Molecular Weight Distribution and Regioisomeric Structure of Triacylglycerols in Some Common Human Milk Substitutes

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ABSTRACT: Triacylglycerol (TAG) molecular weight distribution and regioisomeric structure of selected molecular weight species in human milk and in 32 human milk substitutes was determined. Negative ion chemical ionization mass spectrometry was used to determine the molecular weight distribution and collisionally induced dissociation tandem mass spectrometry applied to identify the *sn*-2 and *sn*-1/3 positions of fatty acids in TAG. The main molecular weight species of human milk TAG in decreasing order of abundance were 52:2, 52:3, 52:1, 54:3, 50:2, 50:1, 54:4, 48:1, 54:2, 48:2, 46:1, 52:4, and 50:3 (acyl carbon number/number of double bonds), constituting 83 mol% of total TAG molecular species. In human milk substitutes, the proportion of the corresponding molecular weight species varied from 33 to 87 mol%. The main TAG regioisomers within the molecular weight species 52:2, 52:3, and 50:1 in human milk were 18:1-16:0-18:1 (83 mol%), 18:1-16:0-18:2 (83 mol%), and 18:1-16:0-16:0 (80 mol%), respectively. In human milk substitutes, the corresponding proportions varied in a wide range of 0–82 mol%, 0–100 mol%, and 0–73 mol%, respectively. Although TAG structures in some human milk substitutes closely resembled those in human milk, the great variation among samples leads to the conclusion that it is still possible to improve the TAG composition in human milk substitutes by applying novel methods to synthesize structured TAG.

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It is well known that fatty acids present in human milk are not randomly distributed in triacylglycerols (TAG) (1–8). The properties of TAG depend on the fatty acid combination constituting the molecule. Winter *et al.* (1) determined 170 fatty acid combinations in human milk TAG, the proportions of which differed from calculated random distribution. In addition to fatty acid combinations, the stereospecific positions of fatty acids within each combination affect the biochemical and nutritional properties of TAG. In human milk, 16:0 is predominantly located in *sn*-2 position of the TAG molecule, whereas 18:0, 18:1, and 18:2 have been reported to be preferentially esterified in *sn*-1/3 positions (2–6). Breckenridge *et al.* (3) reported the molar proportion of palmitic acid to be 16, 58, and 6% of total fatty acids in *sn*-1, *sn*-2, and *sn*-3 positions of human milk TAG, respectively. Christie and Clapperton (4) obtained very similar results (18.7, 57.1, 5.3%) as did Martin *et al.* (5), who concluded that the structure of TAG in colostrum (12.6, 53.5, 11.2%) and mature milk (12.4, 51.2, 11.7%) did not differ significantly. Weber *et al.* (6) reported that 69% of palmitic acid in human milk is esterified in the *sn*-2 position of the TAG, which is in accordance with the corresponding value of 71% reported by Breckenridge *et al.* (3). Correspondingly, 77–93, 87–92, and 78–84% of total 18:0, 18:1, and 18:2 in human milk have been reported to be esterified in *sn*-1/3 positions, respectively (3–5). The above results were obtained by analyzing TAG and/or products of chemical or enzymatic degradation of TAG by a variety of chromatographic methods. Tandem mass spectrometric method has also been used to analyze the regioisomeric structure of human milk TAG (7,8). By means of mass spectrometry, the *sn*-1 and *sn*-3 positions of TAG cannot be distinguished. On the other hand, the fatty acid combinations and regioisomeric structure of specified ACN/DB (acyl carbon number/number of double bonds) species of TAG can be determined rapidly and with minimal sample preparation, which is not easily achieved by other methods. The results obtained for molecular species analyzed by mass spectrometry (7,8) were in accordance with chromatographic results (1–6).

The effect of positional distribution of fatty acids, especially that of palmitic acid, on absorption of fatty acids has been studied extensively (9–17). Tomarelli *et al.* (9) reported that 2-monoacyl-*sn*-glycerols of saturated fatty acids were more readily absorbed than free fatty acids and that high content of 2-palmitoyl TAG in fat facilitated the efficient absorption of fat in the rat. Reports confirming that palmitic acid is absorbed as 2-monoacyl-*sn*-glycerol also in infants have been published (10,11). Filer *et al.* (12) investigated the absorption of lard and randomized lard in infants. The effect of different TAG structures in infant formulas on fat and nutrient absorption also has been studied (13–17). In all the investigations, palmitic acid and other long-chain saturated fatty acids were absorbed more efficiently when located in the *sn*-2 position of TAG. This was explained on the one hand by the more efficient absorption of 2-palmitoyl-*sn*-glycerol compared to free palmitic acid (12–14). On the other hand, the absorption of free palmitic acid released from *sn*-1/3 positions in intestine was also prevented by forma-

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tion of fatty acid mineral soaps that were partially lost in feces (12–14). In parallel with efficient absorption of 2-palmitoyl-*sn*-glycerol, the absorption of stearic acid was increased, which could not be explained by its stereospecific position in TAG. It was suggested that the more efficient micellization in the presence of 2-palmitoyl-*sn*-glycerol would also enhance the absorption of 18:0 (12). In addition, positional isomerism in infant formula TAG has been reported to affect the mineral balance and plasma lipid composition of premature infants (13,15,16).

Since the exceptional distribution of fatty acids in human milk TAG and its importance for infant fat and nutrient absorption have been confirmed by numerous investigations, we analyzed the TAG structure in 32 commercial human milk substitutes. The molecular weight distribution of TAG and the regioisomeric structure of selected ACN/DB species in human milk substitutes were analyzed and the results compared with corresponding results obtained for human milk TAG.

MATERIALS AND METHODS

Sample preparation. A sample of milk was obtained from four Finnish mothers (25–34 yr of age) 3–5 mon postpartum. Milk samples were divided into 1-mL aliquots immediately to avoid separation of fat and frozen to –70°C. Aliquots of milk samples were used to prepare pooled samples for lipid extraction. A total of 30 commonly used commercial human milk substitutes and two oil ingredients used in manufacturing of human milk substitutes were collected from Europe and United States during year 1997 (Table 1). Liquid substitutes were used as such, and substitutes in powder form were reconstituted according to instructions given by the manufacturer before lipid extraction.

Lipids from 2 mL of human milk or human milk substitute were extracted with chloroform/methanol (2:1, vol/vol) according to the modified Folch procedure (18). The solvent was evaporated under a stream of nitrogen, and lipids were redissolved in hexane. Approximately 15 mg of total lipid extract was loaded into a short column filled with Florisil (Fluka Chemie AG, Buchs, Switzerland), and the neutral lipid fraction was eluted from the column with 10 mL of hexane/diethyl ether (4:1, vol/vol). After evaporating the solvent, the neutral lipid fraction was redissolved in hexane to obtain approximately 1 mg/mL lipid solution for mass spectrometric analyses.

