Positional Analysis of Triglycerides and Phospholipids Rich in Long-Chain Polyunsaturated Fatty Acids

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ABSTRACT: Four sources of long-chain polyunsaturated fatty acids (LCP) differing in their chemical structure (triglycerides or phospholipids) and in their origin (tuna triglycerides, fungal triglycerides, egg phospholipids, and pig brain phospholipids) were analyzed to determine the distribution of the component fatty acids within the molecule. Lipase and phospholipase A_2 hydrolysis was performed to obtain 2-monoacylglycerols and lysophospholipids, respectively, which allowed us to determine the distribution of fatty acids between the *sn*-2 and *sn*-1,3 positions of triglycerides or between the *sn*-1 and *sn*-2 position of phospholipids. Fatty acids in the LCP sources analyzed were not randomly distributed. In tuna triglycerides, half of the total amount of 22:6n-3 was located at the *sn*-2 position (49.52%). In fungal triglycerides, 16:0 and 18:0 were esterified to the *sn*-1,3 (92.22% and 91.91%, respectively)18:1 and 18:2 to the *sn*-2 position (59.77% and 62.62%, respectively), and 45% of 20:3n-6 and only 21.64% of 20:4n-6 were found at the *sn*-2 position. In the lipid sources containing phospholipids, LCP were mainly esterified to the phosphatidylethanolamine fraction. In egg phospholipids, most of 20:4n-6 (5.50%, *sn*-2 vs. 0.91%, *sn*-1) and 22:6n-3 (2.89 vs. 0.28%) were located at the *sn*-2 position. In pig brain phospholipids, 22:6n-3 was also esterified to the *sn*-2 (13.20 vs. 0.27%), whereas 20:4n-6 was distributed between the two positions (12.35 vs. 5.86%). These results show a different fatty acid composition and distribution of dietary LCP sources, which may affect the absorption, distribution, and tissue uptake of LCP, and should be taken into account when supplementing infant formulas.

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Long-chain polyunsaturated fatty acids (LCP) play an important role in the structure and function of cellular membranes and are precursors of important lipid mediators. Moreover, some LCP, especially 22:6n-3, account for a large proportion of the polyunsaturated fatty acids in neuronal membranes. Studies with animals have shown an association between very low levels of 22:6n-3 in the retina and central nervous system and measures of visual and behavioral outcomes (1,2). Therefore, several studies have been carried out in preterm and term infants to evaluate the influence of dietary 22:6n-3, and both 20:4n-6 and 22:6n-3 on visual acuity and cognitive development (3). These reports and the observation that infants fed conventional formula have lower erythrocyte and plasma levels of the n-6 and n-3 LCP led us to consider that infants may benefit from dietary supplementation with 22:6n-3 or both 20:4n-6 and 22:6n-3.

A number of highly unsaturated dietary lipid sources are currently available for supplementing infant formulas with LCP, namely: egg yolk lipids, fish oils, and oils from unicellular organisms (i.e., fungi and algae). These lipid sources differ in their structure [triglycerides (TG) or phospholipids (PL); position of fatty acids in their backbone], fatty acid composition, and the presence of other components.

Lipase-catalyzed hydrolysis of triglycerides (TG) occurs in the mouth, stomach, and small intestine. The products of hydrolysis are free fatty acids (FFA) from the *sn*-1 and *sn*-3 positions and *sn*-2-monoacylglycerols (2-MG), which are readily absorbed, reesterified into triglycerides, and secreted into the lymph in the form of chylomicrons (4). The distribution of fatty acids in the outer and *sn*-2 positions within the molecule governs the lumenal partition between the free and 2-MG forms (5). Under normal dietary conditions, dietary PL constitute a minor portion of the PL presented to the gut for intestinal absorption; the majority are of biliary origin. Biliary PL (primary phosphatidylcholine, PC) are absorbed from the lumen as lysophosphatidylcholine (lysoPC) and are reacylated to PC for the formation of chylomicrons (6). A number of studies have focused on the importance of fatty acids esterified to the *sn*-2 position of TG (7–9). Fat and mineral absorption was higher in rat and infants fed diets containing structured triglycerides with 16:0 mainly in the *sn*-2 position (7). Linoleic acid was also better absorbed when it was esterified to the *sn*-2 position (8). Palmitic acid in the *sn*-2 position may also have some effects on cholesterol metabolism (9). Little information is available about the positional distribution of fatty acids in PL sources and its influence on the absorption and distribution of fatty acids. Recent studies carried out in preterm infants fed breast milk or formula with LCP derived from PL or from TG showed that absorption of 22:6n-3 and n-3 LCP was greater from PL-

