JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Model for Human Milk Fat Substitute Evaluation Based on Triacylglycerol Composition Profile

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ABSTRACT: Being the dominant components in human milk fat (HMF), triacylglycerol (TAG) composition might be the best approximation index to represent the composing characteristics of HMF. In this study, TAG composition of HMF from different lactation stages was analyzed by RP-HPLC-APCI-MS, and the establishment of a model for the precise evaluation of human milk fat substitutes (HMFSs) based on TAG composition was indirectly realized by employment of fatty acid composition and distribution and polyunsaturated fatty acid (PUFA) and TAG compositions. The model was verified by the selected fats and oils with specific chemical compositions, and the results revealed the degrees of similarity of these fats and oils in different evaluation aspects reflected their differences in corresponding chemical composition with HMF. The newly established evaluation model with TAG composition as a comparison base could provide a more accurate method to evaluate HMFSs and might have some inspirations for HMFS production in the future.

KEYWORDS: *human milk fat substitutes, evaluation model, triacylglycerol composition, RP-HPLC-APCI-MS, lactation stages, human milk fat*

INTRODUCTION

Human milk is considered as the best food for newborn infants, containing 3−5% fat, 0.8−0.9% protein, 6.9−7.2% carbohydrate, and 0.2% mineral, vitamin, and other physiological substances.[1] Human milk fat (HMF) is composed of triacylglyerols (TAGs) (>98%), phospholipids (0.4−1.0%), cholesterols (12.0−16.6 mg/dL), and many others,[2−4] and the contents of these lipids vary with such factors as lactation stage, dietary habit, season, genetics, and individual conditions.[5,6] The TAGs in HMF have specific composition and structure. From the view of fatty acid composition of TAGs, the major fatty acids (>1%) reported are capric acid (1−2%), lauric acid (3−7%), myristic acid (4−9%), palmitic acid (20−30%), palmitoleic acid (1−3%), stearic acid (5−9%), oleic acid (25−35%), and linoleic acid (10−20%), and the long-chain polyunsaturated fatty acids (LC-PUFA) reported were docosahexaenoic acid (DHA, ω-3), arachidonic acid (AA, ω-6), docosapentaenoic acid (DPA, ω-3), eicosapentaenoic acid (EPA, ω-3), etc.[7−12] A balanced ratio between ω-3 and ω-6 fatty acids and suitable contents of LC-PUFA in HMF are important to ensure the healthy growth of infants. From the view of fatty acid distribution of TAGs in HMF, saturated fatty acids, especially palmitic acid (>60%), are mainly located at the sn-2 position, and unsaturated fatty acids are esterified at the sn-1,3 positions of the glycerol backbone. This special fatty acid distribution of TAGs in HMF enhances the absorption of fat and calcium and has an influence on the subsequent TAG metabolism in infants.[13−16]

The fat used in infant formulas should be based on the characteristics of HMF. On the basis of fatty acid composition and distribution of HMF, many studies have reported the preparation of human milk fat substitutes (HMFSs) by using different starting materials, reactors, and enzymes,[17−21] and a commercial product termed Betapol from Loders Croklaan was also reported.[22] These HMFSs derived from different processes have different chemical compositions. Their qualities or degrees of similarity to HMF are seldom reported. Wang et al.[23] established a model for the evaluation of HMFSs based on the fatty acid composition and distribution of HMF, and the degrees of similarity could be digitized by this model from the fatty acid profiles. However, due to the randomization of fatty acid distribution in different TAG molecules, even though two types of HMFSs have similar fatty acid composition and distribution, their TAG compositions could be different. HMF is ingested as TAGs by infants, and newborn infants, especially preterm infants, have a reduced capacity to hydrolyze dietary lipids because of their low levels of pancreatic lipase and bile salts, and thus whether or not TAG species have an influence on the digestion and metabolism of fat in infants remains unknown. However, some studies did report that different types of fat might regulate the pancreatic lipase gene expression and affect pancreatic lipase activity.[24,25] On the basis of the principle that the greatest imitation of chemical composition of HMF is the golden rule for HMFS preparation, the TAG composition might be the ultimate index for HMFS evaluation. Due to the large amount of TAG isomers existing in HMF, complete separation of TAG species in HMF is difficult. According to the characteristics of the current analytical techniques and TAG intramolecular structure, indirect methods can be applied to describe TAG composition and to evaluate HMFSs. Currently, the separation of TAG species is

Received: September 22, 2012
Revised: December 5, 2012
Accepted: December 10, 2012
Published: December 10, 2012

mainly performed on C18 or Ag^+ columns.[26−29] The elution order of TAGs on the C18 column is in accordance with the order of their equivalent carbon numbers (ECN).[30] Therefore, TAG species with the same ECN, especially TAG isomers, cannot be easily separated, and the data from the C18 column cannot be used for HMFS evaluation individually. However, if the data from TAG composition and the data from fatty acid profiles of HMF are combined to evaluate HMFSs, the accuracy of the evaluation results can be increased. Meanwhile, HMF contains some PUFA, which account for a small proportion of HMF (<1%, individually) but of great importance to the development of infants. These PUFA cannot be reflected when major fatty acid profiles and TAG composition are used as evaluation indices, and thus evaluation for PUFA should be taken into account.

Therefore, on the basis of the above-mentioned analysis, the objective of this study was to establish a model for the precise evaluation of the degrees of similarity of HMFSs to HMF from the viewpoint of TAG composition by employment of the data from fatty acid composition and distribution and PUFA and TAG composition of HMF at different lactation stages, and the validity of the established model was verified by the selected fats and oils with specific chemical compositions.

MATERIALS AND METHODS

Samples and Reagents. Forty-five human milk samples from different lactation stages (colostrum, 1−5 days; transitional milk, 6−15 days; mature milk, after 16 days) were individually donated by apparently healthy Danish mothers in Aarhus University Hospital, who had been well informed before participating in the project. The samples were stored at −20 °C within 24 h after collection. TAG standards including 1,3-dioleoyl-2-palmitoylglycerol (OPO), 1,2-dioleoyl-3-palmitoylglycerol (OOP), 1,2-dipalmitoyl-3-oleoylglycerol (PPO), 1,3-dipalmitoyl-2-oleoylglycerol (POP), triolein (OOO), 1,2-dioleoyl-3-stearolglycerol (OOS), 1,3-stearol-2-oleoylglycerol (SOS), 1,3-stearol-2-oleoylglycerol (SSO), tripalmitin (PPP), and 1-palmitoyl-2-oleoyl-3-stearolglycerol (POS) were purchased from Larodan Fine Chemicals AB (Malmö, Sweden). Methanol, glacial acetic acid, hexane, acetonitrile, and isopropanol were all of high-performance liquid chromatograph (HPLC) purity. Hydrochloric acid and diethyl ether were of analytical grade.

