# Positional Distribution of Δ5-Acids in Triacylglycerols from Conifer Seeds As Determined by Partial Chemical Cleavage

**Pierre Blaise[a], Veronique Tropini[a], Marie Farines[a], and Robert L. Wolff[b,*]**

[a]Laboratoire de Chimie Organique des Substances Naturelles, Université de Perpignan, Perpignan, France, and [b]ISTAB, Université Bordeaux 1, Talence, France

**ABSTRACT:** The positional distribution of various Δ5-acids in the seed triacylglycerols from several conifer species has been established after partial chemical degradation with Grignard reagent. The species studied were representative of four conifer families and were specially selected for their particularly high Δ5-acid contents. These species were *Taxus baccata* (Taxaceae; 5,9-18:2 acid, 11.9%), *Larix decidua* (Pinaceae; 5,9,12-18:3 acid, 28.5%), *Sciadopytis verticillata* (Taxodiaceae; 5,11,14-20:3 acid, 16.7%), and *Juniperus communis* (Cupressaceae; 5,11,14,17-20:4 acid, 19.8%). Calculations from the fatty acid compositions of triacylglycerols and of the mixture of 1,2- and 2,3-diacylglycerols generated by the Grignard reagent indicated that, for the four species, there was a considerable enrichment of Δ5-acids (generally more than ten times) in the 1,3-positions as compared to the 2-position, where Δ5-acids represented always less than 2% of total fatty acids esterified to triacylglycerols. This distribution was practically independent from the species (four families studied), the chainlength (18 or 20 carbon atoms), and the number of ethylenic bonds (two to four) in the Δ5-acids. Similar distributions were established for triacylglycerols from the seeds of three pine species that are available on a ton-scale: *Pinus pinea, P. koraiensis,* and *P. pinaster*. These observations confirm and extend previous studies conducted with other conifer species by similar techniques or by $^{13}$C-nuclear magnetic resonance spectroscopy. Consequently, the almost exclusive location of Δ5-acids in the external positions of triacylglycerols is now well established and appears to be a general feature of conifer seed oils.

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In the search for Δ5-acids, some of which have structures closely related to those of arachidonic and eicosapentaenoic acids, and following a systematic study of conifer seed oils (1,2), four species could be selected with a high content of one of the following Δ5-acids: 5,9-18:2, 5,9,12-18:3, 5,11,14-20:3, and 5,11,14,17-20:4 acids (double bonds in the *cis* configuration). These species were *Taxus baccata*, *Larix decidua*, *Sciadopytis verticillata*, and *Juniperus communis*, respectively. Moreover, each of these species is representative of one of the four conifer families, Taxaceae, Pinaceae, Taxodiaceae, and Cupressaceae, respectively. A biosynthetic pathway for these acids has recently been proposed (2), based on a Δ5-desaturase activity upon 9-18:1, 9,12-18:2, 11,14-20:2, and 11,14,17-20:3 acids as substrates.

Previous $^{13}$C-nuclear magnetic resonance (NMR) spectroscopy investigations (3,4) have shown that Δ5-acids were present in the external positions of triacylglycerols (TAG) from the seeds of all species analyzed, with apparently no Δ5-acids in the 2-position, at least within the limits of the technique (Δ5-acids in amounts less than 3% could not be detected). These observations were in general agreement with those of Takagi and Itabashi (5), who used chemical degradative techniques, except for *Podocarpus nagi*, rich in 5,11,14-20:3 acid (*ca*. 24%), which contained about 10% of this acid in the 2-position. This was not observed by $^{13}$C-NMR spectroscopy of *S. verticillata* seed oil (4), which is also relatively rich in 5,11,14-20:3 acid (*ca*. 14%) (2).

Moreover, it has been shown for the seed oils from two pine species (*Pinus koraiensis* and *P. pinaster*) that TAG species containing Δ5-acids generally contained only one molecule of such an acid per molecule of TAG (6). These features would indicate that Δ5-acids might be esterified to only one of the 1- or 3-positions in conifer seed TAG.

As a first approach to test this hypothesis, the aim of the present study was to verify and complete results obtained by $^{13}$C-NMR spectroscopy (3,4) with a different technique, and to confirm and extend data previously published by Takagi and Itabashi (5). For these purposes, TAG purified from *T. baccata*, *L. decidua*, *S. verticillata*, and *J. communis* seed oils were partially cleaved with ethylmagnesium chloride (Grignard reagent), and the fatty acid compositions of the 1,3-positions on the one hand, and of the 2-position on the other hand, were established by appropriate calculations. To this series, we have added the seed oils from *P. koraiensis* and *P. pinea*, which are available commercially, and the seeds from *P. pinaster* that are produced on a ton-scale for forest planting in the southwest of France and are a potential commercial source of 5,9,12-18:3 and 5,11,14-20:3 acids (*ca*. 8% each).

