

Effect of Dietary N-3 Fatty Acids Upon the Phospholipid Molecular Species of the Monkey Retina

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Purpose. To characterize the molecular species composition of ethanolamine glycerophospholipids (EGP) in the primate retina and to examine the effects of different dietary fats, the authors fed rhesus monkeys diets containing widely ranging amounts of n-3 fatty acids.

Methods. From birth, infant monkeys were fed either a control soybean oil diet, containing 8% of total fatty acids as 18:3(n-3), or a safflower oil-based n-3 fatty acid deficient diet containing <0.4% 18:3(n-3). A subset of the n-3 deficient group was later repleted with 1.6% ethyl docosahexaenoate, 22:6(n-3), starting at 10 months of age. Tissues were taken from all monkeys upon termination at 21 to 51 months of age. The diacyl, alkenylacyl, and alkylacyl EGPs were quantitated by high-pressure liquid chromatography (HPLC).

Results. Twenty-eight molecular species were identified in the retina of control monkeys. Ether phospholipids comprised 36% of the retinal ethanolamine glycerophospholipids. Species containing polyunsaturated fatty acids in both the sn-1 and sn-2 positions (dipolyenes) were present only in the diacyl subclass and comprised 16% of the total species. Species having n-3 fatty acids in the sn-2 position contributed 59%, 36%, and 70% of total species in the diacyl, alkenylacyl, and alkylacyl subclasses, respectively. In the molecular species of the n-3 fatty acid deficient monkeys, the major change was the loss of most of the 18:0-22:6(n-3) species and its partial replacement with 18:0-22:5(n-6). In contrast, the species 18:1-22:6(n-3) decreased only slightly, from 6.2% to 4.8% of total diacyl species. Although the total concentration of dipolyenes (15% to 20% of the total species) was not affected by diet, their fatty acid compositions were changed drastically. The dipolyene species 22:6(n-3)-22:6(n-3) nearly disappeared in the n-3 deficient monkeys. Concomitantly, two new species, 22:5(n-6)-22:6(n-3) and 22:5(n-6)-22:5(n-6), appeared at 2.6% and 2.0%, respectively. Deficient monkeys given the ethyl ester of 22:6(n-3) in the diet recovered to a near-normal molecular species composition, except in the ether lipids, in which 16:0-20:4 remained low.

Conclusion. Diets of differing n-3 fatty acid content had profound qualitative and quantitative effects on the molecular species of retinal phospholipids, and the replacement of 22:6(n-3) by 22:5(n-6) in the retinas of n-3 deficient monkeys was asymmetric and functionally incomplete. Invest Ophthalmol Vis Sci. 1994;35:794-803.

The phospholipids of cell membranes represent a heterogeneous population of molecular species that occur in characteristic proportions. Different molecular species have different metabolic and physical proper-

ties and thereby influence membrane fluidity and the function and activity of membrane-bound proteins.^{1,2} Analysis of the molecular species of retinal phospholipids, which gives information about the pairing of fatty acids in membrane lipids, can provide the foundation for studies on the biosynthesis of retinal membrane lipids and their relationship to membrane function.

The phospholipid molecular species of the retinas of rainbow trout,³ frog,^{4,5} cow,⁶⁻⁸ and rat⁹ have been studied by several investigators. Retinal membranes, particularly those of photoreceptor outer segments, were found to contain the most highly unsaturated phospholipids of all vertebrate tissues and to be

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Supported by NIH grants DK29930, RR00334 (General Clinical Research Centers), RR00163, DK40566 (Clinical Nutrition Research Units), and DK40935, and by a grant from the National Retinitis Pigmentosa Foundation. This is publication No. 1860 of the Oregon Regional Primate Research Center. Submitted for publication July 21, 1993; revised September 27, 1993; accepted October 1, 1993.

Proprietary interest category: N.

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unique in containing molecular species with polyunsaturated fatty acids in both the sn-1 and sn-2 positions. Docosahexaenoic acid [DHA, 22:6(n-3)] is the major polyunsaturated fatty acid of retinal lipids and is preferentially taken up by photoreceptor cells.¹⁰ This fatty acid is most concentrated in the ethanolamine and serine glycerophospholipids but is also present in the choline and inositol glycerophospholipids. No previous data are available on the phospholipid molecular species composition of the primate retina.