Extraction of Total Lipids. Total lipids were extracted from the freeze-dried samples by homogenization with chloroform/methanol (2:1, vol/vol) as described by Folch et al.[31] The extract was shaken and equilibrated with one-fourth volume of saline solution (NaCl 0.86%, w/w). The solvent phase was filtered and evaporated under vacuum, and the obtained total lipids were stored at −20 °C for further analyses.

Separation and Identification of TAG Species. TAG species were analyzed by using a reverse-phase high-performance liquid chromatograph (RP-HPLC), equipped with an evaporative light-scattering detector (ELSD). The ELSD was set at 55 °C at a nitrogen nebulizer gas flow rate of 1.8 mL/min and a gain of 1. The separation was carried out on a Lichrospher C18 column (5 μm, 4.6 × 250 mm; Hanbon Science and Technology Co., Ltd., Jiangsu, China) and eluted with a binary gradient of acetonitrile (A) and isopropanol (B) at a flow rate of 0.8 mL/min with a linear gradient of solvent A from 70 to 60% in the first 30 min, then to 55% in 40 min, staying at 55% for 20 min, and then to 70% in 5 min. The sample concentration was 20 mg/mL, and the injection volume was 10 μL.

The TAG identification was carried out on a HPLC−atmospheric pressure chemical ionization mass spectrometry (HPLC-APCI-MS). The MS conditions were as follows: APCI source block and probe temperatures, 100 and 400 °C, respectively; MS multiplier voltage, 700 V; measurement range, m/z 250−1200.

Separation of TAG Isomers by Ag-HPLC. TAG species were first separated by RP-HPLC as aforementioned, and fractions corresponding to POO and PPO were manually collected. The collected fractions were concentrated by nitrogen and redissolved with hexane to 50 μL. The TAG isomers were then separated on a ChromSpher 5 Lipids analytic silver-impregnated column (5 μm, 4.6 mm × 250 mm; Varian Inc., Middelburg, The Netherlands) using an isocratic elution of 0.5% acetonitrile in hexane at a flow rate of 1 mL/min. The injection volume was 10 μL.

Fatty Acid Composition Analysis. Fatty acid methyl esters (FAMEs) were prepared according to AOCS method Ce-1b 89 (2007) and subsequently analyzed on a gas chromatograph (GC) (Thermo-Fisher Scientific, Waltham, MA, USA) equipped with an autosampler, a flame ionization detector, and an ionic liquid capillary column (Supelco SLB-IL 100, 60 m × 0.25 mm ×0.2 μm, Sigma-Aldrich, St. Louis, MO, USA). Helium was used as the carrier gas at a flow rate of 1 mL/min. The column oven temperature was kept at 170 °C, and the running time for each sample was 60 min. The injection port and detector temperatures were both set at 250 °C. The FAMEs were identified by comparing retention time with the standards, and the relative contents expressed as mole percent were then calculated.

Sn-2 Fatty Acid Composition Analysis. Hydrolysis of TAGs to sn-2 monoacylglycerols (MAGs) was carried out according to the method detailed by Luddy et al.[32] The hydrolytic products were separated on silica gel G TLC plates with a developing solvent system comprising hexane/diethyl ether/acetic acid (50:50:1, v/v/v). The band corresponding to sn-2 MAGs was scraped off and extracted twice with diethyl ether. The solvent was then removed by nitrogen, and the residue was methylated and analyzed as described above.

Statistical Analysis. The data were subjected to analysis of variance (ANOVA) using the Statistical Analysis System software (SAS, Cary, NC, USA). The significance level tested was $\alpha = 0.05$, and differences were considered to be significant at $P < 0.05$.

RESULTS AND DISCUSSION

Separation and Identification of TAG Species in HMF. Many studies have reported that the contents of some fatty acids in HMF varied significantly with the progress of lactation.[7,8] Because the fatty acids are basic components of TAGs, it could be speculated that the contents of some TAG species might vary significantly at different lactation stages as well. On the basis of our previous study,[33] the major fatty acids (>1%) in HMF were C10:0, C12:0, C14:0, C16:0, C16:1 ω-7, C18:0, C18:1 ω-9, and C18:2 ω-6. These fatty acids were randomly combined to form DAGs and TAGs, and their molecular weights were used as the database to analyze the data obtained from RP-HPLC-APCI-MS. The randomly selected chromatographs of fat from colostrum and transitional and mature milks are presented in Figure 1. On the basis of analysis of large amounts of milk samples from different lactation stages, we found that even though the relative contents of TAGs were different, their species were consistent, which, to some extent, indicated the suitability to establish the evaluation model based on TAG composition.

According to Figure 1, some TAGs with saturated fatty acids existing in HMF such as POL, POO, PPO, PPL, and POLa might be composed of different isomers, which have the same molecular formula but different fatty acids on the positions of glycerol backbone. These isomers with the same ECN and similar structure cannot be separated on RP-HPLC, whereas the relative contents of isomers are of great importance to the absorption and subsequent metabolism of HMF in infants. To prove the existence of TAG isomers in HMF at different lactation stages, two types of TAG species, POO and PPO, were selected and analyzed by APCI-MS (Figure 2). Different types of fragment ions can be produced from TAGs after ionization.[30] The abilities of TAGs to form different fragment ions during ionization vary with their structure. The energy required to form sn-1,3 $[DAG]^+$ is more than that to form sn-1,2 $[DAG]^+$, and thus the relative abundances of sn-1,2 $[DAG]^+$ are more than

