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Optimization and validation of HRLC-MS method to identify and quantify Triacylglycerol Molecular Species in human milk

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ABSTRACT

Study on the determination of triacylglycerols (TAG) molecular species in human milk is necessary for understanding absorption of human milk fat as well as designing milk fat in infant formulas. The aim of the present study was to optimize fat extraction and validate a high resolution liquid chromatography-mass spectrometry (HRLC-MS) method to identify and quantify TAG in human milk. Intensity, repeatability, intermediate reproducibility, and recovery values were calculated and a large sample set of human milk analyzed. Each value for matrix effect of internal standard (IS) or standard solution (STD) in human milk during fat extraction ranged from 78 to 106% and from 56 to 107%, respectively, indicating no matrix effect was found except CCC. For linearity of the method, correlation coefficient (r^2) values were found to be ranged from 0.9991 to 0.9999. Recovery values were 88 and 116% for each STD at three different concentrations. Except c'c'c' and LLL, repeatability and intermediate reproducibility values within were 20% and 30%, respectively, indicating that the method was precise. The validated HRLC-MS method was applied to quantify TAG molecular species from human milk, as a quality control (QC). Among quantified 21 TAG, POL, PPO, PLS and OOP were predominant, ranging from 0.01 to 11.0 mg/L. TAG having short chain acyl such as c'c'c', MOB, and LLL were quantified with low amounts in QC (between 0.01 and 0.06 mg/L). Results from current study, validated fat extraction method followed by HRLC-MS efficiently identify and quantify TAG molecular species in human milk samples.

***Abbreviation of TAG**

CCC=Tricaproin, ooo=Triocatanoin, c'c'c'=Tricaprin, lll= Trilaurin, MMM= Trimyristin, LLL= Trilinolein, PPP= Tripalmitin, SSS= Tristearin, M = Myrisitic, B = Butyric, O = Oleic, P = Palmitic, L = Linoleic, S = Stearic, B' = Behenic.

INTRODUCTION

Human milk which is a complex mixture of nutrients and non-nutritional factors such as vitamins, minerals, protein and carbohydrates provide the best nutrition for infants, and is being designed to provide perfectly balanced nutrition to fulfill the necessity of the growing infant in the first 6 months after birth [1-6]. Humans including infants are exposed to heavy metals such as cadmium (Cd), mercury (Hg), arsenic (As), and lead (Pb) [7]. Vitamins can chelation of heavy metal as well as lessening of oxidative stress caused by heavy metal [7]. It can be divided into colostrum (1 – 5 days post-delivery), transitional milk (6 – 15 days post-delivery), and mature milk (over 15 days post-delivery) according to the stage of lactation [2, 4]. Colostrum contains the highest concentration of proteins, mostly immunoglobulins and lactoferrin. Fat content in colostrum is lower than that in mature milk [4]. Fat from human milk is the main source of energy for infants and about 40~50% of energy in human milk is provided by fatty acids and triacylglycerol (TAG) [1, 5, 6]

TAGs accounts for 98% of the fat in human milk [4, 8]. They are composed of three fatty acids esterified to a glycerol backbone with the glycerol carbons stereo-specifically numbered as *sn*-2 (center) and *sn*-1,3 (outer) [1, 8] (Figure 1). The most common fatty acids in the diet

have an even number of carbons between 8 and 22 carbons with up to 6 double bonds [8]. Sub-division of fatty acids into classes is usually based on carbon chain length, position, and orientation of any double bonds and this reflects both physicochemical and functional properties [8]. According to fatty acids, it has various health beneficial roles such as lower blood cholesterol, reducing main risk factor for the development of cardiovascular disease [9].

The molecular profile of TAGs is a key for understanding the hydrolysis and absorption of human milk fat. Previous study determined 170 fatty acid combinations for TAGs in human milk according to properties and random distribution [11]. In addition to fatty acid combinations, the stereospecific position of fatty acids influences on the biochemical and nutritional properties of TAGs [11]. Human milk is a remarkable example of stereo-specific positioning of fatty acids with TAGs structures that are both highly conserved and unusual in the enrichment of the saturated fatty acid at the *sn*-2 position, rather than at the *sn*-1,3 positions [1, 8]. This special fatty acid distribution in human milk TAGs enhanced the absorption of fat and calcium, influencing on the subsequent TAGs metabolism in infants [1, 8, 10]. Especially, usual positioning of saturated fatty acid such as palmitate in human milk triglycerides promotes absorption of mineral, saturated fatty acid, and calcium in term and preterm infants [1, 8, 10]. Due to recent advances in lipid technology, triglycerides can now be synthesized with palmitate in the *sn*-2 position [12].

Up to now many studies extracted fat from human milk by using Folch method that required lots of human milk sample as well as carried out single fat extraction without completing extraction, revealing low efficiency [3, 4, 10, 13]. Extraction method that can provide advantages of not only less use of human milk but also more precise extraction has to be further developed. For the determination of TAG in human milk, liquid chromatograph-mass spectrometry prior to separating by C18 column has been well established[3, 4, 13].

The aim of the present study was to optimize fat extraction and validate a high resolution liquid chromatography-mass spectrometry (HRLC-MS) method to identify and quantify TAG in human milk.

EXPERIMENTAL

Chemicals and standards

Ultra liquid chromatography (ULC) grade ammonium-formate, methanol and isopropanol were obtained from Chemie Brunschwing AG (Basel, Switzerland). LC grade sodium-formate, acetone and n-hexane were purchased from Sigma-Aldrich (Buchs, Switzerland). Mass spectrometry (MS) reference standard, d5-TG internal standard mixture I (catalogue number LM 6000) was obtained from Avanti Polar Lipids Inc (Alabaster, Alabama, US). TAG standards were purchased from Larodan/Chimie Brunschwig AG (Basel, Switzerland).

Human milk collection

Human milk was provided by Lee Bio (St. Louis, Missouri USA).

Standard preparation

Stock solutions of non-labeled TAG were solublized at 10 mg/mL in acetone and methanol (4:1, v:v).