In our previous studies, we found that rhesus monkeys deficient in n-3 fatty acids before and after birth had an 80% to 90% decrease in n-3 fatty acids in the phospholipids of the brain and retina, combined with a compensatory increase in n-6 fatty acids. These reciprocal changes involved primarily the interchange of 22:5(n-6) for 22:6(n-3). Deficient monkeys had impaired visual acuity by 4 weeks of age^{11,12} and abnormal electroretinograms by 3 months of age.^{13,14} At later ages, they showed changes in behavior, including polydipsia.¹⁵

We reported recently that dietary fats with different fatty acid compositions had profound effects on the molecular species of brain phospholipids of rhesus monkeys.¹⁶ In the present study, we characterized the phospholipid molecular species composition in the retina of nonhuman primates. To assess the effects of dietary fats with different amounts and types of n-3 fatty acids, we analyzed the molecular species of retinal ethanolamine glycerophospholipids of monkeys fed four different diets: control (soybean oil-based), n-3 fatty acid deficient (safflower oil-based), 22:6(n-3)-enriched safflower oil, or commercial monkey chow.

MATERIALS AND METHODS

Diets and Animals

Four groups of rhesus monkeys were studied. The first (deficient group, $n = 4$) was made deficient in n-3 fatty acids after birth by feeding a semipurified liquid diet with safflower oil as the only dietary fat source from the day of birth. Their mothers had been fed a standard stock diet (Purina Monkey Chow, Animal Specialties, Hubbard, OR). The second group (control group, $n = 3$) was fed a similar liquid diet with soybean oil as the only fat source. These animals were part of a study of prenatal n-3 fatty acid deficiency in which their mothers had been fed a semipurified safflower oil diet throughout pregnancy. However, plasma and red blood cell levels of n-3 fatty acids rose to control levels by 4 to 8 weeks after birth, and all other biochemical and functional parameters, including the overall fatty acid composition of the retina, were similar to control groups in previous studies in which both mothers and offspring received soybean oil diets. Both the deficient and control groups were killed at 3 to 4 years of age for

detailed biochemical analyses of tissues. A third group, the 22:6(n-3) repletion group ($n = 2$), was made deficient in n-3 fatty acids both before and after birth. Their mothers were fed a safflower diet throughout pregnancy, and the infants received the safflower oil liquid diet from birth until 10 months of age, after which time their diet was supplemented with 22:6(n-3) ethyl ester at 0.25% by weight. These animals were killed at 21 months of age. Our previous measurements of monkey retina and cerebral cortex have shown no difference in overall fatty acid composition or in the degree of 22:6(n-3) depletion in deficient animals in the age range between 10 months and 4 years. A fourth group of monkeys (chow group, $n = 3$), consuming a standard stock diet (Purina Monkey Chow), was included for comparison; they were 2 to 14 years old at termination. All monkeys were cared for according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, as approved by the Animal Care Committee of the Oregon Regional Primate Research Center.

The composition of the experimental diets has been described in detail previously.^{12,13} The fat content of the diets was 5% by weight (13.4% of calories) for pregnant females and 15% by weight (30% of calories) for the offsprings' liquid diets. The fatty acid composition of the experimental diets is presented in Table 1. Safflower oil contains high levels (76%) of linoleic acid [18:2(n-6)] and very low levels (0.3%) of linolenic acid [18:3(n-3)], and the resulting high ratio of these two fatty acids (255:1) exacerbates the effect of the deficient diet by suppressing conversion of linolenic acid to its longer-chain products. The soybean oil control diet provided 53.1% of total fatty acids as linoleic acid and 7.7% as linolenic acid, for a ratio of 6.9:1. The 22:6(n-3) repletion diet contained 1.6% 22:6(n-3) in addition to the low level of linolenic acid. The stock diet supplied 2.3% linolenic acid plus 0.7% longer-chain n-3 fatty acids, including 0.3% 22:6(n-3) derived from fish meal.

Biochemical Analysis

Ethanolamine glycerophospholipid from bovine brain, phospholipase C from *Bacillus cereus*, benzoic anhydride, and 4-dimethylaminopyridine were purchased from Sigma (St. Louis, MO). Chloroform, acetonitrile, 2-propanol, methanol, hexane, and benzene were HPLC grade from Burdick & Jackson (Muskegon, MI), and anhydrous ethyl ether was from Mallinckrodt (Paris, KY).