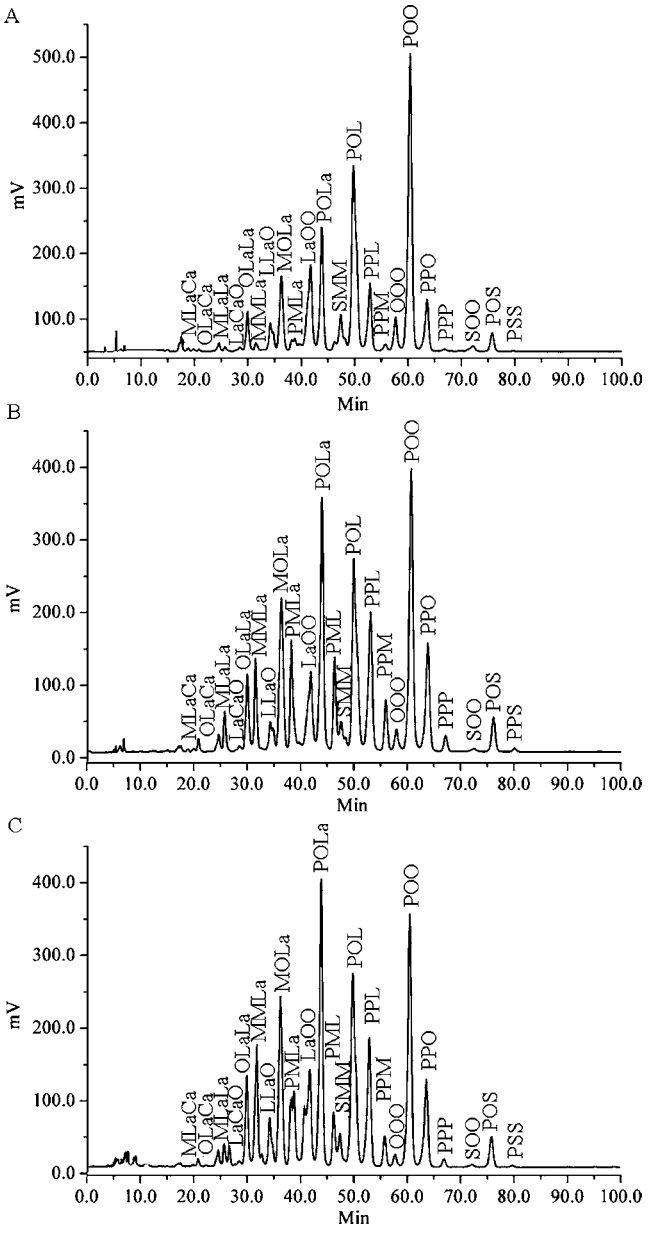

Figure 1. RP-HPLC of colostrum (A) and transitional milk (B) and mature milk (C) fat. Abbreviations: Ca, capric acid; La, lauric acid; M, myristic acid; P, palmitic acid; Po, pamitoleic acid; S, stearic acid; O, oleic acid; L, linoleic acid; Ln, linolenic acid.

those of sn-1,3 $[DAG]^+$. Therefore, asymmetric and symmetric TAG isomers (AAB and ABA) are generally identified by their relative abundances of sn-1,2 and sn-1,3 $[DAG]^+$. The mass spectra of sn-POO, sn-OPO, sn-PPO, and sn-POP are shown in Figure 3. Both sn-OPO and sn-OOP had $[OP]^+$ and $[OO]^+$ after APCI-MS analysis, whereas the relative abundances of each $[DAG]^+$ were different, which were related to the positions of O and P on the glycerol backbone. The relative abundances of $[OP]^+$ and $[OO]^+$ from sn-OPO were 100 and 21.7, whereas the relative abundances of $[OP]^+$ and $[OO]^+$ from sn-OOP were 100 and 73.1, respectively. Sn-PPO and sn-POP got similar results after APCI-MS analysis. The relative abundances of $[PO]^+$ and $[PP]^+$ from sn-PPO were 100 and 82.5, whereas the relative abundances of $[PO]^+$ and $[PP]^+$ from sn-POP were 100.0 and 27.3, respectively. The relative abundances of $[DAG]^+$ in APCI-MS of a specific TAG depend both on the type of instrumentation and experimental conditions. When isomers are mixed together, the relative abundances of different $[DAG]^+$ change with the variation of the relative contents of isomers. Some studies have reported that the relative abundances of isomers in mixtures with different ratios of two standards were similar to those calculated from pure isomers, differing within only a 1−2% range.[29] Therefore, the relative abundances of $[DAG]^+$ reflect the relative contents of isomers in mixture. As seen in Figure 2, the relative abundances of $[DAG]^+$ from POO and PPO in HMF at different lactation stages are similar to those of standard isomers (sn-OPO and sn-PPO), which indicates that the relative contents of sn-OPO and sn-PPO at the peaks of POO and PPO are very high. To precisely quantify the contents of different isomers in POO and PPO, the corresponding fractions from RP-HPLC were collected and subjected to Ag-HPLC analysis, and the Ag-HPLC of POO and PPO from HMF at different lactation stages are shown in Figure 4. The second dimension analysis allowing the separation of TAG isomers was based on a different retention mechanism. In the isomers of POO and PPO from colostrum and transitional and mature milk fat, the contents of sn-OPO and sn-PPO accounted for a large proportion, which was in agreement with the results from APCI-MS analysis. Because the qualification and quantitation of TAG isomers in HMF are not involved with the establishment of the evaluation model, the TAG isomer separations will not be reported in the subsequent experiments.

The TAG composition of HMF at different lactation stages is presented in Table 1. To our best knowledge, although some studies have reported the TAG composition in mature milk fat, the present study is the first to report the variation of TAG composition with the progress of lactation. As seen in Table 1, TAGs in HMF at different lactation stages can be classified into four classes on the basis of their relative contents. The first TAG class was POO and POL with average contents of >15%. The content of POO in colostrum was 24.68 ± 2.33%, significantly higher than these of transitional and mature milk fat. The contents of POL were significantly different among colostrum and transitional and mature milk fat. The second TAG class in HMF was MOLa, LaOO, POLa, PPL, and POP with contents of >5%. The contents of MOLa and POLa in mature and transitional milk fat were significantly higher than those of colostrum fat. The content of LaOO in colostrum fat was significantly higher than that of mature milk fat, whereas no significant difference was observed between transitional milk fat and colostrum fat and mature milk fat. With regard to PPL, the content in transitional milk fat was significantly higher than those of colostrum fat and mature milk fat. The contents of POP were not significantly different among different lactation stages. The third TAG class was OLaLa, MMLa, LLaO, PMLa, MPL, SMM, PPM, OOO, POO, and POS with contents in the range of 1−5%. The contents of most TAGs of this class were significantly different among different lactation stages, in which the contents of OLaLa, SMM, OOO, and POO in transitional and mature milk fat were significantly higher than those of colostrum fat. In terms of other TAG species, the contents of LLaO in colostrum fat and transitional milk fat were 2.02 ± 0.90 and 1.38 ± 0.23%, respectively, significantly higher than that of mature milk fat, and the contents of PMLa and PPM in transitional milk fat were significantly higher than those of colostrum fat and mature milk fat. As for the contents of POS, no significant difference was observed among different lactation stages. The fourth TAG class in HMF was MCaLa, OLaCa, MLaLa, PPO, PPP, SOO, and PPS with contents of <1%. On the basis of the above-mentioned

