Triacylglycerol Analysis in Human Milk and Other Mammalian Species: Small-Scale Sample Preparation, Characterization and Statistical Classification Using HPLC-ELSD Profiles

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ABSTRACT

In this work, a method for the separation of triacylglycerols (TAGs) present in human milk and other mammalian species by reversed-phase high performance liquid chromatography using a core-shell particle packed column with UV and evaporative light-scattering detectors is described. Under optimal conditions, a mobile phase containing acetonitrile/*n*-pentanol at 10 ºC gave an excellent resolution between more than 50 TAG peaks. A small-scale method for fat extraction in these milks (particularly of interest for human milk samples) using minimal amounts of sample and reagents was also developed. The proposed extraction protocol and the traditional method were compared, giving similar results, with respect to the total fat and relative TAG contents. Finally, a statistical study based on linear discriminant analysis on the TAG composition of different types of milks (human, cow, sheep and goat) was carried out to differentiate the samples according to its mammalian origin.

KEYWORDS: *Human milk, mammalian milk triacylglycerols, fat extraction, HPLC-ELSD, Linear Discriminant Analysis*

INTRODUCTION

According to the World Health Organization, breastfeeding is the recommended way of providing to young infants the nutrients for a healthy growth and development. This recommendation is based on knowledge that breast milk from healthy and well-fed mothers, provides sufficient energy and proper profile of nutrients to support normal growth and development of term infants, without any additional foods through the first 4 to 6 months of life.[1] Human milk constitutes a very complex fluid, which contains carbohydrates and salts in solution, caseins in colloidal dispersion, cells and cellular debris, and lipids mostly in emulsified globules.[2] Since lipids are the main energy source in human milk, contributing in 40-55% to the total energy intake, its compositional and physiological aspects have been of interest and research in the last decades and recently reviewed.[3] According to Koletzko *et al*.,[4] the average amount of fat contained in human milk is *ca*. 3.8-3.9 g/100 mL, but this value varies widely. Thus, human milk is a dynamic system whose lipid composition is influenced by factors such as mother's diet,[5] stage of lactation,[6,7] phase of the feeding[2] and breast.[2,8] However, any difference has been observed with regard to frequency of breastfeedings[9,10] and time of day.[11,12]

In spite of the good features in human milk, research to find valuable alternatives, especially when breastfeeding is not possible or may not be advisable, constitutes a high priority. To be nutritionally adequate, any substitute should have the same nutritional characteristics as breast milk. In addition, it should be hypoallergenic and palatable.[13] In

this sense, commercial infant formulas, usually based on mammalian milks such as cow, buffalo, donkey, sheep, camel, and goat milk, may represent an alternative to fulfill the nutritional needs of newborns.[14]

However, these milks are different from human milk in terms of chemical composition (*e.g.* protein and fat contents), which may cause nutritional and immunological problems.[15] Regarding fat content, limited studies have been done so far to systematically compare the lipid composition in different mammalian milks.[14]

Triacylglycerols (TAGs) represent 98% of total lipid fraction, and despite the changes in human milk composition, some TAGs, such as lauric acid-oleic acid-linoleic acid (LaOL), myristic acid-oleic acid-linoleic acid (MOL), can be considered as markers of the mature human milk.[16] Moreover, fatty acids (FAs) represent 90% of these TAGs, that is, 88% of total lipid,[2] and a balance ratio between ω-3 and ω-6 FAs in human milk is important to ensure the healthy growth of infants.[14] Although the analysis of FAs in human milk is much easier than TAGs evaluation, milk FAs are secreted, consumed and hydrolyzed as TAGs in globules.[2] For this reason, it is of great importance to achieve a reliable TAGs determination.

The most widely used techniques for fat extraction are based on the method developed by Folch *et al*.,[17] or its modification, developed by Chen *et al*.;[18] and the AOAC Official Method 989.05[19] based on the study of Barbano *et al*..[20] Nevertheless, these extraction methods are time-consuming and therefore, its automation is barely

possible. Modern trends in analytical chemistry move towards the simplification and miniaturization of sample preparation. This can be simply achieved by scaling down the size of previous systems or by developing new set ups and techniques.[21] Different extraction methods to accelerate and miniaturize the process (sample size and organic solvent volumes), searching for cost-effective solutions have been evaluated.[22] In spite of these benefits, few protocols[23] have been developed in human milk matrices to cover this demand.

Regarding TAGs determination in milk, several techniques such as, thin-layer chromatography (TLC),[7,24] silver ion adsorption-TLC,[25] RP-HPLC,[16,23,26–28] or with silver ions (Ag-HPLC),[14,29] two-dimensional LC,[30] gas chromatography (GC)[25] and tandem MS with ammonia negative ion chemical ionization,[31] have been employed. From all these techniques, RP-HPLC has been widely used, since it provides a better resolution of individual TAG molecules. Thus, these compounds are separated according to both chain length and degree of unsaturation of the FAs.[32] However, RP-HPLC for TAGs separation has been usually performed using a binary gradient acetonitrile (ACN)-isopropanol[14,27] or a linear ternary gradient ACN-dichloromethane-acetone.[16,23,28] These latter gradients led to low resolution of TAGs peaks, and the first-mentioned gave long analysis time (90 min).

