Positional Distribution of Δ5-Olefinic Acids in Triacylglycerols from Conifer Seed Oils: General and Specific Enrichment in the *sn*-3 Position

Robert L. Wolff[a,*], Eric Dareville[a], and Jean-Charles Martin[b]

[a]ISTAB, Université Bordeaux 1, Talence, and [b]INRA, Unité de Nutrition Lipidique, Dijon, France

ABSTRACT: Triacylglycerols (TAG) were purified from the storage lipids extracted from the seeds of several conifer species (*Taxus baccata, Larix decidua, Sciadopytis verticillata,* and *Juniperus communis*), each species belonging to one of the four families Taxaceae, Pinaceae, Taxodiaceae, and Cupressaceae, respectively. Each species was characterized by a high content of 5,9-18:2, 5,9,12-18:3, 5,11,14-20:3, or 5,11,14,17-20:4 acids, respectively. TAG were partially deacylated with ethylmagnesium bromide, and the resulting 1,2-, 2,3-diacylglycerols (DAG), and 2-monoacylglycerols (MAG) were purified by thin-layer chromatography. 1,2- and 2,3-DAG were further fractionated by chiral column high-performance liquid chromatography of the 3,5-dinitrophenylurethane derivatives. Alternately, TAG were subjected to porcine pancreatic lipase, and the resulting 2-MAG were purified for further analysis. Gas–liquid chromatography of fatty acid methyl esters prepared from the separated DAG and MAG, coupled with appropriate calculations, indicated that the Δ5-olefinic acids, irrespective of the species, chainlengths and number of ethylenic bonds, were considerably enriched in the *sn*-3 position of TAG where they accounted for *ca.* 35 to 74 mole% of fatty acids esterified to this position (depending on the initial level of total Δ5-olefinic acids in TAG), which corresponded to 79–94% of Δ5-olefinic acids esterified to the three positions. On the other hand, Δ5-olefinic acids were less than 10% in the *sn*-2 position and less than 6% in the *sn*-1 position of TAG. This specific enrichment of Δ5-olefinic acids in the *sn*-3 position thus appears to be a general characteristic of conifer seed TAG. These results were extended to TAG from the seeds of two pine species (*Pinus koraiensis* and *P. pinaster*) that are rich in Δ5-olefinic acids and available commercially on a ton-scale.

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Δ5-Unsaturated polymethylene-interrupted fatty acids (Δ5-UPIFA, or Δ5-olefinic acids) are characteristic components of conifer seed storage lipids (1–3). Depending on the family considered, some of the following fatty acids may be present: 5,9-18:2, 5,9,12-18:3, 5,9,12,15-18:4, 5,11-20:2, 5,11,14-20:3, and 5,11,14,17-20:4 (1,3,4). These fatty acids have been characterized by several techniques: ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectroscopy, capillary gas–liquid chromatography (GLC) on several different stationary phases, silver-ion thin-layer chromatography (TLC), mass spectrometry, and chemical degradation. Apparently, the Δ5-desaturation of 9-18:1, 9,12-18:2, 9,12,15-18:3 acids and of their elongation products, 11-20:1, 11,14-20:2 and 11,14,17-20:3 acids, would be a terminal step in the biosynthesis of Δ5-UPIFA (3), though some data would indicate that the 5,9,12-18:3 (pinolenic) acid might be elongated to a small extent to 7,11,14-20:3 (bishomo-pinolenic) acid (Wolff, R.L., W.W. Christie, and D. Coakley, unpublished results), at least in Pinaceae.

Recent experiments have shown that the Δ5-UPIFA are almost exclusively esterified to the *sn*-1 and/or *sn*-3 positions of triacylglycerols (TAG) from several conifer seeds. This was evidenced by ^{13}C NMR spectroscopy (5–7), partial chemical deacylation with Grignard reagents (1,8), or pancreatic lipase hydrolysis (7). Moreover, a study by reversed-phase high-performance liquid chromatography (HPLC) of TAG purified from the seeds of *Pinus koraiensis* and *P. pinaster* has shown that there is generally only one molecule of Δ5-UPIFA in individual TAG species (9). Finally, it has been observed (Wolff, R.L., L.G. Deluc, and A.M. Marpeau, manuscript in preparation) that total Δ5-UPIFA never exceeded 34% of total fatty acids in the seeds from about 70 different conifer species. All these data would suggest that Δ5-UPIFA are esterified to only one of the external positions of the glycerol moiety in conifer seed TAG.

The aim of the present study was to test this hypothesis. After their purification, TAG from the seeds of four species (*Taxus baccata*, *Larix decidua, Sciadopytis verticillata*, and *Juniperus communis*), each being representative of the four conifer families Taxaceae, Pinaceae, Taxodiaceae, and Cupressaceae, respectively, and each being selected for its particularly high content of Δ5-UPIFA (5,9-18:2, 5,9,12-18:3, 5,11,14-20:3, and 5,11,14,17-20:4 acids, respectively), were subjected to partial chemical degradation with ethylmagnesium bromide (EMB, Grignard reagent). The resulting 1,2- and 2,3-diacylglycerols (DAG) were separated by chiral col-

*To whom correspondence should be addressed at ISTAB, Laboratoire de Lipochimie Alimentaire, Université Bordeaux 1, Allée des Facultés, 33405 Talence cedex, France.

umn HPLC as their 3,5-dinitrophenylurethane (DNPU) derivatives. Moreover, the 2-monoacylglycerol (MAG) were also isolated, and TAG were further subjected to partial hydrolysis with porcine pancreatic lipase to prepare 2-MAG by another means. Appropriate calculations applied to the fatty acid compositions of DAG and MAG from both origins indicated that the Δ5-UPIFA are considerably enriched in the *sn*-3 position of TAG. This was independent of botanical family, chainlength, and number of ethylenic bonds. Similar results were obtained with TAG from the seeds of two *Pinus* species that are potential sources of Δ5-UPIFA and are available commercially on a ton-scale (*P. koraiensis* and *P. pinaster*).

EXPERIMENTAL PROCEDURES

Conifer seed oil samples. The oil samples were available from previous investigations by one of us (2,3,8,9). Their commercial sources and preparation have been described in detail elsewhere (2,3).