Analysis of molecular weight distribution of TAG. Chemical ionization with ammonia (≥99.998%; Prax Air, Oevel, Belgium) was used to analyze the molecular weight distribution of TAG in negative ion mode as reported earlier (19). All the mass spectrometric analyses were performed using Finnigan MAT TSQ-700 triple quadrupole mass spectrometer (Finnigan MAT, San Jose, CA) equipped with a combined electron ionization/chemical ionization ion source. The pressure of ammonia (8500 mtorr), the ion source temperature (200°C), the electron energy (70 eV), and the filament current (400 μA) were selected based on method optimization as published earlier (19). An aliquot of 0.5 μL of hexane solution of TAG was applied to the rhenium wire of the direct exposure probe. After evaporation of the solvent, the probe was introduced into the ion source, and the rhenium wire was heated at the rate of 40 mA/s to vaporize the sample. The $[M - H]^-$ ions of TAG were detected by scanning *m/z* values from 500 to 1000. Response correction factors for low molecular weight TAG at acyl carbon number (ACN) 34–46 were determined using TAG standard compounds. The correction factors for these ACN molecular species were 0.6 (ACN 34), 0.7 (ACN 36), 0.7 (ACN 38), 0.8 (ACN 40), 0.8 (ACN 42), 0.9 (ACN 44), and 0.9 (ACN 46), whereas no response correction was required for molecular species at ACN 48–56 under optimized conditions (19). Each sample was analyzed four times, and averaged results were presented. Spectra interpretation and processing of results were performed using automatic software developed earlier in our laboratory (20).

Analysis of regioisomeric structure of TAG. Tandem mass spectrometric analyses to determine the regioisomeric structure of TAG possessing certain ACN/DB ratios were performed

TABLE 1
Analyzed Human Milk Substitutes

Sample number	Trade name	Manufacturer[a]
1	Baby Semp 1[b]	Arla Foods AB, Sweden
2	Formulat 1[c]	Belgomilk, Belgium
3	Bona[b]	Chymos OY, Finland
4	Cow & Gate Galactomin[c]	Cow & Gate Ltd., England
5	Pre Gallia[c]	Diepal-NSA, France
6	Gallia 1[c]	Diepal-NSA, France
7	Gallia 2[c]	Diepal-NSA, France
8	Gallia HA[c]	Diepal-NSA, France
9	Blédina Alma[c]	Diepal-NSA, France
10	Gallia 1 Nursilat[c]	Diepal-NSA, France/Italy
11	Vivena 1[c]	Dieterba, Italy
12	Gerber Baby Formula[c]	Gerber Products Co., USA
13	Peptidi Tutteli[c]	Kuivamaito OY, Finland
14	Enfamil Lactofree[c]	Mead Johnson Co., USA
15	Enfalac Infant Formula[c]	Mead Johnson Co., USA
16	Bébédor 1[c]	Milupa, France
17	Milu-Milk[b]	Milupa, Switzerland
18	Aptamil 1[c]	Milupa GmbH & Co., Germany
19	Milumil 2[c]	Milupa GmbH & Co., Germany
20	Carnation Good Start[c]	Nestlé Canada Inc.
21	Alsoy[c]	Nestlé, Italy
22	Plasmon Primigiorni HA[c]	Plasmon Dietetici Alimentari, Italy
23	Isomil Soy Formula[b]	Ross Products Division, Abbott Laboratories, USA
24	Similac Advance[c]	Ross Products Division, Abbott Laboratories, USA
25	Similac with Iron[c]	Ross Products Division, Abbott Laboratories, USA
26	Piltti[c]	Valio OYj, Finland
27	Tutteli B[b]	Valio OYj, Finland
28	Bonamil[c]	Wyeth Ayerst Inc., Canada
29	Nursoy[c]	Wyeth Ayerst Inc., Canada
30	SMA[c]	Wyeth Ayerst Inc., Canada
31	Betapol 45[d]	Loders Croklaan, Holland
32	Betapol 60[d]	Loders Croklaan, Holland

[a]Based on information given on packages.
[b]Liquid formula.
[c]Powder formula.
[d]Oil ingredient.

using the same instrumentation and the same analysis parameters as in molecular weight distribution determinations. Collision gas (argon, ≥99.998%; AGA, Lidingö, Sweden) pressure was 1.4 mtorr, and the collision energy was 15 eV. The fragment ions of selected TAG ACN/DB species were detected by scanning *m/z* values from 100 to 1000. The fatty acid combinations and regioisomeric structure of selected TAG molecular species were determined on the basis of formation of specific $[RCO_2]^-$ and $[M - H - RCO_2H - 100]^-$ fragment ions as reported earlier (8,21–23). Spectra interpretation and regioisomeric structure calculations were performed using previously developed automatic software (8,20).

RESULTS AND DISCUSSION

Molecular weight distribution of TAG. Table 2 lists the molecular weight distribution of human milk and human milk substitutes according to ACN/DB ratios of TAG molecular species. ACN/DB species exceeding 2 mol% of total TAG in human milk and the corresponding molecular species in analyzed human milk substitutes are presented. The total proportion of these molecular species in human milk was 83 mol% (Table 2). In the human milk substitutes, the corresponding proportion varied in the range of 33–87 mol% indicating the wide variation in TAG molecular weight species compositions. Molecular species with 52 acyl carbons constituted the majority of human milk TAG (42.9 mol%). Of the single TAG molecular weight species, the proportions of 52:2, 52:3, 52:1, and 52:4 (ACN/DB) were 21.6, 10.2, 8.3, and 2.8 mol%, respectively. In the milk substitutes, these proportions varied in the range of 2.7–33.3, 3.1–12.2, 0–4.5, and 0–10.1 mol%, respectively (Table 2). ACN 54 molecular species were composed of 54:3 (6.5 mol%), 54:4 (4.5 mol%), and 54:2 (3.8 mol%) while the corresponding proportions in human milk substitutes were in the range of 3.5–53.0, 4.0–16.5, and 1.0–4.6 mol%, respectively. The major molecular species of ACN 50 in human milk consisted of 50:2 (6.6 mol%), 50:1 (6.0 mol%), and 50:3 (2.4 mol%). In the substitutes, the proportions of corresponding molecular species were in the range of 0.6–8.0, 0–22.1, and 0–1.1 mol%, respectively. Figure 1

TABLE 2
Triacylglycerol Molecular Weight Distribution (mol%) in Human Milk and in Human Milk Substitutes as Determined by Ammonia Negative Ion Chemical Ionization Mass Spectrometry[a]