At the time of autopsy, the neural retinas were rapidly dissected, separated from the retinal pigment epithelium, and stored at -70°C until analyzed. Retinal lipids were extracted by the method of Folch et

TABLE 1. Fatty Acid Composition of Experimental Diets

<i>Fatty Acids</i>	<i>Control (Soy Oil)</i>	<i>Deficient (Safflower Oil)</i>	<i>Repletion (DHA + Safflower Oil)</i>	<i>Commercial Monkey Chow</i>
16:0	10.7	7.1	6.0	19.6
18:0	4.2	2.5	1.8	7.3
18:1 (n-9)	23.7	13.3	9.3	26.3
18:2 (n-6)	53.1	76.0	80.1	37.7
Total n-6	53.4	76.5	80.6	37.7
18:3 (n-3)	7.7	0.3	0.2	2.3
20:5 (n-3)				0.4
22:6 (n-3)			1.6	0.3
Total n-3	7.7	0.3	1.8	3.3
n-6/n-3	6.9	255.0	44.8	11.4

Values are percentage of total fatty acids.

al,¹⁷ and butylated hydroxytoluene (5 mg/100 ml) was added as an antioxidant.¹⁸ Retinal phospholipids were separated by thin-layer chromatography.¹⁹ Ethanolamine glycerophospholipids were extracted from gel scrapings with two washes of 5 ml chloroform-methanol (1:1, vol/vol), followed by one wash with 5 ml chloroform-methanol-water (65:45:12 by volume) and one more with 5 ml chloroform-methanol (1:1, vol/vol).²⁰ Molecular species of ethanolamine glycerophospholipids were analyzed based on the method described by Blank et al.²¹ Briefly, ethanolamine glycerophospholipids were hydrolyzed with phospholipase C for 4 hours at room temperature.²² Diradylglycerols were extracted from the hydrolysate by the Bligh and Dyer method,²³ and benzoate derivatives were prepared by reaction with benzoic anhydride and 4-dimethylaminopyridine for 1 hour at room temperature.²⁴ The reaction was stopped with concentrated ammonium hydroxide, and the resulting diradylglycerobenzoates were extracted with hexane.

Diradylglycerobenzoates were separated into the alkenylacyl, alkylacyl, and diacyl subclasses by thin-layer chromatography on silica gel G with benzene-hexane-ethyl ether (50:45:4; vol/vol/vol). Bands were scraped into a 1:1 ethanol and water mixture, and the diradylglycerobenzoates were extracted with hexane. The samples were then filtered (Millex-HV 0.45 μ m filter unit, Millipore Corp. Bedford, MA), dried under nitrogen, and redissolved in acetonitrile-isopropanol (70/30 vol/vol) for HPLC injection.

Separation of molecular species was accomplished with a Perkin-Elmer Model 410 LC BioPump system fitted with a μ Bondapak C18 precolumn insert and a 3.9 mm \times 30 cm analytical column packed with Nova-pak C18 (Water Associates, Milford, MA). Peaks were monitored at 230 nm with a Perkin-Elmer LC-235 diode array detector and quantitated on a Perkin-Elmer LCI-100 integrator. Molecular species within the diacyl-, alkenylacyl- and alkylacyl-glycerobenzoates

were separated by isocratic elution with acetonitrile-isopropanol in the ratios of 70:30, 65:35, and 63:37 (vol/vol), respectively. Column flow rate was 1 ml/min.

Identification of molecular species was accomplished by comparison with retention times in control samples of bovine brain ethanolamine glycerophospholipid, as established by Blank et al.,²¹ and by gas chromatographic analysis²⁵ of the collected peaks. The elution profile was similar to that obtained by Blank et al.,²¹ except that four additional species were identified as described in our previous paper.¹⁶ The dipolyunsaturated fatty acid molecular species (dipolyenes) were identified by the retention times reported by Louie et al.²⁰ and Stinson et al.⁹ and by gas chromatographic analysis of the collected peaks from HPLC. The stereospecific position of the fatty acids on the glycerol backbone of the dipolyenes was not determined. Such species are reported arbitrarily with the shortest and/or least unsaturated fatty acid in the first position.