Fat extraction from human milk

In order to find the optimal concentration of TAG to be injected into HRLC-MS, different volumes of human milk and distilled water were tested. The internal standard and standard solution were added before and after fat extraction for checking matrix effect. The

recovery was calculated by analyzing spiked sample in duplicate, on three different days, by the same analyst, and with the same equipment.

A 100 μL of human milk were diluted in 2 mL distilled water at 40 °C and sonicated for 10 min. An aliquot amount of diluted human milk solution (100 μL) was mixed with 2.9 mL distilled water and 250 μL internal standard solution. For fat extraction, 0.5 mL of 25% ammoniac, 2 mL EtOH, 5 mL diethyl ether, and 5 mL petroleum ether were subsequently added into human milk and vortex. Sample was centrifuged for 10 min at 2500 rpm. After centrifugation, the upper phase was transferred into another tube and solvent was evaporated under gentle stream of nitrogen. The second extraction was performed with same procedure described above. Add volume was differed from first fat extraction; 1 mL EtOH, 3 mL diethyl ether, and 3 mL of petroleum ether for fat extraction. Sample was centrifuged for 10 min at 2500 rpm. The third extraction was performed as second extraction without addition of EtOH. Sample was centrifuged for 10 min at 2500 rpm. After centrifugation, the upper phase was transferred and combined with previous one and solvent was evaporated under gentle stream of nitrogen. Finally, fat samples were solubilized in 1 mL acetone and methanol (4:1, v:v) and a aliquot amount (10 μL) was injected for TAG analysis by HRLC-MS.

Separation of TAGs by high resolution liquid chromatography (HRLC)

An Dionex ultimate 3000 (Thermofisher scientific, Bremen, Germany) equipped with a Aglient poroshell 120 EC-C18 (2.7 μm particle size, 2.1 × 250 mm) was used for separation of analytes of TAGs. The A solvent was n-hexane mixed with isopropanol (1:1, v:v). The solvent B was methanol added by 1 mM ammonium-formate and 2 μM sodium-formate.

The reason for adding of 1 mM ammonium-formate and 2 μM sodium-formate was to allow generation of predominant ammonium adducts and abundant sodium adducts from TAG [14]. The gradient of mobile phase was as followed: 100% B- 3 min hold at 0.6 mL/min, linear to

70% B and 30% A- 50 min hold at 0.6 mL/min; linear to 5% B and 95% A- 7 min hold at 0.4 mL/min; maintained for another 10 min; equilibrium to 5% B- hold 3 min at 0.6 mL/min; linear to 100% B-hold 7 min at 0.6 mL/min.

Identification of TAGs by mass spectrometry (MS)

An LTQ-Orbitrap XL hybrid mass spectrometer (Thermofisher scientific, Bremen, Germany) was used for identification of TAG regiosiomers. Electrospray ionization in a positive ion mode was employed to form ions at 300 °C nebulizer temperature and 4.5 kV capillary voltage. Both nebulizer and auxiliary gases were nitrogen at 40 and 20 units, respectively. Tube lens was adjusted to 110 V and accumulation time was 60 min. Other parameters were optimized during calibration with typical values. The Orbitrap was operated at 60,000 resolution in a mass to charge ratio (*m/z*) of 200-1500 range. Data dependent events were triggered according to an inclusion list containing the accurate masses of ammoniated TAG, applying parent mass width criteria of ± 5 ppm.

Analytical method validation

Method validation was performed to assess the linearity, limit of quantification (LOQ), accuracy, and precision.

Linearity. The linearity of the method was assessed by analyzing seven different concentrations of standard solutions of TAG covering ranges from 10 to 8000 µg/mL (Table 1). A calibration curve for each TAG was made. The calibration curves were plotted as peak areas of TAG (y) vs. concentrations of the standard solutions (x).

Limit of quantification (LOQ). The LOQ was defined as the lowest validated concentration. LOQ is the lowest quantity of a substance that can be distinguished from the absence of that substance (a blank value) within a stated confidence limit (generally 1%).

Accuracy. Recovery of added certified TAG standards was studied at three levels. Ten mg of certified TAG standards were mixed with 1 mL of n-hexane:chloroform:acetone (1:1:1, v:v:v). In order to obtain 1, 3, and 5 mg/L of TAG standard solution, these were further diluted to the required concentration with acetone:methanol (4:1, v:v). These certified reference material solutions were added to the sample.

Precision. The precision of the method was evaluated by calculating the repeatability (r) and the intermediate reproducibility (iR). Repeatability represents the variability of independent results obtained in the same laboratory, with the same analyst, on the same equipment, and in a short interval of time. Intermediate reproducibility represents the variability of independent results obtained in the same laboratory, on different days, with the same analyst, different calibrations, and same equipment. Repeatability and intermediate reproducibility were calculated by analyzing spiked samples in duplicate, on six different days, by the same analyst, with the same equipment, and with different solution preparations. All results for precision were evaluated by using Q-Stat software (Nestle, Lausanne, Switzerland).

Statistical analysis

Results were presented as mean ± standard deviation (SD). Each experiment was repeated at least three times to ensure results reliability. Statistical analysis of variance with *t* test was done to evaluate significant differences among samples at the significant level of 5% by using Graphpad Prism 3.0 software (Graphpad, San Diego, Ca, USA).

RESULTS AND DISCUSSION

Optimization of fat extraction method for HRLC-MS

Fat extraction method prior to analysis of HRLC-MS was optimized to quantify the most abundant TAG in human milk. According to previous study [14], reasonable intensity was around 3.5E8 for quantifying TAG in human milk. In order to adjust the best signal intensity of HRLC-MS for TAG in human milk, various dilution conditions ranged from 300 to 1200 times were examined. The values of peak intensity were between 3.01E8 to 8.31E8 (date were not shown). Among diverse condition, the intensity value of 600 times dilution condition was 3.41E8 in the current study. Result from the current study found that 600 times dilution for fat extraction having intensity signal of 3.41E8 was appeared to be optimal condition.