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Abbreviations: EFA, essential fatty acids; FFA, free fatty acids; HPTLC, high-performance thin-layer chromatography; LCP, long-chain polyunsaturated fatty acids; LysoPC, lysophosphatidylcholine; LysoPE, lysophosphatidylethanolamine; 2-MG, 2-monoacylglycerols; MONO, monounsaturated fatty acids; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PL, phospholipids; SAT, saturated fatty acids, TG, triglycerides; TLC, thin-layer chromatography.

LCP formula than from breast milk or TG-LCP formula (10). On the other hand, data from animal studies suggest that in the enterocytes, dietary fatty acids provided as TG are predominantly incorporated into chylomicrons, whereas dietary fatty acids provided as PL seemed to be predominantly incorporated into intestinal very low density lipoproteins. If such differences exist, the LCP source could influence the distribution and bioavailability of dietary LCP (11).

Stereospecific analysis of TG has received much attention by many authors in the last few years (12–14). Some methods involving chemical and enzymatic reactions have been reported to analyze positional fatty acid distribution. The chemical method using the Grignard degradation is one of the most reliable methods for obtaining representative diacylglycerols, but it does not give reliable results for the composition of 2-MG because of acyl migration during the reaction (13). Therefore, this method is usually combined with an alternative procedure to obtain the *sn*-2 composition, such as lipase hydrolysis, hydrolysis of PL derived from diacylglycerides, and separation of chiral isomers (15,16). This means *sn*-1, *sn*-2, and *sn*-3 fatty acid composition can be determined separately. Lipase hydrolysis does not give the complete structure of the TG molecule, but it offers reliable information about the *sn*-2 position, which is physiologically the most important. It has been reported that LCP such as 20:4n-6, 20:5n-3, and 22:6n-3 are resistant to lipase hydrolysis when linked to the outer position of the glycerol molecule (13). However, when prolonged incubation times are used, leading to nearly complete hydrolysis, representative 2-MG are obtained (12). Phospholipase A_2 hydrolysis has been used not only to study positional distribution of fatty acids in fungi (17) and bacteria (18) but also to analyze TG structure together with the Grignard reaction (12).

In the present study, we measured lipid and fatty acid composition and fatty acid distribution between the inner and outer positions of TG and PL molecules in four LCP dietary lipid sources currently available for use in infant formulas.

EXPERIMENTAL PROCEDURES

Samples. Two sources of LCP-enriched TG were analyzed: fungal TG and tuna TG. In addition, two sources of LCP-enriched PL were analyzed: one from egg lipids (egg PL) and the other from pig brain (pig brain PL).

Materials. *Rhizopus arrhizus* lipase (EC 3.1.1.3) and bee venom phospholipase A_2 (EC 3.1.1.4) were obtained from Boehringer Mannheim (Mannheim, Germany). TLC plates coated with a 0.25-mm thin layer of silica gel G60 and acetyl chloride were purchased from Sigma Chemicals Co. (St. Louis, MO). High-performance thin-layer chromatography (HPTLC) precoated silica gel 60 plates were purchased from Merck (Darmstadt, Germany).