In order to improve chromatographic performance in terms of throughput and/or resolution, particularly when numerous complex food extracts have to be analyzed, recent

advances in LC instrumentation could be beneficial.[33] In this context several analytical strategies related to column technology have been developed in HPLC, including the use of monolithic supports, packed columns with sub-2 μm particles operating at ultra-high pressure (UHPLC) or with core-shell or fused-core particles. These latter core-shell particle columns are capable of maintaining high efficiencies at increasing flow rates with the subsequent reduction of analysis time. Also, these columns operate comfortably within the pressure limits of conventional LC instruments, rivaling the performance obtained with sub-2 columns on UHPLC instruments. However, the use of these columns in conventional LC systems for TAGs separation in human milk samples has not been reported to date.

Multivariate data analysis can be used to obtain more information on major, minor, and trace components in foods.[34] Within these statistical tools, linear discriminant analysis (LDA) is probably one of the best known methods and it has been successfully used for the identification/differentiation of several foods, such as dairy products, oils, wines and others.[36,37]

In this work, the development of analytical conditions for the extraction and RP-HPLC separation of milk fat TAGs is described. For this purpose, a small-scale fat extraction protocol (with reduced consumption of reagents and processing time, in consistency with the recent trends in green chemistry) and in combination with the use of a fused-core HPLC column is presented and compared. In addition, on the basis of TAG profiles of milk samples from different mammalian species (human, bovine, caprine and ovine), a LDA model is applied to differentiate these matrices according to its species

origin.

MATHERIALS AND METHODS

Chemicals. TAG standards including trilaurin (LaLaLa), trimyristin (MMM), tripalmitin (PPP), tripalmitolein (PaPaPa) and triolein (OOO) from Sigma (St. Louis, MO, USA) were employed. The following analytical grade reagents were also used: HPLC-grade ACN and methanol (MeOH) were purchased from VWR Chemicals (Barcelona, Spain); reagent-grade dichloromethane, *n*-pentanol and *n*-hexane, HPLC-grade 2-propanol and *n*-butanol and anhydrous sodium sulfate, were supplied by Scharlau (Barcelona, Spain). Butylhydroxytoluene (BHT) was purchased from Fluka (Buchs SG, Switzerland).

Samples. Human milk samples (n = 15) were kindly donated by healthy well-nourished mothers in different stages of lactation (3 colostrum (1-5 days); 5 transitional milk (6-15 days); 7 mature milk (after 16 days)). All mothers, who were Caucasian, middle-class, and lived within the urban area of Valencia, consumed an unrestricted omnivorous diet. The samples were collected between the baby's feed by manual expression using a Medela Harmony^{TM} Breastpump (Zug, Switzerland).

Bulk raw milk samples of Holstein-Friesian cows (n = 20) were collected by milking machines while milk samples of Cartera goats (n = 20) and Murciano-Granadina sheeps (n = 20) were collected by manual expression from animals in midlactation stage. All these samples were kindly donated by different farms located at *La Comunitat Valenciana*, Spain. All animals were free from mastitis or any other inflammatory diseases. They were grazed in the morning and in the afternoon were reared in stables and

fed with hay, fodder grass and vegetables. After collection, milk samples were rapidly heated to 80 ºC and held at this temperature for 1.5 min in order to inactivate the lipases and to avoid TAGs hydrolysis.[38]

Sample Preparation. A lipid extraction method (Method I) was developed in this study. It consisted of a modification of the traditional gravimetric method.[16] Briefly, a well-mixed milk aliquot (150 μL) was placed in a centrifuge tube and 2.5 mL of a dichloromethane-MeOH solution (2:1, v/v), containing 5 μg mL^{-1} BHT to prevent lipid oxidation,[24] was added. The mixture was sonicated for 10 min, vortexed for 1 min, placed in the fridge (4 ºC) for 15 min and centrifuged at 10000 rpm for 8 min. Then, 800 μL of distilled water was added, sonicated for 10 min, mixed for 1 min in the vortex and centrifuged at 10000 rpm for 8 min. The organic layer was washed with 800 μL of a saturated solution of sodium chloride, sonicated for 10 min, vortexed for 1 min and centrifuged 8 min at 10000 rpm. The organic fraction was dried with anhydrous sodium sulfate and the fat solution was then filtered with a syringe through a 0.45 μm filter, collected in a pre-weigh HPLC vial and dried under a nitrogen stream. The fat was dissolved in a 2:2:1 ACN/2-propanol/*n*-hexane (v/v/v) ternary mixture and injected in the LC system. This method was compared with that described by Morera *et al.*[16] (Method II).

High-Performance Liquid Chromatography and Mass Spectrometry. An 1100 series liquid chromatograph (Agilent Technologies, Waldbronn, Germany) provided with a quaternary pump, a degasser, a thermostated column compartment, an automatic sampler, a UV-Vis diode array detector online coupled to an Agilent 385-ELSD was

employed. Separation was carried out with a Kinetex^{TM} C18 100 Å column (150 mm × 4.6 mm, 2.6 μm; Phenomenex, Torrance, CA, USA). The optimized separation conditions were: isocratic elution with a 80:20 ACN/*n*-pentanol mixture for 45 min, followed by a gradient of ACN/*n*-pentanol up to ratio 60:40 in 20 min; column temperature, 10 ºC; flow rate, 1.0 mL min^{-1} and injection volume, 10 μL. UV detection was performed at 205 ± 10 nm (360 ± 60 nm as reference). The ELSD parameters were: evaporation and nebulization temperature, 55 ºC; gas flow rate, 1.2 Standard Liters per Minute (SLM); gain factor, 1.