Solvents and reagents. Hexane of analytical grade was redistilled before use. Diethyl ether was HPLC-grade, and TLC plates precoated with silica gel (20 × 20 cm, 0.25 mm thick) were from SDS (Peypin, France). Pyridine and BF_3 in methanol (14% w/w), were from the Sigma Chemical Company (St. Louis, MO). Boric acid and EMB were purchased from Aldrich (Milwaukee, WI). Porcine pancreatic lipase (E.C. 3.1.1.3, type VI-S) was from the Sigma Chemical Company.

Purification of TAG. An aliquot of crude oil (300 mg) was dissolved in 2 mL of chloroform/methanol (2:1, vol/vol). Two hundred µL of this solution were spotted on a TLC plate, and TAG were isolated with pure chloroform as a migration solvent. After having sprayed the plate with a 2′,7′-dichlorofluorescein (DCF) solution in methanol (0.2%, wt/vol), the TAG band was visualized under ultraviolet (UV) light. The TAG were then eluted from the gel with chloroform/methanol (2:1, vol/vol). After removal of the solvents under a stream of N_2, TAG were dissolved in anhydrous diethyl ether (10 mg in 1 mL).

Partial degradation of TAG with Grignard reagent. This method is based on the random generation of 1,3-DAG, 1,2- and 2,3-DAG, along with 1-, 2- and 3-MAG, by partial deacylation of TAG with EMB. Purified TAG were partially deacylated according to Becker *et al.* (10), except that EMB was used instead of allyl magnesium bromide. Briefly, 20 mg of purified TAG were dissolved in diethyl ether (1.4 mL) in a glass tube, and EMB (3 M in 100 µL of diethyl ether) was added. After 30 s of vigorous agitation, the reaction was stopped by addition of 4 mL of an acidic buffer (mixture of 1 vol of 37% HCl and 36 vol of a 0.4 M boric acid solution in water). Then, 5 mL diethyl ether was added, and the organic phase was washed with a 0.4 M boric acid solution in water. The aqueous phase was discarded, and an additional washing with the same boric acid solution was performed. The organic phase was extracted, dried with anhydrous Na_2SO_4, and evaporated to dryness under a stream of N_2. The residue was dissolved in chloroform (100 µL) and applied to a boric acid-impregnated TLC plate (11). The reaction products were fractionated with chloroform/acetone (96:4, vol/vol). The fractions were visualized under UV radiation after spraying the DCF solution. The following bands were observed: 1- and 3-MAG (unresolved), R_f = 0.06; 2-MAG, R_f = 0.12; 1,2- and 2,3-DAG (unresolved), R_f = 0.45; 1,3-DAG, R_f = 0.64; unreacted TAG, R_f = 0.82. In addition, tertiary alcohols of the liberated fatty acids were observed with an R_f of 0.71. All bands corresponding to acylglycerols were scraped off the plate, and except for the 1,2- plus 2,3-DAG fraction, were used as such, in the presence of silica gel, for further transmethylation. Concerning the 1,2- and 2,3-DAG fraction (*rac*-DAG), the band was vertically divided into three parts: one-third was used for further fatty acid analysis (direct transmethylation), and the remaining two-thirds were prepared for fractionation by chiral-phase HPLC. For this purpose, DAG were eluted from the gel with a biphasic solvent system made with ethanol (2 mL), hexane/diethyl ether (2:1, vol/vol; 2 mL), distillled water (2 mL), and a few drops of concentrated ammonia. The upper organic phase was withdrawn, briefly dried, and evaporated under a stream of N_2. The dry residue was used for further fractionation by chiral-column HPLC.

Partial degradation of TAG with porcine pancreatic lipase. 2-MAG were generated from TAG upon partial hydrolysis of the acyl moieties in the 1- and 3-positions of the glycerol backbone with porcine pancreatic lipase (12). Briefly, TAG purified as described above were resuspended by vigorous shaking in 0.5 mL of Tris/HCl buffer (1 M, pH 8.0) that contained arabic gum (10%, wt/vol). The suspension was subjected to pancreatic lipase hydrolysis (5000 units dissolved in 0.5 mL of buffer, 10 min at 37°C with vigorous stirring) (11). The reaction products were extracted with diethyl ether, and the 2-MAG were purified on boric acid-impregnated TLC plates with hexane/isopropyl ether/acetic acid (60:40:4, vol/vol/vol) as a developing solvent. The gel band that contained 2-MAG was scraped off the plate and directly used for further transmethylation.

3,5-DNPU derivatization and HPLC fractionation of enantiomeric DAG. Enantiomeric 1,2- and 2,3-DAG (*ca*. 1 mg), evaporated to dryness, were reacted with *ca*. 2 mg of 3,5-isocyanate in 400 µL dry toluene, in the presence of 40 µL dry pyridine, for 3 h at room temperature (13). The resulting 3,5-DNPU were taken to dryness under N_2 and purified by TLC on silicic acid plates that contained a fluorescent indicator (Alltech, Templeuve, France). The reaction mixture, dissolved in 400 µL of chloroform, was spotted on the plate and developed with the solvent mixture petroleum ether/1,2-dichloroethane/ethanol (40:10:3, vol/vol/vol). Bands were visualized under UV light at 254 nm, and the 3,5-DNPU derivatives fraction was scraped off the plate. Pure 3,5-DNPU derivatives were recovered from the absorbent with diethyl ether.

The resolution of enantiomeric *sn*-1,2 and *sn*-2,3 DAG as their 3,5-DNPU derivatives was performed with a Spectra Physics SP 8810 (San Jose, CA) isocratic pump, equipped

with a chiral column (25 cm × 4.6 mm i.d.) that contained (*R*)-(+)-1-(1-naphthyl)-ethylamine polymeric phase covalently bonded to 300 A wide-pore spherical silica particles (5 µm, YMC-Pack A-K03; YMC Inc., Kyoto, Japan). The analysis was done isocratically at 8°C with *n*-hexane/1,2-dichloroethane/ethanol (40:10:1, vol/vol/vol) as a mobile phase at a constant rate of 0.5 mL/min. Usually, 10–20 µg of 3,5-DNPU derivatives, dissolved in the same solvent as the mobile phase, was injected onto the column. Peaks were monitored at 254 nm with a Uvikon 730-LC UV-spectrophotometer (Kontron Instruments, Milano, Italy), and the fractions were collected manually at the outlet of the apparatus.

