed to participate in the study and signed the Free and Informed Consent Form were included. Sample collection was performed in the first 48 h postpartum by a manual milking procedure in a sterile bottle. Approximately 2 mL aliquots of colostrum sample were collected in a sterile environment and frozen in an ultra-freezer at −80 °C until analyses. Information related to the neonatal delivery mode was collected by a doctor and associated with the coded samples. The project was approved by the Ethics and Research Council on Human Beings (no. 1,804,463).

Lipid extraction and content

Folch extraction [19] was applied to extract and quantify the lipid fraction from human colostrum samples. Aliquots of 1 g of human colostrum sample were mixed with chloroform/methanol (2:1 v/v) (10 and 5 mL, respectively). The methanol contained 0.6 mg/mL butylated hydroxytoluene (BHT, Sigma-Aldrich, Palo Alto, CA, US). After vortexing for 2 min, 3.75 mL of potassium chloride (KCl, Synth, Diadema,

SP, Brazil) solution (0.88%(w/v)) was added to the mixture. The extraction was performed twice. The combined lower phases containing the lipids were transferred into a pre-weighed glass tube and evaporated to dryness under nitrogen flux. All solvents used were of analytical grade unless stated otherwise. Folch extraction was performed in triplicate and lipid content was determined gravimetrically after evaporating a measured aliquot of the combined chloroform phase to dryness under nitrogen.

Derivatization

The influence of the different derivatization methods on the total fatty acid recovery rate (expressed as % of total lipids) was accessed. Two commonly applied derivatization approaches [13] were tested: (i) base-catalyzed transesterification using sodium methoxide with incubation. Briefly, 50 mg of lipids was dissolved in 500 µL of dichloromethane containing tricosanoic methyl ester (C23:0, Nu-Chek-Prep, MN, USA) as the internal standard at a final concentration of 1 mg/mL [20]. Then, 2 mL of 0.5 M sodium methoxide (Sigma-Aldrich, Palo Alto, CA, USA) in methanol was added and the solution was incubated for 10 min at two different temperatures, at 50 °C and room temperature to assess the effects of heating on the derivatization. The solution was cooled for 5 min in running water and the reaction was stopped by the addition of 100 µL of glacial acetic acid and 5 mL of deionized water. The FAME were extracted with 5 mL of hexane (2x). Samples were dried under nitrogen flux and reconstituted with hexane to 500 µL. (ii) Acid-catalyzed esterification using boron trifluoride-methanol solution. 50 mg of lipids were mixed with 2 mL of 0.5 M methanolic potassium hydroxide solution and heated for 15 min at 90 °C. After cooling for 5 min, 2 mL of a 12% (w/v) boron trifluoride-methanol (Sigma-Aldrich, Palo Alto, CA, USA) solution was added and the mixture was heated for 30 min at 95 °C. After cooling to room temperature. The FAME were extracted with 5 mL of hexane (2×). Samples were dried under nitrogen flux and reconstituted with hexane to 500 µL. All solvents used were of analytical grade unless stated otherwise. The recovery rate (expressed as % of total lipids) was calculated based on the ratio between the total fatty acid concentration and the amount of lipids used.

Optimization of chromatographic conditions

GC×GC analyses were performed on a TRACE 1310 chromatograph coupled to two detectors: an ISQ single quadrupole mass spectrometer (QMS) and a flame ionization detector (FID) (Thermo Scientific, Waltham, MA, USA). The modulation was performed by a differential flow modulator using an INSIGHT modulator (SepSolve Analytical—Peterborough, UK). The column sets tested consisted of two wall-coated open tubular (WCOT) capillary columns. Four different column sets were tested using 4 different ^{1}D columns with an HP-5 (5.5 m × 0.25 mm, 0.25 µm) (Agilent Technologies—Santa Clara, CA, USA) as the ^{2}D column. For each of the chromatographic columns tested, the flow rate of the carrier gas and auxiliary gas and temperature ramp were optimized. The final conditions tested were (i) SGE SolGel-WAX capillary column (30 m × 0.25 mm, 0.25 µm) (Trajan, Ringwood, Australia) × HP-5. Oven temperature ramp was programmed as follows: initial temperature of 170 °C, with an increase of 3 °C/min until 300 °C. Modulation period was set to 6.0 s with a flush pulse of 250 ms. Helium was used as the carrier and auxiliary gas at constant flow rates of 1.00 mL/min and 12.8 mL/min, respectively. (ii) SLB-IL60i capillary column (30 m × 0.25 mm, 0.20 µm) (Supelco, Bellefonte, PA, USA) × HP-5. Oven temperature ramp was programmed as follows: initial temperature of 150 °C, with an increase of 3 °C/min until 280 °C. Modulation period was set to 6.0 s with a flush pulse of 250 ms. Helium was used as the carrier and auxiliary gas. The carrier gas was set at 1.1 mL/min for 1 min, after the flow was changed to 0.785 mL/min and kept constant until the end of the run, the auxiliary gas flow was kept at 10.3 mL/min. (iii) SLB-IL76i capillary column (30 m × 0.25 mm, 0.20 µm) (Supelco, Bellefonte, PA, USA) × HP-5. Oven temperature ramp was programmed as follows: initial temperature of 150 °C, with an increase of 5 °C /min until 280 °C. Modulation period was set to 6.0 s with a flush pulse of 250 ms. Helium was used as the carrier and auxiliary gas. The carrier gas was set at 1.1 mL/min for 1 min, after the flow was changed to 0.785 mL/min and kept constant until the end of the run, the auxiliary gas flow was kept at 10.3 mL/min. (iv) IL111i capillary column (30 m × 0.25 mm, 0.20 µm) (Supelco, Bellefonte, PA, USA) × HP-5. Oven temperature ramp was programmed as follows: initial temperature of 150 °C, with an increase of 5 °C/min until 250 °C. Modulation period was set to 8.0 s with a flush pulse of 250 ms. Helium was used as the carrier and auxiliary gas. The carrier gas was set at 1.0 mL/min for 2 min, after the flow was changed to 0.785 mL/min and kept constant until the end of the run, the auxiliary gas flow was kept at 10.2 mL/min.