Table 1 and figure 2 show that matrix effect of internal standard (IS) or standard solution (STD) in human milk, and recovery of fat extraction method. The matrix effect of IS values ranged from 78 to 106%. In case of matrix effect of STD, its value ranged from 56 to 107%. Among TAG, c'c'c' having six carbons without double bond was found to have 78% of matrix effect of IS, indicating it interact with IS during fat extraction of human milk. c'c'c', 1-MOB, and MMM were revealed to have 67, 56, and 67% of matrix effect of STD, respectively. Interval of matrix effect below 80% was probably due to the low concentration of TAG in the sample. The results indicate that these TAG interact with STD during fat extraction of human milk. The recoveries were between 93 and 115% for TAG. The difference values in recoveries lower than 20% for recovery indicated that the recovery was not significantly different from 100%. Recovery values of all TAG in the range of 80 and 120%, revealing that the fat extraction method performed in the current study can provide accuracy.

Identification of TAGs in human milk

The retention time, corresponding molecular structure, and mass fragmentation pattern for each standard of TAG are listed in Table 2. Stock solution of TAG were analyzed by

HRLC-MS. All TAG was identified by its positive charged molecular ion $[M+NH_4]^+$. c'c'c' was eluted at 0.77 min of retention time and produced $[M+NH_4]^+$ at *m/z* 404.3. Mass fragmentation pattern was found at *m/z* 271.19, 271.19, and 271.19. OOO (Retention time = 1.16 min) was identified by its $[M+NH_4]^+$ at *m/z* 488.39 with profile of fragmentation pattern at *m/z* 327.25, 327.25, and 327.25. c'c'c' eluted at 2.30 min retention time produced $[M+NH_4]^+$ at *m/z* 572.49 and ionized *m/z* 388.32, 388.32, and 388.32. MOB (Retention time = 4.76 min) was identified by its $[M+NH_4]^+$ at *m/z* 654.57 with profile of fragmentation pattern at *m/z* 549.49, 355.28, and 409.33. lll eluted at 5.54 min retention time produced $[M+NH_4]^+$ at *m/z* 656.58 and ion were subsequently fragmented at *m/z* 439.38, 439.38, and 439.38. MMM (Retention time = 13.55 min) was identified by its $[M+NH_4]^+$ at *m/z* 740.68 with profile of fragmentation pattern at *m/z* 495.44, 495.44, and 495.44. LLL eluted at 17.22 min retention time produced $[M+NH_4]^+$ at *m/z* 896.77 and ionized at *m/z* 599.50, 599.50, and 599.50. LOL (Retention time = 20.06 min) was identified by its $[M+NH_4]^+$ at *m/z* 898.79 with profile of fragmentation pattern at *m/z* 601.52, 599.50, and 601.52. MOP eluted at 21.14 min retention time produced $[M+NH_4]^+$ at *m/z* 882.75 and ion were subsequently fragmented at *m/z* 577.2, 523.47, and 549.45. PPL (Retention time = 22.50 min) was identified by its $[M+NH_4]^+$ at *m/z* 848.77 with profile of fragmentation pattern at *m/z* 575.5, 575.5, and 551.50. POL was identified at 22.79 min retention time and produced $[M+NH_4]^+$ at *m/z* 874.79. Mass fragmentation pattern was found at *m/z* 601.52, 575.5, and 577.52. OOL (Retention time = 23.00 min) was identified by its $[M+NH_4]^+$ at *m/z* 900.80 with profile of fragmentation pattern at *m/z* 601.52, 601.52, and 603.53. LSL was identified at 23.00 min retention time produced $[M+NH_4]^+$ at *m/z* 900.80 and ionized at *m/z* 603.53, 599.50, and 603.53. PPP (Retention time = 24.56 min) was identified by its $[M+NH_4]^+$ at *m/z* 824.77 with profile of fragmentation pattern at *m/z* 551.50, 551.50, and 551.50. PPO eluted at 25.00 min retention time produced $[M+NH_4]^+$ at *m/z* 850.79 and ion were subsequently fragmented at *m/z* 577.52, 577.52, and

551.50. PLS (Retention time = 25.50 min) was identified by its $[M+NH_4]^+$ at *m/z* 876.8 with profile of fragmentation pattern at *m/z* 603.53, 579.53, and 575.50. OOP was identified at 25.53 min retention time and produced $[M+NH_4]^+$ at *m/z* 876.80, and fragmentation pattern at *m/z* 603.53, 577.52, and 577.52. OOO (Retention time = 25.74 min) was identified by its $[M+NH_4]^+$ at *m/z* 902.82 with profile of fragmentation pattern at *m/z* 603.53, 603.53, and 603.53. PPS eluted at 28.50 min retention time produced $[M+NH_4]^+$ at *m/z* 852.80 and mass fragmentation pattern at *m/z* 579.53, 579.53, and 551.50. PSO (Retention time = 28.90 min) was identified by its $[M+NH_4]^+$ at *m/z* 878.82 with profile of fragmentation pattern at *m/z* 605.55, 577.52, and 579.53. OOS was identified at 29.32 min retention time and produced $[M+NH_4]^+$ at *m/z* 904.83, and fragmentation pattern at *m/z* 603.53, 605.55, and 605.55. SPS (Retention time = 32.45 min) was identified by its $[M+NH_4]^+$ at *m/z* 880.83 with profile of fragmentation pattern at *m/z* 579.50, 607.57, and 579.53. MOB eluted at 32.83 min retention time and produced $[M+NH_4]^+$ at *m/z* 906.85 and ionized at *m/z* 661.61, 607.57, and 549.49. SSS (Retention time = 36.16 min) was identified by its $[M+NH_4]^+$ at *m/z* 908.86 with profile of fragmentation pattern at *m/z* 607.57, 607.57, and 607.57.

Validation of HRLC-MS method for TAGs

Limit of Quantification. The LOQ was considered as the lowest validated concentration which ranged from 1 to 10 µg/L for TAG.

Linearity. The calibration curve is essential for quantifying the different TAG and 24 calibration curves were made correspond with each TAG. The response of seven concentration levels ranging from 10 to 8000 µg/mL for each 24 TAG mixture were plotted for establishing the best regression model to quantify TAG. The calibration curves of standard mixture solution were plotted by measuring for three days (Table 3). The amount of each TAG was calculated according to the following formula as $y = a x^2 + b x + c$. If the value of

correlation coefficient (r^2) is above 0.9000, it is considered appropriate calibration curve for analysis by HRLC-MS [15, 16]. The correlation coefficient (r^2) values were found to be ranged from 0.9991 to 0.9999, indicating that each TAG calibration curves were suitable for quantification of TAG.

