Distribution of Δ5-Olefinic Acids in the Triacylglycerols from *Pinus koraiensis* and *Pinus pinaster* Seed Oils

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ABSTRACT: Purified triacylglycerols (TAG) from *Pinus koraiensis* and *P. pinaster* seed oils, which are interesting and commercially available sources of Δ5-olefinic acids (i.e., *cis*-5,*cis*-9,*cis*-12 18:3 and *cis*-5,*cis*-11,*cis*-14 20:3 acids) were fractionated by reversed-phase high-performance liquid chromatography, and each fraction was examined by capillary gas–liquid chromatography for its fatty acid composition. A structure could be assigned to more than 92% of TAG from both oils. In both instances, ca. 48% of the TAG were shown to contain at least one Δ5-olefinic acid. In the great majority of TAG, our data showed that there is only one molecule of Δ5-olefinic acid per molecule of TAG. This is compatible with theoretical calculations based on the proportion of total Δ5-olefinic in the oils. The *cis*-5,*cis*-9,*cis*-12 18:3 acid (14.2 and 8.6% of total fatty acids in the seed oils of *P. koraiensis* and *P. pinaster*, respectively) and the *cis*-5,*cis*-11,*cis*-14 20:3 acid (1.1 and 8.1% of total fatty acids in the seed oils of *P. koraiensis* and *P. pinaster*, respectively) are preferentially associated with two molecules of linoleic acid, and to a lesser extent, to one molecule of linoleic acid and one molecule of oleic acid, or two oleic acid molecules. However, several other combinations occur, each in low amounts. The distribution of Δ5-olefinic acids in TAG is evidently not random. Combining these results with the known preferential esterification of Δ5-olefinic acids to the 1,3-positions of TAG would suggest that most of these acids are present at only one of these positions at a time.

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KEY WORDS: *cis*-5 Olefinic acids, distribution profile, *Pinus koraiensis, pinaster*, reversed-phase high-performance liquid chromatography, triacylglycerols.

In addition to the common 18:1n-9, 18:2n-6, and 18:3n-3 acids, conifer seed oils contain several *cis*-Δ5-unsaturated polymethylene-interrupted fatty acids (Δ5-UPIFA, or Δ5-olefinic acids) with various structures (1–8). All conifer seed oils contain *cis*-5,*cis*-11 20:2 acid in small amounts, and *cis*-5,*cis*-11,*cis*-14 20:3 acid may be a major constituent in some species. Pinaceae seed oils are characterized by relatively high levels of *cis*-5,*cis*-9 18:2 and particularly of *cis*-5,*cis*-9,*cis*-12 18:3 acids, with practically no 18:3n-3 acid, whereas Cupressaceae and Taxodiaceae seed oils contain high amounts of 18:3n-3 and Δ5-UPIFA derived from this acid: traces of *cis*-5,*cis*-9,*cis*-12,*cis*-15 18:4 acid, and in greater amounts, *cis*-5,*cis*-11,*cis*-14,*cis*-17 20:4 acid (6). Cupressaceae and Taxodiaceae seed oils also contain the elongation product of 18:3n-3 acid, the *cis*-11,*cis*-14,*cis*-17 20:3 acid (6). All of these acids have been structurally characterized without ambiguity by several techniques (1–8), including capillary gas–liquid chromatography (GLC) on columns with several different stationary phases, mass spectrometry of appropriate derivatives, ^{1}H- and ^{13}C-nuclear magnetic resonance (NMR) spectroscopy, silver-ion thin-layer chromatography (TLC), and convenient chemical degradative procedures.

The Δ5-UPIFA are more concentrated in the 1,3-positions of triacylglycerols (TAG) than in their 2-position. This was demonstrated by Takagi and Itabashi (3) who used chemical degradative techniques, and more recently by Gunstone *et al.* (7) through ^{13}C-NMR spectroscopy. In the latter study, no Δ5-UPIFA could be detected, within the limits of the method, in the 2-position of TAG from the seeds of seven *Pinus* species, including *P. koraiensis* and *P. pinaster* (the method cannot detect Δ5-UPIFA if they are less than 3% of fatty acids esterified to a given position).

Some conifer (i.e., *Biota orientalis*, *P. koraiensis*, and *P. pinaster*) seed oils exhibit peculiar biochemical properties. They are hypocholesterolemic and hypotriglyceridemic, at least in the rat (9,10, and C.C. Bayard, and R.L. Wolff, unpublished observations), and these effects have been attributed to the presence of Δ5-UPIFA in these oils. The *cis*-5,*cis*-11,*cis*-14 20:3 acid present in *B. orientalis* seed oil can replace arachidonic acid in mice hepatic phosphatidylinositol (11). Such oils might thus have some pharmaceutical applications in the future, or at least, serve as new tools to explore fatty acid metabolism.

However, conifer seed oils are not easily available for animal experiments. In France, only three kinds of conifer seeds are used or produced on a multiton scale. The seeds from *P. koraiensis* (imported from China) are used for edible purposes (pine nuts), but also for the production of an oil with some cosmetic applications. *Pinus koraiensis* seeds are relatively rich in pinolenic (*cis*-5,*cis*-9,*cis*-12 18:3) acid (*ca.* 15%). *Pinus pinea* seeds (imported from several Mediterranean countries) have the same applications as those from *P.*

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koraiensis, but their Δ5-UPIFA content is low—less than 4% (5). Consequently, *P. pinea* seeds are not interesting as a source of Δ5-UPIFA. *Pinus pinaster* seeds are harvested in the southwest region of France for forest planting. The forest of *P. pinaster*, called "pignada," covers approximately 2.3 million acres and is exploited for its wood. In addition to pinolenic acid (7–8%), *P. pinaster* seed oil contains 7–8% of *cis*-5,*cis*-11,*cis*-14 20:3 acid (5), an acid sharing three double bonds in common with arachidonic acid. Several other conifer species might also be of interest as Δ5-UPIFA sources (6), but their seeds are not yet harvested, except for two Cupressaceae species, *Juniperus communis* in Europe and *Biota orientalis* in China, for edible or pharmaceutical purposes.

To better characterize conifer seed oils, we have focused our attention in the present study on the distribution profile of TAG in the seed oils from both *P. koraiensis* and *P. pinaster*. For this purpose, we have used a combination of reversed-phase high-performance liquid chromatography (RP-HPLC) of TAG and of GLC of fatty acid methyl esters (FAME) that were prepared from the resulting fractionated TAG. One of our main observations was that pine seed oil TAG that contain Δ5-UPIFA generally contain only one *cis* Δ5-olefinic acid molecule per molecule of TAG. Consequently, the concentration of TAG that contain Δ5-UPIFA may be high in pine seed oils (almost one-half).