Fatty acid analysis. Fatty acids from acylglycerols were converted into methyl esters with BF_3 in methanol (14%, w/w) in the presence of methanol and hexane (14). After completion of the reaction (100°C for 30 min), fatty acid methyl esters (FAME) were extracted with hexane and analyzed by GLC with an HP 5890 gas chromatograph (Hewlett-Packard, Palo Alto, CA), equipped with a split-splitless injector and a flame-ionization detector. Separation of FAME was realized with a BPX 70 fused-silica capillary column (50 m × 0.32 mm i.d., 0.25 µm film; Scientific Glass Engineering, Melbourne, Australia). The helium flow rate was 2 mL/min, and samples were injected in the splitless mode. The oven temperature was initially held at 60°C for 1.1 min, then increased at a rate of 20°C/min up to 175°C, and maintained at this temperature for 35 min. The injector and the detector were heated at 250°C. Results obtained with an SP 4400 integrator, coupled to an SP Chemstation (Spectra Physics), were converted into mole% prior to calculations. Identifications of Δ5-UPIFA were realized according to Wolff *et al*. (3).

Determination of TAG structures. The positional distribution of fatty acids in TAG was deduced from the fatty acid compositions of acylglycerols according to the following formulas:

$$sn\text{-}1\ (\text{mole}\%) = [2 \times sn\text{-}1,2\text{-DAG}\ (\text{mole}\%)] - [sn\text{-}2\text{-MAG}\ (\text{mole}\%)]$$

$$sn\text{-}3\ (\text{mole}\%) = [2 \times sn\text{-}2,3\text{-DAG}\ (\text{mole}\%)] - [sn\text{-}2\text{-MAG}\ (\text{mole}\%)]$$

sn-2-MAG were generated either with Grignard reagent or with porcine pancreatic lipase, whereas *sn*-1,2 and *sn*-2,3-DAG were obtained after deacylation of TAG with Grignard reagent and fractionation of the 3,5-DNPU derivatives by chiral-column HPLC.

RESULTS AND DISCUSSION

A specific enrichment of Δ5-UPIFA in the external positions (α-chains) of conifer seed TAG was shown to occur in the twenty-four species listed in Table 1. These data were obtained by partial chemical cleavage with Grignard reagents, ^{13}C NMR spectroscopy, or by use of pancreatic lipase. With perhaps the exception of *Podocarpus nagi* (1), Δ5-UPIFA are generally less than 5% of fatty acids esterified to the *sn*-2 position, and it thus appears that a specific enrichment of Δ5-UPIFA in the external positions is a widespread characteristic of conifer seed TAG. This applies to 5,9-18:2, 5,9,12-18:3, 5,11,14-20:3, and 5,11,14,17-20:4 acids.

TABLE 1
Conifer Species for Which an Important Enrichment of Δ5-Unsaturated Polymethylene-Interrupted Fatty Acids in the α-Chains of Seed Triacylglycerols Was Observed

Family	Species	Method[a]	Reference
Podocarpaceae	*Podocarpus nagi*	GR	(1)
Taxaceae	*Torreya nucifera*	GR	(1)
	Taxus cuspidata	GR	(1)
	T. baccata	GR, NMR	(5,8)
Pinaceae	*Pinus koraiensis*	GR, NMR	(1,5,8)
	P. sylvestris	NMR	(5)
	P. mughus	NMR	(5)
	P. nigra	NMR	(5)
	P. griffithii	NMR	(5)
	P. pinaster	GR, NMR	(5,8)
	P. pinea	GR	(8)
	Larix decidua	GR, NMR	(6,8)
	L. leptolepis	NMR	(6)
	Picea jezoensis	GR	(1)
	P. abies	NMR	(6)
	P. sitchensis	NMR	(6)
	Cedrus atlantica	NMR	(6)
	Abies concolor	NMR	(6)
Taxodiaceae	*Sciadopytis verticillata*	GR, NMR	(6,8)
	Cryptomeria japonica	GR	(1)
Cupressaceae	*Thuja occidentalis*	NMR	(6)
	Juniperus virginiana	NMR	(6)
	J. communis	GR	(8)
	Biota orientalis	PL, NMR	(7)

[a]GR, Grignard reagent; NMR, ^{13}C nuclear magnetic resonance spectroscopy; PL, pancreatic lipase.

In the present study, we have added a supplementary step in the analysis of the distribution of Δ5-UPIFA, i.e., the separation of 1,2- and 2,3-DAG generated by Grignard reagent by chiral-column HPLC of their 3,5-DNPU derivatives. Examples of such separations are given in Figure 1, which clearly shows the asymmetry between the 1,2- and 2,3-DAG peaks, thus indicating a probable nonrandom distribution of fatty acids between the three positions for the four species analyzed. This separation, together with the analysis of fatty acids in 2-MAG, allowed the location of fatty acids on each of the three positions of the glycerol backbone for a few selected species.

Results for these species are detailed in Tables 2 to 7. In these tables, we have recapitulated all experimental data for TAG, DAG, and MAG obtained with EMB, along with the calculated compositions of fatty acids esterified to each of the *sn*-1, *sn*-2, and *sn*-3 positions. We also give the arithmetically reconstituted fatty acid compositions of TAG based on these data. No statistical differences (Mann and Whitney rank sum test) were noted between the fatty acid compositions of 2-MAG obtained by the chemical or enzymatic procedures ($0.50 \le P \le 0.96$). Apparently, the lipase did not discriminate against Δ5-UPIFA for the reaction period used. Consequently, for calculations, the fatty acid compositions in the *sn*-2 position of TAG were obtained by averaging results obtained with