Recovery. The accuracy of the analytical method was evaluated by recovery from spiking certified TAG STDs into human milk samples. Recovery was calculated by quantification of spiking TAG in duplicate, on three different days, by the same analyst, and with the same equipment. Recovery values were compared with reference values. A *t-test* was performed to check if recovery was significantly different from 100%. Values of above 80% or below 120% for recovery were regarded as the method has accuracy [15, 16]. Three different concentrations of STD for TAG (1.0, 3.0, and 5.0 mg/L) were added into human milk samples. The recoveries ranged from 89 to 125% for 1 mg/L of STD, 86 to 116% for 3 mg/L of STD, and 88 to 160% for 5 mg/L of STD (Table 4). The recovery values of 1 mg/L of CCC, 1 mg/L of c'c'c', and 5 mg/L of CCC were above 20%, indicating its recovery was relatively low. These short chain TAG have until now failed to provide quantitative data because TAG including short acyl chains had not only less specific of fragmentation but also low concentration in human milk [17]. Except short chain TAG including CCC and c'c'c', recovery values of all TAG were within interval (80-120%), revealing that it was not significantly different from 100%.

Repeatability (r) and intermediate Reproducibility (iR). The precision of method was evaluated by calculating the simple repeatability and the intermediate reproducibility (Table 5). Standard deviation of repeatability (SD(r)) means measures the amount of variation between samples from the average, standard deviation of intermediate reproducibility (SD(iR)) indicates measurement of the amount of variation between samples and different days from the average, coefficient variation of repeatability (CV(r)) shows normalized measure of

dispersion of a frequency distribution between samples, and coefficient variation of intermediate of the reproducibility (CV(iR)) means normalized measure of dispersion of a frequency distribution between samples and different days. Values of below 20% and 30% for CV(r) (%) and CV(iR) (%), respectively, were regarded as method has precision for analyzing TAG by using HRLC-MS [15]. CV(r) (%) values ranged from 0.80 to 27.04%. Except c'c'c' having 27.04% of CV(r), the rest of TAG showed CV(r) values lower than 20%, indicating that method has precision between each samples in one day. CV(iR) values ranged from 3.30 to 62.78%. All CV(iR) values of TAG were below 30% except c'c'c' and LLL. Therefore, it could be considered that method has a precision in duplicate on different days. CV(r) and CV(iR) values for short chain TAG such as c'c'c' (C16:0) and LLL (C18:2) were shown to have less precision, implying due to the low concentration of TAG in the human milk.

Quantification of TAGs from human milk by validated method of HRLC-MS

Many studies have been reported that human milk is a critical factor for infant and the amount of TAG changes depending on different lactation stages because different nutritional requirements of infant to support their specific growth and development patterns [5, 18-20]. TAG in human milk are source of energy as well as of essential fatty acids [20]. For example, maternal diet is the only source of linoleic acid that cannot be synthesized in the body [18]. TAG provide the most caloric value among all nutrients [18]. In fact, energy content and total lipid content have been observed to have similar pattern over time [18].

Thus, the validated HRLC-MS method was applied to quantify TAG molecular species from human milk, as a quality control (QC). The calibration curves were made for quantification TAG with ranged from 10 to 40000 µg/mL. The quantification TAG from QC are shown in Table 6. The absolute concentration of TAG in QC ranged from 0.01 to 11.0 mg/L. Except CCC, ooo, and SSS, 21 TAG were detected and quantified for QC. In

particularly, POL (16:0-18:1-18:2), PPO (16:0-16:0-18:1), PLS (16:0-18:2-18:1), and OOP (18:1-18:1-16:0) were the most abundant in QC. On the other hand, TAG having short chain acyl such as c'c'c', MOB, and lll were quantified with low amounts. Relative error (RE) values ranged from 1 to 130%. RE value within 20% were regarded as the method has repeatability [21]. As shown in table 6, result from quantification of QC had repeatability except c'c'c' and lll. In case of c'c'c' and lll, these TAG exist low amounts in QC. For this reason, c'c'c' and lll were quantified imprecisely and values of RE were above 20%.

Similar to previous studies [3, 4, 10, 13], the current study for identified and quantified major TAG in human milk such as POL, MOP, PPO, and PPP were quantified. Optimized fat extraction and validated HRLC-MS even quantified PLS, PPL, OOP, and PSO which were not able to detect by previous method [3, 4, 10, 13].

Fatty acids of TAG in human milk, palmitic acid and linoleic acid accounts for 20-25% and 4-26% of total milk fatty acids, respectively [22, 23]. In fact, TAG belong to linoleic acid (C18:2) and palmitic acid (C16:0) were also the most abundant in the current study.

CONCLUSIONS

In this study, a fat extraction method from human milk was optimized and HRLC-MS method was validated for analysis TAG. Fat from human milk was extracted using various organic solvent such as ammoniac, EtOH, diethyl ether, and petroleum ether with smaller amount than Folch method was then separated on C18 column followed by detecting HRLC-MS and quantified on orbitrap hybrid mass spectrometer. The matrix effect of IS and STD values were ranged from 78 to 106% and 56 to 107%, respectively. Twenty four TAGs were identified and correlation coefficient (r^2) values were found to be ranged from 0.9991 to 0.9999 for linearity, recoveries ranged from 88 to 160% for STD of three concentration levels,

and CV(r) and CV(iR) values ranged from 0.80 to 27.04 % and from 3.30 to 62.78%, respectively.

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LEGENDS OF FIFURE AND TABLES

Figure 1. The structure of triacylglycerol (TAG) molecular species.

Figure 2. Chromatogram of peak intensity for optimization of fat extraction method prior to HRLC-MS analysis for TAG molecular species depends on condition of dilution.