For TAG identification, a UPLC^{TM} binary pump system (Acquity, Waters, Milford, USA) was interfaced to a triple quadrupole mass spectrometer (TQD, Waters, Manchester, UK) through an Atmospheric Pressure Chemical Ionization (APCI) source. The MS working conditions were as follows: probe temperature, 600 ºC; corona discharge current, 20 μA; source temperature: 120 ºC; desolvation gas flow, 800 L h^{-1}; cone gas flow, 60 L h^{-1}. Drying as well as nebulizing gas was nitrogen (Praxair, Valencia, Spain). The mass spectrometer scanned within the m/z 150-1000 range in the positive ionization mode at one scan per second.

Data Treatment and Statistical Analysis. The area of selected TAG peaks was measured from ELSD detector, and a data matrix was constructed (see Results and Discussion section). After normalization of the variables, statistical data treatment was performed using SPSS (v. 15.0, Statistical Package for the Social Sciences, Chicago, IL, USA). Using a stepwise algorithm the predictors to be included in the LDA model were selected. According to this algorithm, a predictor is considerable eligible for its inclusion

in the model if the significance of its F-test exceeds the specified probability level given by the entrance threshold, F_{in}. However, the entrance of a new predictor modifies the significance of those predictors that are already present in the model. For this reason, after the inclusion of a new predictor, a rejection threshold, F_{out}, is used to decide if one of the other predictors should be removed from the model. This sequential process continues until no more predictors are eligible for entrance or removal from the model. The default probability values of F_{in} and F_{out}, 0.05 and 0.10, respectively, were adopted.

RESULTS AND DISCUSSION

Optimization of the Separation of TAGs. The initial separation conditions were adapted from a previous work for TAGs separation in vegetables oils.[39] The optimization study was performed using human milk samples. Using a flow rate of 1.5 mL min^{-1} and a column temperature of 10 ºC, a similar optimization study of mobile phase, including several binary mixtures of ACN with different alcohols (2-propanol, *n*-butanol and *n*-pentanol) at 70:30 ratio, was conducted. Using 2-propanol, poor resolution and relative long analysis time (*ca*. 50 min) were obtained. Using *n*-butanol and *n*-pentanol, analysis time and resolution improved significantly, although most peaks still overlapped. Despite this overlapping, ACN/*n*-pentanol mixture gave shorter analysis time with respect to *n*-butanol and therefore, *n*-pentanol was selected for the following studies.

Next, the influence of the content of *n*-pentanol in the 20-30% range on TAGs separation was studied. As shown in Figure S1 (see Supporting Information), resolution was improved with decreasing of *n*-pentanol content. Broad peaks with separation times

higher than 45 min were obtained with less than 20% *n*-pentanol. Thus, an 80:20 (v/v) ACN/*n*-pentanol mixture was selected. This mobile phase also provided a satisfactory response in ELSD. Then, the effect of column temperature on TAGs separation and detection response of UV (Figure S2, left) and ELSD (Figure S2, right) detectors was performed under isocratic conditions. As it can be seen, the TAG resolution increased when temperature decreased, with a concomitant increase both in backpressure (from 22.4 to 25.4 MPa along the 5-20ºC range) and analysis time. Within the studied temperature interval, the TAG peaks showed satisfactory peak widths (measured at 50% peak height) ranging between 6.3-53.0 s (Figure S2, left) and 6.2-42.2 s (Figure S2, right). Regarding to detector response, UV and ELSD detection did not show significant changes in the response (measured as peak area) over the temperature range investigated. These observations in ELSD response were different from those reported in literature, where a positive[40] or negative[41] correlation with column temperature for peak area could be found. This behavior could be explained by the narrow temperature interval (5-20 ºC) considered in this study. Thus, a column temperature of 10ºC provided the highest number of resolved peaks (30) (with peak widths ranging between 5.4 and 29.1 s) in < 65 min with a reasonable backpressure (24.1 MPa), being selected for further studies.

Next, the influence of flow rate on TAGs separation was also studied. A flow rate decrease from 1.5 mL min^{-1} to 1.0 mL min^{-1} led to an improvement in the resolution at expense of an increase of analysis time. Lower flow rates (< 1.0 mL min^{-1}) provided a significant peak broadening (peak widths ranged between 10.2 and 80.3 s and asymmetry factors comprised between 0.85-1.60) at very long separation time (> 100 min). In

addition, a decrease of the ELSD response with increasing flow rate was found, which was consistent with findings reported by several authors.[40,42] As a result, a flow rate of 1.0 mL min^{-1} was selected for further studies. Under these conditions, the late-eluting compounds were barely distinguished from baseline. In order to decrease the retention time and improve detection (peak shape), a solvent gradient step was established. Thus, the *n*-pentanol content was increased from 20% to 40% in 20 min after the first 45 min of isocratic elution with 80:20 ACN/*n*-pentanol. Figure 1 shows the ELSD chromatogram obtained under these conditions, where satisfactory peak shapes for highly retained TAGs were obtained.