Statistical analysis of diet-induced differences in levels of individual molecular species was done by one-way analysis of variance, followed by post hoc testing with the appropriate *t*-statistic.²⁶ Only molecular species showing a significant overall diet effect by analysis of variance ($P < 0.05$) were subjected to the pair-wise post hoc testing. The Bonferroni inequality²⁷ was used to control the overall alpha level of the post hoc testing. This was done by adding pair-wise *P* values less than 0.05. The sum was constructed by ranking the pair-wise *P* values from lowest to highest, progressively adding each *P* value in turn, starting with lowest, until either the sum exceeded 0.05 or until all *P* values had been added. Where the sum exceeded 0.05, the last pair-wise *P* value was discarded, and this last comparison was declared insignificant. In cases where the sum only slightly exceeded the traditional level of significance ($P = 0.05$), the value is nevertheless shown to indicate a trend in the data. Thus, the *P* values listed in the tables represent Bonferroni adjusted *P* values that

apply to the claims of significant differences indicated with appropriate superscripts.

RESULTS

Twenty-eight molecular species were identified in the retinas of control monkeys (Tables 2 to 4). Retinal ethanolamine glycerophospholipids are composed of 64%, 31%, and 5% of the diacyl, alkenylacyl, and alkylacyl subclasses, respectively (Table 5). Dipolyenes were found only in the diacyl subclass and comprised 16% of the total species. The species with n-3 fatty acids at the sn-2 position contributed 59%, 36%, and 70% of the total species in the diacyl, alkenylacyl, and alkylacyl subclasses, respectively.

The n-3 fatty acid deficient safflower oil diet had its greatest effect upon the molecular species contain-

ing 22:6(n-3) in the diacyl subclass of the ethanolamine glycerophospholipids of the retina (Table 2, Fig. 1). The deficient diet affected the monoplyene molecular species (species containing only one polyunsaturated fatty acid), as well as dipolyene molecular species (species containing two polyunsaturated fatty acids). Most notably, the 22:6(n-3)-22:6(n-3) species nearly disappeared, and the 18:0-22:6(n-3) species decreased from 43% to 10% after safflower oil feeding. Among the n-3 fatty acid-containing species, 18:1-22:6(n-3) was the least affected by the safflower oil diet, showing only a slight decrease from 6.2% to 4.8%, which was not statistically significant. These changes were accompanied by reciprocal increases in species containing 22:5(n-6), especially 18:0-22:5(n-6), which increased from 2.6 to 27.1% (Table 2). New dipolyene species, 22:5(n-6)-22:6(n-3) and 22:5(n-6)-22:5(n-6), were detected. However, the relative pro-

TABLE 2. Diet-Induced Changes in the Major Molecular Species of Diacyl Ethanolamine Glycerophospholipid in Monkey Retina