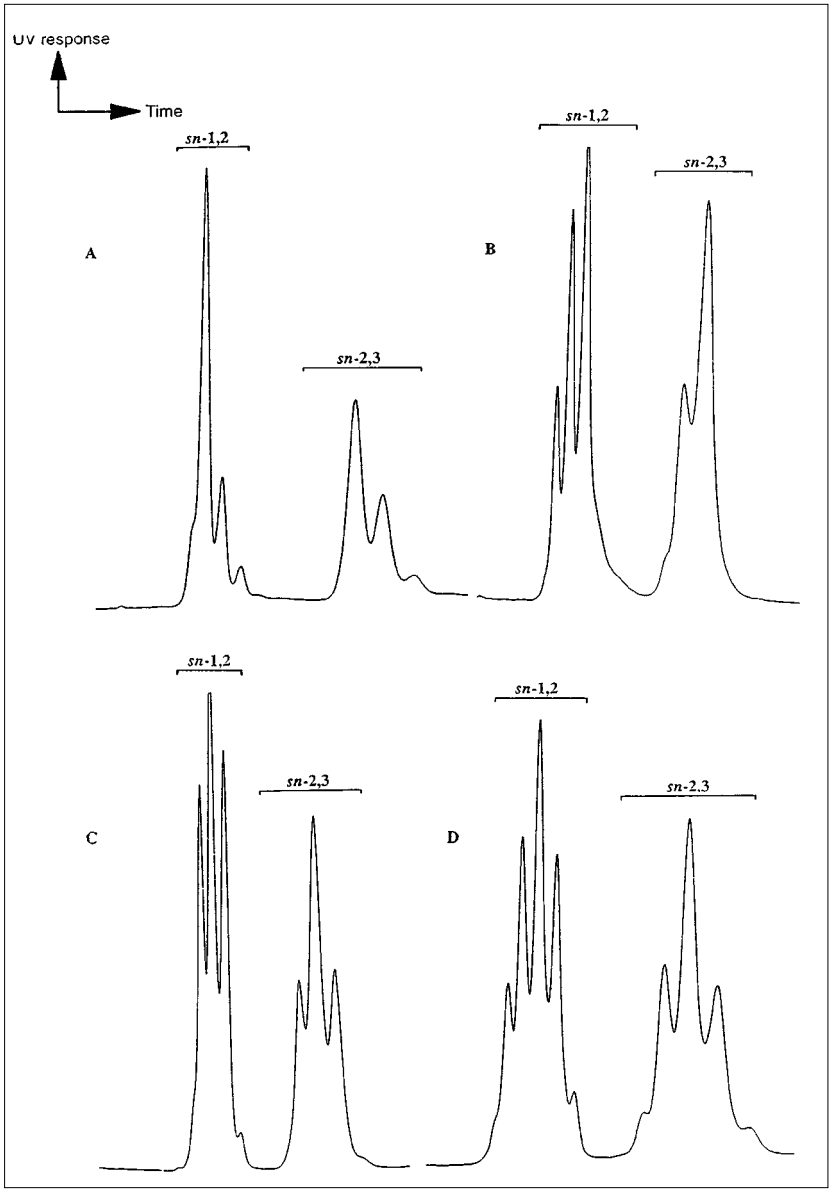

FIG. 1. Chromatograms of the 3,5-dinitrophenylurethane derivatives of 1,2- and 2,3-diacylglycerols (DAG) generated by partial deacylation with ethyl magnesium bromide of purified conifer seed triacylglycerols. Analyses by high-performance liquid chromatography on a chiral column under conditions are described in the Experimental Procedures section. Seed oils from: A, *Taxus baccata*; B, *Larix decidua*; C, *Sciadopytis verticillata*, and D, *Juniperus communis*; UV, ultraviolet.

2-MAG generated either with Grignard reagent or with pancreatic lipase.

Taxaceae from the genus *Taxus* (*T. cuspidata*, *T. canadensis*, *T. baccata*) are characterized by a relatively high level of 5,9-18:2 acid (more than 11%) (1,3,8,15) and small amounts of both 5,9,12-18:3 and 5,11,14-20:3 acids. Because 5,9-18:2 acid is the most abundant Δ5-UPIFA in the seeds from the genus *Taxus*, we propose the trivial name "taxoleic" acid. In a previous study of *T. baccata* seed TAG (8), we could observe that the 5,9-18:2 acid was predominantly esterified to the *sn*-1,3 positions, with only 2% being present in the *sn*-2 position. This was in agreement with data published earlier for *T. cuspidata* (1). It is now clear from Table 2 that taxoleic acid is almost exclusively esterified to the *sn*-3 position (31.2%). Δ5-UPIFA other than 5,9-18:2 acid are also mainly esterified to this position, which also confirms previous data (1,8) that indicated that these acids were located in the external positions of TAG. It should be noted that the precursor of 5,9-18:2 acid, oleic acid, is low in the *sn*-3 position (34%) as compared to either the *sn*-1 (62%) or the *sn*-2 (77%) posi-

TABLE 2
Fatty Acid Compositions of Monoacylglycerols (MAG), Diacylglycerols (DAG), and Triacylglycerols (TAG) (experimental and calculated), and Distribution of Fatty Acids in the *sn*-1, *sn*-2, and *sn*-3 Positions of TAG from *Taxus baccata* Seed Oil (results expressed as mole%)

Fatty acid	2-MAG[a]	DAG *rac*[b]	DAG *rac*[c]	1/3-MAG	1,3-DAG	1,2-DAG	2,3-DAG	*sn*-1	*sn*-2	*sn*-3	TAG[d]	TAG[e]
16:0	0.7	2.3	4.0	5.7	4.5	5.7	2.2	10.8	0.7	3.8	3.5	5.1
18:0	0.7	2.7	3.6	4.9	5.3	5.3	1.9	10.0	0.7	3.0	3.3	4.6
9-18:1	77.4	63.3	62.6	50.4	50.2	69.6	55.6	61.8	77.4	33.7	57.1	57.7
11-18:1	0.0	0.2	0.2	0.4	0.3	0.5	0.0	0.9	0.0	0.0	0.3	0.3
5,9-18:2	1.1	8.3	8.5	15.3	14.6	0.9	16.1	0.7	1.1	31.2	11.0	11.0
9,12-18:2	18.5	19.4	17.9	18.9	19.5	15.7	20.1	13.0	18.5	21.8	20.0	17.7
5,9,12-18:3	0.0	0.2	0.3	0.5	0.5	0.2	0.5	0.3	0.0	0.9	0.4	0.4
9,12,15-18:3	1.0	1.3	1.1	1.3	1.5	1.1	1.2	1.1	1.0	1.3	1.5	1.1
20:0	0.1	0.0	0.0	0.1	0.3	0.0	0.0	0.0	0.1	0.0	0.0	0.0
11-20:1	0.4	0.9	0.6	1.0	1.3	0.7	0.5	1.1	0.4	0.6	1.1	0.7
5,11-20:2	0.0	0.2	0.0	0.1	0.2	0.0	0.2	0.0	0.0	0.3	0.1	0.0
11,14-20:2	0.0	0.3	0.2	0.3	0.5	0.1	0.3	0.2	0.0	0.5	0.4	0.2
5,11,14-20:3	0.0	0.9	0.8	1.1	1.3	0.0	1.5	0.1	0.0	2.8	1.3	1.0
11,14,17-20:3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
5,11,14,17-20:4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