The performance of the developed RPLC method was compared to those of previously reported for analysis of TAGs in human milk. Most of these studies were usually conducted using conventional microparticulate columns packed with 5-µm silica particles,[23,27,28] however, it should be mentioned that data of efficiency or other analytical parameters were not provided. In any case, an estimation of the number of resolved peaks of some of these works was done. These values were comprised between 17 and 22,[23,27,28] which were significantly lower than those obtained in this work (35). Also, packed columns of sub-2 µm particles operating at UPLC conditions have been applied to these samples.[43,44] To our knowledge, the work of Beccaria *et al.*[43] is the only one that has reported performance data (peak capacities values up to 170 for 114-min gradient) using three-serially coupled core-shell type C18 columns. However, the number of resolved peaks was similar to our method, which is accomplished in shorter analysis (< 65 min) using a RP column in a conventional high pressure system.

Identification of TAGs. Generally, the elution of TAGs in RP-HPLC occurs according the partition number (PN).[32,45] The PN for each individual TAG can be calculated as follows: PN = CN – 2ND, where CN is the number of carbon atoms and ND is the number of double bonds in the FAs attached to the glycerol molecule (see Table 1). However, the procedure for identifying the TAGs based on the PN is complicated due to the large number of FA constituents and rather limited to the coincidence of this parameter for several TAGs. For this reason, to achieve a reliable TAG identification, a fragmentation study resulting from the APCI-MS analysis of TAGs in the positive ionization mode was performed.[46,47] The APCI mass spectra of TAGs exhibited a protonated molecule, $[M+H]^+$, whose intensity depends on the degree of unsaturation of the TAG molecule, and $[M+H–R_{1,2,3}COOH]^+$ ions resulting from the loss of fatty acyl moieties (diacylglycerol (DAG) ions). The intensities of the protonated molecules $[M+H]^+$ formed from saturated TAGs were low or even no detected (for fully saturated TAGs), and its identification was performed according to their respective DAG ions. These results were consistent with those previously reported.[45,48] Table 1 shows the *m/z* values of the protonated molecules and DAG ions found in the human milk samples studied. No distinction was made between the *sn*-1, *sn*-2 and *sn*-3 positions in the TAG species identified. It should be noted that in spite of the satisfactory resolution achieved in this work, some cases of co-elution of TAGs were observed (see Table 1 and Figure 1).

With regard to the number of TAGs here identified, this can be favorably

compared to that reported in previous human milk studies. For example, in the work of Morera *et al.*[16] up to 43 different TAGs were reported, whereas in recent studies of Linderborg *et al.*[44] and Mondello's group,[43] a number of 37 and 51 different TAGs, respectively, were positively identified. However, these values were lower than those reported by Haddad *et al.*;[49] where up to 98 TAGs were identified by using two serially coupled C18 columns in combination with tandem ESI-MS and subsequently confirmed by GC-MS.

Small-Scale Extraction Method and Quantification. First, the evaluation of fat content using the proposed small-scale extraction method was performed. In order to evaluate the efficiency of fat extraction, the method developed here (Method I) was compared to the conventional protocol (Method II). Thus, fat was extracted and gravimetrically determined[25,26,41,42] from twelve mature human milk aliquots, six by Method I and six by Method II. The fat content obtained by Method I and II ranged between 0.021-0.029 and 0.025-0.029 g mL^{-1}, respectively. Table 2 shows the fat content (expressed as mean ± SD) obtained for both methods. No significant differences in the fat milk content between both extraction procedures were observed ($p > 0.05$, Student's *t*-test) and the results found were consistent with those described in the literature.[3,23] At sight of these results, the evaluation of TAG content was next considered.

When performing quantification in ELSD, several authors have established that its response is linear for a wide range of concentrations.[38,50] However, some studies[51,52]

have demonstrated that the response from HPLC–ELSD follows a non-linear empirical model: $A = am^b$, where A is the area of chromatographic peak, m the mass of analyte, being a and b two experimental parameters related to the ELSD configuration. Thus, each change in the instrumental working conditions would require a new estimation of these parameters. This equation can be easily linearized as follows: $\log A = b \log m + \log a$. However, the application of this equation requires the availability of each TAG identified in the target sample, which is not commercially feasible. To overcome this limitation, Heron *et al.*[52] have developed an empirical methodology to evaluate the TAG content in vegetable oils based on the previous equation using a reduced number of standards. However, the method provided a high variability (between 1 and 40%) with respect to the real mass percentage. Another approach proposed by several authors[28,51] is based on the application of the internal normalization method as measurement of mass percentage, where a similarity in the response factors of TAGs (relative response factors, RRF) with respect to triolein (OOO) ranged between 0.83-1.21) was assumed. In order to confirm this assumption, calibration curves of homologous TAG standards were performed and fitting equations of both linear and power models to detector response (area) versus mass of lipid injected were obtained. As shown in Table 3, the peak areas were well-fitted by power model equations in the mass range studied (0.5-100 μg). The limits of detection (LOD) and limits of quantification (LOQ) were also estimated for signal-to-noise ratios of 3 and 10, respectively. The results obtained for homogeneous TAG standards were: LOD (9.2-13.1) ng and LOQ (30.6-43.3) ng (see Table 3). Then, the RRF calculated for pure homogeneous TAG standards in relation to OOO were obtained, giving values close