Sample[b]	46:1[c]	48:1	48:2	50:1	50:2	50:3	52:1	52:2	52:3	52:4	54:2	54:3	54:4	Total[d]
Human milk	3.0	4.1	3.3	6.0	6.6	2.4	8.3	21.6	10.2	2.8	3.8	6.5	4.5	83
1	1.2	2.8	1.0	19.8	6.5	0.7	4.5	14.7	6.9	3.3	1.9	6.8	5.3	75
2	—	—	—	1.0	0.6	—	—	7.2	4.0	—	1.3	53.0	16.5	83
3	0.7	1.2	0.6	11.9	3.7	—	2.8	12.3	6.0	3.0	3.2	13.7	7.4	66
4	—	—	—	8.8	2.4	—	1.1	14.9	5.5	1.9	2.0	30.6	11.1	78
5	—	1.3	0.6	12.6	6.4	0.6	3.1	16.8	7.7	4.1	4.5	13.7	4.8	76
6	1.0	1.4	0.7	10.3	3.5	—	2.2	10.2	5.5	3.3	3.5	14.2	4.5	60
7	—	1.3	—	18.9	6.5	0.5	3.3	17.2	8.6	4.4	2.2	5.0	4.5	72
8	1.5	3.1	1.1	19.2	6.3	0.7	4.5	15.0	6.8	2.8	2.4	6.3	4.4	74
9	0.8	1.2	0.6	10.7	3.5	—	2.5	10.7	5.5	3.3	3.0	11.1	4.8	58
10	—	0.8	—	16.1	4.8	—	1.2	22.2	10.3	4.0	2.1	5.9	5.6	73
11	0.7	1.3	0.6	7.6	2.7	—	1.3	12.4	4.1	2.3	4.6	38.5	4.6	81
12	—	1.1	—	15.5	4.7	—	1.3	17.4	7.8	3.9	2.0	16.9	5.3	76
13	0.5	1.8	0.7	16.6	8.0	0.7	4.3	25.4	9.2	2.4	3.9	8.5	4.0	86
14	—	0.7	—	13.5	4.5	—	1.5	16.2	8.9	4.4	2.3	18.6	5.6	76
15	—	0.9	—	14.6	4.7	—	1.8	17.0	7.3	4.9	2.9	16.4	5.7	76
16	—	1.0	—	18.9	5.8	—	2.3	21.6	9.5	3.3	2.6	9.6	7.8	82
17	2.3	3.6	1.7	4.4	4.5	1.1	2.8	5.8	5.2	5.7	2.2	4.1	6.4	50
18	—	1.3	—	22.1	6.1	—	2.5	20.5	9.4	3.4	2.4	7.8	5.2	81
19	—	0.9	—	19.7	5.5	—	1.9	21.7	9.5	3.1	2.4	9.6	7.2	82
20	—	1.0	—	16.3	4.9	—	1.9	18.6	7.4	5.9	2.2	9.7	6.2	74
21	—	0.6	—	11.3	3.0	—	0.8	24.8	12.2	5.4	3.0	7.2	7.7	76
22	—	0.5	—	7.6	3.1	—	1.1	15.8	5.1	2.0	4.5	43.2	4.5	87
23	0.7	—	—	—	1.7	—	—	2.7	5.2	9.6	1.0	3.5	9.1	33
24	—	—	—	—	1.0	—	—	4.8	3.1	4.5	1.4	32.4	14.7	62
25	—	—	—	—	0.8	—	—	3.5	4.0	5.9	1.6	17.3	15.9	49
26	—	1.3	0.6	12.5	5.9	—	0.8	24.5	10.2	3.0	2.5	8.9	5.7	76
27	1.4	3.5	1.0	12.4	6.2	0.7	3.7	19.0	7.4	2.9	2.9	6.8	4.4	72
28	0.7	—	—	—	1.9	—	—	3.1	5.1	10.1	1.5	3.6	8.7	35
29	0.6	1.3	0.9	3.4	3.3	0.5	2.9	14.7	4.5	3.6	3.9	20.4	7.9	68
30	0.7	1.8	1.0	3.8	3.5	0.6	2.6	14.2	5.3	3.6	4.1	18.3	7.7	67
31	1.5	2.7	1.7	14.5	5.3	0.6	3.2	27.1	9.7	2.3	2.6	7.8	5.2	84
32	1.3	2.9	1.4	7.7	4.7	0.7	2.9	33.4	10.5	2.0	2.8	7.1	4.1	82

[a]The proportions of molecular weight species exceeding 2 mol% in human milk are presented.
[b]See Table 1 for sample codes.
[c]ACN/DB, acyl carbon number/number of double bonds.
[d]Total proportion of presented molecular weight species in each sample (mol%).