Table 1. The effect of matrix among human milk, internal standard (IS), and standard solution (STD). The recovery of each TAG molecular species among human milk, internal standard (IS), and standard solution (STD). * N = the matrix effect was 100%, the range of values interval 80-120% for matrix effect were regarded as the method hasn't matrix effect. Y = the recovery was 100%, the range of values interval 80-120% for recovery were regarded as the method has accuracy. * M = Myrisitin, B = Butyrin, O = Olein, P = Palmitin, L = Linolein, S = Stearin, B' = Behenin

Table 2. Mass fragment pathway of TAG standards at each retention time by using HRLC-MS. * M = Myrisitin, B = Butyrin, O = Olein, P = Palmitin, L = Linolein, S = Stearin, B' = Behenin

Table 3. The calibration curves from various concentrations of standard mixture solution injected for 3 days. * M = Myrisitin, B = Butyrin, O = Olein, P = Palmitin, L = Linolein, S = Stearin, B' = Behenin

Table 4. Median and recovery of 23 TAG spiked in human milk. *Y = yes, N = no, *Y = the recovery was 100%, the range of values interval 80-120% for recovery were regarded as the method has accuracy. * M = Myrisitin, B = Butyrin, O = Olein, P = Palmitin, L = Linolein, S = Stearin, B' = Behenin

Table 5. Standard deviation of repeatability (SD(r)), relative standard deviation of repeatability (CV(r)), standard deviation of intermediate reproducibility (SD(iR)), and relative standard deviation of intermediate reproducibility (CV(iR)) of TAG in human milk. *Standard deviation of repeatability (SD(r)) means measures the amount of variation between samples from the average, Standard deviation of intermediate reproducibility (SD(iR)) indicates measurement of the amount of variation between samples and different days from the average, coefficient variation of repeatability (CV(r)) shows normalized measure of dispersion of a frequency distribution between samples, and coefficient variation of intermediate of the reproducibility (CV(iR)) means normalized measure of dispersion of a frequency distribution between samples and different days. * M = Myrisitin, B = Butyrin, O = Olein, P = Palmitin, L = Linolein, S = Stearin, B' = Behenin

Table 6. Quantification of TAG in human milk collected for quality control (QC) of HRLC-MS. * M = Myrisitin, B = Butyrin, O = Olein, P = Palmitin, L = Linolein, S = Stearin, B' = Behenin

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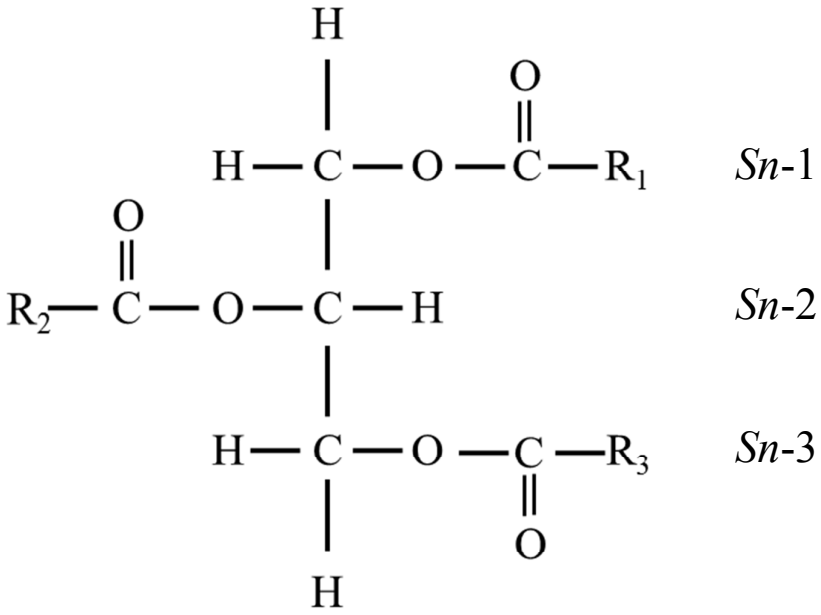

Figure 1. The structure of triacylglycerol (TAG).

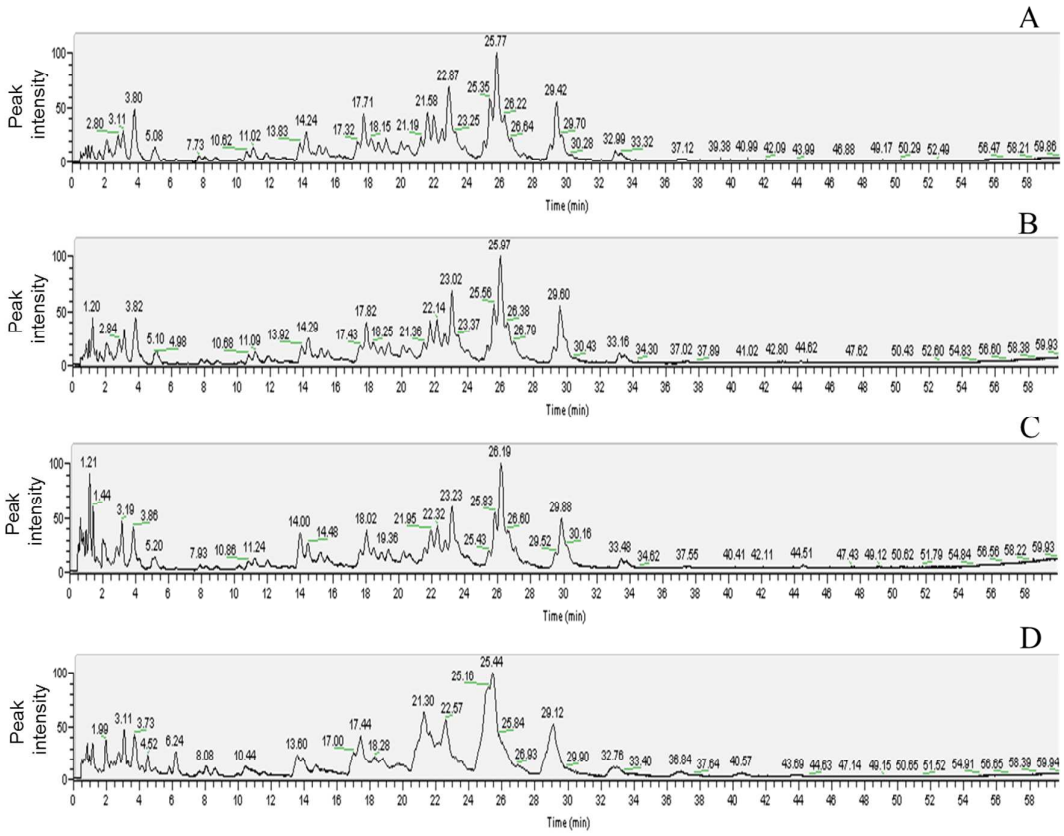

Figure 2. Mass spectrum of TAGs in human milk. The peak intensities of TAG are $8.31 \times e^8$ (A, 300 times dilution), $4.98 \times e^8$ (B, 400 times dilution), $3.41 \times e^8$ (C, 600 times dilution), and $3.01 \times e^8$ (D, 1200 times dilution).