dx.doi.org/10.1021/jf304094p | J. Agric. Food Chem. 2013, 61, 167−175

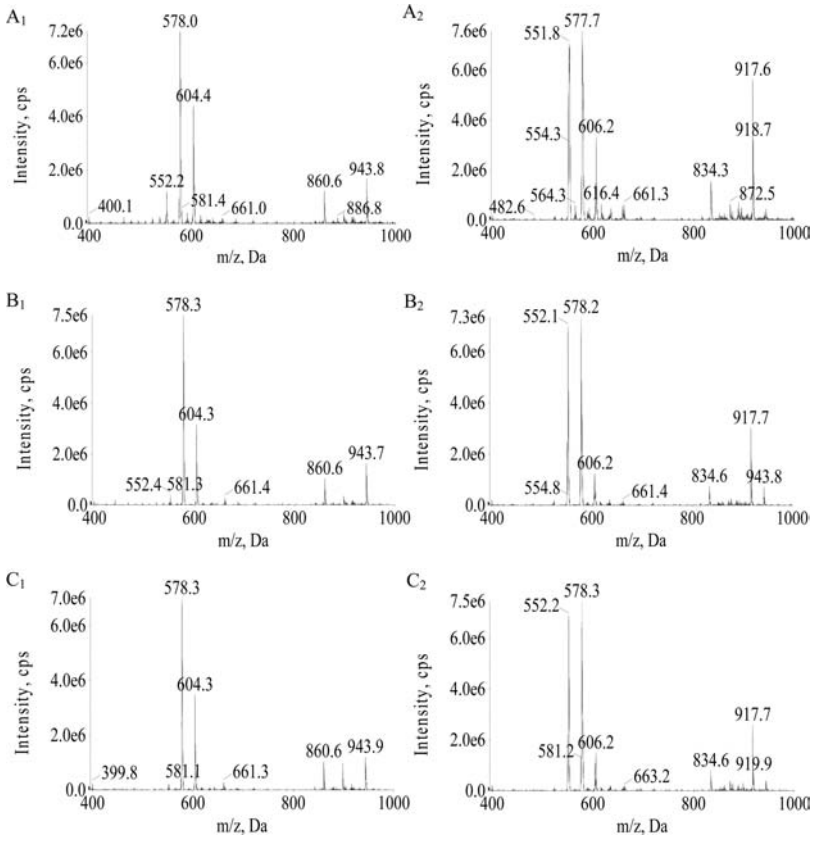

Figure 2. Positive-ion APCI mass spectra of POO (A_1, B_1, and C_1) and PPO (A_2, B_2, and C_2) in colostrum (A_1 and A_2) and transitional milk (B_1 and B_2) and mature milk (C_1 and C_2) fat. Abbreviations: P, palmitic acid; O, oleic acid.

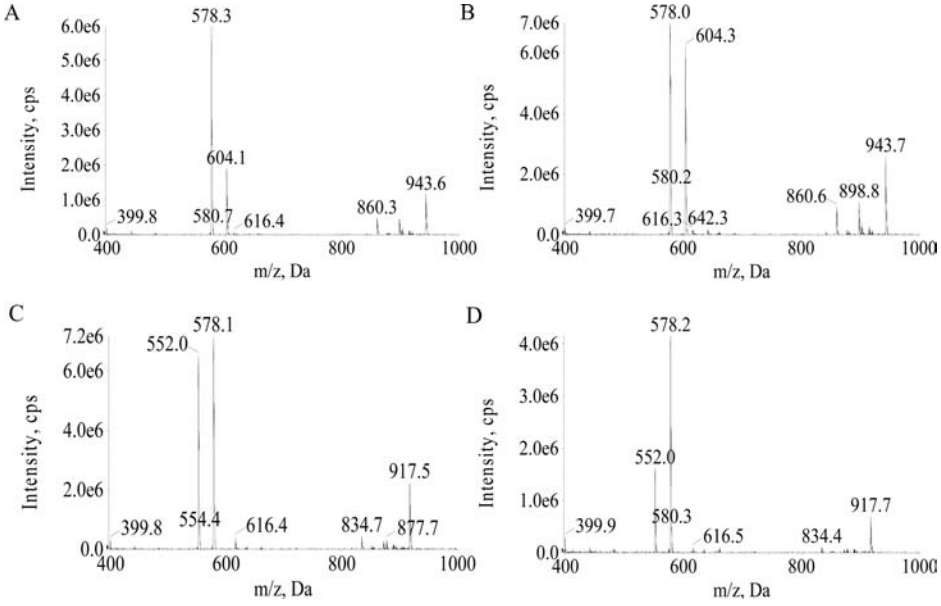

Figure 3. Positive-ion APCI mass spectra of sn-OPO (A), sn-OOP (B), sn-PPO (C), and sn-POP (D). Abbreviations: P, palmitic acid; O, oleic acid.

results, the major TAGs with contents of >1% in HMF were OLaLa (2.58, 0.59−3.31), MMLa (1.85, 0.56−5.59), LLaO (1.99, 1.21−2.65), MOLa (6.65, 2.27−8.44), PMLa (2.42, 1.70−5.27), LaOO (6.54, 2.19−9.01), POLa (10.39, 5.80−13.52), MPL (1.77, 0.73−3.97), SMM (2.56, 1.17−4.64), POL (16.93, 12.69−21.25), PPL (7.15, 5.80−8.87), PPM (1.35, 0.69−2.44), OOO (2.04, 1.14−3.75), POO (21.52, 15.09−28.46), PPO (6.12,4.57−7.89), and POS (2.28, 1.65−3.08), and their average contents and ranges were used as indices to evaluate the degrees of similarity of HMFSs to HMF.

Establishment of the Evaluation Model. As aforementioned, it is impossible to separate all TAG species in HMF by a monodimensional approach due to the existence of large amounts of TAG isomers. Therefore, to obtain more accurate evaluation results, fatty acid composition and distribution of HMF have to be employed as the indices for the evaluation