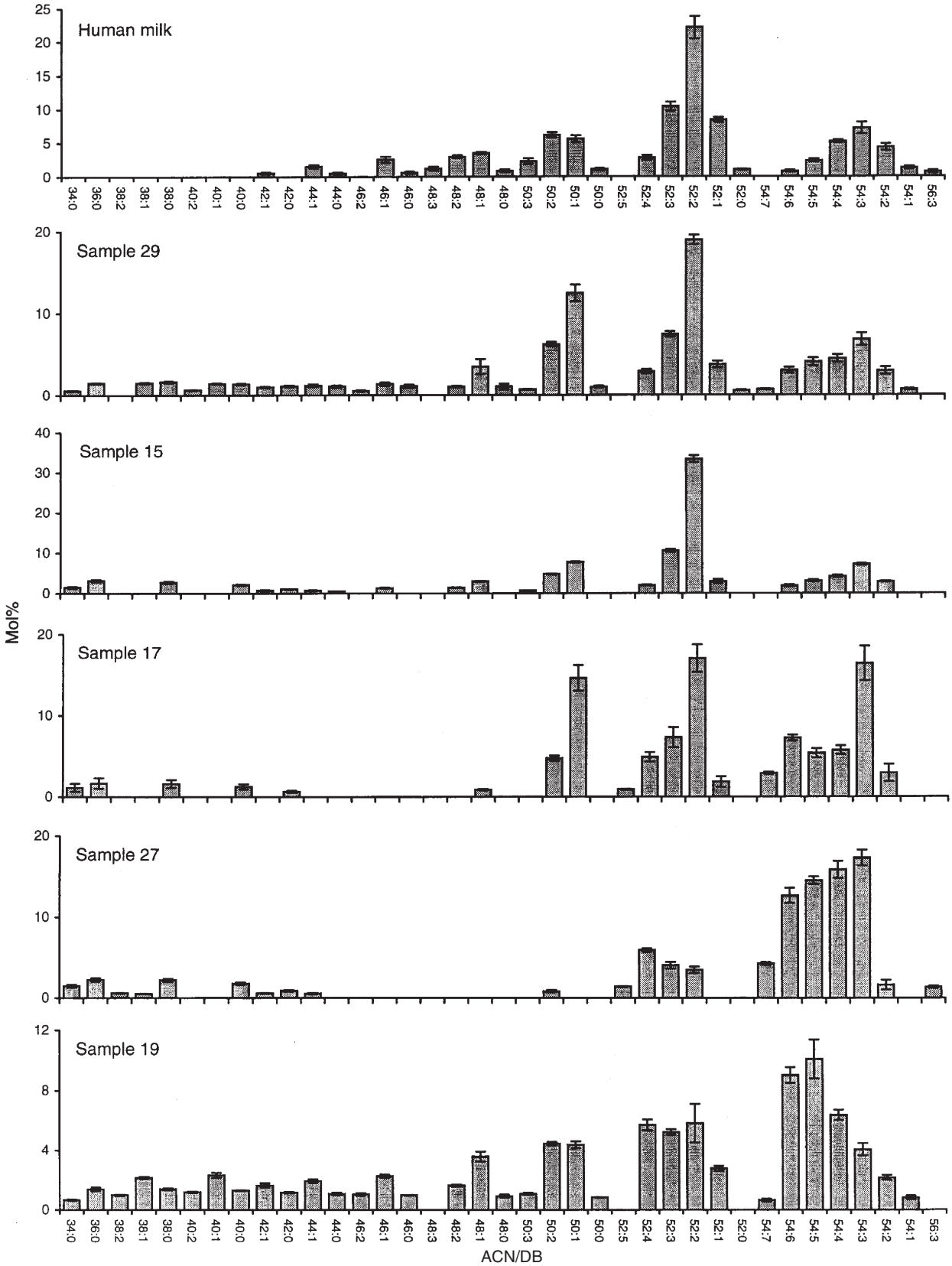

FIG. 1. Examples of molecular weight distribution of human milk and some human milk substitute triacylglycerols as determined by ammonia negative ion chemical ionization mass spectrometry. The values presented are means ± standard deviation (n = 4). ACN, acyl carbon number; DB, number of double bonds. Sample 29: Nursoy (Wyeth Ayerst Inc., Canada); sample 15: Enfalac Infant Formula (Mead Johnson Co., USA); sample 17: Milu-Milk (Milupa, Switzerland); sample 27: Tutteli B (Valio OYj, Finland); sample 19: Milumil 2 (Milupa GmbH & Co., Germany).

shows the TAG molecular weight distribution of human milk and some examples of different human milk substitutes. The relative deviation of four independent analyses was typically 2–5% of the proportion of major molecular species as illustrated in Figure 1. The proportions of ACN 50 and ACN 54 species were around one-third each of that of the major species (ACN 52) (Fig. 1). The molecular weight distribution of TAG in samples 27 and 32 closely resembled the distribution in human milk. The distribution profile in sample 15 was close to that in human milk except for the high proportions of 50:1 and 54:3 species. In samples 17 and 25, ACN 54 species formed the clear majority. In addition, sample 17 contained a wide variety of low molecular weight TAG at ACN values 34–46, which were not present in human milk (Fig. 1).

Regioisomeric structure of TAG. The regioisomeric structure of 52:2, 52:3, 50:1, 54:4, and 54:2 (ACN/DB) TAG in human milk and human milk substitutes was determined. These molecular species constituted 46.1 mol% of total TAG molecular species in human milk. The main regioisomers of the most abundant molecular weight species 52:2 (21.6 mol%) in human milk were 18:1-16:0-18:1 (82.5 mol%) and 18:0-16:0-18:2 (11.1 mol%) as listed in Table 3 (in this manuscript 18:0-16:0-18:2 is used to denote *sn*-18:0-16:0-18:2 + *sn*-18:2-16:0-18:0 and correspondingly for other fatty acid combinations). Regioisomers 18:1-16:0-18:2 (83.1 mol%) and 18:1-18:2-16:0 (10.8 mol%) composed the majority of ACN/DB 52:3 molecular species (Table 4) and 18:1-16:0-16:0 (70.0 mol%) and 18:0-14:0-18:1 (13.4 mol%) were the main regioisomers found in ACN/DB 50:1 (Table 5). Molecular species of 54:4 consisted of regioisomers 18:1-18:2-18:1 (54.5 mol%) and 18:1-18:1-18:2 (45.5 mol%) as shown in Table 6, and the main ACN/DB 54:2 regioisomers were 18:0-18:1-18:1 (77.7 mol%) and 18:1-18:0-18:1 (22.3 mol%) (Table 7). The results were in accordance with corresponding previous results obtained for 52:2 and 52:3, while the small differences in 50:1 and 54:2 may be due to natural variation of human milk samples of different origin (8). The distribution of 16:0, 18:0, 18:1, and 18:2 between *sn*-2 and *sn*-1/3 positions in human milk TAG was calculated using the molecular weight distribution results (Table 2) and regioisomeric structure of single ACN/DB molecular species (Tables 3–7). The proportions of these fatty acids in *sn*-2 position were: 16:0 (81 mol%), 18:0 (11 mol%), 18:1 (10 mol%), and 18:2 (25 mol%). The proportions of corresponding fatty acids in *sn*-2 position calculated on the basis of previously published results (3–6) were in the range of: 16:0 (66–73 mol%), 18:0 (7–23 mol%), 18:1 (8–13 mol%), and 18:2 (16–23 mol%), respectively. Although the regioisomeric structure of only 46.1 mol% of total molecular species in human milk was analyzed in the present study, the comparison shows that the results obtained by different methods are well in accordance. The outcome of the enzymatic and chromatographic methods is usually the total proportions of fatty acids in *sn*-positions of the TAG mixture. Even though it is not possible to distinguish *sn*-1 and *sn*-3 positions of TAG by the mass spectrometric method, the regioisomeric structure of single ACN/DB molecular species can be resolved, which is of importance when nutritional properties of TAG mixtures are considered.