EXPERIMENTAL PROCEDURES

Oil samples. The oil from *P. koraiensis* seeds (cold-pressed) was provided by the Bertin Society (Lagny-le-Sec, France). Seeds from *P. pinaster* were given by the D'a Noste Society (Vendays-Montalivet, France). The seeds (undehulled) were cold-pressed at the Bertin Society's facilities. Except for filtration, the oils were not further processed.

TAG purification and fractionation. TAG were purified by silicic-acid column chromatography according to Hirsch and Ahrens (12). For this purpose, 45 g of silicic acid was introduced in a 1-cm i.d. glass column and washed successively with acetone, diethyl ether and pentane (100 mL each). The crude oils (500 mg in pentane) were fractionated with 200 mL of several mixtures of diethyl ether in pentane (3, 8, 20, and 30% by vol), 200 mL of pure diethyl ether, and 200 mL of methanol. Fractions of 25 mL were collected, and their compositions were assessed by TLC. All those fractions that contained pure TAG were pooled and concentrated.

To fractionate TAG by RP-HPLC, a Model 6000A solvent delivery system and an R401 differential refractometer (Waters Associates, Milford, MA) were used. The 250 × 4.0 mm i.d. Hibar LichroCART Superspher RP-18 (4-μm particles) column and the Lichrosorb RP-18 precolumn were purchased from Merck (Darmstadt, Germany). The fractionations were carried out isocratically at a constant temperature of 15°C with acetone/acetonitrile (55:45, vol/vol) as the mobile phase at a rate of 1 mL/min. The solvents (analytical grade; Merck) were vacuum-degassed for 1 min before use. For each fractionation, approximately 2 mg of TAG in 50 μL acetone were injected. Peak areas were measured by means of an Enica 21 (Delsi Instruments, Paris, France) recorder–integrator. Each detected fraction was collected manually at the outlet of the refractometer for further processing (13). A known amount of triheptadecanoin was added as an internal standard to each fraction for quantitation of TAG by GLC.

FAME preparation and analysis. The fatty acid composition of the collected TAG fractions (including the internal standard) was determined by GLC of the methyl esters prepared from 0.5M sodium methoxide in methanol (14). The analyses were performed on a Becker-Packard Model 417 (Packard, Rungis, France) gas chromatograph, equipped with a 30 m × 0.4 mm i.d. laboratory-made capillary column coated with Carbowax 20M (Applied Sciences Labs., State College, PA), operated at a constant temperature of 195°C with a nitrogen flow-rate of 3 mL/min. Injections were made with a moving glass-needle injector (SPIRAL, Dijon, France). Detection was through a flame-ionization detector. Peak areas were measured with an Enica 21 integrator (Delsi Instruments). Calibration factors for quantitative determinations were calculated with standard mixtures of FAME (Nu-Chek-Prep, Elysian, MN).

To help in the identification of TAG that contained a Δ5-octadecatrienoic acid as compared to those containing a Δ9-octadecatrienoic acid, methyl esters of these acids were analyzed by RP-HPLC for the determination of their elution characteristics. The column was the same as for TAG, but the operating conditions were: acetone/bidistilled water (90:10, vol/vol) as a mobile phase at a flow rate of 1 mL/min and a temperature of 30°C (15).

Calculations. To identify TAG peaks, several determinations were carried out for each collected TAG peak from its fatty acid composition. The mean carbon number (CN) was

TABLE 1
Fatty Acid Compositions (mole %) of Triacylglycerols Purified from the Seed Oils of *Pinus koraiensis* and *P. pinaster*

Fatty acid[a]	*P. koraiensis*	*P. pinaster*
16:0	4.4	3.8
9-16:1	0.1	0.2
18:0	2.3	2.8
9-18:1[b]	29.4	18.8
5,9-18:2	2.2	1.1
9,12-18:2	43.9	52.2
5,9,12-18:3	14.2	8.6
9,12,15-18:3	0.2	1.3
20:0	0.3	0.3
11-20:1	1.2	1.1
5,11-20:2	0.1	0.8
11,14-20:2	0.6	0.9
5,11,14-20:3	1.1	8.1
ΣΔ5[c]	17.6	18.6

[a]Ethylenic bonds are in the *cis* configuration. Fatty acids are listed according to their elution order from the Carbowax 20M capillary column (Applied Sciences Labs., State College, PA).
[b]Contains small amounts of 11-18:1 acid.
[c]Sum of fatty acids containing a Δ5-ethylenic bond.

calculated from the acyl carbon number of all fatty acids and their percentages in the mixture. The mean unsaturation number (UN) was calculated from the number of ethylenic bonds of each component fatty acid and its percentage in the mixture. The equivalent carbon number (ECN) was calculated according to formula in Equation 1 (16):

$$ECN = CN - 2 \times UN \quad [1]$$

For both oils, a random TAG composition was calculated by assuming that the fatty acids were randomly distributed between the three positions of the glycerol moiety. The formulas used were those proposed by Bailey as shown in Equations 2–4 (17):

$$\%\ \text{of TAG (A A A)} = (a \times a \times a) \times 10^{-4} \quad [2]$$

$$\%\ \text{of TAG (A A B)} = (a \times a \times b) \times 3 \times 10^{-4} \quad [3]$$

$$\%\ \text{of TAG (A B C)} = (a \times b \times c) \times 6 \times 10^{-4} \quad [4]$$

in which a, b, and c are the percentages of fatty acids A, B, and C, respectively, relative to total fatty acids in unfractionated TAG.

RESULTS AND DISCUSSION

Fatty acid composition of TAG. The fatty acid compositions of purified TAG from the seed oils of *P. koraiensis* and *P. pinaster* (as mole %), based on identifications according to Wolff and Bayard (5) and Wolff *et al.* (6), are given in Table 1. They do not differ significantly from the corresponding fatty acid compositions previously established for the crude oils (5). This means that the oils are essentially TAG, an observation that was confirmed by TLC examination of the fractions collected after silicic-acid column chromatography of the crude oil. Only trace amounts of components with R_f identical to those of sterol esters and free fatty acids could be detected. These results confirm that *P. koraiensis* seed oil TAG are characterized by a high amount of *cis*-5,*cis*-9,*cis*-12 18:3 acid (*ca.* 14%), whereas the seed oil TAG from *P. pinaster* contain both this acid and *cis*-5,*cis*-11,*cis*-14 20:3