to unity, which allows a quantitative estimation of TAGs on the basis of the percentage peak area. The relative content of each TAG (expressed as mean ± SD) obtained is given in Table 4. As shown in Table 4, no significant differences ($p > 0.05$, Student's *t*-test) were observed between results obtained with both methods for more than 85% of the TAGs. From this study, it can be derived that Method I provided satisfactory results, both in the total fat content as in the quantitative analysis of representative TAGs. Moreover, the Method I has several advantages over the Method II. Table 5 summarizes the essential features of both sample preparation methods. The Method I uses much smaller volumes of the sample and the chemical solutions (90% reduction), thus, the expense and chemical hazards are greatly reduced. Although other operational steps for both methods were quite similar, the small-scale protocol requires the use of materials such as micropipettes or small syringes, which simplifies the handling of more samples simultaneously and speeds up the isolation process of fat. In particular, the Method I allowed processing 120 samples per day (in an 8 h-working day), whereas a rate of 30 samples per day could be achieved with the Method II. A comparison between our method and the traditional Folch method for milk samples[43,49] was also done. In this protocol, the extraction step employs larger volumes of solvents (40-180 mL of chloroform-methanol (2:1, v/v), followed by several re-extractions and washings, which results in larger experimental effort, time and amounts of residues of harmful solvents generated than the developed protocol. Consequently, the present procedure is suitable to be applied to systematic and routine characterization of lipid composition in human milk samples. Additionally, due to the small sample volume required, the proposed method offers the possibility of analyzing separate individual milk sample portions from within one feeding, allowing the

characterization of possible fluctuations in the TAG composition.

As shown in Table 4, from 69 TAG molecular species identified, the six major TAGs found in lipid fraction in both protocols were: POO, POL, LaPO + MMO, MOO, PSO (see Table 1 for abbreviations) with 20, 10, 7, 6 and 5%, respectively. The amounts of these most abundant TAGs were similar to those obtained in the literature[16,27,49]. For example, the studies of Zou *et al.*,[27] in milk from Danish women, pointed out that the TAGs were mainly composed of POO (21.52%) and POL (16.93%). These small discrepancies can be attributed to natural factors such as randomization in human milk, the physiological stage,[16] and the susceptibility of human milk TAGs to dietary habits.[5,44] It is also important to remark that the molecular weight distribution of TAGs (expressed as CN:ND groups[44]) in human milk reported in this work (see Figure S3) is in agreement with the findings of studies previously reported.[44,49]

Taking into account the advantages described above for the Method I, it was extended to the other milk mammalian species (cow, sheep and goat). Thus, the total fat content varied within the ranges 0.023-0.046, 0.046-0.057 and 0.034-0.076 g mL^{-1} for cow, sheep and goat milks, respectively. These milk samples were also analyzed using the developed LC method with an excellent resolution/elution time ratio compared to those previously reported[14,16,23,27,28] (Figure 2). Similar TAG profiles (particularly for the main TAG peaks) were found than those reported in literature for these samples.[14] From these profiles, 22 common peaks, which could be easily integrated, were selected for the

four mammalian species, and used for statistical treatment.

Classification of Mammalian Milks Using TAG Profiles with LDA Model. First, to reduce the variability associated to total amount of TAGs recovered from milk samples, and to minimize the sources of variance also affecting the sum of the areas of all the peaks, normalized rather than absolute peak area were used. In order to normalize the variables, the area of each peak was divided by each one of the areas of the other 21 peaks; in this way, and taking into account that each pair of peaks should be consider only once, (22 x 21)/2 = 231 non-redundant peak area ratios were obtained to be used as predictors. Using the normalized variables, an LDA model capable of classifying the milk samples according to their respective mammalian specie was constructed. A matrix containing 75 objects which corresponded to all the milk samples analyzed was constructed. Thus, this matrix was divided in two groups of objects to constitute the training and evaluation sets. The training set was composed by 67 objects (18 milks 3 animal mammalian species, and 13 human milk samples), while the evaluation set was constituted by the remaining samples (8 objects). A response column, containing the categories corresponding to the 4 mammalian species, was added to these matrices. When the LDA model was constructed, an excellent resolution between all the category pairs was achieved (Wilks' lambda, λ_w < 0.01). The variables selected by the SPSS stepwise algorithm, and the corresponding standardized coefficients of the model, showing the predictors with large discriminant capabilities, are given in Table 6. As shown in Figure 3a, a large resolution between the human milk from the other mammalian milks was achieved along the first discriminant function (df). As deduced from Table 6, first df was mainly constructed with the peak area ratios OOL/PaOO and POO/(POAra+SSO) (peaks

labelled as 24/25 and 33/41, respectively). The projection on the second df shows a lack of capability to resolve between cow and goat milks. However, both milks were clearly distinguished from sheep and human milks. In this case, second df was mainly constructed with the peak area ratios (CaMS+CaPP+LaLaS+LaMP+MMM+OLL)/(MOO+PPaO) and (LaPO+MMO)/PSO. (peaks labelled as 16/27 and 22/39, respectively). According to Figure 3b, along the third df, it is not possible to distinguish between sheep and human milk, but both were markedly differentiated from cow and goat milks. As shown in Table 6, the third df was mainly constituted with the peak area ratios (CaPO+LaMO)/(CaPS+LaMS+LaPP+MMP) and (CaMS+CaPP+LaLaS+LaMP +MMM+OLL) (peaks labelled as 15/23 and 16/24, respectively). Finally, as illustrated in Figure 3c, by using a plane oblique to the three first discriminant functions, all the possible pair of categories were very well resolved from each other.