TABLE 3
Regioisomeric Structure of ACN/DB[a] 52:2 Triacylglycerols in Human Milk and Human Milk Substitutes[b]

Sample[c]	*sn*-18:1-16:0-18:1	*sn*-18:0-16:0-18:2 + *sn*-18:2-16:0-18:0	*sn*-18:1-18:1-16:0 + *sn*-16:0-18:1-18:1	*sn*-18:0-18:2-16:0 + *sn*-16:0-18:2-18:0	Total[d]
Human milk	82.5	11.1	3.8	2.7	100
1	17.5	1.0	81.5	—	100
2	21.0	—	79.0	—	100
3	—	—	100	—	100
4	—	—	100	—	100
5	17.7	—	82.3	—	100
6	53.1	—	46.9	—	100
7	11.4	—	88.7	—	100
8	21.9	—	78.1	—	100
9	29.2	—	70.8	—	100
10	7.1	—	92.9	—	100
11	4.0	—	96.0	—	100
12	3.1	—	97.0	—	100
13	5.0	—	95.0	—	100
14	—	—	100	—	100
15	1.5	—	98.5	—	100
16	43.0	—	57.0	—	100
17	88.1	—	11.9	—	100
18	—	—	100	—	100
19	7.9	—	92.1	—	100
20	1.4	—	98.6	—	100
21	1.2	—	98.8	—	100
22	0.5	—	99.5	—	100
23	8.9	—	91.1	—	100
24	—	—	100	—	100
25	25.5	—	74.5	—	100
26	—	—	100	—	100
27	23.1	—	76.9	—	100
28	29.2	1.6	55.8	9.2	96
29	4.1	—	95.9	—	100
30	4.0	—	96.0	—	100
31	61.1	—	39.0	—	100
32	82.2	—	17.9	—	100

[a]For abbreviation see Table 2.
[b]Mol% of total ACN/DB 52:2 molecular species.
[c]See Table 1 for sample codes.
[d]Total proportion of presented molecular weight species.

Table 3 lists the regioisomers of ACN/DB 52:2 TAG in human milk and in the substitutes. The regioisomeric structure of 52:2 species showed the typical 93.6 mol% location of 16:0 in *sn*-2 position in human milk. The corresponding proportion in the substitutes varied in the range of 0–88.1 mol% (Table 1). The distinctive feature in the substitute TAG was that 18:0-16:0-18:2 was not present in most of the samples at all, but 18:1-18:1-16:0 was the major regioisomer in many samples. This is worth notice since the nutritional effect of regiospecific position of palmitic acid in the TAG pool has to be considered. The molar proportion of the main TAG regioisomer 18:1-16:0-18:1 in oil ingredient samples 31 and

TABLE 4
Regioisomeric Structure of ACN/DB[a] 52:3 Triacylglycerols in Human Milk and Human Milk Substitutes[b]

Sample[c]	*sn*-18:1-16:0-18:2 + *sn*-18:2-16:0-18:1	*sn*-18:1-18:2-16:0 + *sn*-16:0-18:2-18:1	*sn*-18:1-18:1-16:1 + *sn*-16:1-18:1-18:1	*sn*-18:2-18:1-16:0 + *sn*-16:0-18:1-18:2	*sn*-18:1-16:1-18:1	Total[d]
Human milk	83.1	10.8	6.2	—	—	100
1	—	100	—	—	—	100
2	33.3	27.2	3.3	2.8	33.3	100
3	8.3	52.7	0.2	38.7	—	100
4	11.4	84.6	0.6	3.4	—	100
5	14.6	31.8	—	53.7	—	100
6	24.3	9.1	—	66.7	—	100
7	6.2	44.2	—	49.6	—	100
8	24.2	51.5	—	24.4	—	100
9	35.0	37.8	—	26.7	—	100
10	33.2	20.7	—	46.1	—	100
11	17.8	59.3	4.5	16	2.5	100
12	1.2	53.6	1	44.2	—	100
13	10.4	80.6	0.8	8.2	—	100
14	1.1	69.5	—	29.5	—	100
15	6.6	62.5	—	30.9	—	100
16	—	92.6	—	7.3	—	100
17	28.7	67.5	2.3	1.4	—	100
18	0.6	63.6	—	35.8	—	100
19	4.0	79.6	—	16.4	—	100
20	8.5	69.2	—	22.3	—	100
21	3.2	52.8	—	44	—	100
22	12.6	51.7	2.4	33.4	—	100
23	—	—	—	—	—	—
24	8.1	68.6	—	23.3	—	100
25	10.4	57.9	1.4	5.3	25	100
26	12.1	70.7	—	17.3	—	100
27	11.7	77.4	—	10.9	—	100
28	—	77.2	—	22.8	—	100
29	21.5	58.0	12.9	4.2	3.4	100
30	20.5	61.3	13.6	3.0	0.9	100
31	100	—	—	—	—	100
32	82.0	71.0	0.3	10.6	—	100

[a]For abbreviation see Table 2.
[b]Mol% of total ACN/DB 52:3 molecular species.
[c]See Table 1 for sample codes.
[d]Total proportion of presented molecular weight species.

TABLE 5
Regioisomeric Structure of ACN/DB[a] 50:1 Triacylglycerols in Human Milk and Human Milk Substitutes[b]

Sample[c]	*sn*-18:1-16:0-16:0 + *sn*-16:0-16:0-18:1	*sn*-16:0-18:1-16:0	*sn*-18:0-14:0-18:1 + *sn*-18:1-14:0-18:0	Total[d]
Human milk	70.0	6.9	13.4	90[e]
1	18.3	81.8	—	100
2	—	—	—	
3	60.4	39.7	—	100
4	—	100	—	100
5	41.0	59.1	—	100
6	69.9	30.1	—	100
7	13.7	86.3	—	100
8	37.4	62.7	—	100
9	73.4	26.7	—	100
10	2.4	97.6	—	100
11	14.1	85.9	—	100
12	0.8	99.2	—	100
13	10.8	89.2	—	100
14	4.6	95.5	—	100
15	0.4	99.6	—	100
16	18.0	82.0	—	100
17	88.6	—	11.5	95
18	2.5	97.5	—	100
19	2.2	97.8	—	100
20	9.3	90.7	—	100
21	8.8	91.2	—	100
22	—	100	—	100
23	—	100	—	100
24	—	—	—	—
25	—	—	—	—
26	8.9	91.1	—	100
27	35.3	64.8	—	100
28	—	—	—	—
29	31.4	63.6	—	95
30	28.2	57.1	10.1	95
31	51.3	48.7	—	100
32	73.2	26.9	—	100

[a]For abbreviation see Table 2.
[b]Mol% of total ACN/DB 50:1 molecular species.
[c]See Table 1 for sample codes.
[d]Total proportion of presented molecular weight species.
[e]Also contained *sn*-18:0-16:0-16:1 + *sn*-16:1-16:0-18:0 (4.6 mol%) and *sn*-18:1-18:0-14:0 + *sn*-14:0-18:0-18:1 (4.5 mol%).

32, and in human milk substitute samples 6 and 17 best resembled the corresponding proportion in human milk (Table 3). The total proportion of 52:2 in samples 6 and 17 was, however, significantly lower than in human milk (Table 2).