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

## EXPERIMENTAL PROCEDURES

*Conifer seed oil samples*. The oil samples were available from previous studies by one of us (1,2). Their commercial sources and preparation have been described elsewhere (1,2).

*Purification of TAG*. TAG were purified from 300 mg of each oil by columnn chromatography on silica gel (Kieselgel 60, 70-230 mesh; Merck, Darmstadt, Germany) according to the I.U.P.A.C. procedure (7) with benzene as a solvent.

*Partial degradation of TAG*. Purified TAG (100 mg) were partially cleaved according to the methods described by Brockerhof (8) and Takagi and Ando (9). Purified TAG were partially hydrolyzed according to Taylor *et al*. (10) by using 300 µL ethyl magnesium chloride (Fluka, Buchs, Switzerland). The reaction products, dissolved in 800 µL of dry chloroform, were fractionated by thin-layer chromatography on two plates coated with Kieselgel 60 (0.2 mm thickness) and impregnated with boric acid (10%, w/w). The fractionation was performed twice with chloroform/acetone (96:4, vol/vol). The 1,2- and 2,3-diacylglycerols (DAG) ran as a single band ($R_f = 0.48$), well-resolved from other lipids. They were recovered by extraction from the gel with hot chloroform (three times for 15 min). The chloroform suspensions were filtered on 2V Whatman filters (Springfield Mill, United Kingdom) and evaporated under reduced pressure. The 2-monoacylglycerols (MAG) ($R_f = 0.20$) were collected in the same way.

*Fatty acid analysis*. Fatty acids from TAG and DAG were transmethylated with 0.5 M sodium methoxide in methanol (11). The resulting fatty acid methyl esters (FAME) were analyzed by gas–liquid chromatography (GLC) in a DI200 chromatograph (Delsi-Nermag, Paris, France), equipped with a BPX 70 capillary column (25 m × 0.25 mm i.d., 0.25 µm film; SGE, Melbourne, Australia). The oven temperature was programmed from 120 to 200°C at a rate of 3°C/min, and helium was used as a carrier gas. Injections were made with a glass-needle evaporator injector (Ross type). The flame-ionization detector was maintained at 250°C. Ordinary FAME were identified by comparison with authentic standards (Sigma Chemical Company, St. Louis, MO), whereas Δ5-acids were identified according to Wolff and Bayard (1) and Wolff *et al*. (2).

*Calculations*. Peak area percentages were converted into mole percentage prior to calculation of the distribution of fatty acids between the 1,3- and 2-positions of TAG. This was achieved according to Becker *et al*. (12) with the formulas:

$$\text{(1,3)-MAG} = 3 \times \text{TAG} - 2 \times \text{1,2(2,3)-DAG} \quad [1]$$

$$\text{2-MAG} = 4 \times \text{1,2(2,3)-DAG} - 3 \times \text{TAG} \quad [2]$$

## RESULTS AND DISCUSSION

The fatty acid compositions of TAG, purified from conifer seed oils, and of the corresponding mixtures of 1,2- and 2,3-DAG generated by Grignard reagent, are given in Table 1. This table also shows the calculated compositions of fatty acids esterified to the 1,3- and 2-positions of TAG, as mole percentage of total fatty acids in TAG. Though not reported in Table 1, the experimentally determined fatty acid compositions of 2-MAG fully supported calculated data for fatty acids in the 2-position. The fatty acid compositions of purified TAG from all species are in good agreement with the fatty acid compositions of crude oils reported previously (1,2).

In *T. baccata*, where 5,9-18:2 acid represents 11.9% of total fatty acids, 94% of this acid is esterified to the 1,3-positions. For *L. decidua* seed oil, in which 5,9,12-18:3 acid is a main component (28.5%), 94% of this acid is present in the external positions. For *S. verticillata*, rich in 5,11,14-20:3 acid (16.7%), and *J. communis*, with a high level of 5,11,14,17-20:4 acid (19.8%), more than 90% of both acids are esterified to the 1,3-positions of TAG. As a general rule, Δ5-acids are present in position 2 in amounts less than 2% of total fatty acids. It thus clearly appears that the specific enrichment of Δ5-acids in the 1,3-positions as compared to the 2-position is practically independent from the botanical family, the chainlength (18 or 20 carbon atoms), and the number of ethylenic bonds (2 to 4). This evidences the nonrandom distribution of Δ5-acids in TAG from conifer seeds.

These data, obtained by partial chemical cleavage, confirm previous determinations of the positional distribution of Δ5-acids by $^{13}$C-NMR spectroscopy of crude seed oils from several conifer species (3,4). They also emphasize the power and usefulness of $^{13}$C-NMR spectroscopy in this domain, though this technique seems to have a threshold of *ca*. 3% below which Δ5-acids cannot be detected. The present study also confirms and extends previous results by Takagi and Itabashi (5) for some other conifer seed oils, which would indicate that the preferential location of Δ5-acids in the external positions of TAG is a general feature of conifer seed storage lipids.