Using this model and leave-one-out validation, all the objects of the training set were correctly classified. On the other hand, the prediction capability of the model was evaluated using the evaluation set. In this case, all the objects (represented with a cross symbol in Figure 3) were correctly assigned within 95% probability level. It should be outlined that in the case of human milk samples, different period of lactation were taken, and in all cases, an excellent classification within this category was accomplished, which supports the robustness of the developed LDA model.

In summary, a highly efficient RP-HPLC-UV-ELSD method of TAGs in human milk samples has been developed. Also, a small-scale sample preparation method has been established, being particular important for human milk samples. The combination of this sample extraction protocol with HPLC-UV-ELSD technique could provide a methodology highly recommended in studies on breastfeeding and its contribution to

infant growth and development. Finally, the possibility of classifying milks according to their mammalian origin by using TAG profiles obtained by HPLC-ELSD has been successfully demonstrated.

SUPPORTING INFORMATION AVAILABLE:

Figure S1. Influence of *n*-pentanol content on TAGs separation in mature human milk: 70:30 (a), 75:25 (b) and 80:20 (c) ACN/*n*-pentanol. Chromatographic conditions: isocratic elution; column temperature, 10 ºC; flow rate, 1.5 mL min^{-1}.

Figure S2. Influence of column temperature on TAGs separation in mature human milk using UV (left) and ELSD detector (right): 20 (a), 15 (b), 10 (c) and 5 ºC (d). Chromatographic conditions: isocratic elution, 80:20 ACN/*n*-pentanol; flow rate, 1.5 mL min^{-1}.

Figure S3. TAG molecular weight distribution (expressed as CN:ND groups) in human milk.

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Fundings:

Project CTQ2014-52765-R (MINECO of Spain and FEDER). I. T-D thanks the MINECO for an FPU grant for PhD studies.

FIGURE CAPTIONS

Figure 1. TAG profile of mature human milk. Chromatographic conditions: isocratic elution for 45 min at 80:20 ACN/*n*-pentanol followed by a linear gradient up to 60:40 ACN/*n*-pentanol in 20 min; column temperature 10 ºC; flow rate 1.0 mL min^{-1}.

Figure 2. TAG profile of milk from different mammalian species: cow (a); sheep (b); goat (c). Chromatographic conditions as in Figure 1.

Figure 3. Score plots on the planes of the first and second (a), and second and third discriminant functions (df) (b), and on an oblique plane of the 3-D space defined by the three discriminant functions (c) of the LDA model constructed to discriminate between different mammalian milk species. Evaluation set samples are labeled as indicated in figure legend.

Table 1. TAGs Identified by APCI-HPLC-MS Analysis of Mature Human Milk.

Peak no.[a]	TAGs[b]	PN	[M+H]+	[M+H-R_1COOH]+	[M+H-R_2COOH]+	[M+H-R_3COOH]+
1	CaML + LaLaL	38	719.62	LaLa 439.40 CaM 439.40	LaL 519.44 CaL 491.41	- ML 547.47
2	CaLaO	38	693.60	CaLa 411.34	CaO 493.42	LaO 521.45
3	n. i.					
4	CaMM + LaLaM	38	667.59	CaM 439.40 LaLa 439.40	- LaM 467.41	MM 495.44 -
5	LaLL	40	799.68	LaL 519.44	-	LL 599.50
6	CaOL	40	773.67	CaO 493.42	CaL 491.41	OL 601.52
7	CaPL + LaML	40	747.65	CaP 467.41 LaM 467.41	CaL 491.41 LaL 519.44	PL 575.50 ML 547.47
8	CaMO + LaLaO	40	721.63	CaM 439.40 LaLa 439.40	CaO 493.42 LaO 521.45	MO 549.48 -
9	n. i.					
10	CaLaS + CaMP + LaLaP + LaMM	40	695.61	CaLa 411.34 CaM 439.40 LaLa 439.40 LaM 467.41	CaS 495.44 CaP 467.41 LaP 495.44 -	LaS 523.47 MP 523.47 - MM 495.44

11	MLL/PaPaETE + MPaETE + PLLn	42	827.71 853.73 827.71 853.73	ML 547.47 PaPa 547.47 MPa 521.45 PL 575.50	- PaETE 599.50 METE 573.48 PLn 573.48	LL 599.50 - PaETE 599.50 LLn 597.50
12	LaOL	42	801.70	LaO 521.45	LaL 519.44	OL 601.52
13	CaOO	42	775.68	CaO 493.42	-	OO 603.53
14	LaPL + MML	42	775.68	LaP 495.44 MM 495.44	LaL 519.44 ML 547.47	PL 575.50 -
15	CaPO + LaMO	42	749.67	CaP 467.41 LaM 467.41	CaO 493.42 LaO 521.45	PO 577.52 MO 549.48
16	CaMS + CaPP + LaLaS + LaMP + MMM + OLL	42 44	723.65 881.76	CaM 439.40 CaP 467.41 LaLa 439.40 LaM 467.41 MM 495.44 OL 601.52	CaS 495.44 - LaS 523.47 LaP 495.44 -	MS 551.50 PP 551.50 - MP 523.47 - LL 599.50
17	PaOL	44	855.74	PaO 575.50	PaL 573.48	OL 601.52
18	PLL	44	855.74	PL 575.50	-	LL 599.50
19	MOL	44	829.73	MO 549.48	ML 547.47	OL 601.52
20	LaOO	44	803.71	LaO 521.45	-	OO 603.53
21	MPL	44	803.71	MP 523.47	ML 547.47	PL 575.50
22	LaPO + MMO	44	777.70	LaP 495.44 MM 495.44	LaO 521.45 MO 549.48	PO 577.52 -