dx.doi.org/10.1021/jf304094p | J. Agric. Food Chem. 2013, 61, 167−175

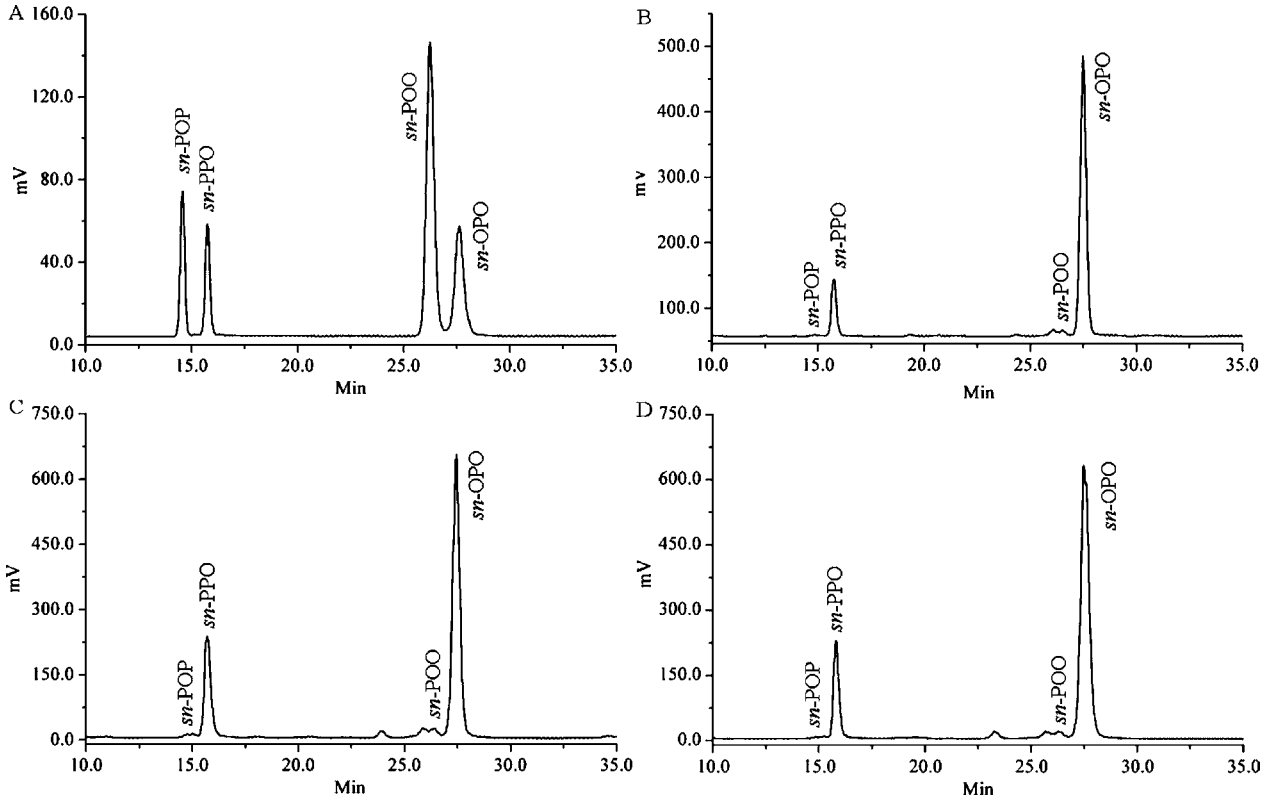

Figure 4. Ag-HPLC of mixed standards (A) of sn-POP, sn-PPO, sn-POO, and sn-OPO and POO and PPO isomers in colostrum (B) and transitional milk (C) and mature milk (D) milk fat. Abbreviations: P, palmitic acid; O, oleic acid.

Table 1. TAG Composition of Colostrum Fat and Transitional and Mature Milk Fat

TAG[a]	ECN	colostrum fat(n = 15)	transitional fat(n = 15)	mature fat(n = 15)	human milk fat(n = 45)	range
MCaLa	36	0.10 ± 0.06a	0.30 ± 0.22b	0.17 ± 0.10a	0.19 ± 0.14	0.06−0.45
OLaCa	38	0.39 ± 0.34a	0.69 ± 0.20b	0.64 ± 0.12b	0.58 ± 0.22	0.14−0.84
MLaLa	38	0.22 ± 0.15a	0.98 ± 0.71b	0.45 ± 0.19a	0.54 ± 0.45	0.12−0.67
OLaLa	40	1.57 ± 1.39a	3.05 ± 0.37b	2.93 ± 0.87b	2.58 ± 1.03	0.59−3.31
MMLa	40	0.45 ± 0.25a	2.44 ± 1.66b	2.38 ± 2.79b	1.85 ± 1.49	0.56−5.59
LLaO	42	2.02 ± 0.90a	1.38 ± 0.23a	2.38 ± 0.31b	1.99 ± 0.61	1.21−2.65
MOLa	42	4.13 ± 2.62a	8.23 ± 0.30b	7.27 ± 2.09b	6.65 ± 2.40	2.27−8.44
PMLa	42	1.32 ± 0.90a	3.74 ± 2.16b	2.28 ± 0.50a	2.42 ± 1.41	1.70−5.27
LaOO	44	7.89 ± 1.59b	6.05 ± 0.51ab	5.96 ± 3.27a	6.54 ± 2.21	2.19−9.01
POLa	44	7.30 ± 2.12a	12.72 ± 0.64b	10.89 ± 3.09b	10.39 ± 3.02	5.80−13.52
MPL	46	0.79 ± 0.08a	3.00 ± 1.37c	1.60 ± 0.78b	1.77 ± 1.17	0.73−3.97
SMM	46	4.03 ± 0.86b	1.63 ± 0.52a	2.20 ± 1.32a	2.56 ± 1.35	1.17−4.64
POL	46	20.20 ± 1.49c	13.94 ± 1.77a	16.75 ± 3.12b	16.93 ± 3.27	12.69−21.25
PPL	46	6.33 ± 0.75a	8.49 ± 0.54b	6.81 ± 0.56a	7.15 ± 1.06	5.80−8.87
PPM	48	0.94 ± 0.36a	1.99 ± 0.64b	1.20 ± 0.25a	1.35 ± 0.56	0.69−2.44
OOO	48	3.31 ± 0.63b	1.24 ± 0.14a	1.40 ± 0.81a	2.04 ± 0.98	1.14−3.75
POO	48	26.67 ± 2.52b	17.79 ± 3.65a	20.57 ± 5.97ab	21.52 ± 5.39	13.69−28.46
sn-POO	48	1.99 ± 0.19b	1.33 ± 0.27a	1.53 ± 0.44a	1.61 ± 0.40	1.02−2.12
sn-OPO	48	24.68 ± 2.33b	16.46 ± 3.38a	19.03 ± 5.51a	19.91 ± 4.99	14.07−26.34
PPO	48	6.53 ± 2.46a	6.73 ± 0.73a	6.12 ± 1.46a	6.41 ± 1.38	4.79−8.27
sn-POP	48	0.30 ± 0.11a	0.31 ± 0.03a	0.28 ± 0.07a	0.29 ± 0.06	0.22−0.38
sn-PPO	48	6.23 ± 2.35a	6.43 ± 0.70a	5.84 ± 1.39a	6.12 ± 1.31	4.57−7.89
PPP	48	0.29 ± 0.14a	0.70 ± 0.30c	0.38 ± 0.05b	0.45 ± 0.22	0.19−0.91
SOO	50	0.82 ± 0.10b	0.57 ± 0.31a	0.46 ± 0.21a	0.59 ± 0.24	0.21−0.89
POS	50	2.14 ± 0.70a	2.41 ± 0.68a	2.29 ± 0.71a	2.28 ± 0.58	1.65−3.08
PPS	50	0.10 ± 0.06a	0.20 ± 0.05b	0.13 ± 0.04a	0.14 ± 0.06	0.05−0.24