[a]Mean experimental values of chemical and enzymatic experiments. [b]Experimental values. [c]Calculated values [(1,2-DAG + 2,3-DAG)/2]. [d]Experimental values. [e]Calculated values [(*sn*-1 + *sn*-2 + *sn*-3)/3].

tions. Finally, Table 2 also shows that 16:0 and 18:0 acids are practically excluded from the *sn*-2 position in *T. baccata* seed TAG. However, and as will be seen later, this is apparently a feature common to all conifer species analyzed.

Larix decidua (Table 3), like most Pinaceae (1–3), is characterized by a high level of 5,9,12-18:3 acid (28.8%) in its seed oil, with a considerably lower level of 5,9-18:2 acid (2.4%). Other Δ5-UPIFA with 20 carbon atoms, 5,11-20:2 and 5,11,14-20:3 acids are minor components. In previous studies, either by ^{13}C NMR spectroscopy (6) or by use of Grignard reagent (8), a specific enrichment of Δ5-UPIFA in the external positions of TAG from the seeds of *L. decidua* was observed. In fact, such an enrichment has been observed in all Pinaceae studied until now (Table 1). In the present study, we have unambiguously demonstrated that pinolenic acid is specifically esterified to the *sn*-3 position (68%), though small amounts of this acid (*ca*. 5–6%) are also present in the *sn*-1 and *sn*-2 positions. At least for the *sn*-2 position, this is in agreement with previous studies conducted with various pine species (1,5) or with *L. decidua* and *L. leptolepis* seed oils (8). The precursor of pinolenic acid, linoleic acid, is low in the *sn*-3 position (6.7%) as compared to the *sn*-1 (51.3%) or the *sn*-2 (64.9%) positions. However, this also holds true for oleic acid (6.5% in the *sn*-3 position vs. 24-26% in the *sn*-1 and *sn*-2 positions). As for *T. baccata* seed TAG, the saturated fatty acids are lower in the *sn*-2 position than in the external positions of *L. decidua* seed TAG.

Sciadopytis verticillata, a member of the Taxodiaceae family, was chosen for its particularly high level (13.8%) of 5,11,14-20:3 acid in its seed oil. The name "podocarpic" was proposed for this peculiar fatty acid, but this was inappropriate, the name having been attributed earlier to a resin acid

TABLE 3
Fatty Acid Compositions of Monoacylglycerols (MAG), Diacylglycerols (DAG), and Triacylglycerols (TAG) (experimental and calculated), and Distribution of Fatty Acids in the *sn*-1, *sn*-2, and *sn*-3 Positions of TAG from *Larix decidua* Seed Oil (results expressed as mole%)

Fatty acid	2-MAG[a]	DAG *rac*[b]	DAG *rac*[c]	1/3-MAG	1,3-DAG	1,2-DAG	2,3-DAG	*sn*-1	*sn*-2	*sn*-3	TAG[d]	TAG[e]
16:0	1.5	1.2	5.7	5.1	3.7	5.6	5.7	9.7	1.5	9.9	3.3	7.1
18:0	0.8	1.0	1.9	2.6	2.0	2.4	1.4	4.0	0.8	2.0	1.4	2.2
9-18:1	24.5	20.6	20.3	18.6	16.8	25.2	15.5	25.8	24.5	6.5	18.2	18.9
11-18:1	0.4	0.7	0.4	1.2	1.1	0.7	0.2	1.0	0.4	0.0	0.8	0.5
5,9-18:2	1.0	2.2	1.8	3.2	3.0	0.6	3.1	0.2	1.0	5.2	2.4	2.1
9,12-18:2	64.9	49.5	47.0	32.7	34.4	58.1	35.8	51.3	64.9	6.7	43.1	41.0
5,9,12-18:3	5.5	22.9	21.2	34.4	37.1	5.5	36.8	5.6	5.5	68.2	28.8	26.4
9,12,15-18:3	0.4	0.5	0.4	0.6	0.5	0.5	0.3	0.7	0.4	0.2	0.6	0.4
20:0	0.0	0.1	0.2	0.3	0.4	0.2	0.3	0.3	0.0	0.5	0.1	0.3
11-20:1	0.3	0.4	0.2	0.4	0.3	0.3	0.1	0.4	0.3	0.0	0.3	0.2
5,11-20:2	0.0	0.1	0.0	0.1	0.1	0.0	0.0	0.0	0.0	0.2	0.0	0.0
11,14-20:2	0.2	0.4	0.3	0.3	0.3	0.3	0.2	0.4	0.2	0.2	0.3	0.3
5,11,14-20:3	0.4	0.6	0.4	0.4	0.3	0.3	0.5	0.2	0.4	0.6	0.5	0.4
11,14,17-20:3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
5,11,14,17-20:4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

[a]Mean experimental values of chemical and enzymatic experiments. [b]Experimental values. [c]Calculated values [(1,2-DAG + 2,3-DAG)/2]. [d]Experimental values. [e]Calculated values [(*sn*-1 + *sn*-2 + *sn*-3)/3].