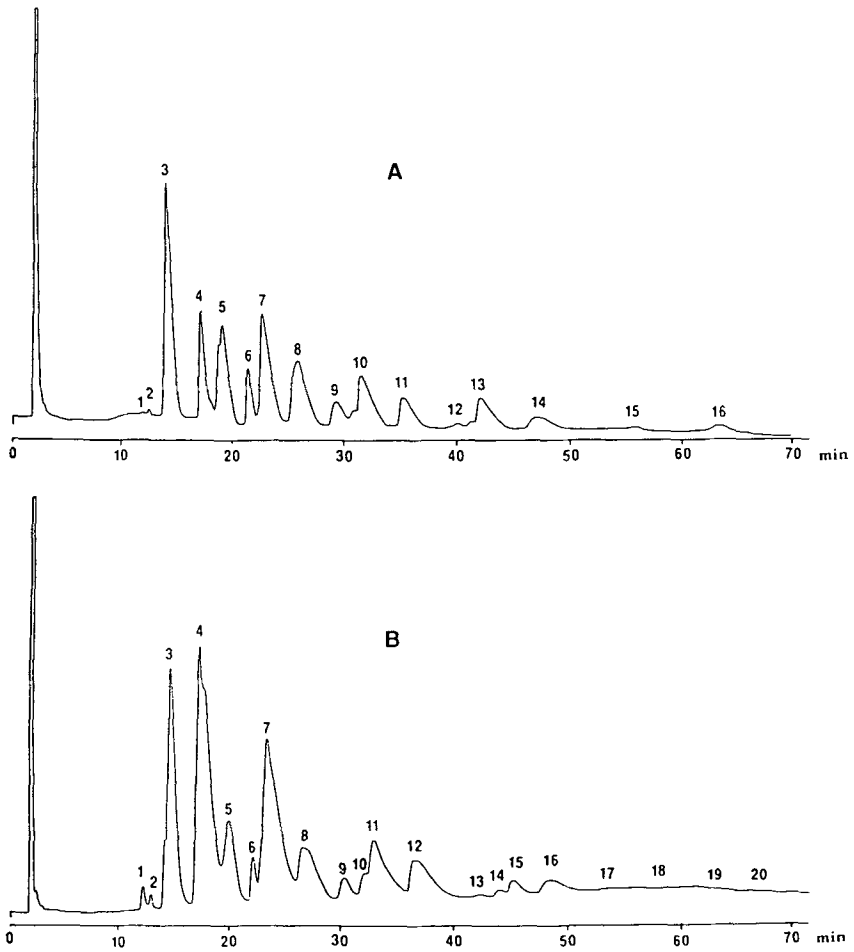

FIG. 1. Chromatographic profiles of triacylglycerols in the oils from *Pinus Koraiensis* (A) and *P. pinaster* (B) seeds after fractionation by reversed-phase high-performance liquid chromatography. Peak numbering refers to Tables 2 to 5. Operating conditions as described in the text.

TABLE 2
Triacylglycerol Distribution in *Pinus koraiensis* Seed Oil After Reversed-Phase High-Performance Liquid Chromatography (mole % of each fraction) and Fatty Acid Composition of Individual Fractions (mole% in each fraction).

	Fraction number (molar %)[b]																
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	Total
Fatty acid[a]	(0.1)	(0.2)	(17.0)	(7.9)	(12.1)	(3.6)	(13.8)	(12.1)	(3.2)	(9.2)	(5.8)	(1.3)	(7.4)	(3.9)	(1.0)	(1.4)	TAG[c]
16:0	—	0.4	0.2	0.2	0.05	27.1	1.0	7.9	15.3	0.9	16.5	14.2	0.5	14.4	12.7	2.5	4.8
16:1	—	0.1	0.1	0.1	0.1	—	0.1	0.2	0.2	0.2	0.05	0.30	0.05	—	0.2	0.5	0.1
18:0	—	3.1	0.1	0.1	0.1	0.5	0.1	0.1	14.4	0.3	8.5	17.8	0.3	12.6	10.4	23.9	2.3
9-18:1	2.0	1.9	0.2	0.7	23.8	1.7	30.4	40.6	12.0	56.6	29.5	20.9	91.5	47.4	41.9	51.3	29.2
5,9-18:2	—	—	0.05	0.4	7.3	0.6	—	3.9	5.3	—	5.1	9.9	—	—	2.5	—	2.2
9,12-18:2	49.0	32.8	64.9	92.2	41.6	35.4	64.2	23.6	23.2	35.2	35.9	16.3	3.5	19.6	9.7	12.0	43.9
5,9,12-18:3	25.3	61.3	34.0	2.4	25.3	33.7	1.7	20.8	27.9	1.1	1.4	14.4	0.2	0.1	1.8	0.4	14.3
9,12,15-18:3	23.6	0.4	0.3	0.1	0.3	0.3	0.1	0.3	0.1	—	0.1	0.4	0.05	—	—	—	0.2
20:0	—	—	—	—	—	—	—	—	—	—	—	1.6	—	3.4	1.1	5.6	0.3
11-20:1	—	—	0.05	—	—	—	0.05	1.4	0.5	3.4	1.2	1.6	3.0	1.6	19.0	3.4	1.1
5,11-20:2	—	—	—	—	—	—	0.3	0.1	—	0.3	0.2	—	0.3	0.3	—	0.1	0.1
11,14-20:2	—	—	—	0.2	0.9	0.7	1.0	0.2	0.9	0.9	0.3	1.7	0.7	0.4	0.3	0.3	0.5
5,11,14-20:3	—	—	0.2	3.6	0.7	0.1	1.2	1.0	0.1	1.0	1.2	0.9	0.05	0.3	0.3	0.1	1.0
CN	54.0	54.0	54.0	54.2	54.1	52.4	54.1	53.7	53.2	54.3	53.2	53.5	54.2	53.5	54.5	54.4	
UN	7.4	7.6	7.0	6.1	6.1	5.3	5.1	4.9	4.7	4.2	3.7	3.8	3.1	2.7	2.8	2.4	
ECN	39.2	38.8	40.0	41.9	42.0	41.8	43.9	43.9	43.8	45.9	45.9	45.9	48.0	48.0	48.9	49.5	

[a]Ethylenic bonds are in the *cis* configuration. CN, carbon number; UN, unsaturation number; ECN, equivalent carbon numnber (ECN = CN − 2 × UN).
[b]Data just beneath the peak numbers (1 to 16) refer to Figure 1 and are the calculated molar percentages of triacylglycerols in each isolated fraction. Values facing fatty acids are the molar percentages of fatty acids in triacylglycerols from each isolated peak.
[c]Values reconstituted by calculation from the molar proportions of individual triacylglycerol (TAG) fractions and from their fatty acid compositions.

acid, each in equivalent amounts (*ca.* 8% for each acid). The sums of all Δ5-UPIFA are practically the same for TAG from both oils (17–18%).