Table 1. The effect of matrix among human milk, internal standard (IS), and standard solution (STD). The recovery of each TAG among human milk, internal standard (IS), and standard solution (STD)

Retention time (min)	TAG	Composition	Matrix effect of IS (%)	Matrix effect of STD (%)	Recovery (%)
0.77	CCC	6:0-6:0-6:0	Y (78)	N (105)	Y (93)
1.16	ooo	8:0-8:0-8:0	N (87)	N (105)	Y (102)
2.30	c'c'c	10:0-10:0-10:0	N (103)	Y (67)	Y (108)
4.76	MOB	14:0-18:1-4:0	N (102)	Y (56)	Y (107)
5.54	lll	12:0-12:0-12:0	N (106)	N (88)	Y (104)
13.55	MMM	14:0-14:0-14:0	N (106)	Y (67)	Y (110)
17.22	LLL	18:2-18:2-18:2	N (104)	N (97)	Y (115)
20.06	LOL	18:2-18:1-18:2	N (103)	N (89)	Y (104)
21.14	MOP	14:0-18:1-16:0	N (106)	N (80)	Y (106)
22.50	PPL	16:0-16:0-18:2	N (103)	N (90)	Y (105)
22.79	POL	18:1-18:1-18:2	N (105)	N (94)	Y (102)
23.00	OOL	18:2-18:0-18:2	N (103)	N (98)	Y (102)
23.00	LSL	16:0-16:0-16:0	N (100)	N (98)	Y (102)
24.56	PPP	16:0-16:0-18:1	N (103)	N (92)	Y (102)
25.00	PPO	16:0-18:2-18:0	N (102)	N (92)	Y (100)
25.50	PLS	18:1-18:1-16:0	N (104)	N (94)	Y (99)

25.53	OOP	18:1-18:1-18:1	N (105)	N (94)	Y (100)
25.74	OOO	16:0-16:0-18:0	N (104)	N (99)	Y (93)
28.50	PPS	16:0-16:0-18:0	N (100)	N (100)	Y (101)
28.90	PSO	16:0-18:0-18:1	N (102)	N (105)	Y (98)
29.32	OOS	18:1-18:1-18:0	N (100)	N (106)	Y (102)
32.45	SSP	18:0-18:0-16:0	N (100)	N (107)	Y (101)
32.83	MOB'	14:0-18:1-22:0	N (100)	N (106)	Y (102)
36.16	SSS	18:0-18:0-18:0	N (102)	N (99)	Y (104)

* N = the matrix effect was 100%, the range of values interval 80-120% for matrix effect were regarded as the method hasn't matrix effect. Y = the recovery was 100%, the range of values interval 80-120% for recovery were regarded as the method has accuracy

* CCC=Tricaproin, ooo=Triocatanoin, c'c'c'=Tricaprin, lll= Trilaurin, MMM= Trimyristin, LLL= Trilinolein, PPP= Tripalmitin, SSS= Tristearin, M = Myrisitic, B = Butyric, O = Oleic, P = Palmitic, L = Linoleic, S = Stearic, B' = Behenic.

Table 2. Mass fragment pathway of TAG standards at each retention time by using HRLC-MS

Retention time (min)	TAG*	Composition	Concentration	Mass to charge ratio (*m/z*)			
				$[M+NH_4]^+$	Fragment 1	Fragment 2	Fragment 3
0.77	CCC	06:0-6:0-6:0	10 mg/mL	404.3	271.19	271.19	271.19
1.16	ccc	08:0-8:0-8:0	10 mg/mL	488.39	327.25	327.25	327.25
2.30	c'c'c	10:0-10:0-10:0	10 mg/mL	572.49	383.32	383.32	383.32
4.76	MOB	14:0-18:1-4:0	10 mg/mL	654.57	549.49	355.28	409.33
5.54	lll	12:0-12:0-12:0	10 mg/mL	656.58	439.38	439.38	439.38
13.55	MMM	14:0-14:0-14:0	10 mg/mL	740.68	495.44	495.44	495.44
17.22	LLL	18:2-18:2-18:2	10 mg/mL	896.77	599.5	599.5	599.5
20.06	LOL	18:2-18:1-18:2	10 mg/mL	898.79	601.52	599.5	601.52
21.14	MOP	14:0-18:1-16:0	10 mg/mL	882.75	577.52	523.47	549.49
22.5	PPL	16:0-16:0-18:2	10 mg/mL	848.77	575.5	575.5	551.5
22.79	POL	16:0-18:1tr-18:2	10 mg/mL	874.79	601.52	575.5	577.52
23.00	OOL	18:1-18:1-18:2	10 mg/mL	900.8	601.52	601.52	603.53
23.00	LSL	18:2-18:0-18:2	10 mg/mL	900.8	603.53	599.5	603.53
24.56	PPP	16:0-16:0-16:0	10 mg/mL	824.77	551.5	551.5	551.5
25.00	PPO	16:0-16:0-18:1	10 mg/mL	850.79	577.52	577.52	551.5
25.50	PLS	16:0-18:2-18:0	10 mg/mL	876.8	603.53	579.53	575.5
25.53	OOP	18:1-18:1-16:0	10 mg/mL	876.8	603.53	577.52	577.52
25.74	OOO	18:1-18:1-18:1	10 mg/mL	902.82	603.53	603.53	603.53
28.50	PPS	16:0-16:0-18:0	10 mg/mL	852.8	579.53	579.53	551.5
28.90	PSO	16:0-18:0-18:1	10 mg/mL	878.82	605.55	577.52	579.53
29.32	OOS	18:1-18:1-18:0	10 mg/mL	904.83	603.53	605.55	605.55
32.45	SSP	18:0-16:0-18:0	10 mg/mL	880.83	579.5	607.57	579.53
32.83	MOB'	14:0-18:1-22:0	10 mg/mL	906.85	661.61	607.57	549.49
36.16	SSS	18:0-18:0-18:0	10 mg/mL	908.86	607.57	607.57	607.57