TABLE 4
Fatty Acid Compositions of Monoacylglycerols (MAG), Diacylglycerols (DAG), and Triacylglycerols (TAG) (experimental and calculated), and Distribution of Fatty Acids in the *sn*-1, *sn*-2, and *sn*-3 Positions of TAG from *Sciadopytis verticillata* Seed Oil (results expressed as mole%)

Fatty acid	2-MAG[a]	DAG *rac*[b]	DAG *rac*[c]	1/3-MAG	1,3-DAG	1,2-DAG	2,3-DAG	*sn*-1	*sn*-2	*sn*-3	TAG[d]	TAG[e]
16:0	0.7	2.3	5.3	5.1	3.9	8.4	2.2	16.1	0.7	3.7	3.8	6.8
18:0	0.3	1.7	3.1	3.7	3.4	4.8	1.4	9.4	0.3	2.6	2.3	4.1
9-18:1	33.3	24.9	24.6	17.5	17.8	29.1	20.2	24.9	33.3	7.0	21.5	21.7
11-18:1	0.0	0.3	0.4	0.6	0.6	0.6	0.2	1.2	0.0	0.3	0.4	0.5
5,9-18:2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
9,12-18:2	58.3	49.5	49.3	41.4	41.3	48.5	50.1	38.7	58.3	41.9	47.1	46.3
5,9,12-18:3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
9,12,15-18:3	0.8	1.8	1.7	2.8	2.7	2.3	1.1	3.9	0.8	1.3	2.3	2.0
20:0	0.0	0.2	0.2	0.3	0.3	0.2	0.1	0.4	0.0	0.2	0.2	0.2
11-20:1	0.3	0.9	1.1	1.5	1.4	0.7	1.5	1.1	0.3	2.6	1.0	1.3
5,11-20:2	0.3	0.7	0.5	0.8	1.0	0.1	0.9	0.0	0.3	1.4	0.7	0.6
11,14-20:2	2.1	4.2	3.0	5.8	5.6	2.5	3.6	2.9	2.1	5.0	4.3	3.3
5,11,14-20:3	3.3	11.6	9.2	17.7	18.8	1.8	16.6	0.2	3.3	30.0	13.8	11.2
11,14,17-20:3	0.2	0.2	0.2	0.3	0.3	0.3	0.2	0.5	0.1	0.2	0.3	0.3
5,11,14,17-20:4	0.4	1.7	1.4	2.6	2.9	0.7	2.1	0.9	0.4	3.8	2.2	1.7

[a]Mean experimental values of chemical and enzymatic experiments. [b]Experimental values. [c]Calculated values [(1,2-DAG + 2,3-DAG)/2]. [d]Experimental values. [e]Calculated values [(*sn*-1 + *sn*-2 + *sn*-3)/3].

(16). Consequently, we propose the trivial name "sciadonic" acid, to recall its origin and its structure closely related to that of arachidonic acid. ^{13}C NMR (6) and partial deacylation with Grignard reagent (8) had shown that this acid was considerably enriched in the external positions of TAG. A similar observation was made with *Cryptomeria japonica* (1), another Taxodiaceae species. As shown in Table 4, 5,11,14-20:3 acid is more specifically attached to the *sn*-3 position of *S. verticillata* seed TAG, where it accounts for 30%. A small amount (3.3%) is also present in the *sn*-2 position, but practically none in the *sn*-1 position. Other minor Δ5-UPIFA with 20 carbon atoms are also preferentially esterified to the *sn*-3 position, whereas oleic acid is low in this position (7%) as compared to the *sn*-1 and *sn*-2 positions (24.9 and 33.3%, respectively). Saturated fatty acids are unevenly distributed, being higher in the *sn*-1 position than in the *sn*-3 position, and practically excluded from the *sn*-2 position.

Among Cupressaceae, *J. communis* seed TAG are characterized by a high content of 5,11,14,17-20:4 acid (17.2%) (Table 5), a structural analog of eicosapentaenoic (timnodonic) acid. For this reason, we suggest that this acid could be named "juniperonic" acid. Along with this acid are nonnegligible amounts of 5,11,14-20:3 acid (6.6%). Takagi and Itabashi (1) did not establish the positional distribution of these acids in Cupressaceae seed TAG, but Gunstone and Wolff (6), using ^{13}C NMR spectroscopy, have observed that neither sciadonic acid nor juniperonic acid was present in the β-chains of *J. virginiana* seed crude oil, at least within the limits of detection of the method (3%). Blaise *et al.* (8) have shown, after partial deacylation of TAG with Grignard

TABLE 5
Fatty Acid Composition of Monoacylglycerols (MAG), Diacylglycerols (DAG), and Triacylglycerols (TAG) (experimental and calculated), and Distribution of Fatty Acids in the *sn*-1, *sn*-2, and *sn*-3 Positions of TAG from *Juniperus communis* Seed Oil (results expressed as mole%)

Fatty acid	2-MAG[a]	DAG *rac*[b]	DAG *rac*[c]	1/3-MAG	1,3-DAG	1,2-DAG	2,3-DAG	*sn*-1	*sn*-2	*sn*-3	TAG[d]	TAG[e]
16:0	0.5	3.6	5.3	7.6	9.2	8.9	1.7	17.3	0.5	2.9	4.6	6.9
18:0	0.2	2.3	2.7	4.0	4.2	4.3	1.1	8.4	0.2	1.9	2.2	3.5
9-18:1	15.6	10.6	11.3	6.2	5.9	12.6	10.1	9.6	15.6	4.6	8.2	9.9
11-18:1	0.0	0.2	0.3	0.4	0.5	0.5	0.0	0.9	0.0	0.2	0.2	0.4
5,9-18:2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
9,12-18:2	56.8	40.5	40.7	25.4	26.6	43.1	38.3	29.4	56.8	19.7	34.1	35.3
5,9,12-18:3	0.0	0.2	0.3	0.4	1.8	0.0	0.5	0.0	0.0	1.0	0.3	0.3
9,12,15-18:3	16.2	19.5	18.9	22.5	24.6	19.8	18.1	23.4	16.2	20.0	21.5	19.8
20:0	0.0	0.4	0.3	0.6	0.6	0.5	0.2	1.0	0.0	0.4	0.4	0.4
11-20:1	0.0	0.7	0.5	1.3	0.8	0.9	0.1	1.7	0.0	0.2	0.8	0.7
5,11-20:2	0.3	0.3	0.3	0.5	1.9	0.1	0.4	0.0	0.3	0.5	0.3	0.3
11,14-20:2	0.8	1.9	1.6	3.3	2.2	1.9	1.2	3.1	0.8	1.6	2.2	1.8
5,11,14-20:3	2.6	5.2	5.1	7.4	5.7	1.6	8.6	0.6	2.6	14.6	6.7	5.9
11,14,17-20:3	0.4	1.1	0.8	1.9	1.3	1.2	0.5	2.0	0.4	0.5	1.3	1.0
5,11,14,17-20:4	6.4	13.4	11.9	18.4	14.8	4.6	19.2	2.7	6.4	31.9	17.2	13.7