The main regioisomers of 52:3 in human milk TAG were 18:1-16:0-18:2 (83.1 mol%) and 18:1-18:2-16:0 (10.8 mol%) followed by 18:1-18:1-16:1 (6.2 mol%) (Table 4). The corresponding highly variable proportions in human milk substitutes were 0–100, 0–100, and 0–13.6 mol% (Table 4). In most of the substitutes, the main regioisomer was 18:1-18:2-16:0, and they contained significant amounts of 18:2-18:1-16:0, which was not present in human milk (Table 4). Also, in 52:3, palmitic acid was generally more abundant in *sn*-2 position in human milk than in the investigated human milk substitutes (Table 4). Only the oil ingredients 31 (100 mol%) and 32 (82.0 mol%) contained over 50 mol% of 18:1-16:0-18:2, the main regioisomer in human milk (83.1 mol%) (Table 4). Also, the proportion of total 52:3 in samples 31 and 32 resembled that in human milk (Table 2).

Figure 2 illustrates the regioisomeric structure of 52:2 and 52:3 in human milk and some human milk substitute samples and also shows the deviation of the analysis, which was typically 5–15% of the proportion of the major molecular species. The proportions of 52:2 in human milk and in sample 10 were close to each other, whereas the proportion in sample 17 was significantly lower and in sample 32 higher than in human milk (Fig. 2A). Therefore, there was great variation in the

TABLE 6
Regioisomeric Structure of ACN/DB[a] 54:4 Triacylglycerols in Human Milk and Human Milk Substitutes[b]

Sample[c]	*sn*-18:1-18:2-18:1	*sn*-18:1-18:1-18:2 + *sn*-18:2-18:1-18:1	*sn*-18:0-18:2-18:2 + *sn*-18:2-18:2-18:0	*sn*-18:2-18:0-18:2	Total[d]
Human milk	54.5	45.5	—	—	100
1	48.3	51.2	0.4	—	100
2	20.7	79.3	—	—	100
3	2.3	97.7	—	—	100
4	36.1	64.0	—	—	100
5	45.0	46.9	8.2	—	100
6	42.2	42.3	9.4	6.1	100
7	43.2	43.4	10.1	3.2	100
8	48.7	49.8	1.6	—	100
9	49.6	46.9	3.6	—	100
10	23.7	70.9	5.5	—	100
11	44.0	44.5	8.4	3.2	100
12	32.0	55.2	10.2	2.7	100
13	50.1	48.0	—	—	98
14	19.2	68.5	12.3	—	100
15	51.0	33.6	7.5	7.9	100
16	68.9	31.3	—	—	100
17	20.8	79.2	—	—	100
18	59.2	32.5	6.5	1.9	100
19	63.7	29.2	5.9	1.1	100
20	39.0	43.1	14.3	3.6	100
21	30.6	63.4	5.9	—	100
22	29.0	68.0	3	—	100
23	50.8	18.9	24.9	5.3	100
24	33.3	63.2	3.4	—	100
25	60.4	35.5	4.2	—	100
26	36.3	53.9	9.8	—	100
27	19.3	80.7	—	—	100
28	30.0	39.9	23.1	7	100
29	26.3	69.7	1.2	2	96
30	34.9	55.6	6.3	3.2	100
31	75.5	21.3	3.2	—	100
32	42.3	53.4	4.2	0.2	100

[a]For abbreviation see Table 2.
[b]Mol% of total ACN/DB 54:4 molecular species.
[c]See Table 1 for sample codes.
[d]Total proportion of presented molecular weight species.

TABLE 7
Regioisomeric Structure of ACN/DB[a] 54:2 Triacylglycerols in Human Milk and Human Milk Substitutes[b]

Sample[c]	*sn*-18:0-18:1-18:1 + *sn*-18:1-18:1-18:0	*sn*-18:1-18:0-18:1	Total[d]
Human milk	77.7	22.3	100
1	100	—	100
2	100	—	100
3	66.7	33.3	100
4	88.5	11.5	100
5	85.1	15.0	100
6	100	—	100
7	50.3	49.7	100
8	78.4	21.6	100
9	78.2	21.9	100
10	93.2	—	93
11	66.6	33.5	100
12	83.7	16.3	100
13	93.6	6.4	100
14	91.8	8.2	100
15	95.3	4.7	100
16	100	—	100
17	100	—	100
18	100	—	100
19	90.0	8.9	99
20	85.4	14.6	100
21	100	—	100
22	91.1	8.9	100
23	73.4	19.0	92
24	100	—	100
25	100	—	100
26	81.2	18.8	100
27	70.8	26.7	98
28	100	—	100
29	80.2	19.8	100
30	100	—	100
31	41.2	56.5	98
32	62.4	37.6	100

[a]For abbreviation see Table 2.
[b]Mol% of total ACN/DB 54:2 molecular species.
[c]See Table 1 for sample codes.
[d]Total proportion of presented molecular weight species.

proportion of 18:1-16:0-18:1 between human milk, sample 17, and sample 32 even though the relative molar proportions of 18:1-16:0-18:1 of total ACN/DB 52:2 were close to each other (human milk, 83 mol%; sample 17, 82 mol%; and sample 32, 88 mol%). Although the proportions of total 52:2 TAG in human milk and in sample 10 were almost equal, the regioisomeric structures differed clearly since 18:1-16:0-18:1 comprised the majority of 52:2 in human milk and 16:0-18:1-18:1 was dominant in sample 10 (Fig. 2A).

Figure 2B shows that the proportions of total 52:3 in human milk, in sample 16, in sample 21, and in sample 32 were approximately at the same level. The regioisomeric structure of 52:3 in these samples was nevertheless clearly different. The regioisomer 18:2-16:0-18:1 constituted the majority of 52:3 in human milk and in sample 32, whereas in sample 16, 52:3 consisted mainly of 16:0-18:2-18:1 and in sample 21 of 16:0-18:2-18:1 (53 mol%) and 18:1-16:1-18:1 (44 mol%) (Fig. 2B). These examples clearly show that TAG mixtures having similar molecular weight distributions may still have highly significant differences in fatty acid combinations and in regioisomeric structure of TAG.