* CCC=Tricaproin, ooo=Triocatanoin, c'c'c'=Tricaprin, lll= Trilaurin, MMM= Trimyristin, LLL= Trilinolein, PPP= Tripalmitin, SSS= Tristearin, M = Myrisitic, B = Butyric, O = Oleic, P = Palmitic, L = Linoleic, S = Stearic, B' = Behenic.

Table 3. The calibration curves from various concentrations of standard mixture solution injected for 3 days.

Retention time (min)	TAG*	Composition	Correlation coefficient (R^2)		
			Day 1	Day 2	Day 3
0.77	CCC	6:0-6:0-6:0	0.9999	0.9997	0.9999
1.16	ccc	8:0-8:0-8:0	0.9997	0.9996	0.9997
2.30	c'c'c	10:0-10:0-10:0	0.9999	0.9998	0.9999
4.76	MOB	14:0-18:1-4:0	0.9995	0.9994	0.9996
5.54	lll	12:0-12:0-12:0	0.9993	0.9994	0.9993
13.55	MMM	14:0-14:0-14:0	0.9994	0.9990	0.9994
17.22	LLL	18:2-18:2-18:2	0.9995	0.9986	0.9995
20.06	LOL	18:2-18:1-18:2	0.9996	0.9986	0.99968
21.14	MOP	14:0-18:1-16:0	0.9994	0.9987	0.9994
22.50	PPL	16:0-16:0-18:2	0.9995	0.9993	0.9995
22.79	POL	18:1-18:1-18:2	0.9997	0.9993	0.9997
23.00	OOL	18:2-18:0-18:2	0.9998	0.9991	0.9998
23.00	LSL	16:0-16:0-16:0	0.9998	0.9991	0.9998
24.56	PPP	16:0-16:0-18:1	0.9991	0.9985	0.9991
25.00	PPO	16:0-18:2-18:0	0.9997	0.9995	0.9997
25.50	PLS	18:1-18:1-16:0	0.9997	0.9992	0.9997
25.53	OOP	18:1-18:1-18:1	0.9997	0.9992	0.9997

25.74	OOO	16:0-16:0-18:0	0.9995	0.9989	0.9995
28.50	PPS	16:0-16:0-18:0	0.9992	0.9992	0.9992
28.90	PSO	16:0-18:0-18:1	0.9994	0.9992	0.9994
29.32	OOS	18:1-18:1-18:0	0.9993	0.9994	0.9993
32.45	SSP	18:0-18:0-16:0	0.9997	0.9995	0.9996
32.83	MOB'	14:0-18:1-22:0	0.9994	0.9991	0.9994
36.16	SSS	18:0-18:0-18:0	0.9996	0.9995	0.9996

* CCC=Tricaproin, ooo=Triocatanoin, c'c'c'=Tricaprin, lll= Trilaurin, MMM= Trimyristin, LLL= Trilinolein, PPP= Tripalmitin, SSS= Tristearin, M = Myrisitic, B = Butyric, O = Oleic, P = Palmitic, L = Linoleic, S = Stearic, B' = Behenic.

Table 4. Median and recovery of 23 TAG regioisomers spiked in human milk.

Retention time (min)	TAG*	Composition	Median (mg/L)	Recovery (%)	Median (mg/L)	Recovery (%)	Median (mg/L)	Recovery (%)
0.77	CCC	6:0-6:0-6:0	1.1	N** (125)	2.5	Y (106)	4.1	N (126)
1.16	ooo	8:0-8:0-8:0	1.2	Y (100)	3.2	Y (98)	5.0	Y (95)
2.30	c'c'c	10:0-10:0-10:0	1.0	N (122)	3.1	Y (97)	4.9	Y (102)
4.76	MOB	14:0-18:1-4:0	1.0	Y (99)	3.0	Y (94)	4.8	Y (99)
5.54	lll	12:0-12:0-12:0	1.0	Y (99)	3.2	Y (91)	5.1	Y (98)
13.55	MMM	14:0-14:0-14:0	1.0	Y (113)	3.1	Y (93)	5.0	Y (106)
17.22	LLL	18:2-18:2-18:2	1.1	Y (112)	3.1	Y (107)	5.1	Y (107)
20.06	LOL	18:2-18:1-18:2	1.0	Y (96)	3.1	Y (95)	5.0	Y (103)
21.14	MOP	14:0-18:1-16:0	1.0	Y (99)	3.1	Y (93)	5.1	Y (98)
22.50	PPL	16:0-16:0-18:2	1.1	Y (116)	3.2	Y (108)	4.9	Y (95)
22.79	POL	16:0-18:1-18:2	1.0	Y (89)	3.0	Y (93)	5.1	Y (104)
23.00	OOL	18:1-18:1-18:2	0.9	Y (100)	2.9	Y (107)	5.0	Y (111)
23.00	LSL	18:2-18:0-18:2	0.9	Y (100)	2.9	Y (107)	5.0	Y (111)
24.56	PPP	16:0-16:0-16:0	1.0	Y (100)	3.0	Y (92)	4.9	Y (97)
25.00	PPO	16:0-16:0-18:1	1.0	Y (94)	3.2	Y (86)	5.2	Y (88)
25.50	PLS	16:0-18:2-18:0	1.2	Y (96)	3.8	Y (90)	6.2	Y (98)
25.53	OOP	18:1-18:1-16:0	1.0	Y (91)	3.0	Y (95)	5.1	Y (104)
25.74	OOO	18:1-18:1-18:1	1.0	Y (110)	3.1	Y (93)	5.1	Y (105)
28.50	PPS	16:0-16:0-18:0	1.0	Y (103)	3.0	Y (102)	4.9	Y (103)
28.90	PSO	16:0-18:0-18:1	1.0	Y (94)	3.0	Y (102)	5.1	Y (101)
29.32	OOS	18:1-18:1-18:0	1.0	Y (96)	3.1	Y (99)	5.1	Y (100)
32.45	SSP	18:0-18:0-16:0	1.0	Y (105)	3.1	Y (100)	5.0	Y (104)
32.83	MOB'	14:0-18:1-22:0	1.0	Y (100)	3.0	Y (98)	5.0	Y (98)