[a]Mean experimental values of chemical and enzymatic experiments. [b]Experimental values. [c]Calculated values [(1,2-DAG + 2,3-DAG)/2]. [d]Experimental values. [e]Calculated values [(*sn*-1 + *sn*-2 + *sn*-3)/3].

reagent, that both acids were considerably enriched in the external positions of TAG purified from *J. communis* seed oil. Table 5 shows that 5,11,14-20:3 and 5,11,14,17-20:4 acids are preferentially esterified to the *sn*-3 position (14.6 and 31.9%, respectively), though they are not completely absent from position 2 (2.6 and 6.4%, respectively). *Biota orientalis*, another Cupressaceae species, displays a somewhat lower level of Δ5-UPIFA than *J. communis*, and the overall content in the *sn*-2 position of seed TAG is less than 2% (7). In contrast to other species analyzed in the present study, TAG from the seeds of *J. communis* are rich in 18:3n-3 acid (21.5%), the initial precursor of juniperonic acid (3), and this acid is almost evenly distributed between the three positions (Table 5). Oleic and linoleic acids are lower in the *sn*-3 position than in the two other positions. As for all conifer seed TAG analyzed here, the saturated fatty acids are extremely low in the internal position, with a particular enrichment in the *sn*-1 position as compared to the *sn*-3 position.

Pinus koraiensis seed oil, to our knowledge, is the only conifer seed oil rich in pinolenic acid that is available commercially. It is produced in France and Japan, at least upon request (2,17). Moreover, pinolenic acid is believed to have favorable effects in the regulation of various lipid variables in the rat (17). It was thus interesting to analyze *P. koraiensis* seed oil. As shown in Table 6, the 5,9,12-18:3 acid is almost exclusively esterified to the *sn*-3 position (50.2% vs. less than 1% in the two other positions). This result fully supports data from previous studies, which showed both by ^{13}C NMR spectroscopy (5) and by partial chemical deacylation (1,8), that pinolenic acid was almost exclusively esterified to the external positions of TAG. Moreover, a reversed-phase HPLC study of purified *P. koraiensis* seed TAG (9) had shown that TAG species esterified with 5,9,12-18:3 acid generally contained only one such molecule per molecule of TAG. This is now explained by the fact that pinolenic acid is exclusively esterified to the *sn*-3 position of TAG. As for *L. decidua*, the contents of oleic and linoleic acids in the *sn*-3 position are low as compared to those in the *sn*-1 or *sn*-2 positions. Total saturated fatty acids are less than 2% of fatty acids in the *sn*-2 position, but they are comparatively high in both the *sn*-1 and *sn*-3 positions, as for all other conifer seed TAG.

Pinus pinaster is interesting because its seeds are harvested on an industrial scale for forest planting and because the seed oil contains both pinolenic and sciadonic acids. This last acid, also present in *Biota orientalis* seed oil, has been shown to replace arachidonic acid in mice hepatic phosphatidylinositol (18). In previous studies, it was established that Δ5-UPIFA in *P. pinaster* seed crude oil [by ^{13}C NMR spectroscopy (5)] or in purified seed TAG [by partial chemical deacylation (8)] were concentrated in the external positions of the glycerol backbone. Moreover, a reversed-phase HPLC study had indicated (9) that there was generally one single molecule of Δ5-UPIFA in individual TAG species, which was indicative of a possible esterification of only one external position at a time. This is now confirmed (Table 7): all individual Δ5-UPIFA are preferentially esterified to the *sn*-3 position of TAG. Oleic acid is almost evenly distributed between the three positions, but linoleic acid is mainly esterified to the *sn*-2 (65.7%) and *sn*-1 (45.6%) positions, with only 19.3% in the *sn*-3 position. Saturated fatty acids are practically excluded from the *sn*-2 position.

All results established in the present study show that Δ5-UPIFA, irrespective of their chainlength (18 or 20 carbon atoms) and of their number of ethylenic bonds (2 to 4), are specifically esterified to the *sn*-3 position of conifer seed TAG. As shown in Table 8, 79 to 96% of total Δ5-UPIFA are esterified to the *sn*-3 position. This is apparently independent of the species considered, which would indicate that this structural feature is a general characteristic of conifers, and perhaps more generally of gymnosperms (1). It is noteworthy that in the Northern Hemisphere, approximately one tree out of two is a conifer, which emphasizes the importance of our

TABLE 6
Fatty Acid Composition of Monoacylglycerols (MAG), Diacylglycerols (DAG), and Triacylglycerols (TAG) (experimental and calculated), and Distribution of Fatty Acids in the *sn*-1, *sn*-2, and *sn*-3 Positions of TAG from *Pinus koraiensis* Seed Oil (results expressed as mole%)

Fatty acid	2-MAG[a]	DAG *rac*[b]	DAG *rac*[c]	1/3-MAG	1,3-DAG	1,2-DAG	2,3-DAG	*sn*-1	*sn*-2	*sn*-3	TAG[d]	TAG[e]
16:0	0.7	3.2	6.9	7.0	6.6	9.3	4.5	17.9	0.7	8.3	5.5	9.0
18:0	0.5	1.7	2.5	3.6	3.2	2.9	2.1	5.3	0.5	3.7	2.2	3.2
9-18:1	29.8	28.4	24.9	28.4	27.2	26.9	23.0	24.1	29.8	16.1	26.8	23.3
11-18:1	0.1	0.4	0.4	0.5	0.6	0.5	0.3	0.9	0.1	0.4	0.4	0.5
5,9-18:2	0.3	1.7	1.4	2.6	2.7	0.0	2.8	-0.3	0.3	5.3	2.0	1.8
9,12-18:2	66.4	51.0	48.9	34.5	35.8	58.3	39.6	50.1	66.4	12.7	45.0	43.1
5,9,12-18:3	1.0	11.3	13.2	19.8	20.5	0.8	25.6	0.7	1.0	50.2	15.5	17.3
9,12,15-18:3	0.2	0.2	0.3	0.0	0.2	0.3	0.3	0.5	0.2	0.4	0.1	0.3
20:0	0.0	0.2	0.2	0.5	0.4	0.1	0.3	0.2	0.0	0.6	0.3	0.3
11-20:1	0.0	0.8	0.5	1.4	1.3	0.4	0.5	0.8	0.0	1.1	0.9	0.6
5,11-20:2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
11,14-20:2	0.5	0.5	0.2	0.5	0.6	0.2	0.2	0.0	0.5	-0.1	0.5	0.1
5,11,14-20:3	0.3	0.6	0.5	0.9	0.9	0.2	0.8	0.0	0.3	1.3	0.7	0.5
11,14,17-20:3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
5,11,14,17-20:4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

[a]Mean experimental values of chemical and enzymatic experiments. [b]Experimental values. [c]Calculated values [(1,2-DAG + 2,3-DAG)/2]. [d]Experimental values. [e]Calculated values [(*sn*-1 + *sn*-2 + *sn*-3)/3].