The ^{2}D effluent was divided between the FID and MS using a three-port microfluidic planar device (Trajan—Victoria, Australia) that uses a deactivated fused silica capillary (5 m × 0.32 mm-id for FID and 5 m × 0.18 mm-id for the MS). The injection of the samples was performed using a split injection system (1:10) with 1 min of sampling time and injector temperature of 250 °C. The MS conditions were ionization source at 200 °C, ionization by EI (70 eV), transfer line temperature of 300 °C, acquisition of 50-350 *m/z*, and acquisition rate of 35.7 Hz. The FID conditions were detector temperature at 300 °C, synthetic air flow at 350 mL/min, nitrogen flow at 35 mL/min, makeup gas flow at

40 mL/min, and rate of acquisition of 200 Hz. Instrument control and data acquisition were performed using Xcalibur (Thermo Scientific—Waltham, MA, USA) software.

GC Image (Zoex—Houston, TX, USA) was used in combination with standards (Supelco 37 FAME standard mix (Supelco, Bellefonte, PA, USA) and GLC 85 standard mix (Nu-Chek-Prep, MN, US) for the identification of analytes by combining mass spectrum similarity searches and Linear Temperature Programmed Retention Indices (LTPRI) comparison. Blob detection was done by setting the following parameters in GC Image: minimum area of 40, minimum volume of 50, and minimum peak value of 50. Identification was performed by standard retention time (in the first and second dimension), similarity match of 90%, and ± 10 LTPRI using the NIST14 MS library (National Institute of Standards—Gaithersburg, MD, USA).

Statistical analysis

The results are given as the means ± standard deviation. Data were analyzed in the Statistica™ Software (TIBCO Software Inc, Palo Alto, CA, US) using a one-way ANOVA and Tukey's post hoc with $p < 0.05$. Principal component analysis (PCA) was performed to visualize the clusters of FAME profile of human colostrum samples from different neonatal delivery modes. The PCA was performed using the peak height of each identified FAME, and the data were