TAG distribution profile. TAG from *P. koraiensis* gave sixteen identifiable peaks upon RP-HPLC fractionation, whereas those from *P. pinaster* gave twenty fractions (Fig. 1). Their molar proportions and their fatty acid compositions (mole %) are reported in Tables 2 and 3. Interestingly, the arithmetically reconstituted fatty acid compositions of total TAG, based on the proportions and the compositions of individual fractions, closely agree with those experimentally determined with unfractionated TAG (compare last columns in Tables 2 and 3 with Table 1). This emphasizes the quantitative aspect of the procedures used in this study. Although all CN lie in the narrow range 52.4–55.0 (which was expected, due to the reduced diversity of chain lengths, with a large majority of C_{18} acids), the experimental ECN span from *ca.* 39 for the fast-eluting TAG fraction to 49.5 for the last-eluting TAG fraction. This is due to a progressive decrease in the mean number of ethylenic bonds in the TAG fractions, a normal situation in RP-HPLC.

For both oils, identification of TAG present in each collected fraction was tentatively achieved from its fatty acid composition, from the values of CN, UN, and ECN, and from the general rules of TAG elution order by RP-HPLC. For TAG species with a Δ5-olefinic acid, analysis by RP-HPLC of the methyl esters of Δ5- and Δ9-octadecatrienoic acids showed that the elution volume of the Δ5 isomer was higher than that for the Δ9 isomer (results not shown). This property is similar to that observed with octadecenoic isomers (18). Accordingly, a TAG species with a Δ5-olefinic acid will elute later than the corresponding TAG species with a Δ9 isomer under isocratic conditions.

Considering the complex fatty acid pattern of several fractions, it is clear that not all of them correspond to a pure TAG species. A pure TAG fraction should theoretically contain at most three different fatty acids, in molar proportions equal to or multiple of 33%. Only a few peaks show fatty acid compositions compatible with such values [peaks 2, 3, 4, 6, 7, 10, and 13 for *P. koraiensis* (Table 2), peaks 6, 7, 11, and 15 for *P. pinaster* (Table 3)]. This situation is explained by the fact that, in RP-HPLC, TAG species with different CN and a different number of double bonds, but the same ECN, may elute under the same peak. Moreover, some slight cross-contamination between adjoining peaks may have occurred during collection of TAG fractions.

Hereafter, and for the sake of clarity, the following abbreviations for fatty acids will be used in the text: 18:2(Δ5), 5,9-18:2; 18:2(Δ9), 9,12-18:2; 18:3(Δ5), 5,9,12-18:3; 18:3(Δ9), 9,12,15-18:3; 20:3(Δ5), 5,11,14-20:3 (all ethylenic bonds are in the *cis* configuration). Tables 4 and 5 list the TAG species found in *P. koraiensis* and *P. pinaster* seed oils, respectively. The percentages of TAG species in the collected fractions were calculated from the percentages of the three component fatty acids. Based on such calculations, the proportions of 50 different TAG species could be determined in *P. koraiensis*, accounting for 94% of total TAG. The corresponding figures for *P. pinaster* were 57 TAG species and 91.8% of total TAG. Among these TAG species, 45.6 and 44.1% contained at least one Δ5-UPIFA in *P. koraiensis* and *P. pinaster* seed oils, respectively. This corresponds, on a relative basis, to 48.5 and 48.0% of identified

TABLE 3
Triacylglycerol Distribution in *Pinus pinaster* Seed Oil After Reversed-Phase High-Performance Liquid Chromatography (mole % of each fraction) and Fatty Acid Composition of Individual Fractions (mole % in each fraction)

	Fraction number (molar %)[b]										
Fatty acid[a]	1 (0.7)	2 (0.6)	3 (14.1)	4 (19.0)	5 (7.7)	6 (2.2)	7 (19.5)	8 (7.8)	9 (2.3)	10 (1.6)	11 (8.1)
16:0	5.2	8.6	0.4	0.2	1.1	27.9	0.9	18.3	9.7	3.8	1.0
16:1	4.6	7.5	0.5	0.2	0.8	1.9	0.4	0.7	1.6	1.9	0.5
18:0	2.3	3.9	0.1	0.1	0.2	1.1	0.2	0.8	20.3	2.0	0.3
9-18:1	6.6	10.8	0.8	1.2	23.3	4.2	27.8	17.8	8.5	13.5	55.7
5,9-18:2	0.7	0.7	0.3	0.2	5.3	0.4	—	2.7	3.3	0.1	—
9,12-18:2	36.8	26.1	60.7	79.5	35.0	31.8	56.1	40.0	27.2	46.5	29.0
5,9,12-18:3	21.7	36.0	30.2	1.4	22.8	28.8	1.4	9.1	25.8	4.3	0.4
9,12,15-18:3	20.3	2.2	4.3	1.1	1.9	1.8	0.4	1.2	0.7	0.9	0.1
20:0	—	0.5	—	0.05	0.4	0.4	—	—	0.1	0.05	0.3
11-20:1	0.5	—	—	—	—	—	0.2	1.5	0.4	16.7	3.0
5,11-20:2	—	—	—	0.1	0.6	—	2.0	0.6	—	1.2	3.0
11,14-20:2	—	—	—	0.9	0.7	0.9	2.1	0.2	1.6	7.9	0.3
5,11,14-20:3	1.2	1.9	2.7	15.2	5.9	0.9	8.4	7.6	1.2	1.2	6.5
CN	53.5	53.2	54.2	55.0	54.3	52.4	54.7	53.5	53.5	55.3	54.7
UN	6.5	5.9	7.1	6.5	6.1	5.0	5.4	4.8	4.7	4.9	4.3
ECN	40.5	41.4	40.0	42.0	42.2	42.3	43.9	43.9	44.1	45.5	46.0