HPTLC analysis. HPTLC was used to analyze the lipid composition of each lipid source. Plates were washed with chloroform/methanol/water (60:35:1.5, by vol) and activated by heating for 30 min at 100°C. Five μg of oils was placed on the HPTLC plates and developed using chloroform/methanol/acetic acid/water (65:50:1:4, by vol) for polar lipids (19), and hexane/isopropyl ether/acetic acid (75:25:1.5, by vol) for neutral lipids (20). After migration, plates were treated with a solution of 3% copper acetate in 8% *o*-phosphoric acid and heated at 180°C for 10 min. The relative percentage of each lipid was measured by photodensitometry in a Shimadzu CS 9000 scanning densitometer (Kyoto, Japan).

Fatty acid composition of oils. The total fatty acid compositon of the lipid sources were obtained by direct transmethylation according to the method of Lepage and Roy (21). The fatty acid composition of the main PL classes in the sources containing PL were measured after separation of species by TLC (developing solvent: chloroform/methanol/acetic acid/water, 60:40:1:2, by vol) and methylation.

Lipase hydrolysis of TG. The lipase reaction method of Fischer *et al.* (22), with minor modifications was used to hydrolyze the TG sources. Six mg of the lipid sources was sonicated for 10 min with Tris-HCl buffer (40 mM, pH 7.2) containing 50 mM sodium borate (to reduce positional migration of fatty acids) and 0.5% sodium taurocholate. One hundred to 120 units of lipase was added to the mixture and incubated at 22°C for up to 1 h with continuous shaking. The reaction was stopped by the addition of 0.5 mL 0.1 N acetic acid. Lipids were extracted from the assay with chloroform/methanol (2:1, vol/vol). After evaporation of the solvent under N_2, lipid extracts were applied to TLC plates and developed with hexane/isopropyl ether/acetic acid (75:25:1.5, by vol). Bands were visualized by iodine vapors and the band corresponding to 2-MG (*sn*-2) was scraped into test tubes. The lipid fractions were methylated as above (21).

Phospholipase A_2 hydrolysis of phospholipids. Phospholipase A_2 reaction was performed according to a modified procedure of Griffith *et al.* (23). Five mg of the sample was dissolved in 1 mL of diethylether, and 1 mL borate buffer (100 mM, pH 8.9) was added. The mixture was then sonicated for 15 min. Eighty to 200 units of phospholipase A_2 was added to the mixture and incubated with continuous shaking for 30 min at 37°C. Diethylether was then evaporated under N_2, and samples were extracted with chloroform/methanol (2:1, vol/vol). To obtain the different lipid fractions, lipid extracts were subjected to single-dimension double development with hexane/isopropanol/acetic acid (75:25:1.5, by vol) followed by chloroform/methanol/acetic acid/water (65:50:1:4, by vol). The spots were visualized with iodine vapors and the bands corresponding to lysoPC, lysophosphatidylethanolamine (lysoPE), and free fatty acids (FFA) were scraped and collected into tubes and methylated (21). Bee venom phospholipase A_2 hydrolyzes not only PC but also phosphatidylethanolamine (PE), phosphatidylinositol, and phosphatidylserine (24).

Gas–liquid chromatography. Fatty acid methyl esters were separated and quantified by gas–liquid chromatography, using a Hewlett-Packard 5890 gas chromatograph (Hewlett-Packard Co., Palo Alto, CA) equipped with a flame-ionization detector and a 60 m × 0.32 mm internal diameter SP-2330 capillary column (Supelco, Bellefonte, PA). Nitrogen at a flow rate of 1 mL/min was used as the carrier gas, and the

split ratio was 1:40. Temperature programming started at 165°C for 3 min, then was raised 2°C/min to 195°C, held for 2 min at 195°C, increased again at 3°C/min to 211°C, held for 6 min at 211°C, and then decreased at 15°C/min to return to 165°C. The injector and detector temperatures were 275°C. Peaks were identified by comparison with known standards (Sigma, St. Louis, MO).