[a]Abbreviations: Ca, capric acid; La, lauric acid; M, myristic acid; P, palmitic acid; Po, pamitoleic acid; S, strearic acid; O, oleic acid; L, linoleic acid; Ln, linolenic acid.

dx.doi.org/10.1021/jf304094p | J. Agric. Food Chem. 2013, 61, 167−175

Table 2. Fatty Acid Profiles of Different Selected Fats and Oils

fatty acid	H1[a]	H2	H3	S1	S2	S3	S4	S5	S6	S7	IF1	IF2	IF3	L	P	A
major fatty acid composition																
C10:0	1.9	1.1	1.3							0.3	1.1	0.9	1.3			
C12:0	9.1	5.1	4.9							1.0	5.57	11.5	9.2		0.2	
C14:0	14.7	8	5.5	1.2	1.2	0.6	0.4	0.4	1.2	3.1	5.1	4.8	4.4	1.5	1.1	8.7
C16:0	28.3	20.6	24.1	21.9	21.4	16.3	15	14.2	56.1	47.9	23.9	19	21.8	24.9	44.0	24.7
C16:1 ω-7	2.9	1.8	2	1.8	1.6	0.9	0.7	0.5			0.8	0.2	0	2.2	0.1	0.8
C18:0	5.5	6.8	6.3	10.3	9.4	6.6	6	4.9	2.8	2.3	6.7	3.5	4.1	12.2	4.5	0.7
C18:1 ω-9	26	28.8	39.6	35.8	33.7	28.8	27.7	25.5	6.7	11.5	38.1	42.1	34.6	39	39.3	1.4
C18:2 ω-6	8.1	21.1	10.5	23.8	27.2	40.1	43.4	47.7	12.4	8.2	13.6	12.7	20.6	15.7	10.1	2.4
PUFA composition																
C18:3 ω-3	0.73	1.58	1.53	1.73	2.31	4.21	4.72	5.12	4.31	3.56	1.23	1.25	1.93	0.61	0.47	0.32
C20:2 ω-6	0.03	0.46	0.20	0.52	0.45						0.06	0.06		0.66		
C20:3 ω-6	0.25	0.38	0.30								0.04	0.01	0.07			0.38
C20:4 ω-6	0.28	0.56	0.34								0.22	0.03	0.75			0.51
C20:5 ω-3	0.07	0.11	0.14								0.05	0.05				
C22:2 ω-6	0.04	0.05	0.03													
C22:4 ω-6	0.05	0.08	0.07													
C22:5 ω-6	0.17	0.13	0.11													0.62
C22:5 ω-3	0.32	0.36	0.24								0.04	0.03				13.7
C22:6 ω-3	0.22	0.32	0.50							11.35	0.14	0.13	0.52			47.8
sn-2 fatty acid composition																
C12:0	11.7	7.1	9.6							0.9	9.9	10.6	22.3		0.3	
C14:0	21.6	13.8	15.2						1.2	3.1	7.1	3.9	4.6	2.4	0.5	
C16:0	50.6	52.6	58.0	43.7	38.7	16.6	22.6	19.1	57.4	55.0	15.1	22.1	20.0	67.3	6.4	13.1
C16:1 ω-7	0.8	3.2	1.0								0.2	0.1		1	0	0.6
C18:0	1.1	1.4	1.2	8.8	6.7	7.8	1.6	1.1	2.8	2.7	2.1	1.7	1.9	8.3	6.5	
C18:1 ω-9	5.3	10.3	6.2	14.9	19.6	43.1	45.9	52	6.6	9.6	41	37.6	30.8	12.8	63.4	1.1
C18:2 ω-6	3.8	5.6	3.3	6.8	8.7	4.8	3.5	2.1	12.4	7.7	20.6	18.9	17.9	5.7	22.1	2.1
relative contents of sn-2 fatty acids[b]																
C12:0	42.9	46.4	65.3							30.0	59.2	30.7	80.8		50.0	
C14:0	49.0	57.5	92.1						33.3	33.3	46.4	27.1	34.8	53.3	15.2	
C16:0	60.6	85.1	80.2	66.5	60.3	33.9	50.2	44.8	34.1	38.3	21.1	38.8	30.6	90.1	4.8	17.7
C16:1 ω-7	9.2	59.3	16.7								8.3	16.7		15.2	0.0	25.0
C18:0	6.7	6.9	6.3	28.5	23.8	39.4	8.9	7.5	33.3	39.1	10.4	16.2	15.4	22.7	48.1	
C18:1 ω-9	6.8	11.9	5.2	13.9	19.4	49.9	55.2	68.0	32.8	27.8	35.9	29.8	29.7	10.9	53.8	26.2
C18:2 ω-6	12.7	8.8	10.5	9.5	10.7	4.0	2.7	1.5	33.3	31.3	50.5	49.6	29.0	12.1	72.9	29.2

[a]H1− H3 were three HMF samples; S1−S5 were HMFSs obtained by interesterification of lard with soybean oil at ratios of 8:2, 7:3, 4:6, 3:7, and 2:8, respectively, reported by da Silva et al.;[34] S6 and S7 were HMFSs obtained by acidolysis of tripalmitin with free fatty acids reported by Teichert et al.;[35] IF1−IF3 were infant formulas reported by Lopez−Lopez et al.;[36] L, lard; P, palm oil; A, algal oil. [b]Values were calculated as 100% × sn-2 fatty acid/(3 × total fatty acid).

dx.doi.org/10.1021/jf304094p | J. Agric. Food Chem. 2013, 61, 167−175

Table 3. TAG Composition of Different Selected Fats and Oils

TAG[a]	H1[b]	H2	H3	S1	S2	S3	S4	S5	S6	S7	L	P	A
OLaLa	3.3	3.0	2.0										
MMLa	3.6	1.0	0.6										
LLaO	1.2	2.0	2.5										
MOLa	8.4	7.9	5.0	1.2	1.2						1.7	0.1	
PMLa	5.3	1.7	2.5										
LaOO	5.7	7.9	7.8									0.3	
POLa	12.3	11.7	7.5										
MPL	4.0	1.3	1.0	11.4	12.6	10.8	14.9	14.4	4.5	0.39	5.6	3.4	12.9
SMM	2.0	1.2	3.7										
POL	12.7	17.7	19.3	15	15.5	11.6	13.6	12.5	0.9	4.57	15.1	0.6	9.5
PPL	8.1	7.0	6.2	5.2	4.7	3.1	4.2	6.2	11.9	12.51	5.5	6.4	2.7
PPM	2.4	1.0	1.1	1.1	1	0	0.5	0			0.8	1.6	
OOO	1.3	1.2	2.3	5.3	5.4	5.2	2.2	2.3			3.6	5.4	2.8
POO	15.2	23.7	24.3	18.3	15.6	12.2	7.1	5.7	1.18	1.48	29.6	22.4	4.4
PPO	5.9	5.3	7.4	6.4	5.8	2.1	2.8	0.3	6.89	9.68	5.6	7.2	1.1
POS	1.9	2.1	3.1	7.1	5.5	4.6	1.6	1.1	2.07		14.2	0.6	0.5