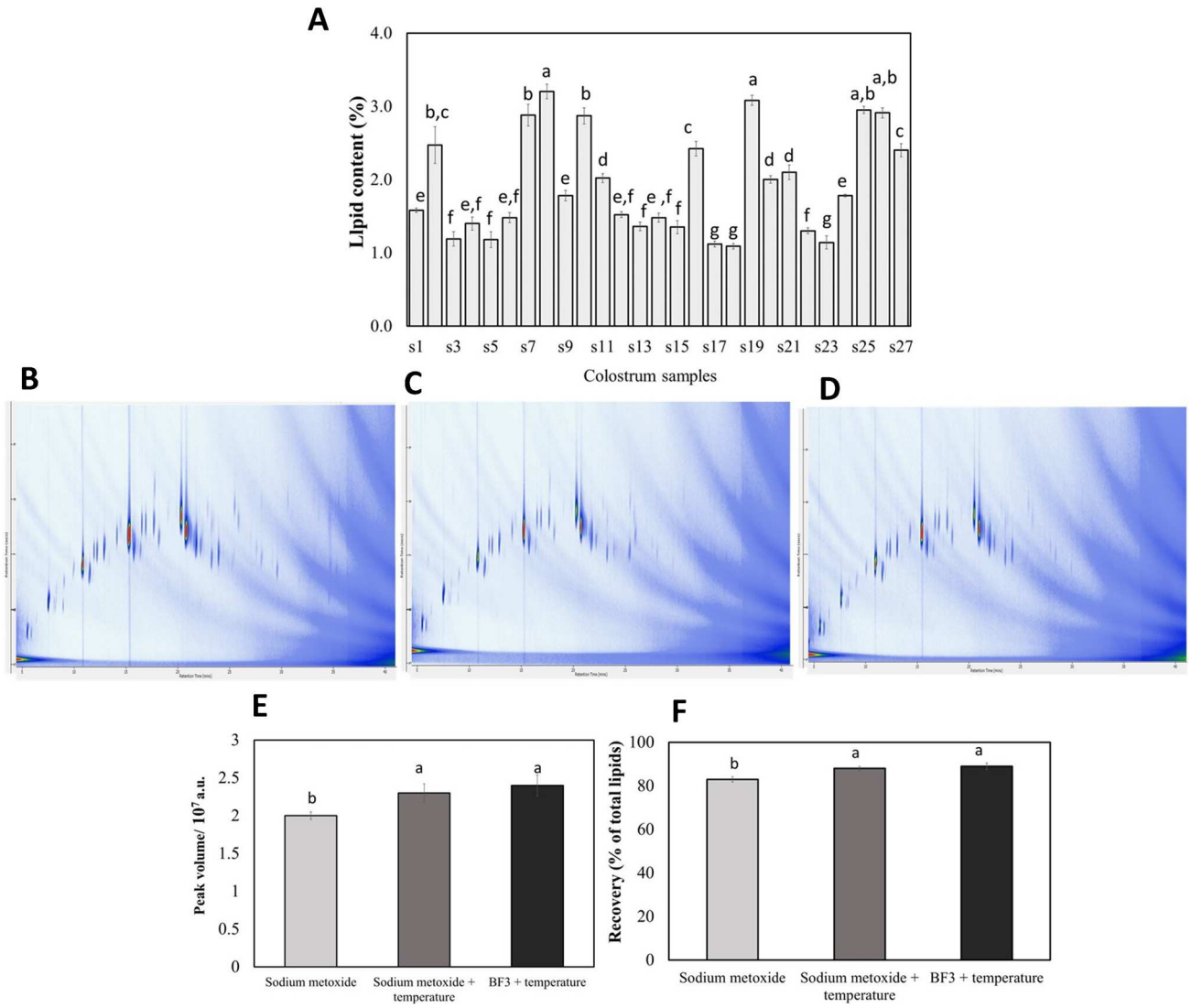

Fig. 1 Lipid content (**A**) of human colostrum samples. GC×GC-MS chromatograms for samples derivatized using sodium methoxide with incubation at room temperature (**B**), sodium methoxide with incubation at 50 °C (**C**), and boron trifluoride (BF_3) (**D**). Total peak volume (**E**) and recovery rates (**F**) for the different derivatization methods. Different letters indicate a significant difference between samples by ANOVA and Tukey test ($p < 0.05$)

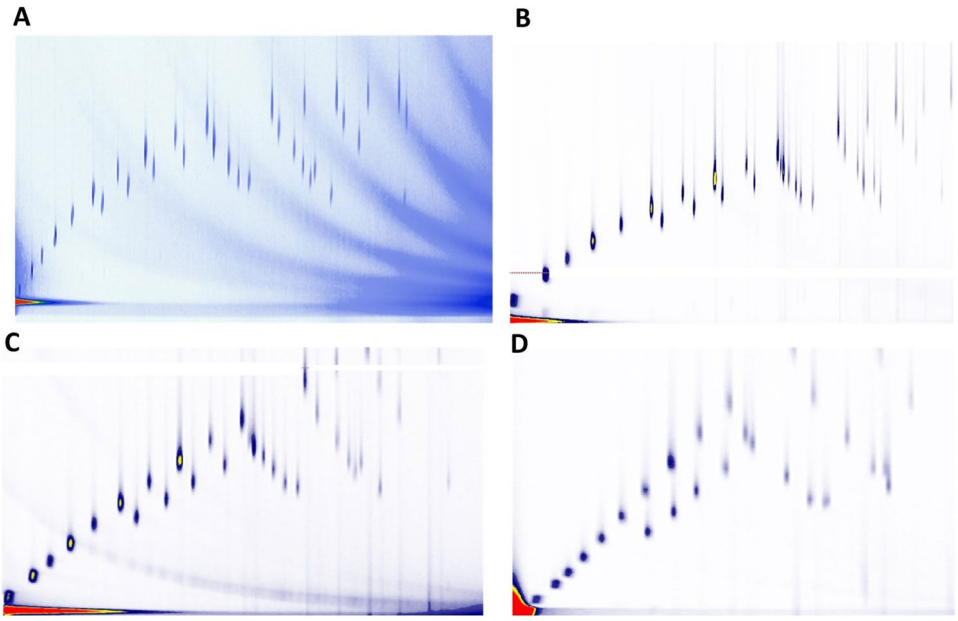

Fig. 2 GC×GC-MS chromatogram of 37 FAME standard mix (1 mg/mL) for each of the column sets (**A**) SolGel-WAX × HP-5, (**B**) SLB-IL 60i × HP-5, (**C**) SLB-IL76i × HP-5, and (**D**) SLB-IL111i × HP-5

mean-centered and analyzed using MetaboAnalyst 5.0 (https://www.metaboanalyst.ca/, accessed on July 15, 2023).

Results and discussion

Lipid content and derivatization

Proper fatty acid profiling in complex samples requires a quantitative extraction of lipids and optimized derivatization since deficiencies in the extraction and derivatization steps could directly influence the quality of the results. Problems during the esterification are usually related to the formation of artifacts, incomplete esterification, and loss of polyunsaturated fatty acids (PUFA) [21].

The lipid content found for the human colostrum samples ranged from 1.09 to 3.20% (g of lipid per 100 g of colostrum sample) (Fig. 1A) and the majority of the samples (60%) had lipid content below 2%. Those results are in accordance with previous work reporting values ranging from 1.9 to 2.3% in colostrum and from 3.2 to 5.0% in mature milk [6, 22]. Before the fatty acid components of lipids can be analyzed by GC, it