Calculation. Results were expressed as the means of three replicates in mole percentage of total fatty acids. The fatty acid composition of the *sn*-1,3 position in tryglycerides was calculated from the overall fatty acid composition, and from that of the *sn*-2 position, using the formula: [*sn*-1,3] = 1.5[TG] − 0.5[*sn*-2]. The fatty acid composition of the *sn*-2 position in PL was calculated from the overall fatty acid composition of each PL class and from that of the *sn*-1 position using the formula: [FFA] = 2[PL] − [LysoPL]. The contribution of each PL class to the FFA fractions was corrected by their percentage in the original source. Both *sn*-1,3 in TG and *sn*-2 in PL were calculated from the other positions because they were obtained in the FFA fraction after the corresponding hydrolysis. We verified that, by means of structured standards, the composition of this fraction was less reliable because it was contaminated by acyl migration.

RESULTS AND DISCUSSION

Characterization of the lipid sources. The percentage of each lipid fraction in the original dietary lipid sources was assessed by HPTLC. Tuna TG and fungal TG were composed mainly of TG (>99%), and therefore were subjected to lipase hydrolysis without previous purification. Egg PL was composed of 86.76% PC and 11.13% PE. Pig brain PL was a complex mixture of PL in which PC and PE represented 24.43 and 43.75% of the total PL, respectively.

The overall fatty acid compositions of these lipid sources are shown in Figure 1 and in Tables 1–3. Tuna TG were characterized by a high content of 22:6n-3 and a low ratio of 20:5n-3 to 22:6n-3 (0.29). Saturated and monounsaturated fatty acids were also present, with percentages exceeding 30 and 20% mol of the total fatty acids, respectively. On the contrary, fungal TG contained a high proportion of 20:4n-6, without detectable amounts of 22:6n-3. The availability of oils containing a high proportion of n-6 and n-3 LCP, separately, provides the advantage of varying the n-6/n-3 ratio in infant formulas. Egg PL had a 20:4n-6/22:6n-3 ratio of 1.36. Pig brain PL contained a higher proportion of LCP compared to the egg PL source, with a 20:4n-6/22:6n-3 ratio of 1.31.

Positional analysis of TG. Table 1 gives the fatty acid composition of the TG molecule in the lipid sources containing LCP and that of the *sn*-2 and *sn*-1,3 positions. Likewise, Figure 2 shows the percentages of the total amount of selected fatty acids which were located at the *sn*-2 position (calculated according to the formula % *sn*-2 = [(*sn*-2) × 100]/3 TG). If random distribution among the three positions of the TG molecule was expected, 33.33% of the total amount of a considered fatty acid would be located at the *sn*-2 position. How-