* CCC=Tricaproin, ooo=Triocatanoin, c'c'c'=Tricaprin, lll= Trilaurin, MMM= Trimyristin, LLL= Trilinolein, PPP= Tripalmitin, SSS= Tristearin, M = Myrisitic, B = Butyric, O = Oleic, P = Palmitic, L = Linoleic, S = Stearic, B' = Behenic.

**Y = yes, the recovery was 100%, the range of values interval 80-120% for recovery were regarded as the method has accuracy; N = no

Table 5. Standard deviation of repeatability (SD(r)), relative standard deviation of repeatability (CV(r)), standard deviation of intermediate reproducibility (SD(iR)), and relative standard deviation of intermediate reproducibility (CV(iR)) of TAG regioisomers in human milk.

Retention time (min)	TAG*	Composition	SD (r)	CV (r) (%)	SD (iR)	CV (iR) (%)
2.30	CCC	10:0-10:0-10:0	0.002	27.04	0.003	48.83
13.55	MMM	14:0-14:0-14:0	0.050	8.10	0.078	12.60
17.22	LLL	18:2-18:2-18:2	0.004	7.83	0.028	62.78
20.06	LOL	18:2-18:1-18:2	0.004	6.07	0.020	28.92
21.14	MOP	14:0-18:1-16:0	0.120	2.10	0.360	6.20
22.50	PPL	16:0-16:0-18:2	0.073	3.50	0.170	8.20
22.79	POL	16:0-18:1-18:2	0.086	2.00	0.333	7.60
23.00	OOL	18:1-18:1-18:2	0.017	4.50	0.060	15.40
23.00	LSL	18:2-18:0-18:2	0.016	4.20	0.059	15.50
24.56	PPP	16:0-16:0-16:0	0.053	2.60	0.147	7.10
25.00	PPO	16:0-16:0-18:1	0.079	0.80	0.332	3.40
25.50	PLS	16:0-18:2-18:0	0.087	1.90	0.408	9.10
25.53	OOP	18:1-18:1-16:0	0.087	1.90	0.408	9.10
25.74	OOO	18:1-18:1-18:1	0.039	2.20	0.184	10.60
28.50	PPS	16:0-16:0-18:0	0.025	1.10	0.050	4.20

28.90	PSO	16:0-18:0-18:1	0.120	2.60	0.200	4.30
29.32	OOS	18:1-18:1-18:0	0.020	1.10	0.057	3.30
32.45	SSP	18:0-18:0-16:0	0.016	4.80	0.026	7.80
32.83	MOB'	14:0-18:1-22:0	0.008	2.03	0.023	5.93

*Standard deviation of repeatability (SD(r)) means measures the amount of variation between samples from the average, Standard deviation of intermediate reproducibility (SD(iR)) indicates measurement of the amount of variation between samples and different days from the average, coefficient variation of repeatability (CV(r)) shows normalized measure of dispersion of a frequency distribution between samples, and coefficient variation of intermediate of the reproducibility (CV(iR)) means normalized measure of dispersion of a frequency distribution between samples and different days

* CCC=Tricaproin, ooo=Triocatanoin, c'c'c'=Tricaprin, lll= Trilaurin, MMM= Trimyristin, LLL= Trilinolein, PPP= Tripalmitin, SSS= Tristearin, M = Myrisitic, B = Butyric, O = Oleic, P = Palmitic, L = Linoleic, S = Stearic, B' = Behenic.

Table 6. Quantification of TAG in human milk collected for quality control (QC) of HRLC-MS.

Retention time (min)	Analyte		Concentration (mg/L)	Relative error (%)
	TAG*	Composition	Mean± SD	
2.30	CCC	10:0-10:0-10:0	0.02±0.03	130%
4.76	MOB	14:0-18:1-4:0	0.01±0.00	1%
5.54	lll	12:0-12:0-12:0	0.13±0.13	98%
13.55	MMM	14:0-14:0-14:0	1.18±0.12	10%
17.22	LLL	18:2-18:2-18:2	0.06±0.01	10%
20.06	LOL	18:2-18:1-18:2	1.20±0.06	5%
21.14	MOP	14:0-18:1-16:0	3.81±0.22	6%
22.50	PPL	16:0-16:0-18:2	4.40±0.21	5%
22.79	POL	16:0-18:1-18:2	10.14±0.62	6%
23.00	OOL	18:1-18:1-18:2	2.41±0.08	3%
23.00	LSL	18:2-18:0-18:2	2.42±0.08	3%
24.56	PPP	16:0-16:0-16:0	1.58±0.09	5%
25.00	PPO	16:0-16:0-18:1	11.00±0.57	5%
25.50	PLS	16:0-18:2-18:0	9.87±0.65	7%
25.53	OOP	18:1-18:1-16:0	9.87±0.64	7%
25.74	OOO	18:1-18:1-18:1	1.97±0.12	6%
28.50	PPS	16:0-16:0-18:0	1.11±0.04	3%
28.90	PSO	16:0-18:0-18:1	6.66±0.19	3%
29.32	OOS	18:1-18:1-18:0	2.66±0.09	3%
32.45	SSP	18:0-18:0-16:0	0.86±0.05	6%
32.83	MOB'	14:0-18:1-22:0	0.56±0.03	6%

* CCC=Tricaproin, ooo=Triocatanoin, c'c'c'=Tricaprin, lll= Trilaurin, MMM= Trimyristin, LLL= Trilinolein, PPP= Tripalmitin, SSS= Tristearin, M = Myrisitic, B = Butyric, O = Oleic, P = Palmitic, L = Linoleic, S = Stearic, B' = Behenic.