Molecular Species	Control (Soy Oil)	Deficient (Safflower Oil)	Repletion (DHA + Safflower Oil)	P Value
Dipolyenes	15.6 ± 3.6	17.6 ± 8.0	20.8 ± 2.5	
20:3(n-6)-22:6	4.4 ± 1.7	2.5 ± 1.1	4.5 ± 1.1	
20:4-22:6	3.4 ± 0.3	2.2 ± 0.4	3.8 ± 2.7	
22:4-22:6	3.9 ± 1.5	5.8 ± 2.5	3.6 ± 0.8	
22:5(n-6)-22:5(n-6)	0.0 ± 0.0	2.0 ± 2.4	0.0 ± 0.0	
22:5(n-6)-22:6	0.0 ± 0.0	2.6 ± 3.3	0.0 ± 0.0	
22:6-22:6	2.4 ± 0.7 ^a	0.6 ± 1.3 ^a	7.9 ± 4.7 ^b	0.042
Unknown	1.5 ± 1.3	2.0 ± 1.4	1.1 ± 15.	
N-3	58.9 ± 8.7 ^a	16.9 ± 1.6 ^b	46.2 ± 2.5 ^c	0.039
16:0-22:5	0.8 ± 0.3 ^a	0.3 ± 0.3 ^b	0.0 ± 0.0 ^b	0.049
18:0-22:5	0.9 ± 0.6	0.3 ± 0.6	1.2 ± 0.1	
16:0-22:6	7.8 ± 0.8 ^a	2.0 ± 0.7 ^b	5.8 ± 0.7 ^c	0.022
18:0-22:6	43.1 ± 8.8 ^a	9.6 ± 1.4 ^b	35.0 ± 3.6 ^a	0.002
18:1-22:6	6.2 ± 0.1	4.8 ± 1.1	4.2 ± 0.6	
N-6	20.7 ± 3.4 ^a	59.7 ± 9.7 ^b	22.7 ± 2.3 ^a	0.001
16:0-18:2	0.2 ± 0.2	0.6 ± 0.7	0.0 ± 0.0	
18:0-18:2	0.8 ± 0.2	1.0 ± 0.7	2.0 ± 1.4	
16:0-20:3	0.8 ± 0.8	0.6 ± 0.4	1.0 ± 0.3	
18:0-20:3	0.7 ± 0.3	1.0 ± 0.3	1.4 ± 0.3	
16:0-20:4	1.9 ± 0.8	2.1 ± 0.1	1.6 ± 0.2	
18:0-20:4	8.3 ± 0.3 ^a	9.6 ± 0.8 ^b	7.1 ± 0.3 ^a	0.042
18:1-20:4	3.4 ± 0.5	6.0 ± 3.6	5.5 ± 1.4	
16:0-22:4	0.2 ± 0.3	1.4 ± 0.9	0.9 ± 0.2	
18:0-22:4	1.0 ± 0.5	3.7 ± 2.0	1.8 ± 0.6	
18:1-22:5	0.1 ± 0.1 ^a	1.7 ± 0.9 ^b	0.4 ± 0.1 ^a	0.061
16:0-22:5	0.6 ± 0.6 ^a	5.0 ± 0.9 ^b	0.0 ± 0.0 ^a	0.001
18:0-22:5	2.6 ± 1.8 ^a	27.1 ± 8.2 ^b	1.1 ± 0.3 ^a	0.004
N-9/saturated	4.4 ± 2.6	5.4 ± 3.7	8.1 ± 2.1	
16:0-16:0	0.2 ± 0.3	0.8 ± 1.0	1.7 ± 2.0	
17:0-18:1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	
16:0-18:1	2.1 ± 0.9	2.7 ± 2.3	1.4 ± 0.3	
18:0-18:1	1.2 ± 1.1	1.6 ± 1.1	2.7 ± 0.4	
18:1-18:1	0.9 ± 0.5 ^a	0.5 ± 0.6 ^a	2.3 ± 0.0 ^b	0.030

Values are mean ± SD (mol %). Values with unlike superscripts within a given row are different at the indicated *P* value, which reflects a Bonferroni adjustment for all possible pairwise comparisons after ANOVA.

TABLE 3. Diet-Induced Changes in the Major Molecular Species of Alkenylacyl Ethanolamine Glycerophospholipid in Monkey Retina