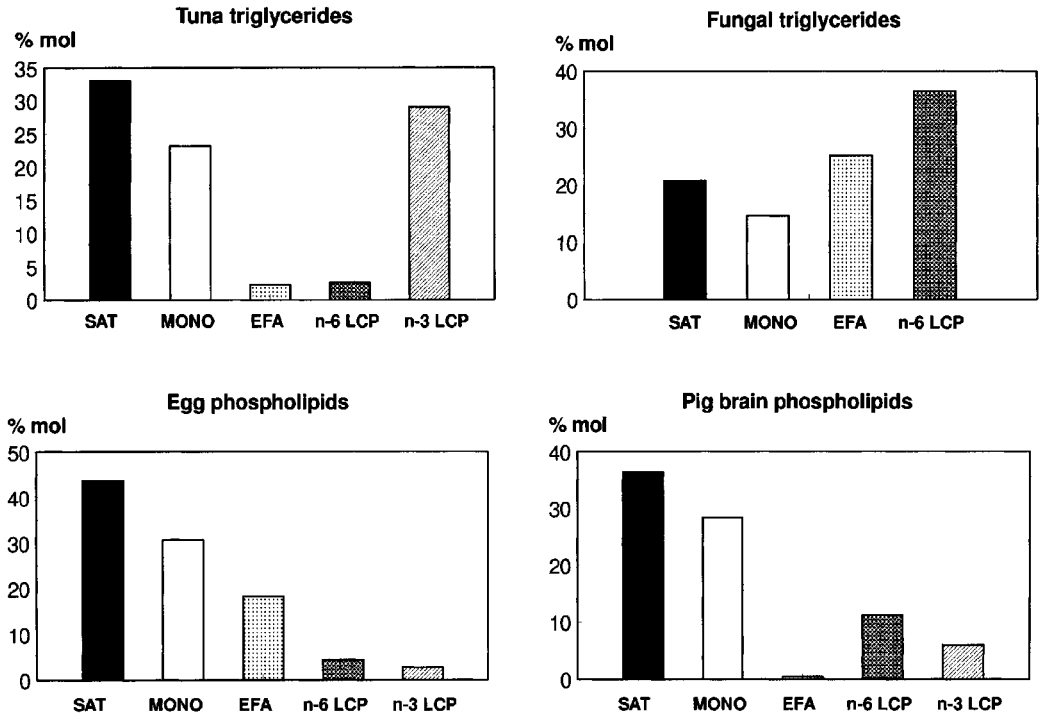

FIG. 1. Fatty acid profile of long-chain polyunsaturated fatty acid (LCP)-enriched dietary lipid. SAT, saturated fatty acids; MONO, monounsaturated fatty acids; EFA, essential fatty acids.

TABLE 1
Fatty Acid Composition of Intact Triglycerides, *sn*-2 and *sn*-1,3 Positions of Triglycerides in LCP-Enriched Dietary Lipid Sources (mol %)[a]

	Tuna TG			Fungal TG		
Fatty acid	TG	*sn*-2	*sn*-1,3	TG	*sn*-2	*sn*-1,3
14:0	4.53	5.12	4.24	—	—	—
16:0	23.25	26.06	21.84	14.49	338	20.05
18:0	5.29	4.66	5.61	6.26	1.52	8.64
18:1n-9	23.21	12.59	28.52	14.65	26.27	8.84
18:2n-6	1.68	2.8	1.12	22.84	42.91	12.8
18:3n-6	0.25	—	0.37	2.5	2.28	2.6
18:3n-3	0.6	0.94	—	2.39	2.64	2.26
18:4n-6	1.48	1.28	1.57	—	—	—
20:2n-6	0.33	—	0.49	0.42	0.54	0.36
20:3n-6	0.21	—	0.32	3.25	4.5	2.63
20:4n-6	2.1	3.12	1.59	28.37	18.42	33.35
20:5n-3	6.53	5.15	6.79-	—	—	—
22:4n-6	0.3	0.59	0.16	4.18	—	6.27
24:1n-9	0.94	1.31	0.76	—	—	—
22:6n-3	22.54	31.13	18.24	—	—	—

[a]TG, fatty acid composition of the total triglyceride; *sn*-2, fatty acid composition of the *sn*-2 monoglycerides obtained after lipase hydrolisis; *sn*-1,3, fatty acid composition of *sn*-1 and *sn*-3 positions obtained as followed [*sn*-1,3] = 1.5[TG] – 0.5[*sn*-2]; LCP, long-chain polyunsaturated fatty acids.

ever, the *sn*-2 position in tuna TG contained 49.52% of the total 22:6n-3. Eicosapentaenoic acid and palmitic acid were almost equally distributed between the *sn*-2 and *sn*-1,3 positions. In fungal TG, 16:0 and 18:0 were mainly esterified to the *sn*-1,3 positions, whereas the opposite was found for 18:1 and 18:2. Forty-five percent of 20:3n-6 and only 21.64% of 20:4n-6 were found at the *sn*-2 position.

Only one work is available on the stereospecific distribution of highly unsaturated oils (12). This study showed the analysis of six polyunsaturated oils containing triglycerides, two of which were quite similar in the total fatty acid and *sn*-2 fatty acid composition to the tuna TG and fungal TG reported here, and were also of tuna and fungal origin.