Table 5 shows the regioisomers composing the ACN/DB 50:1 TAG molecular species. The main regioisomers of human milk were 18:1-16:0-16:0 (70.0 mol%), 18:0-14:0-18:1 (13.4 mol%), and 16:0-18:1-16:0 (6.9 mol%). The proportions in human milk substitutes varied in the range of 0–83.6, 0–100, and 0–11.5 mol%, respectively. As shown in Table 5, the relative proportion of 18:1-16:0-16:0 was close

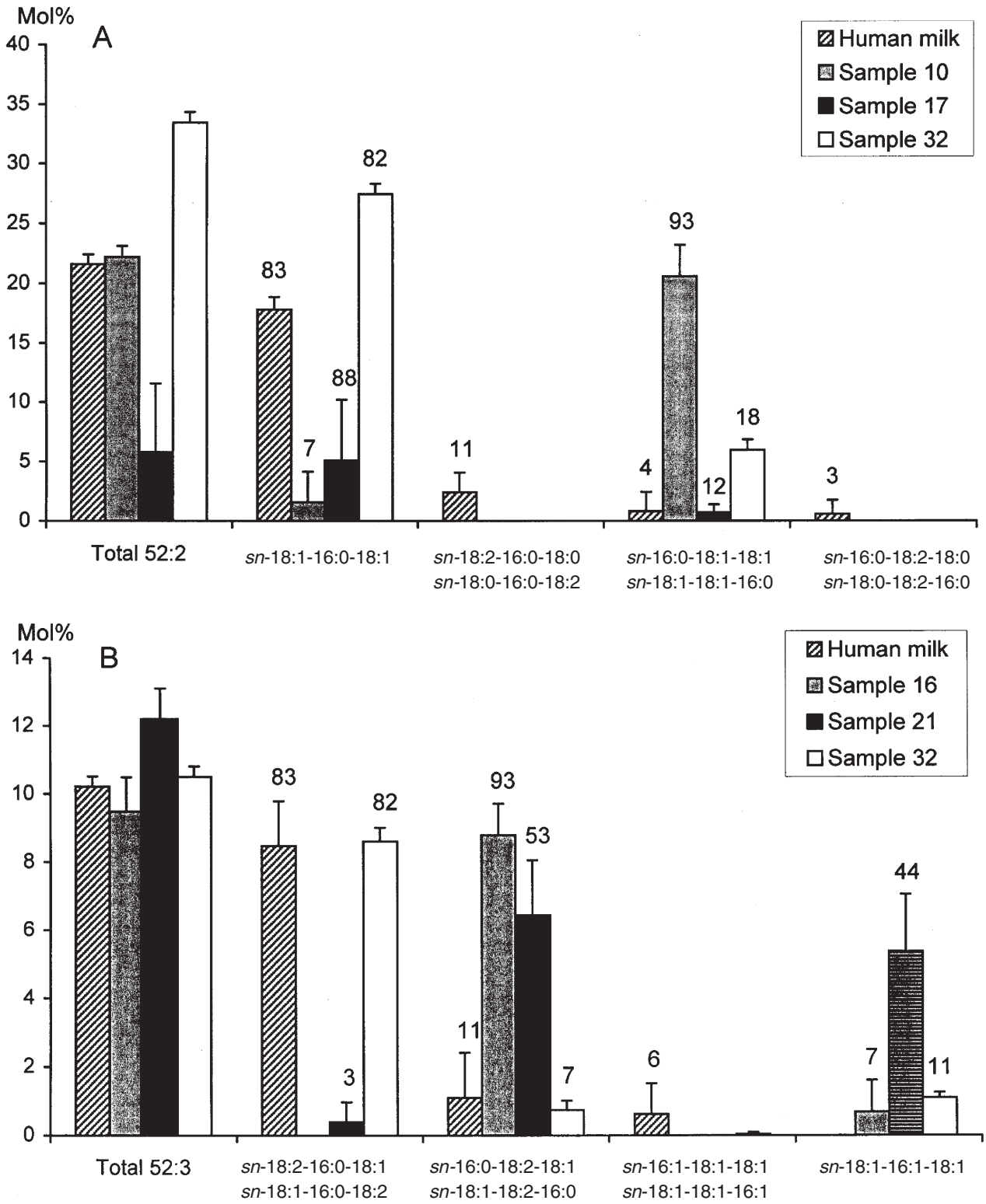

FIG. 2. Total content and regioisomeric structure of ACN/DB 52:2 (A) and 52:3 (B) triacylglycerol molecular species in human milk and in some human milk substitutes as determined by ammonia negative ion chemical ionization mass spectrometry/tandem mass spectrometry. The values presented are means ± standard deviations (n = 4). The numbers above the bars represent the percentage of regioisomers of the total ACN/DB species. Sample 10: Gallia 1 Nursilat (Diepal-NSA, France/Italy); sample 32: Betapol 60 (Loders Croklaan, Holland); sample 16: Bébédor (Milupa, France); sample 21: Alsoy (Nestlé, Italy). See Figure 1 for abbreviations and Sample 17.

to that in human milk in samples 3, 6, 9, 17, and 32, and also the proportion of total ACN/DB 50:1 species in these samples was at the same level as in human milk (Table 2). In many cases, 16:0-18:1-16:0 was the major regioisomer among the substitutes, and 18:0-14:0-18:1 was present only in samples 17 and 30 (Table 5).

The typical feature of ACN 50 and 52 TAG molecular species in human milk was the preferential location of 16:0 in *sn*-2 position (Tables 3–5). Palmitic acid is not present in ACN 54 molecular species. Instead, the preferential location of 18:2 in *sn*-2 position is observed when compared to 18:1, as indicated by the regioisomeric structures of 54:4 in Table 6. The regioisomers were 18:1-18:2-18:1 (54.5 mol%) and 18:1-18:1-18:2 (45.5 mol%). Correspondingly, the preferential location of 18:1 in *sn*-2 position was observed when compared to 18:0 in ACN 54:2 molecular species that consisted of 18:0-18:1-18:1 (77.7 mol%) and 18:1-18:0-18:1 (22.3 mol%) as shown in Table 7. The species of 54:4 in the substitutes consisted mainly of the same regioisomers as in human milk. Some samples also contained 18:2-18:0-18:2, which was not present in human milk TAG (Table 6). The relative proportion of 18:1-18:2-18:1 was generally lower in the substitutes (2.3–75.5 mol%) than in human milk (54.5 mol%), and that of 18:1-18:1-18:2 in human milk substitutes (18.9–97.7 mol%) was higher when compared to human milk (45.5 mol%) (Table 6). TAG 54:2 in the substitutes consisted mainly of the same regioisomers as in human milk (Table 7). The proportion of 18:0-18:1-18:1 was generally higher (41.2–100 mol%) and the proportion of 18:1-18:0-18:1 lower (0–56.5 mol%), as in human milk, in which the corresponding proportions were 77.7 and 22.3 mol%, respectively (Table 7).

The authors want to emphasize that the investigation was not performed to rank the human milk substitutes, but rather to study the differences between TAG in human milk and in the substitutes. It should also be remembered that in addition to lipid composition numerous other quality parameters affect the nutritional value of the products. However, the positional distribution of fatty acids in TAG has been shown to affect infant fat absorption, plasma lipid composition, and mineral balance (9–17). Therefore, the regioisomeric structure of human milk substitute TAG was investigated by applying the tandem mass spectrometric method (8,21–23) and a newly developed automatic spectra interpretation software (19), which allowed fast processing of large quantities of mass spectral data. The results showed that TAG mixtures possessing similar molecular weight distributions may still have significant differences in fatty acid combinations comprising the TAG and in regioisomeric structure of TAG. Great variation in molecular weight distributions and regioisomeric structures of TAG among human milk substitutes was observed. Although the TAG in some substitutes resembled the composition in human milk quite well, none of the substitutes investigated had TAG structures identical with human milk. Because novel methods have enabled the production of specific structured TAG (24,25), there are great possibilities to further develop the TAG composition in human milk substitutes.