<i>Molecular Species</i>	<i>Control (Soy Oil)</i>	<i>Deficient (Safflower Oil)</i>	<i>Repletion (DHA + Safflower Oil)</i>	<i>P Value</i>
N-3	36.2 ± 3.4 ^a	13.7 ± 3.2 ^b	38.4 ± 0.6 ^a	0.000
16:0-22:5	1.1 ± 0.4 ^a	0.2 ± 0.2 ^b	0.0 ± 0.0 ^b	0.007
18:0-22:5	1.6 ± 0.5 ^a	0.0 ± 0.0 ^b	1.9 ± 0.7 ^a	0.005
16:0-22:6	7.2 ± 0.5 ^a	2.1 ± 0.3 ^b	5.6 ± 0.5 ^c	0.004
18:0-22:6	20.9 ± 2.1 ^a	6.9 ± 1.0 ^b	24.5 ± 2.1 ^a	0.000
18:1-22:6	5.3 ± 2.8	4.5 ± 2.3	6.5 ± 1.5	
N-6	55.8 ± 4.2 ^a	78.6 ± 5.2 ^b	48.1 ± 4.2 ^a	0.001
16:0-18:2	0.5 ± 0.4	0.9 ± 1.1	0.0 ± 0.0	
18:0-18:2	1.6 ± 0.2	1.3 ± 0.9	2.7 ± 1.1	
16:0-20:3	1.5 ± 0.9	1.1 ± 0.2	1.4 ± 0.3	
18:0-20:3	1.4 ± 0.4	1.3 ± 0.7	2.0 ± 0.4	
16:0-20:4	11.7 ± 1.2 ^a	7.0 ± 2.2 ^b	7.4 ± 0.6 ^b	0.047
18:0-20:4	30.9 ± 4.2	31.6 ± 3.9	22.8 ± 1.6	
18:1-20:4	4.7 ± 0.9	4.5 ± 0.5	6.0 ± 1.6	
16:0-22:4	0.7 ± 0.7 ^a	2.9 ± 1.1 ^b	1.3 ± 0.2 ^{a,b}	0.021
18:0-22:4	2.0 ± 0.8	5.3 ± 3.4	2.8 ± 2.1	
18:1-22:5	0.9 ± 0.2 ^a	4.9 ± 1.0 ^b	1.9 ± 0.1 ^a	0.003
16:0-22:5	0.0 ± 0.0 ^a	5.1 ± 0.9 ^b	0.0 ± 0.0 ^a	0.000
18:0-22:5	0.0 ± 0.0	12.8 ± 1.9	0.0 ± 0.0	
N-9/Saturated	7.2 ± 1.5	7.0 ± 5.4	11.1 ± 2.3	
16:0-16:0	0.1 ± 0.2	0.5 ± 0.8	0.0 ± 0.0	
17:0-18:1	1.1 ± 0.8	1.3 ± 1.6	3.6 ± 1.7	
16:0-18:1	4.0 ± 1.0	3.4 ± 2.9	2.0 ± 0.4	
18:0-18:1	1.4 ± 1.0	1.2 ± 1.2	2.8 ± 0.6	
18:1-18:1	0.6 ± 0.9 ^a	0.7 ± 0.7 ^a	2.8 ± 0.4 ^b	0.038

Values are mean ± SD (mol %). Values with unlike superscripts within a given row are different at the indicated *P* value, which reflects a Bonferroni adjustment for all possible pairwise comparisons after ANOVA.

portion of total dipolyene molecular species was not significantly affected by the deficient diet.

The molecular species compositions of the alkenylacyl and alkylacyl ethanolamine glycerophospholipids are shown in Tables 3 and 4, respectively. Interestingly, there were no dipolyene fatty acid species found in these subclasses regardless of dietary background. For the monopolyene fatty acid species, the two subclasses were affected by diet in a manner similar to the diacyl group, except for the unexpected result that the 16:0-20:4 alkenylacyl species was significantly reduced in the safflower oil group.

Feeding 22:6(n-3)-supplemented safflower oil to n-3 fatty acid-deficient monkeys almost restored their retinal phospholipid molecular species composition to the pattern found in control monkeys (Tables 2 to 4). However, some abnormalities remained. In the alkenylacyl subclass, 16:0-20:4(n-6) remained at the lower level induced by safflower oil feeding. In other words, this species did not return to control levels when the n-3 fatty acid deficiency was alleviated. In the alkylacyl subclass, a minor component of the ethanolamine glycerophospholipids, the reversal was the least pronounced. In fact, the differences in total n-3 and total n-6 molecular species between the deficient and reple-

tion diets did not reach statistical significance. In addition, feeding 22:6(n-3) had some effects that went beyond reversal of n-3 fatty acid deficiency. In the diacyl subclass, the proportion of 22:6(n-3)-22:6(n-3) was increased over that found in control animals. Another curious effect of the 22:6(n-3)-supplemented diet was a small but significant rise in the proportion of 18:1-18:1 in the diacyl and alkenyl subclasses and 18:0-18:2 in the alkylacyl subclass, compared to both control and deficient groups.

The ratio of sn-1 18:0 species to sn-1 16:0 species, for a given sn-2 fatty acid, was different in the different subclasses of ethanolamine glycerophospholipid. For instance, in both the diacyl and alkenylacyl subclasses, the predominant fatty acid paired with sn-2 22:6(n-3) was 18:0. In the alkylacyl subclass, on the other hand, sn-1 18:0 and 16:0 were about equally paired with 22:6(n-3). For diacyl species with 20:4 in the sn-2 position, sn-1 18:1 was more prevalent than 16:0. The opposite was true in the alkenyl and alkyl subclasses. These relationships were not affected by diet.

Because the HPLC detector response is proportional to the molar concentration of the glycerobenzoate derivatives of the different molecular species,²¹

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