	Fraction number (molar %)[b]									
Fatty acid[a]	12 (6.3)	13 (0.9)	14 (1.0)	15 (2.2)	16 (3.6)	17 (1.0)	18 (0.1)	19 (1.2)	20 (0.2)	Total TAG[c]
16:0	14.2	23.4	9.0	2.4	10.3	23.7	—	6.1	17.4	4.2
16:1	0.7	3.7	5.6	1.2	1.2	8.0	11.7	4.2	17.6	0.2
18:0	15.3	12.5	2.0	1.24	15.9	13.5	17.5	16.6	12.6	2.7
9-18:1	17.7	15.6	32.8	83.0	35.3	20.7	38.0	39.4	34.7	18.8
5,9-18:2	1.2	5.4	0.7	—	—	1.1	—	0.7	—	1.1
9,12-18:2	40.7	20.2	24.0	2.7	26.2	17.7	—	17.4	17.8	52.6
5,9,12-18:3	1.0	10.8	1.1	0.2	0.3	4.4	—	—	—	8.7
9,12,15-18:3	0.3	—	0.2	—	—	—	—	0.2	—	1.4
20:0	0.2	2.7	0.2	0.1	3.4	3.0	—	7.1	—	0.3
11-20:1	0.8	2.8	18.8	2.6	1.4	5.4	20.8	3.6	—	1.0
5,11-20:2	1.1	—	0.9	2.4	1.6	0.7	—	0.6	—	0.7
11,14-20:2	0.3	2.2	3.7	2.1	1.4	0.6	—	—	—	0.8
5,11,14-20:3	7.2	0.9	1.0	1.7	3.0	1.1	—	4.1	—	7.4
CN	53.7	52.9	54.6	54.3	54.0	52.7	54.5	54.3	50.4	
UN	3.9	3.4	3.7	3.2	3.2	2.7	2.8	2.9	2.2	
ECN	45.9	46.1	47.2	47.9	47.6	47.3	48.9	48.5	46.0	

[a]Ethylenic bonds are in the *cis* configuration. CN, carbon number; UN, unsaturation number; ECN, equivalent carbon number (ECN = CN −2 × UN).
[b]Data just beneath the peak numbers (1 to 20) refer to Figure 1 and are the calculated molar percentages of triacylglycerols in each isolated fraction. Values facing fatty acids are the molar percentages of fatty acids in triacylglycerols from each isolated peak.
[c]Values reconstituted by calculation from the molar proportions of individual triacylglycerol (TAG) fractions and from their fatty acid compositions.

TAG species. If all Δ5-UPIFA were present as an isolated component in TAG species, the theoretical proportions of such TAG species should have been 52.7 and 55.8%, respectively, in the seed oils of *P. koraiensis* and *P. pinaster*. This indicates that almost 90% of the Δ5-UPIFA-containing TAG species are esterified by only one Δ5-UPIFA, which was indeed experimentally verified. This observation, together with the fact that Δ5-UPIFA are mainly esterified to positions 1 and 3 of TAG (3,7), would suggest that Δ5-UPIFA are mainly present in TAG species in either the 1- or the-3-position at a time.

In *P. koraiensis* seed oil, where 18:3(Δ5) acid is the major Δ5-UPIFA, the main TAG species (Table 4) are 18:2(Δ9), 18:3(Δ5) (16.4%); 18:1, 18:2(Δ9), 18:3(Δ5) (8.5%); and 18:1, 18:1, 18:3(Δ5) (7.3%). Other abundant species are 18:1, 18:2(Δ9), 18:2(Δ9) (12.5%); 18:1, 18:1, 18:2(Δ9) (7.7%); trilinolein (6.7%); and triolein (6.5%). The minor 18:2(Δ5) acid is also preferentially esterified together with two linoleic or two oleic acid molecules (total, 3.6%). However, several other combinations occur, but in amounts less than 2% of total TAG. TAG species with two Δ5-UPIFA are scanty. From an analytical point of view, trilinolein [18:2(Δ9), 18:2(Δ9), 18:2(Δ9)] (fraction 4) and its isomer 18:2(Δ9), 18:2(Δ9), 18:2(Δ5) (fraction 5) are quite well separated, though both TAG species have the same ECN.

The situation is more complex in *P. pinaster* seed oil (Table

TABLE 4
Triacylglycerol Species Composition of the Oil from *Pinus koraiensis* Seeds

Fraction number	Proportion[a] (mole %)	CN[b]	Class[c]	ECN[d]	TAG species[e]	Concentration in the fraction (mole %)	Concentration in total TAG (mole %)
1	0.1	54	2 3 3	38	18:2(Δ9) 18:3(Δ9) 18:3(Δ5)	72.0	0.06
2	0.2	54	2 3 3	38	18:2(Δ9) 18:3(Δ5) 18:3(Δ5)	92.0	0.20
3	17.1	54	2 2 3	40	18:2(Δ9) 18:2(Δ9) 18:3(Δ5)	96.0	16.44
4	7.9	54	2 2 2	42	18:2(Δ9) 18:2(Δ9) 18:2(Δ9)	85.0	6.68
		56	2 2 3	42	18:2(Δ9) 18:2(Δ9) 20:3(Δ5)	11.0	0.86
		52	1 2 2	42	16:1 18:2(Δ9) 18:2(Δ9)	0.4	0.03
5	12.1	54	1 2 3	42	18:1 18:2(Δ9) 18:3(Δ5)	70.0	8.45
		54	2 2 2	42	18:2(Δ9) 18:2(Δ9) 18:2(Δ5)	21.0	2.53
		56	2 2 3	42	18:2(Δ9) 20:2(Δ11) 18:3(Δ5)	2.4	0.29
		54	1 2 3	42	18:1 18:2(Δ5) 18:3(Δ9)	1.0	0.12
6	3.6	52	0 2 3	42	16:0 18:2(Δ9) 18:3(Δ5)	81.0	2.89
		54	1 2 3	42	18:1 18:2(Δ5) 18:3(Δ5)	2.0	0.07
7	13.9	54	1 2 2	44	18:1 18:2(Δ9) 18:2(Δ9)	90.0	12.47
		54	1 2 3	42	16:1 18:2(Δ9) 20:3(Δ5)	3.0	0.42
		56	2 2 2	44	18:2(Δ9) 18:2(Δ9) 20:2(Δ11)	3.0	0.42
		54	1 2 2	44	16:1 18:2(Δ9) 20:2(Δ5)	0.8	0.11
8	12.1	54	1 1 3	44	18:1 18:1 18:3(Δ5)	60.0	7.28
		52	0 2 2	44	16:0 18:2(Δ9) 18:2(Δ9)	24.0	2.91
		56	1 2 3	44	20:1 18:2(Δ9) 18:3(Δ5)	4.0	0.49
		56	1 2 3	44	18:1 18:2(Δ5) 20:3(Δ5)	3.0	0.36
		50	0 1 2	44	16:0 16:1 18:2(Δ9)	0.5	0.06
9	3.2	54	0 2 3	44	18:0 18:2(Δ9) 18:3(Δ5)	42.0	1.33
		52	0 1 3	44	16:0 18:1 18:3(Δ5)	33.0	1.05
		52	0 2 2	44	16:0 18:2(Δ9) 18:2(Δ5)	15.0	0.48
10	9.2	54	1 1 2	46	18:1 18:1 18:2(Δ9)	84.0	7.69
		56	1 2 2	46	20:1 18:2(Δ9) 18:2(Δ9)	10.3	0.95
		56	1 2 2	46	18:1 18:2(Δ9) 20:2(Δ11)	2.6	0.24
		56	0 2 3	46	18:0 20:2(Δ5) 18:3(Δ5)	0.9	0.08
11	5.8	52	1 1 2	46	16:0 18:1 18:3(Δ9)	50.0	2.89
		54	1 2 2	44	18:1 18:2(Δ9) 18:2(Δ9)	22.0	1.27
		54	1 1 2	46	18:1 18:1 18:2(Δ5)	1.0	1.10
		56	0 2 3	46	18:0 18:2(Δ9) 20:3(Δ5)	3.6	0.21
12	1.3	54	0 1 3	46	18:0 18:1 18:3(Δ5)	2.0	0.27
		52	0 1 2	46	16:0 18:1 18:2(Δ5)	18.0	0.23
		50	0 0 2	46	16:0 16:0 18:2(Δ9)	12.0	0.15
		54	0 2 2	46	18:0 18:2(Δ9) 18:2(Δ5)	9.0	0.12
		54	0 2 3	46	18:0 18:2(Δ9) 18:3(Δ5)	5.0	0.06
13	7.4	54	1 1 1	48	18:1 18:1 18:1	88.0	6.53
		56	1 2 2	46	18:1 18:2(Δ9) 18:2(Δ9)	9.0	0.67
		56	1 1 2	48	18:1 18:1 20:2(Δ5)	0.8	0.06
		56	1 1 2	48	18:1 18:1 20:2(Δ11)	0.8	0.06
14	3.9	52	0 1 1	48	16:0 18:1 18:1	42.0	1.65
		54	0 1 2	48	18:0 18:1 18:2(Δ9)	36.0	1.42
		56	0 2 2	48	20:0 18:2(Δ9)18:2(Δ9)	10.0	0.39
15	1.0	56	1 1 1	50	18:1 18:1 20:1	30.0	0.30
		56	0 1 2	50	18:0 20:1 18:2(Δ9)	30.0	0.30
		50	0 0 0	50	16:0 16:0 16:0	12.7	0.13
16	1.4	52	0 1 1	50	18:0 18:1 18:1	65.0	0.88
		56	0 1 2	50	20:1 18:1 18:2(Δ9)	17.0	0.23
		54	0 1 1	50	16:0 18:1 20:1	9.0	0.12