[a]Abbreviations see Table 1. [b]Abbreviations see Table 2.

model. As previously reported, HMF contained a small amount of PUFA (<1%), which could not be reflected in the TAG and fatty acid evaluation.[33] Due to the importance of these fatty acids to the development of infants, their contents in HMFSs have to be considered. Therefore, the indices selected for the evaluation model were total fatty acid composition (>1%), relative contents of sn-2 fatty acids (sn-2 fatty acid content >1%), PUFA composition (>0.1%), and TAG composition (>1%). The fatty acid profiles of HMF were reported in our previous study, and the data used in this study were reorganized and cited as follows. The data of total fatty acids and PUFA were expressed as average contents and ranges, whereas the data of sn-2 fatty acids were expressed as average contents and ranges of relative contents. Total fatty acids include C10:0 (1.71, 0.44−4.98), C12:0 (6.74, 3.10−10.11), C14:0 (8.54, 5.69−14.74), C16:0 (23.83, 19.60−29.01), C16:1 (2.00, 1.08−2.71), C18:0 (6.09, 5.28−8.66), C18:1 (33.43, 26.03−40.63), and C18:2 (10.57, 7.14−20.15); sn-2 fatty acids include C12:0 (6.26, 15.40−48.43), C14:0 (13.08, 42.37−88.93), C16:0 (52.66, 59.20−89.51), C16:1 (1.91, 9.16−33.72), C18:0 (1.72, 6.35−11.22), C18:1 (9.99, 5.22−17.30), and C18:2 (6.85, 6.61−23.76); PUFA include C18:3 (0.67, 0.47−1.07), C20:2 (0.42, 0.03−0.80), C20:3 (0.42, 0.25−0.81), C20:4 (0.45, 0.3−1.20), C20:5 (0.17, 0.00−0.45), C22:2 (0.08, 0.00−0.20), C22:4 (0.17, 0.05−0.32), C22:5 (0.15, 0.07−0.25), C22:5 (0.28, 0.15−0.44), and C22:6 (0.51, 0.22−0.87). The fatty acid composition and distribution and PUFA and TAG composition of HMF were considered as objectives; that is, the degrees of similarity were 100. The HMFSs with fatty acid composition and distribution and PUFA and TAG composition within the ranges of HMF were considered to be identical with HMF. However, other HMFSs with chemical indices outside the ranges have to be evaluated. The similarity evaluation was carried out by comparison of the major fatty acid profiles and PUFA and TAG composition of HMFSs with the corresponding indices of HMF, and the degrees of similarity were obtained by "deducting score principle".[26] The evaluation model is described as

$$G_{\mathrm{FA/sn\text{-}2FA/PUFA/TAG}} = 100 - \sum_{i=1}^{n} E_{i(\mathrm{FA/sn\text{-}2/PUFA/TAG})} \quad (1)$$

$$E_{i(\mathrm{FA/sn\text{-}2FA/PUFA/TAG})} = 100 \times \left(C_{i(\mathrm{FA/sn\text{-}2FA/PUFA/TAG})} \frac{D_{i(\mathrm{FA/sn\text{-}2/PUFA/TAG})}}{\sum_{i=1}^{n} D_{i(\mathrm{FA/sn\text{-}2FA/PUFA/TAG})}} \right) \quad (2)$$

$$C_{i(\mathrm{FA/sn\text{-}2FA/PUFA/TAG})} = \frac{|B_{i(\mathrm{FA/sn\text{-}2FA/PUFA/TAG})} - A_{i(\mathrm{FA/sn\text{-}2FA/PUFA/TAG})}|}{A_{i(\mathrm{FA/sn\text{-}2FA/PUFA/TAG})}} \quad (3)$$

where $G_{\mathrm{FA/sn\text{-}2FA/PUFA/TAG}}$ is the degree of similarity of HMFSs to HMF in the aspect of total fatty acid composition, relative contents of sn-2 fatty acids, or PUFA or TAG composition; $E_{i(\mathrm{FA/sn\text{-}2FA/PUFA/TAG})}$ is the deducted degree of similarity from the total fatty acid content, relative content of sn-2 fatty acid, or PUFA or TAG content that is outside the range of that of HMF; $(D_{i(\mathrm{FA/sn\text{-}2FA/PUFA/TAG})})/(\Sigma_{i=1}^{n} D_{i(\mathrm{FA/sn-2FA/PUFA/TAG})})$ is the weight of the total fatty acid, sn-2 fatty acid, PUFA, or TAG relative to its total amount; and $C_{i(\mathrm{FA/sn\text{-}2FA/PUFA/TAG})}$ is the floating coefficient, which is dependent on the total fatty acid content, relative content of sn-2 fatty acid, or PUFA or TAG content in HMFSs. $B_{i(\mathrm{FA/sn\text{-}2FA/PUFA/TAG})}$ is the total fatty acid content, relative content of sn-2 fatty acid, or PUFA or TAG content in HMFSs; $A_{i(\mathrm{FA/sn\text{-}2FA/PUFA/TAG})}$ is the upper or lower limit of corresponding total fatty acid content, relative content of sn-2 fatty acid, or PUFA or TAG content in HMF. When B is higher than the upper limit of the corresponding fatty acid content, the relative content of sn-2 fatty acid or PUFA or TAG content, A is selected as the upper limit, and vice versa. If B is within the range, C is set to zero.

On the basis of the established evaluation model, a qualified HMFS has to pass a four-step evaluation, that is, the primary two steps for total and sn-2 fatty acid evaluation and the advanced two steps for PUFA and TAG evaluation.