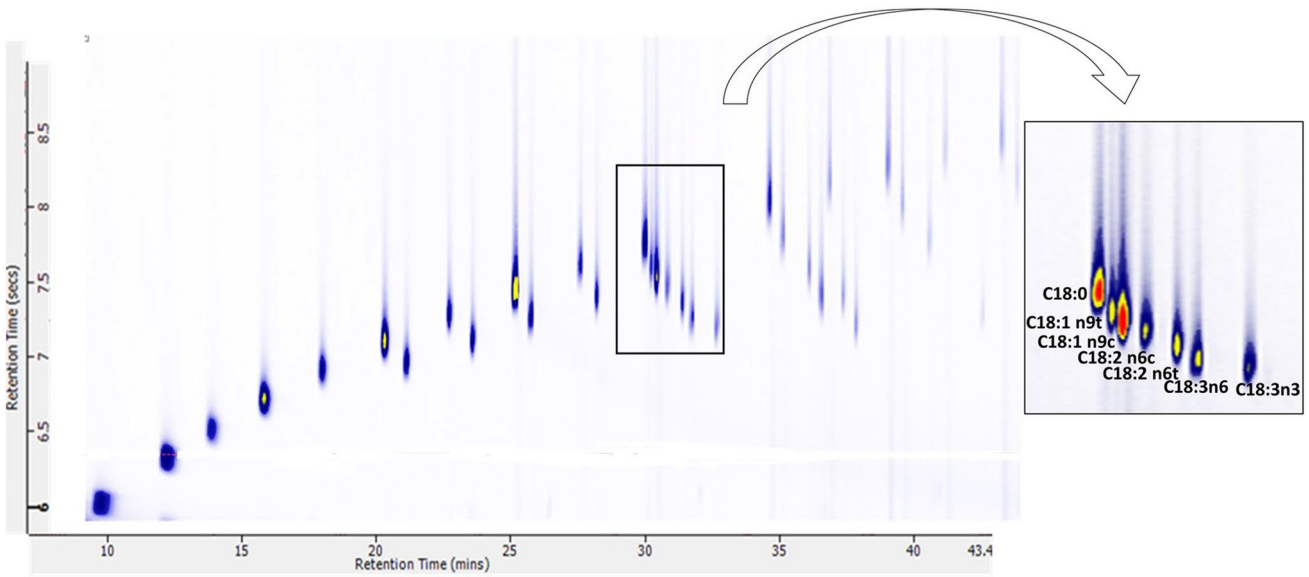

Fig. 3 GC×GC-MS chromatogram obtained for the 37 FAME standard mix (1mg/mL for the SLB-IL60i × HP-5 column set, highlighting the C18 FAME family

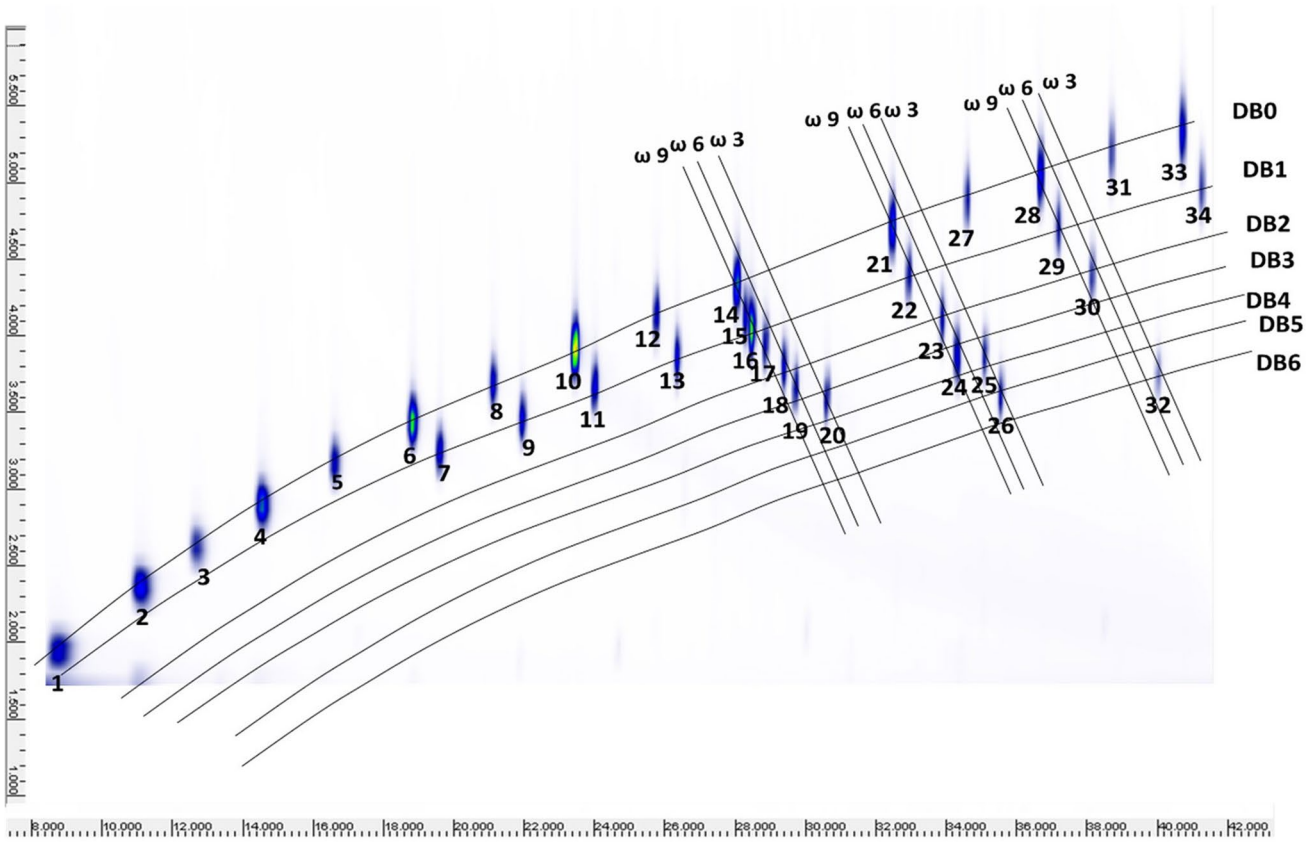

Fig. 4 GC×GC-MS chromatogram for the 37 FAME standard mix (1 mg/mL) with identification of fatty acid methyl esters. DB double bond

Table 1 Identification of the compound in the 37 FAME standard mix using a reverse column set. Retention times (Rt) in the ^{1}D (min) and ^{2}D (s)