It has been reported that polyunsaturated fatty acids are distributed within the TG molecule of human milk in a highly specific manner, with 20:4n-6 and 22:6n-3 being located at the *sn*-2 and *sn*-3 positions (13). Although LCP linked to the outer position of the TG molecule are resistant to pancreatic hydrolysis, breast-fed infants may efficiently hydrolyze TG containing LCP due to gastric lipase and bile salt-stimulated lipase. In contrast to human milk, infant formulas do not contain any lipase activity, and consequently the hydrolysis of the *sn*-1 and *sn*-3 ester bonds of TG may decline. In the case of appropriate lipase hydrolysis, LCP absorbed as 2-MG or FFA may undergo different metabolic processes (25). Some researchers suggest a possible substantial blood absorption pathway for polyunsaturated fatty acids with reference to their hydrosolubility properties (26). On the other hand, it has been reported that 20:5n-3 and 22:6n-3 were more readily and efficiently absorbed when the TG administered had a specific intramolecular structure, with medium-chain fatty acids located in the *sn*-1 and *sn*-3 positions and n-3 LCP located in the *sn*-2 position, compared with TG having a random fatty acid distribution among the three positions (27). Our results showed that nearly 50% of 22:6n-3 and more than 20% of 20:4n-6 were esterified to the *sn*-2 position. Fatty acids in the *sn*-2 position may have some unique properties in terms of facilitating fatty acid intestinal absorption and improving

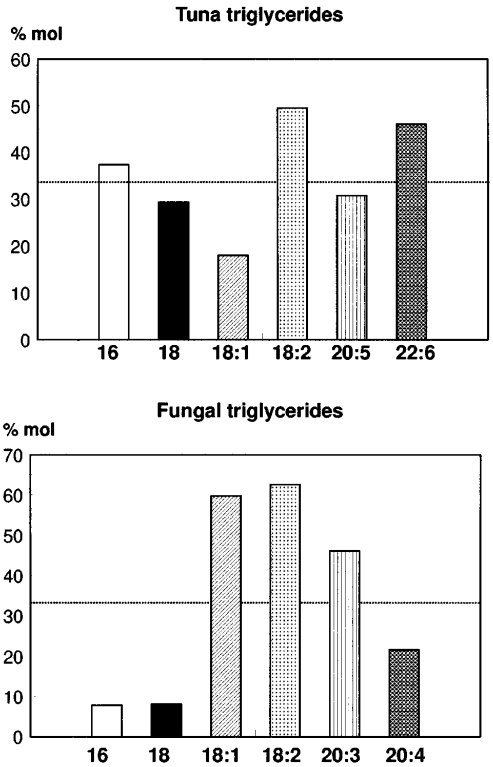

FIG. 2. The *sn*-2 fatty acid composition in triglyceride LCP-enriched lipid sources. The horizontal broken line indicates the proportion of each fatty acid expected (33.33%) on the basis of random distribution. See Figure 1 for abbreviation.

TABLE 2
Fatty Acid Composition of Intact PL, PC, and PE Fractions and the Corresponding Lysophospholipids and Free Fatty Acids in Egg PL (mol%)[a]

				sn-1[b]		
Fatty acids	PL	PC	PE	LysoPC	LysoPE	*sn*-2[c]
16:0	31.56	40.27	23.04	70.49	36.18	9.8
16:1n-9	1.21	1.03	0.7	0.34	—	1.64
18:0	12.1	13.11	26.6	26.26	58.62	0.63
18:1n-9	29.44	27.1	19.17	2.52	2.88	48.65
18:2n-6	17.73	14.75	13.43	0.19	0.77	28.29
18:3n-3	0.56	0.26	0.38	0.1	0.36	0.41
20:2n-6	0.25	0.25	0.34	—	—	0.43
20:3n-6	0.26	0.18	0.42	—	—	0.31
20:4n-6	3.73	1.96	9.26	—	0.28	5.5
22:4n-6	0.22	—	1.06	—	—	0.25
22:6n-3	2.74	1.08	5.6	0.1	0.91	2.89

[a]PL, PC and PE: fatty acid composition of the total phospholipids, phosphatidylcholine and phosphatidylethanolamine fractions, respectively.
[b]Fatty acid composition of the *sn*-1 position obtained from the corresponding lysophospholipid fraction (lysoPC and lysoPE) after phospholipase A_2 hydrolysis.
[c]Fatty acid composition of the *sn*-2 position obtained as follows [FFA]=2[PL] − [LysoPL].

mineral balance (7). However, the influence of this fatty acid distribution on LCP absorption and metabolic fate when supplementing infant formulas needs further research.