[a]Calculated by reference to 17:0 acid methyl ester added as an internal standard prior to gas–liquid chromatography of fatty acid methyl esters prepared with the triacylglycerol (TAG) fraction.
[b]Carbon number: sum of carbon atoms in fatty acids.
[c]Number of double bonds in each of the three fatty acids.
[d]Equivalent carbon number: CN minus twice the total number of ethylenic bonds.
[e]Abbreviations used for TAG species: 18:2(Δ9),9,12-18:2; 18:3(Δ9),9,12,15-18:3; 18:2(Δ5),5,9,18:2; 18:3(Δ5),5,9,12-18:3; 20:2(Δ11),11,14-20:2; 20:2(Δ5),5,11-20:2; 20:3(Δ5),5,11,14-20:3.

TABLE 5
Triacylglycerol Species Composition of the Oil from *Pinus pinaster* Seeds

Fraction number	Proportion[a] (mole %)	CN[b]	Class[c]	ECN[d]	TAG species[e]	Concentration in the fraction (mole %)	Concentration in total TAG (mole %)
1	0.7	54	2 3 3	38	18:2(Δ9) 18:3(Δ9) 18:3(Δ5)	63.0	0.45
2	0.6	54	2 3 3	38	18:2(Δ9) 18:3(Δ5) 18:3(Δ5)	54.0	0.33
3	14.1	54	2 2 3	40	18:2(Δ9) 18:2(Δ9) 18:3(Δ5)	88.0	12.36
		56	2 3 3	40	18:2(Δ9) 18:3(Δ9) 20:3(Δ5)	8.0	1.12
4	19.0	54	2 2 2	42	18:2(Δ9) 18:2(Δ9) 18:2(Δ9)	50.0	9.49
		56	2 2 3	42	18:2(Δ9) 18:2(Δ9) 20:3(Δ5)	46.0	8.73
		56	2 2 3	42	18:2(Δ9) 20:2(Δ11) 18:3(Δ9)	3.0	0.57
5	7.7	54	1 2 3	42	18:1 18:2(Δ9) 18:3(Δ5)	70.0	5.38
		56	2 2 3	42	18:2(Δ9) 18:2(Δ5) 20:3(Δ5)	15.0	1.15
		52	0 2 3	42	16:0 18:2(Δ9) 18:3(Δ9)	4.0	0.31
		56	2 3 2	42	18:2(Δ9) 18:3(Δ9) 20:2(Δ5)	1.8	0.14
6	2.2	52	0 2 3	42	16:0 18:2(Δ9) 18:3(Δ5)	84.0	1.81
		52	1 1 3	42	16:1 18:1 18:3(Δ5)	6.0	0.13
		54	1 2 3	42	18:1 18:2(Δ5) 18:3(Δ5)	1.2	0.03
7	19.6	52	1 2 2	44	18:1 18:2(Δ9) 18:2(Δ9)	71.0	13.90
		58	1 3 3	44	18:1 20:3(Δ5) 20:3(Δ5)	13.0	2.55
		58	2 2 3	44	20:2(Δ5) 20:2(Δ5) 18:3(Δ5)	3.0	0.59
		56	2 2 2	44	18:2(Δ9) 18:2(Δ9) 20:2(Δ11)	3.0	0.59
8	7.8	52	0 2 2	44	16:0 18:2(Δ9) 18:2(Δ9)	55.0	4.31
		56	1 2 3	44	18:1 18:2(Δ9) 20:3(Δ5)	22.0	1.72
		54	1 1 3	44	18:1 18:1 18:3(Δ5)	13.5	1.06
		54	1 2 2	44	18:1 18:2(Δ9) 18:2(Δ5)	3.6	0.28
		56	1 2 3	44	20:1 18:2(Δ5) 18:3(Δ9)	4.5	0.35
9	2.3	54	0 2 3	44	18:0 18:2(Δ9) 18:3(Δ5)	61.0	1.41
		52	0 1 3	44	16:0 18:1 18:3(Δ5)	19.0	0.44
		52	0 2 2	44	16:0 18:2(Δ9) 18:2(Δ5)	10.0	0.23
10	1.6	56	1 2 2	46	20:1 18:2(Δ9) 18:2(Δ9)	50.0	0.81
		56	1 2 2	46	18:1 18:2(Δ9) 20:2(Δ11)	21.0	0.34
		56	1 1 3	46	18:1 20:1 18:3(Δ5)	13.0	0.21
11	8.1	56	1 1 2	48	18:1 18:1 18:2(Δ9)	78.0	6.33
		56	1 2 2	46	18:1 18:2(Δ9) 20:2(Δ5)	9.0	0.73
		56	1 3 3	42	18:1 18:3(Δ9) 20:3(Δ5)	9.0	0.73
12	6.3	52	0 1 2	46	16:0 18:1 18:2(Δ9)	42.0	2.65
		52	0 2 2	48	18:0 18:2(Δ9) 18:2(Δ9)	25.0	1.58
		56	0 2 3	46	18:0 18:2(Δ9) 20:3(Δ5)	22.0	1.39
		56	1 2 2	46	18:1 18:2(Δ5) 20:2(Δ5)	4.0	0.25
13	0.9	50	0 0 2	46	16:0 16:0 18:2(Δ9)	34.5	0.32
		54	0 2 2	46	18:0 18:1 18:3	21.0	0.20
		54	0 1 3	46	18:0 18:2(Δ9) 18:2(Δ9)	15.0	0.14
		56	0 2 3	46	20:0 18:2(Δ9) 18:3(Δ5)	8.5	0.08
14	1.0	56	1 1 2	48	18:1 20:1 18:2(Δ9)	57.0	0.56
		48	0 0 1	46	16:0 16:0 16:1	14.0	0.14
		56	1 1 2	48	18:1 18:1 20:2(Δ11)	12.0	0.12
15	1.4	54	1 1 1	48	18:1 18:1 18:1	76.0	1.03
		54	0 1 2	48	16:0 20:1 18:2(Δ9)	8.0	0.11
		56	1 1 2	48	18:1 18:1 20:2(Δ5)	7.0	0.09
		54	0 1 2	48	16:0 18:1 20:2(Δ11)	6.0	0.08
16	3.6	54	0 1 2	48	18:0 18:1 18:2(Δ9)	40.0	1.42
		52	0 1 1	48	16:0 18:1 18:1	30.0	1.07
		56	0 2 2	48	20:0 18:2(Δ9) 18:2(Δ9)	10.0	0.36
		56	0 1 3	48	18:0 18:1 20:3(Δ5)	9.0	0.32
17	0.9	52	0 0 2	48	18:0 16:0 18:2(Δ9)	40.0	0.39
18	0.05	56	1 1 1	50	18:1 18:1 20:1	40.0	0.02
		52	0 0 1	50	18:0 18:0 16:1	26.0	0.01
19	1.2	54	0 1 1	50	18:0 18:1 18:1	48.0	0.56
		56	0 1 2	50	20:0 18:1 18:2(Δ9)	21.0	0.24
		52	0 0 1	50	16:0 16:0 20:1	9.0	0.10
20	0.15				Undetermined		