No.	Fatty acid	Rt ^{1}D (min)	Rt ^{2}D (s)	#	Fatty acid	Rt ^{1}D (min)	Rt ^{2}D (s)
1	C9:0	7.6	1.1	18	C18:2 n 6t	30.0	3.2
2	C10:0	10.1	1.5	19	C18:3 n 6	30.4	3
3	C11:0	11.8	1.8	20	C18:3 n 3	31.3	2.9
4	C12:0	13.9	2.1	21	C20:0	33.4	4.2
5	C13:0	16.1	2.5	22	C20:1	33.9	3.9
6	C14:0	18.5	2.7	23	C20:2	34.9	3.5
7	C14:1	19.4	2.5	24	C20:3 n 6	35.4	3.2
8	C15:0	21.0	3	25	C20:3 n 3	35.7	4.4
9	C15:1	21.9	2.8	26	C20:5 n 3	36.2	3.2
10	C16:0	23.6	3.3	27	C21:0	36.7	2.9
11	C16:1	24.2	3	28	C22:0	37.9	4.6
12	C17:0	26.1	3.6	28	C22:1 n 9	38.5	4.2
13	C17:1	26.7	3.2	30	C22:2	39.5	3.8
14	C18:0	28.6	3.8	31	C22:6 n 3	40.1	4.7
15	C18:1 n 9t	28.8	3.5	32	C23:0	41.6	3.1
16	C18:1 n 9c	29.0	3.4	33	C24:0	42.3	4.9
17	C18:2 n 6c	29.4	3.3	34	C24:1	42.6	4.5

is necessary to convert them to low molecular weight nonpolar derivatives, such as methyl esters. Saponification followed by methylation is a classical method for the preparation of FAME. Saponification is a process involving the hydrolysis of fats in its reaction with alkali, thereby leading to the formation of salts of fatty acids and glycerol [13]. Conventionally, FAME are prepared by base- or acid-catalyzed esterification [13]. The influence of different derivatization methods on the total fatty acid recovery rate (expressed as % of total lipids) was studied in three experimental assays. Alkaline-catalyzed derivatization using sodium methoxide in methanol with incubation and room temperature (Fig. 1B) and at 50 °C (Fig. 1C) were tested. Acid-catalyzed derivatization method with boron trifluoride in methanol (Fig. 1D) was also studied. Acid-catalyzed derivatization and alkaline-catalyzed derivatization with incubation at 50 °C had similar chromatograms and peak volume, while the incubation at room temperature showed a slight reduction in peak volume, highlighting the need for mild heat to favor the reaction (Fig. 1E). Moreover, both acid and alkali-catalyzed at 50 °C derivatizations achieved about 90% of recovery rates (Fig. 1F). Similarly, Kohn et al. [9] reported analogous results (about 90% conversion of lipids) for FAME analysis in infant milk formula using sodium methoxide. However, the authors reported 81% and 97% of conversion for derivatization of human milk using sodium hydroxide and boron trifluoride, respectively. Derivatization with sodium methoxide has the advantage of being a fast and milder transesterification method of fatty acids linked to alcohols (cholesterol, glycerol) (10 min at 50 °C *vs.* 30 min at 95 °C for alkali and acid-catalyzed derivatization, respectively). The acid derivatization method, in addition to requiring more analysis time, has the disadvantage of promoting the polymerization of double bonds of polyunsaturated fatty acids, which can generate artifacts [13]. It is important to highlight that alkaline-catalyzed derivatization does not methylate free fatty acids or transesterify fatty acids from amide bonds as in phospholipids. Which is not an issue for human colostrum since the majority of the fatty acids (>95%) are present in the