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REFERENCES

1. Winter, C.H., E.B. Hoving, and A.J. Muskiet, Fatty Acid Composition of Human Milk Triglyceride Species, *J. Chromatogr. 616*:9–24 (1993).
2. Kuksis, A., and W.C. Breckenridge, Triglyceride Composition of Milk Fats, in *Dairy Lipids and Lipid Metabolism*, edited by M.F. Brink and D. Kritchevsky, AVI Publishing, Westport, CT, 1968, pp. 28–98.
3. Breckenridge, W.C., L. Marai, and A. Kuksis, Triglyceride Structure of Human Milk Fat, *Can. J. Biochem. 47*:761–769 (1969).
4. Christie, W.W., and J.L. Clapperton, Structures of the Triglycerides of Cows' Milk, Fortified Milks (including infant formulae), and Human Milk, *J. Soc. Dairy Technol. 35*:22–24 (1982).
5. Martin, J.-C., P. Bougnoux, J.-M. Antoine, M. Lanson, and C. Couet, Triacylglycerol Structure of Human Colostrum and Mature Milk, *Lipids 28*:637–643 (1993).
6. Weber, K., E. Schulte, and H.-P. Thier, Comparison Between the Triglyceride Patterns of Human Milk and Infant Milk Formulae, *Fat Sci. Technol. 3*:113–115 (1989).
7. Currie, G.J., and H. Kallio, Triacylglycerols of Human Milk: Rapid Analysis by Ammonia Negative Ion Tandem Mass Spectrometry, *Lipids 28*:217–222 (1993).
8. Kallio, H., and P. Rua, Distribution of the Major Fatty Acids of Human Milk Between *sn*-2 and *sn*-1/3 Positions of Triacylglycerols, *J. Am. Oil Chem. Soc. 71*:985–992 (1994).
9. Tomarelli, R.M., B.J. Meyer, J.R. Weaber, and F.W. Bernhart, Effect of Positional Distribution on the Absorption of the Fatty Acids of Human Milk and Infant Formulas, *J. Nutr. 95*:583–590 (1968).
10. Innis, S.M., R. Dyer, and C.M. Nelson, Evidence That Palmitic Acid Is Absorbed as *sn*-2 Monoacylglycerol from Human Milk by Breast-Fed Infants, *Lipids 29*:541–545 (1994).
11. Nelson, C.M., and S.M. Innis, Plasma Lipoprotein Fatty Acids Are Altered by the Positional Distribution of Fatty Acids in Infant Formula Triacylglycerols and Human Milk, *Am. J. Clin. Nutr. 70*:62–69 (1999).
12. Filer, L.J., F.H. Mattson, and S.J. Fomon, Triglyceride Configuration and Fat Absorption by the Human Infant, *J. Nutr. 99*:293–298 (1969).
13. Carnielli, V.P., I.H.T. Luijendijk, J.B.G. Goudoever, E.J. Sulkers, A.A. Boerlage, H.J. Degenhart, and P.J.J. Sauer, Feeding Premature Newborn Infants Palmitic Acid in Amounts and Stereoisomeric Position Similar to That of Human Milk: Effects on Fat and Mineral Balance, *Am. J. Clin. Nutr. 61*:1037–1042 (1995).
14. Lien, E.L., The Role of Fatty Acid Composition and Positional Distribution in Fat Absorption in Infants, *J. Pediatr. 125*:S62–S68 (1994).
15. Carnielli, V.P., I.H.T. Luijendijk, R.H.T. van Beek, G.J.M. Boerma, H.J. Degenhart, and P.J.J. Sauer, Effect of Dietary Triacylglycerol Fatty Acid Positional Distribution on Plasma Lipid Classes and Their Fatty Acid Composition in Preterm Infants, *Am. J. Clin. Nutr. 62*:776–781 (1995).
16. Lucas, A., P. Quinlan, S. Abrams, S. Ryan, S. Meah, and P.J. Lucas, Randomised Controlled Trial of a Synthetic Triglyceride Milk Formula for Preterm Infants, *Arch. Dis. Child. 77*:178–184 (1997).
17. Lien, E.J., F.G. Boyle, R. Yuhas, R.M. Tomarelli, and P. Quinlan, The Effect of Triglyceride Positional Distribution on Fatty

Acid Absorption in Rats, *J. Pediatr. Gastroenterol. Nutr. 25*: 167–174 (1997).
18. Folch, J., M. Lees, and G.H. Sloane-Stanley, A Simple Method for the Isolation and Purification of Total Lipides from Animal Tissues, *J. Biol. Chem. 226*:497–509 (1957).
19. Laakso, P., and H. Kallio, Optimization of the Mass Spectrometric Analysis of Triacylglycerols Using Negative-Ion Chemical Ionization with Ammonia, *Lipids 31*:33–42 (1996).
20. Kurvinen, J.-P., P. Rua, O. Sjövall, and H. Kallio, A Software (MSPECTRA) for Automatic Interpretation of Triacylglycerol Molecular Weight Distribution Spectra and Collision Induced Dissociation Daughter Ion Spectra Obtained by Ammonia Negative Ion Chemical Ionization Mass Spectrometry, *Rapid Commun. Mass Spectrom. 15*:1084–1091 (2001).
21. Kallio, H., and G. Currie, Analysis of Low Erucic Acid Turnip Rapeseed Oil (*Brassica campestris*) by Negative Ion Chemical Ionization Tandem Mass Spectrometry: A Method Giving Information on the Fatty Acid Composition in Positions *sn*-2 and *sn*-1/3 of Triacylglycerols, *Lipids 28*:207–215 (1993).
22. Kallio, H., and and G. Currie, Analysis of Natural Fats and Oils by Ammonia Negative Ion Tandem Mass Spectrometry—Triacylglycerols and Positional Distribution of Their Acyl Groups, in *Handbook of Chromatography*, edited by K.D. Mukherjee and N. Weber, CRC Press, Boca Raton, 1993, pp. 435– 458.
23. Kallio, H., and G. Currie, A Method of Analysis, EP Patent 0566599 (1997).
24. Xu, X., Enzymatic Production of Structured Lipids: Process Reactions and Acyl Migration, *inform 11*:1121–1131 (2000).
25. Xu, X.B., Production of Specific-Structured Triacylglycerols by Lipase-Catalyzed Reactions: A Review, *Eur. J. Lipid Sci. Technol. 102*:287–303 (2000).

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