[a]Calculated by reference to 17:0 acid methyl ester added as an internal standard prior to gas–liquid chromatography of fatty acid methyl esters prepared with the triacylglycerol (TAG) fraction.
[b]Carbon number: sum of carbon atoms in fatty acids.
[c]Number of double bonds in each of the three fatty acids.
[d]Equivalent carbon number: CN minus twice the total number of ethylenic bonds.
[e]Abbreviations used for TAG species: 18:2(Δ9),9,12-18:2; 18:3(Δ9),9,12,15-18:3; 18:2(Δ5),5,9,18:2; 18:3(Δ5),5,9,12-18:3; 20:2(Δ11),11,14-20:2; 20:2(Δ5),5,11-20:2; 20:3(Δ5),5,11,14-20:3.

TABLE 6
Comparison of the Proportions of the Main[a] Triacylglycerol Species in *Pinus koraiensis* and *P. pinaster* Seed Oils as Determined Experimentally or Calculated Assuming a Random Distribution of Fatty Acids

	P. koraiensis		*P. pinaster*	
Main TAG species[b]	Exp.	Random	Exp.	Random
18:1 18:2(Δ9) 18:2(Δ9)	12.47	16.88	13.90	15.58
18:2(Δ9) 18:2(Δ9) 18:3(Δ5)	16.44	8.26	12.36	7.24
18:2(Δ9) 18:2(Δ9) 18:2(Δ9)	6.68	8.48	9.49	14.54
18:2(Δ9) 18:2(Δ9) 20:3(Δ5)	0.86	0.57	8.73	6.17
18:1 18:1 18:2(Δ9)	7.69	11.20	6.33	5.57
18:1 18:2(Δ9) 18:3(Δ5)	8.45	10.96	5.38	5.18
16:0 18:2(Δ9) 18:2(Δ9)	2.91	2.80	4.31	3.49
16:0 18:1 18:2(Δ9)	2.89	3.71	2.65	2.50
18:1 20:3(Δ5) 20:3(Δ5)	—	0.01	2.55	0.62
16:0 18:2(Δ9) 18:3(Δ5)	2.89	1.82	1.81	1.16
18:0 18:2(Δ9) 18:2(Δ9)	1.27	1.34	1.58	2.26
18:1 18:2(Δ9) 20:3(Δ5)	—	0.75	1.72	4.41
18:0 18:1 18:2(Δ9)	1.42	1.77	1.42	1.62
18:0 18:2(Δ9) 18:3(Δ5)	1.33	0.87	1.41	0.75
18:0 18:2(Δ9) 20:3(Δ5)	0.21	0.06	1.39	0.64
18:2(Δ9) 18:2(Δ5) 20:3(Δ5)	—	0.06	1.15	0.26
18:2(Δ9)18:3(Δ9) 20:3(Δ5)	—	0.01	1.12	0.32
16:0 18:1 18:1	1.65	1.23	1.07	0.45
18:1 18:1 18:3(Δ5)	7.28	3.64	1.06	0.92
18:1 18:1 18:1	6.53	2.48	1.03	0.66
16:0 18:1 18:3(Δ5)	1.05	1.02	0.44	0.41
18:1 18:1 18:2(Δ5)	1.10	0.56	—	—
18:2(Δ9) 18:2(Δ9) 18:2(Δ9)	2.53	1.27	—	—
Σ Δ5-UPIFA[c]	42.14	28.85	39.12	28.08