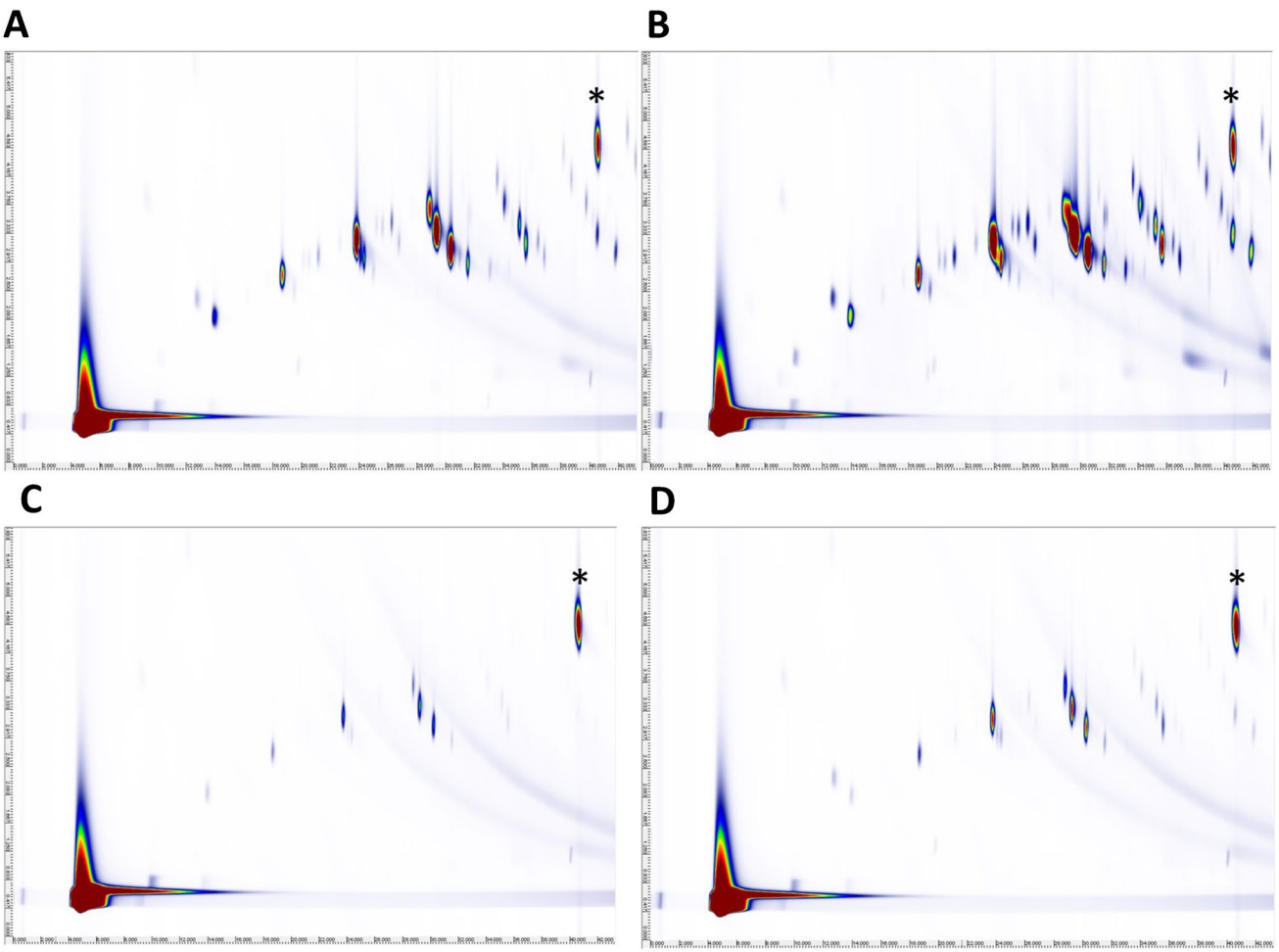

Fig. 5 GC×GC-MS chromatogram of colostrum samples: *s6* (**A**), *s9* (**B**), *s19* (**C**), and *s20* (**D**). The asterisk indicates the internal standard C23:0 (1 mg/mL)

esterified form (triacylglycerol) [22]. Considering these factors, and the shorter time spent with alkaline-catalyzed derivatization, as well as the milder conditions employed, this was the method chosen to continue the analyses.

Optimization of chromatographic condition

Optimal selection of column combinations is crucial for achieving the highest resolution in GC×GC and for displaying discernible patterns in the 2D contour plots [10, 23]. Seeking to maximize the FAME separation, an inverted phase orthogonal set using polar/nonpolar columns was selected, providing a reasonable differentiation in separation mechanisms. Four primary (^{1}D) different polar columns (Solgel-WAX < SLB-IL60i < SLB-IL76i < SLB-IL111i) were tested while the secondary column was the same for all tests (HP-5). The HB-5 column was selected due to its separation capability of grouping the FAME with similar carbon chain lengths [10]. Initial tests were conducted using the 37 FAME mix standard. For each column set, several preliminary were conducted to optimize the carrier gas, auxiliary gas flow, and modulation time.

Figure 2 illustrates the GC×GC analysis of the 37 FAME standard mixture obtained for each set of columns tested after optimizing the chromatographic conditions. The best-performing GC×GC configuration was selected based on the elution pattern of the standard mixture. Similar elution patterns were observed for the column sets Solgel-WAX × HP-5 (Fig. 2A) and SLB-IL60i × HP-5 (Fig. 2B) with the latest showing an improved peak capacity. SLB-IL76i × HP-5 (Fig. 2C) and SLB-IL111i × HP-5 (Fig. 2D) exhibited a wrap-around effect and poor utilization of the elution space, probably due to the high polarity of the ^{1}D columns.

The column set SLB-IL60i × HP-5 provided superior separation, good peak shape, and utilization of elution space. Moreover, the use of a combination of SLB-IL60i and HP-5 improved the separation of the C18 FAME family (Fig. 3). The C18 FAME family in human milk is a very complex mixture of isomeric compounds. By using GC×GC, the C18:1, and trans-C18:1 isomers were clearly separated (Fig. 3). The elution order within the same FAME families depends on the column set. Using an inverted set (polar/nonpolar) it was observed that the nonsaturated fatty acids were eluted after the saturated ones and that the cis isomers were eluted after the trans ones. Similar observations were reported by Manzano et al. [16] when using inverted phase orthogonal sets with a polar first dimension BPX-70 column (30 m × 0.25 mm × 0.25 µm) and a nonpolar ZB5-MS second dimension column (10, 5 and 2 m × 0.25 mm × 0.25 µm). Figure 4 shows the identification of compounds in the 37 FAME standard mix using the SLB-IL60i × HP-5 column set. The diagonal