23	CaPS + LaMS + LaPP + MMP	44	751.68	CaP 467.41 LaM 467.41 LaP 495.44 MM 495.44	CaS 495.44 LaS 523.47 - MP 523.47	PS 579.53 MS 551.50 PP 551.50 -
24	OOL	46	883.76	OO 603.53	OL 601.52	-
25	PaOO	46	857.76	PaO 575.50	-	OO 603.53
26	POL	46	857.76	PO 577.52	PL 575.50	OL 601.52
27	MOO + PPaO	46	831.74	MO 549.48 PPa 549.48	- PO 577.52	OO 603.53 PaO 575.50
28	PPL	46	831.74	PP 551.50	PL 575.50	-
29	LaSO + MPO	46	805.73	LaS 523.47 MP 523.47	LaO 521.45 MO 549.48	SO 605.55 PO 577.52
30	LaPS + MMS + MPP	46	779.71	LaP 495.44 MM 495.44 MP 523.47	LaS 523.47 MS 551.50 -	PS 579.53 - PP 551.50
31	OOO	48	885.76	OO 603.53	-	-
32	SOL	48	885.76	SO 605.55	SL 603.53	OL 601.52
33	POO	48	859.77	PO 577.52	-	OO 603.53
34	PSL	48	859.77	PS 579.53	PL 575.50	SL 603.53
35	MSO + PPO	48	833.76	MS 551.50 PP 551.50	MO 549.48 PO 577.52	SO 605.55 -

36	LaSS + MPS + PPP	48	807.74	LaS 523.47 MP 523.47 PP 551.50	- MS 551.50 -	SS 607.56 PS 579.53 -
37	n. i.					
38	SSL + SOO	50	887.81	SS 607.56 SO 605.55	SL 603.53 -	- OO 603.53
39	PSO	50	861.79	PS 579.53	PO 577.52	SO 605.55
40	PPS	50	835.76	PP 551.50	PS 579.53	-
41	POAra + SSO	52	889.82	PO 577.52 SS 607.56	PAra 607.57 SO 605.55	OAra 633.58 -
42	PSS	52	863.81	PS 579.53	-	SS 607.56

a Peak identification number according to Figure 4; TAGs identified according to protonated molecule ($[M+H]^+$) and diacylglycerol ions ($[M+H-R_{1,2,3}COOH]^+$) observed in the APCI mass spectrum, and to the relative order of PN.

b Structure indicated by FA composition (*e. g*. OOO for triolein) using the following abbreviations: Ca, capric acid (C10.0); La, lauric acid (C12:0); M, myristic acid (C14:0); P, palmitic acid (C16.0); Pa, palmitoleic acid (C16:1); S, stearic acid (C18:0); O, oleic acid (C18:1); L, linoleic acid (C18:2); Ln, linolenic acid (C18:3); Ara, Arachidic acid (C20:0); ETE, eicosatrienoic acid (C20:3).

Table 2. Fat Content (g mL^{-1}) in Human Milk Obtained Gravimetrically by Methods I and II.

Human milk aliquot	Fat (g mL^{-1})	
	Method I	**Method II**
1	0.0231	0.0270
2	0.0246	0.0249
3	0.0294	0.0258
4	0.0252	0.0256
5	0.0258	0.0279
6	0.0207	0.0291
Mean ± SD	0.0248 ± 0.0029	0.0267 ± 0.0016

Table 3. Calibration Equation Coefficients (Linear Regression and Power Curve Fittings), LODs and LOQs, and RRF values for TAG Standards in the Assayed LC-ELSD Method (x = µg Injected; y = Peak Area in mV).

TAG[a]	Linear regression ($y = ax + b$)				Power curve ($y = bx^a$)			LOD (ng)	LOQ (ng)	RRF[b]
	a	b	r^2		b	a	r^2			
LaLaLa	859.59	-856.49	0.9798		253.51	1.50	0.9995	9.2	30.6	1.0067
PaPaPa	806.75	-929.79	0.9638		198.47	1.56	0.9991	12.4	41.1	1.0475
MMM	711.87	-768.68	0.9646		186.91	1.54	0.9992	13.1	43.3	1.0357
OOO	738.15	-830.13	0.9703		208.15	1.49	0.9976	10.9	36.0	1.0000
PPP	412.05	-246.41	0.9885		217.27	1.26	0.9986	9.4	31.1	0.8471

[a] For abbreviations see Table 1.

[b] RRF values are given in relation to OOO.

Table 4. Relative Content of each TAG in Mature Human Milk Obtained by Methods I and II[a].