Verification of the Evaluation Model. The validity of the evaluation model was verified by analysis of the selected fats and oils with specific chemical compositions, including three HMF samples, seven types of reported HMFSs (S1−S5 were obtained by interesterification of lard with soybean oil at ratios of 8:2, 7:3, 4:6, 3:7, and 2:8, respectively; S6 and S7 were obtained by

dx.doi.org/10.1021/jf304094p | J. Agric. Food Chem. 2013, 61, 167−175

Table 4. Degrees of Similarity of Different Fats and Oils to Human Milk Fat

similarity	H1[a]	H2	H3	S1	S2	S3	S4	S5	S6	S7	IF1[b]	IF2	IF3	L	P	A
G_{total}	99.8	100.0	100.0	82.0	81.2	91.6	65.6	60.8	27.8	47.6	98.4	91.6	89.4	77.4	67.8	40.3
$G_{sn\text{-}2}$	100.0	98.4	97.2	75.4	73.6	40.4	40.4	18.6	47.6	61.2	40.2	58.6	51.4	82.8	−9.0	30.5
G_{PUFA}	100.0	99.8	99.7	6.6	−9.0	−39.0	−48.7	−56.2	−40.9	−11.4	67.2	53.6	28.9	32.8	20.2	−1021.2
G_{TAG}	100.0	100.0	100.0	59.8	59.6	48.0	48.2	38.9	23.4	24.0				57.7	16.6	26.38

[a]Abbreviations see Table 2. [b]TAG compositions of IF1−IF3 in the reference were not given.

acidolysis of tripalmitin with free fatty acids),[34,35] three types of reported infant formula (IF1−IF3),[36] lard (L), palm oil (P), and algal oil (A). The reasons for selecting these fats and oils are as follows: HMF was the standard for HMFSs; S1 and S2 had fatty acid compositions and distributions similar to those of HMF, and probably had similar TAG compositions; the similarity of chemical composition of S3−S5 was gradually decreased with the increase of amounts of soybean oil; S6 and S7 had sn-2 fatty acid compositions similar to that of HMF yet different fatty acid composition; IF1−IF3 had similar fatty acid composition and also contained some PUFA; lard had similar total and sn-2 fatty acid composition, but lacked PUFA; palm oil contained palmitic acid but without special positional distribution; and algal oil contained a high amount of PUFA. Their total and sn-2 fatty acid compositions, relative contents of sn-2 fatty acids, and PUFA and TAG compositions are shown in Tables 2 and 3, respectively.

The chemical compositions of different fats and oils were compared with those of HMF by the evaluation model, and their degrees of similarity in corresponding evaluation aspects were calculated (Table 4). The degrees of similarity of HMF (H1−H3) in the aspects of fatty acid composition and distribution and PUFA and TAG composition were 100 or close to 100. As for the HMFSs derived from lard, the contents of total and sn-2 palmitic acid were significantly decreased and the content of linoleic acid was significantly increased with the increase of the addition of soybean oil. Therefore, the difference between HMFSs and HMF in fatty acid composition and distribution was increased from S1 to S5. The changes in fatty acid profiles of HMFSs were digitized by the evaluation model, which indicated that the degrees of similarity of the products decreased with an increase in the addition of soybean oil. The degrees of similarity of S1−S5 in total fatty acid composition were 82.0, 81.2, 91.6, 65.6, and 60.8 and in relative contents of sn-2 fatty acids were 75.4, 73.6, 40.4, 40.4 and 18.6, respectively. The fatty acid composition and distribution of HMFSs reflected to some extent the TAG composition. However, due to the randomization of fatty acid distribution in different TAG species and the complexity of TAG composition, the similarity was always lower than those of total fatty acid composition and relative contents of sn-2 fatty acids. With an increase in the addition of soybean oil, the contents of POO, PPO, POL, etc., in HMFSs were significantly decreased and other TAGs composed of unsaturated fatty acids from soybean oil were significantly increased, which resulted in a decrease in the degree of similarity. In terms of PUFA composition, because the contents of C18:3 ω-3 in S2, S3, S4, and S5 were much higher than that in HMF, the degrees of similarity of these HMFSs were negative as evaluated by the model. S6 and S7 had similar sn-2 fatty acid compositions but different total fatty acid compositions, whereas IF1−IF3 had similar total fatty acid compositions but different sn-2 fatty acid compositions. After comparison with HMF by the established model, the degrees of similarity of S6 and S7 in sn-2 fatty acid evaluation were low, and the degrees in total fatty acid evaluation were lower. The reason for the low degree of similarity in sn-2 fatty acid evaluation was that even though the sn-2 fatty acid composition was similar to HMF, their relative contents were quite different. Because the contents of C18:3 ω-3 in S6 and S7 were considerably higher than that of HMF and as they lacked other types of PUFA, their degrees in PUFA were negative. IF1−IF3 had high degrees of similarity in total fatty acid composition and low degrees in relative contents of sn-2 fatty acids, which were in agreement with their chemical composition. As IF1 and IF2 had different types of PUFA, their degrees of similarity were higher than those of other HMFSs. IF3 had some PUFA, but their contents were quite different from HMF. Therefore, the degree of similarity was low. In terms of TAG evaluation, due to great differences in fatty acid profiles, S6 and S7 had low degrees of similarity in TAG composition. The cited study did not report the TAG composition of IF1−IF3, where, due to their low degrees of similarity in relative contents of sn-2 fatty acids and the exstence of TAG isomers, TAG evaluation was of little improtance. Lard is the only natural fat with a fatty acid profile close to HMF, and high degrees of similarity in total fatty acid, sn-2 fatty acid, and TAG evalutation were observed. The distribution of palmitic acid of palm oil was different from that of HMF, which led to a low degree of similarity. Algal oil contained a high amount of PUFA. However, because their contents were considerably higher than the upper limits of HMF, the degree of similarity in PUFA composition was negative. The degrees of similarity of the selected fats and oils in different evaluation aspects reflected their differences in corresponding chemical composition with HMF, indicating the validity of the model to evaluate HMFSs. Compared with the previously reported model based on fatty acid profiles,[23] this model obtained from the perspective of TAG composition could provide more precise information with regard to the similarity of HMFSs.

In conclusion, the TAG composition of HMF from different lactation stages was analyzed by RP-HPLC-APCI-MS, and the establishment of a model for precise evaluation HMFSs based on TAG composition of HMF was indirectly realized by employment of four indices, including fatty acid composition and distribution and PUFA and TAG composition (obtained from RP-HPLC). The model was verified by the selected fats and oils with specific chemical compositions, and the results revealed that the degrees of similarity of these fats and oils in different evaluation aspects reflected their differences in corresponding chemical composition with HMF, which indicated the validity of the model for HMFS evaluation. This model, evaluating the similarity of HMFSs from the view of TAG composition, might have some inspirations for future HMFS production.

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Funding

This work was supported by the National Science and Technology Pillar Program during the Twelfth Five-Year Plan Period (No. 2011BAD02B04) and the Major State Basic Research Development Program of China (973 Program, 2012CB720802, 2012CB720806).

Notes

The authors declare no competing financial interest.

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