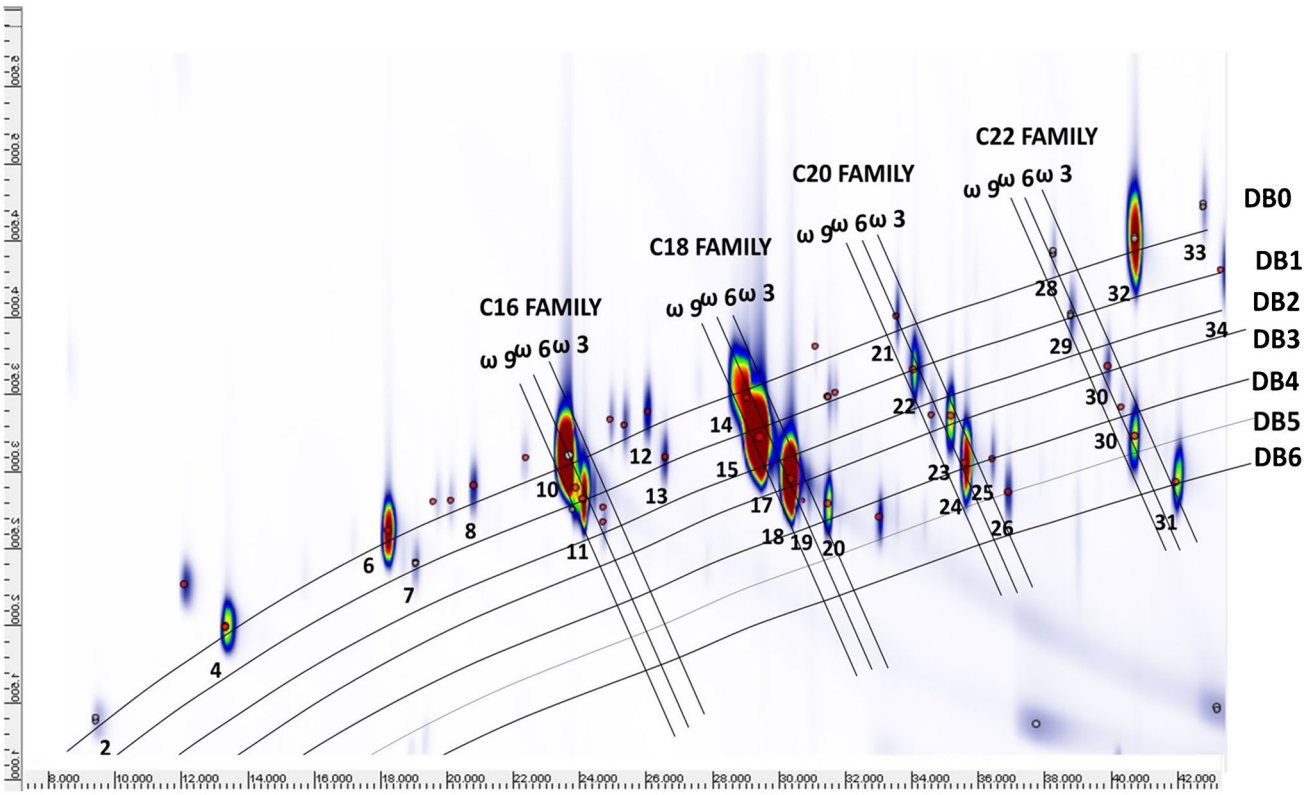

Fig. 6 GC×GC-MS chromatogram of colostrum sample *s9*

and vertical chromatographic structures can be visualized, in which the FAME are grouped according to the number of carbons in the chain (C9:0 to C24:0) and according to the positions of the unsaturations from n-1 to n-9, respectively. The presence of seven diagonal bands related to the number of saturations present in the fatty acids (double bond-DB 0 to 6) was also observed. Table 1 shows compounds identified with retention time in the first and second dimensions.

FAME profiling of human colostrum samples

As a proof of concept, the developed integrated method using IL and FM-GC×GC-MS was applied to access the FAME composition of human colostrum samples. Human colostrum provides a complex array of lipids that contribute to energy, as well as to the essential n-6 and n-3 polyunsaturated fatty acids (PUFA) to support the growth and development of the breastfed infant [24]. Figure 5A to D show the GC×GC-MS chromatograms of four of the 27 analyzed samples. Considering that all samples were obtained from the same mass of colostrum (1 g), it can be noted that the composition in terms of fatty acids of the samples varied considerably. Sample *s19* (Fig. 5C) had the lowest number of FAME at the lowest concentration among all analyzed samples, while sample *s9* (Fig. 5B) was one of the samples with the highest FAME concentrations. The identification of the compounds in *s9* with emphasis on the C16, C18, C20, and C22 families is shown in Fig. 6. Overall, the major