Positional analysis of phospholipids. Tables 2 and 3 give the fatty acid composition of intact PL, the fatty acid composition of the major PL fractions, and the fatty acid composition of the products after phospholipase A_2 hydrolysis in egg PL and pig brain PL. The fatty acid composition of lysoPC and lysoPE represents that of the *sn*-1 position in PC and PE, respectively. The fatty acid composition of FFA represents that of the *sn*-2 position in the complete PL. As shown in Tables 2 and 3, the PC fraction contained more 16:0 and 18:1n-9 and less 18:0 than PE in PL from eggs. In pig brain PL, PC contained more 16:0, 18:1n-9, and 18:0 than PE. LCP were mainly esterified to the PE fraction of PL sources, both from egg and pig brain, and were located at the *sn*-2 positions, because they were nearly absent from lysophospholipids. Arachidonic acid in pig brain PL was also present in the *sn*-1 position. On the contrary, saturated fatty acids were mainly esterified to the *sn*-1 position, but oleic acid to the *sn*-2. These results agree with the general assumption that, in tissues, saturated fatty acids were linked to the *sn*-1 and polyunsaturates to the *sn*-2 position of PL.

TABLE 3
Fatty Acid Composition of Intact PL, PC, and PE Fractions and the Corresponding Lysophospholipids and Free Fatty Acids in Pig Brain PL (mol%)[a]

				sn-1[a]		
Fatty acids	PL	PC	PE	LysoPC	LysoPE	*sn*-2[b]
16:0	15.85	45.5	5.01	51.6	12.69	13.76
16:1n-9	0.57	0.41	—	—	—	0.36
18:0	20.54	21.37	14.38	36.97	60.62	—
18:1n-9	23.51	2.36	21.77	11.43	—	49.66
18:2n-6	0.47	0.82	—	—	—	—
18:3n-3	0.54	0.08	1.25	—	3.59	—
20:4n-6	7.43	1.19	10.97	—	5.86	12.35
24:0	1.49	—	—	—	—	—
22:4n-6	2.96	—	9.44	—	3.72	10.75
24:1n-9	4.28	0.85	1.8	—	13.25	—
22:6n-3	5.66	0.42	9.2	—	0.27	13.20
DMA 16:0	3.36	—	6.18	—	[c]	—
DMA 18:0	4.9	—	8.19	—	[c]	—
DMA 18:1n-9	2.72	—	4.16	—	[c]	—
DMA 18:1n-7	3.91	—	5.92	—	[c]	—

[a]Fatty acid composition of the *sn*-1 position obtained from the corresponding lysophospholipid fraction (lysoPC and lysoPE) after phospholipase A_2 hydrolysis.
[b]Fatty acid composition of the *sn*-2 position obtained as follows: [FFA] = 2[PL] − [lysoPL].
[c]Lyso(alkenyl)PE was lost on the thin-layer chromatography plate. For abbreviations see Table 2.

As far as we know, no study has been made about the positional distribution of PL in sources currently used in infant nutrition. Henderson *et al.* (18) and Kendrick and Ratledge (17) used similar methods to analyze the positional distribution of fatty acids in PL isolated from species of bacteria and fungi which contained LCP. Phospholipids enriched in LCP are an available source of these fatty acids and some of them are used currently to supplement infant formulas. Data of animal studies suggest that in the enterocyte, dietary fatty acids provided as TG are predominantly incorporated into chylomicrons. On the contrary, fatty acids provided as PL seem to be predominantly incorporated into very low density particles synthetized at the enterocytes (28). It has been recently reported that infants fed LCP-supplemented formula from egg PL absorbed 22:6n-3 as efficiently as did breast-fed infants (10,11), and better than did infants fed a formula containing LCP-TG (10).

In conclusion, there are many differences in the chemical structure and fatty acid composition and distribution of the currently available dietary sources of LCP for infant formulas. These differences may affect the absorption, distribution, and tissue uptake of these important fatty acids and should be taken into account when supplementing infant formulas.

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