[a]More than 0.2% of total triacylglycerols either as determined experimentally (Exp.) or calculated by assuming a random distribution (random).
[b]TAG, triacylglycerols. Abbreviations for fatty acids: 18:2(Δ5), 5,9-18:2; 18:2(Δ9), 9,12-18:2; 18:3(Δ5), 5,9,12-18:3; 20:3(Δ5), 5,11,14-20:3. All ethylenic bonds are in the *cis* configuration.
[c]Sum of TAG species that contain at least one molecule of Δ5-unsaturated polymethylene-interrupted fatty acid (UPIFA)

5) because it contains two major Δ5-UPIFA instead of one, i.e., 18:3(Δ5) and 20:3(Δ5) acids (7–8% each). However, here too, both acids are preferentially esterified with two molecules of 18:2(Δ9) acid [18:3(Δ5), 12.4%; 20:3(Δ5), 8.7%; total, 21.1%]. In contrast to *P. koraiensis*, *P. pinaster* seed oil contains a non-negligible amount of TAG species with two 20:3(Δ5) acid molecules (2.6%) or one 18:2(Δ5) and one 20:3(Δ5) acid molecules (1.2%). Abundant TAG species that do not contain Δ5-UPIFA are almost the same as for *P. koraiensis*: 18:1, 18:2(Δ9), 18:2(Δ9) (13.9%); 18:1, 18:1, 18:2(Δ9) (6.3%); trilinolein (9.5%). However, triolein is low (1.0%).

Comparison with a random distribution. Considering the main Δ5-UPIFA-containing TAG in *P. koraiensis* seed oil (those at levels above 0.2% of total TAG), which total 42.1%, calculation indicates that a random distribution should have led to only 29.9% for the same TAG species (Table 6). The same holds true for *P. pinaster* (Table 6), for which a total of 28.1% of Δ5-UPIFA-containing TAG species is obtained assuming a random distribution, instead of the experimental value of 39.1%. Thus, the distributions of Δ5-UPIFA in *P. koraiensis* and *P. pinaster* seed oil TAG species are not random. The discrepancies noted between experimental and random distributions of TAG species suggest some peculiarity in the action mode of the Δ5-desaturase and other enzymes of fatty acid and glycerolipid biosynthesis.

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REFERENCES

1. Plattner, R.D., G.F. Spencer, and R. Kleiman, *cis*-5-Polyenoic Acids in *Larix leptolepis* Seed Oil, *Lipids 10*:413–416 (1975).
2. Madrigal, R.V., and C.R. Smith, Jr., *Taxus baccata* Seed Oil: A New Source of *cis*-5,*cis*-9-Octadecadienoic Acid, *Ibid. 10*:502–504 (1975).
3. Takagi, T., and Y. Itabashi, *cis*-5 Olefinic Unusual Fatty Acids in Seed Lipids of Gymnospermae and Their Distribution in Triacylglycerols, *Ibid. 17*:716–723 (1982).
4. Lie Ken Jie, M.S.F., C. Choi, A. Berger, and R.G. Berger, Re-Examination of the Fatty Acid Composition of *Biota orientalis* by Gas Chromatography–Mass Spectrometry of the Picolinyl Ester Derivatives, *J. Chromatogr. 543*:257–261 (1991).
5. Wolff, R.L., and C.C. Bayard, Fatty Acid Composition of Some Pine Seed Oils, *J. Am. Oil Chem. Soc. 72*:1043–1046 (1995).
6. Wolff, R.L., L.G. Deluc, and A.M. Marpeaux, Conifer Seeds: Oil Content and Fatty Acid Composition, *Ibid. 73*, 765–771 (1996).
7. Gunstone, F.D., S. Seth, and R.L. Wolff, The Distribution of Δ5 Polyene Acids in Some Pine Seed Oils Between the α- and β-Chains by ^{13}C-NMR Spectroscopy, *Chem. Phys. Lipids 78*:89–96 (1995).

8. Berdeaux, O., and R.L. Wolff, Gas–Liquid Chromatography–Mass Spectrometry of the 4,4-Dimethyloxazoline Derivatives of Δ5-Unsaturated Polymethylene-Interrupted Fatty Acids from Conifer Seed Oils, *J. Am. Oil Chem. Soc. 73*, 1996, in press.
9. Ikeda, I., T. Oka, K. Koba, M. Sugano, and M.S.F. Lie Ken Jie, 5*c*,11*c*,14*c*-Eicosatrienoic Acid and 5*c*,11*c*,14*c*,17*c*-Eicosatetraenoic Acid of *Biota orientalis* Seed Oil Affect Lipid Metabolism in the Rat, *Lipids 27*:500–504 (1992).
10. Sugano, M., Comparative Evaluation of Nutritional and Physiological Functions of Octadecatrienoic Acids (Abstract), *INFORM 6*:505 (1995).
11. Berger, A., and J.B. German, Extensive Incorporation of Dietary Δ-5,11,14 Eicosatrienoate into the Phosphatidylinositol Pool, *Biochim. Biophys. Acta 1085*:371–376 (1991).
12. Hirsch, J., and E.J. Ahrens, Jr., The Separation of Complex Lipid Mixtures by the Use of Silicic Acid Chromatography, *J. Biol. Chem. 233*:311–320 (1958).
13. Bézard, J., and M.A. Ouédraogo, Fractionation of Triacylglycerols by Reversed-Phase Liquid Chromatography, *J. Chromatogr. 196*:279–293 (1980).
14. Bannon, L.D., G.J. Brenn, J.D. Craske, N.T. Ha, N.L. Harper, and L. O'Rourke, Analysis of Fatty Acid Methyl Esters with High Accuracy and Reliability. III. Literature Review of and Investigation into the Development of Rapid Procedures for the Methoxide-Catalyzed Methanolysis of Fats and Oils, *J. Chromatogr. 247*:71–89 (1982).
15. Narce, M., J. Gresti, and J. Bézard, A Method for Evaluating the Bioconversion of Radioactive Polyunsaturated Fatty Acids by Reversed-Phase Liquid Chromatography, *J. Chromatog. Sci. 448*:249–264 (1988).
16. Christie, W.W., The Separation of Molecular Species of Glycerolipids, in *High-Performance Liquid Chromatography. A Practical Guide*, edited by Pergamon Press, Oxford, 1987, pp. 169-210.
17. Bailey, A.E., in Industrial Oil and Fat Products, edited by Interscience, New York, 1951.
18. Svensson, L., L. Sisfontes, G. Nyborg, and R. Blomstrand, High-Performance Liquid Chromatography and Glass Capillary Gas–Chromatography of Geometric and Positional Isomers of Long Chain Monounsaturated Fatty Acids, *Lipids 17*:50–59 (1982).

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