TABLE 7
Fatty Acid Composition of Monoacylglycerols (MAG), Diacylglycerols (DAG), and Triacylglycerols (TAG) (experimental and calculated), and Distribution of Fatty Acids in the *sn*-1, *sn*-2, and *sn*-3 Positions of TAG from *Pinus pinaster* Seed Oil (results expressed as mole%)

Fatty acid	2-MAG[a]	DAG *rac*[b]	DAG *rac*[c]	1/3-MAG	1,3-DAG	1,2-DAG	2,3-DAG	*sn*-1	*sn*-2	*sn*-3	TAG[d]	TAG[e]
16:0	1.1	3.7	6.9	8.2	7.2	8.0	5.8	15.0	1.1	10.5	5.8	8.9
18:0	0.5	2.2	3.3	4.5	4.1	3.1	3.5	5.7	0.5	6.5	2.7	4.2
9-18:1	28.2	28.2	27.3	28.3	28.7	27.7	26.9	27.3	28.2	25.7	27.2	27.0
11-18:1	0.0	0.4	0.5	0.6	0.7	0.5	0.4	1.1	0.0	0.9	0.5	0.7
5,9-18:2	0.0	0.6	0.8	1.0	1.0	0.5	1.1	1.0	0.0	2.3	0.8	1.1
9,12-18:2	65.7	52.1	49.1	37.0	40.3	55.6	42.5	45.6	65.7	19.3	46.7	43.5
5,9,12-18:3	0.9	5.1	4.9	8.9	9.5	1.0	8.9	1.1	0.9	16.8	7.3	6.3
9,12,15-18:3	0.7	1.0	1.0	1.3	1.3	1.1	1.0	1.5	0.7	1.2	1.4	1.1
20:0	0.0	0.3	0.3	0.4	0.3	0.3	0.3	0.6	0.0	0.6	0.2	0.4
11-20:1	0.3	0.9	0.5	1.1	0.9	0.4	0.6	0.6	0.3	1.0	0.8	0.6
5,11-20:2	0.2	0.6	0.5	1.5	0.5	0.0	0.8	0.0	0.2	1.4	0.6	0.5
11,14-20:2	0.6	0.6	1.4	0.7	0.7	0.9	2.0	1.2	0.6	3.3	0.7	1.7
5,11,14-20:3	1.8	4.5	3.4	6.5	4.8	0.7	6.2	-0.5	1.8	10.5	5.4	3.9
11,14,17-20:3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
5,11,14,17-20:4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

[a]Mean experimental values of chemical and enzymatic experiments. [b]Experimental values. [c]Calculated values [(1,2-DAG + 2,3-DAG)/2]. [d]Experimental values. [e]Calculated values [(*sn*-1 + *sn*-2 + *sn*-3)/3].

findings. Though a Δ5-unsaturation in vegetable oil fatty acids was considered "uncommon" until recently, it is reasonable now to admit that it is as usual as other unsaturations (Δ9, Δ12, and Δ15) in a vast array of vegetables.

The fact that most Δ5-UPIFA are essentially located in the *sn*-3 position of TAG has several possible metabolic implications. In vegetables, the biosynthesis of TAG proceeds *via* the phosphatidic acid pathway. Because TAG in conifer seeds contain only minor amounts of Δ5-UPIFA in the *sn*-1 and *sn*-2 positions, this would imply that these acids are practically not used for the acylation of glycerol–phosphate to give phosphatidic acid. Alternately stated, fatty acids that are used for the acylation of the *sn*-1 and *sn*-2 positions of phosphatidic acid are not substrate of the Δ5-desaturase. Only those acids that are used for the acylation of the *sn*-3 position contain a Δ5-unsaturation, which might be indicative of a special location of the Δ5-desaturase within the cell, in a compartment different from that containing the more habitual Δ9-, Δ12-, and Δ15- desaturases. Another explanation would be that the acylase that ensures the esterification of the *sn*-3 position of 1,2-DAG has a pronounced affinity for the Δ5-ethylenic bond, in contrast to the two other acylases specific for the *sn*-1 and *sn*-2 positions.

Beside the biochemical aspects discussed above, the fact that most Δ5-UPIFA are essentially located in the *sn*-3 position of conifer seed TAG has other possible nutritional implications. Considering the action mode of pancreatic lipase, a crucial step in the intestinal absorption of TAG, one may surmise that Δ5-UPIFA will be absorbed almost exclusively in their free form, rather than in the form of 2-MAG (19–22). Whether this is of interest for these fatty acids is not known but deserves consideration for further nutritional studies.

TABLE 8
Absolute and Relative Percentages of Total Δ5-Acids in the Three Positions of Triacylglycerols from the Seeds of Some Conifer Species

	Positions		
Species	*sn*-1	*sn*-2	*sn*-3
Taxus baccata	1.1[a]	1.1	35.2
	(2.9)[b]	(2.9)	(94.2)
Larix decidua	6.0	6.9	74.2
	(6.9)	(7.9)	(85.2)
Sciadopytis verticillata	1.1	4.0	35.2
	(2.7)	(9.9)	(87.4)
Juniperus communis	3.3	9.3	48.0
	(5.4)	(15.4)	(79.2)
Pinus koraiensis	0.7	1.6	56.8
	(1.2)	(2.7)	(96.1)
P. pinaster	2.1	2.9	31.0
	(5.8)	(8.1)	(86.1)

[a]Mole%, relative to total fatty acids esterified to the indicated position (absolute percentages).
[b]Mole%, relative to total Δ5-acids in the three positions (relative percentages).

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