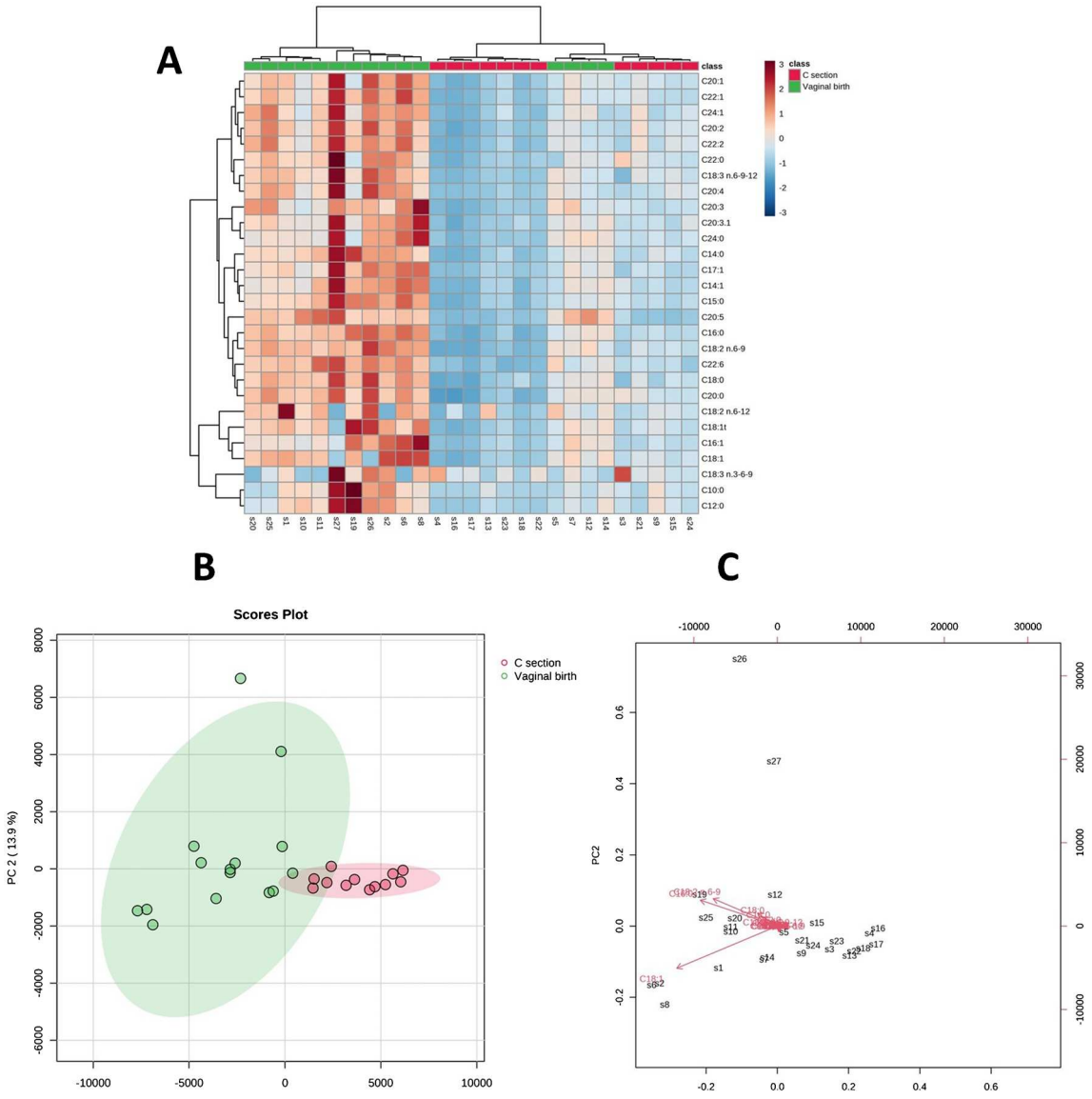

Fig. 7 Heat map of the fatty acid profiling of human colostrum milk (**A**). Score plot (**B**) and loadings plot (**C**) of the first two principal components (PCs) for the human colostrum fatty acids as a function of the neonatal delivery mode

FAME found in the colostrum samples analyzed were palmitic (C16:0, ranging from 19 to 27%), oleic (C18:1n-9, ranging from 29 to 42%), and linoleic (LA; 18:2n-6, ranging from 9 to 12%) (Fig. 7A). The presence of α-linolenic acid (ALA; 18:3n-3), arachidonic acid (ARA; 20:4n-6), eicosapentaenoic acid (EPA, 20:5n-3), and docosahexaenoic acid (DHA, 22:6n-3) was also observed. The distribution of saturated fatty acids in colostrum lipids was similar in all samples, with palmitic (C16:0) acid being represented in the greatest amounts, followed by stearic (C18:0) and myristic acid (C14:0). Fidler et al. [25] compiled 15 different studies reporting the fatty acid composition of human colostrum milk from 16 geographic regions. Similarly, to our findings, the authors reported C16:0 and C18:1n-9 values ranging from 21 to 28%, and 27 to 43%, respectively.

The variation observed in this study in the fatty acid composition can be attributed to a variety of factors, including lifestyle, nutritional status, ethnicity, stage of lactation, maternal diet, gestational age, and mode of neonatal delivery [1, 8]. A principal component analysis was performed aiming to further investigate the potential effects of neonatal delivery modes (C-section *vs*. vagina) in the fatty acid profile of the colostrum samples. Considering the impact of C-section on several aspects of maternal and child health, it is important to understand how the mode of delivery influences the fatty acid composition of human colostrum. Figure 7A represents a heat map of the FAME profile averages of each sample. Figure 7B and C illustrates the score plot and the biplot of the two first principal components (PCs), which together explained 97.8% of the total variance of the data (PC1 = 83.9% and PC2 = 13.9%). The biplots indicate that oleic and linoleic acid can be the main compounds to indicate the potential effects of deliver mode on the fatty acid profile. Overall, higher levels of oleic acid, LA, and ALA were observed in the human colostrum samples from the vaginal delivery mode. Our results are in accordance with the ones reported by Samuel et al. [8], the authors reported that the human milk, from mothers who delivered vaginally, showed significantly higher levels of n-3 PUFA (anti-inflammatory effects) and stearic and palmitoleic acids, whereas human milk from mothers undergoing C-section exhibited high levels of n-6 PUFA (pro-inflammatory effects). PCA results and cluster analysis suggest a possible association between the mode of delivery and the fatty acid profile. Further studies with a larger sample size should be performed to confirm the trend.

Conclusions

We successfully developed and applied an integrated ionic liquids capillary columns and flow-modulated comprehensive two-dimensional gas chromatography coupled to mass spectrometry (FM-GC×GC-MS) method for fatty acid profiling in human colostrum. An efficient, fast, and mild derivatization step with sodium methoxide was selected among the strategies tested. The use of IL60 as the ^{1}D column provided superior separation, good peak shape, and utilization of elution space. Therefore, the reverse set (SLB-IL 60i × HP-5) was selected. As a proof of concept, the developed method was applied to access the effects of the mode of neonatal delivery (vaginal *vs*. C-section) on the fatty acid profile of 27 human colostrum samples, indicating the potential influence of the mode of delivery in the fatty acid composition. This paper demonstrates the application of inverted phase orthogonal sets to improve FAME separation and identification.

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Authors contribution FFGD: methodology, formal analysis, writing—original draft preparation, review and editing; SBJ: conceptualization, supervision, writing—review and editing; RSS: resources and samples, review and editing; MF; resources and samples, review and editing; LWH: conceptualization writing—review and editing, supervision, project administration funding acquisition.

Declarations

Institutional review board The study was conducted in accordance with the Declaration of Helsinki and approved by the Institutional Review Board (or Ethics Committee) (no. 1,804,463).

Informed consent Patient consent was waived due to the nature of the study. No identification can be made by the results shown herein.

Conflict of interest The authors declare no competing interests.

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