Peak no.[b]	TAGs[b]	Method I (n = 3), mean ± SD	Method II (n = 3), mean ± SD	p
1	CaML + LaLaL	0.385 ± 0.005	0.371 ± 0.019	0.057
2	CaLaO	0.612 ± 0.007	0.649 ± 0.017	0.173
3	n. i.	0.066 ± 0.003	0.091 ± 0.019	0.149
4	CaMM + LaLaM	0.508 ± 0.007	0.51 ± 0.02	0.359
5	LaLL	0.140 ± 0.003	0.146 ± 0.014	0.568
6	CaOL	0.242 ± 0.007	0.241 ± 0.006	0.866
7	CaPL + LaML	1.034 ± 0.006	0.977 ± 0.003	0.0001
8	CaMO + LaLaO	2.33 ± 0.02	2.134 ± 0.008	0.0001
9	n. i.	0.092 ± 0.007	0.10 ± 0.02	0.710
10	CaLaS + CaMP + LaLaP + LaMM	0.915 ± 0.017	0.896 ± 0.019	0.266
11	MLL/ PaPaETE + MPaETE + PLLn	0.232 ± 0.009	0.243 ± 0.005	0.124
12	LaOL	2.09 ± 0.04	1.98 ± 0.06	0.055
13	CaOO	0.86 ± 0.05	0.82 ± 0.02	0.309
14	LaPL + MML	1.58 ± 0.04	1.55 ± 0.05	0.362
15	CaPO + LaMO	5.04 ± 0.07	4.86 ± 0.07	0.031
16	CaMS + CaPP + LaLaS + LaMP + MMM + OLL	1.95 ± 0.12	2.03 ± 0.04	0.302
17	PaOL	0.17 ± 0.03	0.18 ± 0.04	0.768
18	PLL	0.96± 0.07	0.96 ± 0.07	0.991
19	MOL	2.77 ± 0.06	2.760 ± 0.08	0.875
20	LaOO	4.74 ± 0.12	4.68 ± 0.07	0.507
21	MPL	1.29 ± 0.05	1.278 ± 0.015	0.795

22	LaPO + MMO	7.39 ± 0.14	7.30 ± 0.10	0.412
23	CaPS + LaMS + LaPP + MMP	1.18 ± 0.03	1.25 ± 0.02	0.051
24	OOL	2.75 ± 0.04	2.82 ± 0.12	0.445
25	PaOO	0.91 ± 0.17	0.9 ± 0.2	0.748
26	POL	9.88 ± 0.21	10.1 ± 0.3	0.335
27	MOO + PPaO	5.410 ± 0.016	5.55 ± 0.05	0.010
28	PPL	1.220 ± 0.014	1.22 ± 0.05	0.947
29	LaSO + MPO	4.27 ± 0.07	4.44 ± 0.03	0.013
30	LaPS + MMS + MPP	1.036 ± 0.006	1.06 ± 0.04	0.402
31	OOO	3.65 ± 0.06	3.7 ± 0.2	0.551
32	SOL	0.97 ± 0.02	1.03 ± 0.03	0.118
33	POO	20.3 ± 0.6	20.21 ± 0.19	0.914
34	PSL	1.117 ± 0.010	1.14 ± 0.08	0.680
35	MSO + PPO	4.09 ± 0.07	4.17 ± 0.16	0.480
36	LaSS + MPS + PPP	0.55 ± 0.03	0.56 ± 0.04	0.946
37	n. i.	0.15 ± 0.02	0.117 ± 0.009	0.068
38	SSL + SOO	1.48 ± 0.04	1.43 ± 0.05	0.246
39	PSO	5.06 ± 0.13	4.9 ± 0.2	0.425
40	PPS	0.34 ± 0.03	0.35 ± 0.03	0.833
41	POAra + SSO	0.133 ± 0.006	0.139 ± 0.011	0.448
42	PSS	0.145 ± 0.002	0.157 ± 0.014	0.282

a Data obtained from three extractions performed the same day for each extraction method.

b Peak identification number, TAG information and abbreviations as indicated in Table 1.

Table 5. Comparison of Sample Preparation Protocols.

	Method I (small-scale)	**Method II**[16]
Sample volume (mL)	0.15	1.5
Extraction i) CH_2Cl_2:MeOH (2:1, v/v) (mL) ii) Water (mL) iii) Saline solution (mL)	 2.5 0.8 0.8	 25 8 8
Material required	10 mL centrifuge tubes, syringes, syringe filters, micropipettes	Falcon™ 50 mL conical centrifuge tubes, separating funnels, filter funnels, filter paper, pipettes, Pasteur pipettes
Samples per day	120	30

Table 6. Predictors Selected and Corresponding Standardized Coefficients of the LDA Model Constructed to Discriminate between Milks Obtained from Different Mammalian Species.

Predictor[a]	1st df	2nd df	3rd df
Peak 15/Peak 23	-0.466	0.219	2.275
Peak 16/Peak 24	0.700	-0.302	-2.742
Peak 16/Peak 27	0.075	-2.114	2.020
Peak 16/Peak 35	-0.262	0.126	1.306
Peak 16/Peak 41	-0.395	1.559	0.128
Peak 22/Peak 39	-0.284	2.067	0.067
Peak 23/Peak 41	-0.230	-1.282	0.055
Peak 24/Peak 25	2.748	0.463	-0.143
Peak 26/Peak 40	0.949	0.263	0.243
Peak 30/Peak 33	-0.115	0.693	1.016
Peak 33/Peak 41	1.662	0.724	0.812

[a] See Table 1 for peak identification.

Figure 1.

Figure 2.

Figure 3.TABLE OF CONTENTS (TOC)