Concerning the oils from *P. koraiensis* and *P. pinaster* seeds, where Δ5-acids are also concentrated in the external positions (Table 1), a previous study had shown that almost half of the TAG species contained at least one molecule of Δ5-acid, and that generally, there was only one molecule of Δ5-acid per molecule of TAG. Previous $^{13}$C-NMR spectroscopy studies (3) and the present observations demonstrate that Δ5-acids are essentially esterified to the 1,3-positions of TAG from both species. This also holds true for *P. pinea*, in which Δ5-acids are low (Table 1). In TAG from *P. pinaster* seed oil, which contains four different Δ5-acids in amounts higher than 0.5% (i.e., 5,9-18:2, 5,9,12-18:3, 5,11-20:2 and 5,11,14-20:3 acids), all of these different Δ5-acids are preferentially esterified to the 1,3-positions.

These facts would thus indicate, at least for the pine species, that Δ5-acids, in general, esterify only one of the external positions of TAG at a time, and seldom both. Another important fact is that during a systematic study of conifer seed oils (Refs. 1,2, and Wolff, R.L., L.G. Deluc, and A.M. Marpeau, unpublished results), more than seventy species were analyzed, and that total Δ5-acids never exceeded 33% of total fatty acids. This might be indicative of an almost exclusive esterification of only one external position of TAG by Δ5-acids. This possibility is under current investigation.

**TABLE 1**
**Compositions of Fatty Acids Esterified to the *sn*-2- and *sn*-1,3-Positions of Some Conifer Seed Triacylglycerols as Calculated from the Fatty Acid Compositions of Intact Triacylglycerols and 1,2- and 2,3-Diacylglycerols Prepared with Ethylmagnesium Chloride**

| Species | Fraction[a] | Fatty acids (mole %)[b] | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 16:0 | 18:0 | 18:1 (Δ9)[c] | 18:1 (Δ11) | 18:2 (Δ5,9) | 18:2 (Δ9,12) | 18:3 (Δ5,9,12) | 18:3 (Δ9,12,15) | 20:0 | 20:1 (Δ11) | 20:2 (Δ5,11) | 20:2 (Δ11,14) | 20:3 (Δ5,11,14) | 20:3 (Δ11,14,17) | 20:4 (Δ5,11,14,17) |
| | TAG | 3.1 | 4.0 | 54.5 | — | 11.9 | 21.0 | — | 1.5 | — | 1.6 | — | 0.6 | 1.8 | — | — |
| *Taxus baccata* | DAG | 2.5 | 3.6 | 58.6 | — | 9.5 | 20.6 | — | 1.7 | — | 1.4 | — | 0.9 | 1.3 | — | — |
| | *sn*-2 | 0.3 | 0.7 | 23.7 | — | 0.7 | 6.5 | — | 0.7 | — | 0.2 | — | 0.6 | — | — | — |
| | *sn*-1,3 | 2.8 | 3.3 | 30.8 | — | 11.2 | 14.5 | — | 0.8 | — | 1.4 | — | — | 1.8 | — | — |
| | TAG | 3.4 | 1.8 | 19.3 | 1.1 | 3.1 | 40.1 | 28.5 | 0.7 | — | 0.5 | — | — | 1.5 | — | — |
| *Larix decidua* | DAG | 3.2 | 1.7 | 21.4 | 1.2 | 2.8 | 44.1 | 22.6 | 1.1 | — | 0.5 | — | — | 1.3 | — | — |
| | *sn*-2 | 0.8 | 0.5 | 9.3 | 0.4 | 0.7 | 18.9 | 1.6 | 0.7 | — | 0.2 | — | — | 0.2 | — | — |
| | *sn*-1,3 | 2.6 | 1.3 | 10.0 | 0.7 | 2.4 | 21.2 | 26.9 | — | — | 0.3 | — | — | 1.3 | — | — |
| | TAG | 3.7 | 2.9 | 21.3 | — | — | 40.2 | — | 2.8 | — | 1.7 | 1.2 | 6.3 | 16.7 | — | 3.2 |
| *Sciadopytis* | DAG | 3.0 | 2.3 | 24.5 | — | — | 43.2 | — | 2.3 | — | 1.4 | 1.8 | 5.5 | 13.5 | — | 2.5 |
| *verticillata* | *sn*-2 | 0.3 | 0.3 | 11.5 | — | — | 17.3 | — | 0.2 | — | 0.2 | 1.2 | 1.0 | 1.3 | — | 0.1 |
| | *sn*-1,3 | 3.4 | 2.6 | 9.8 | — | — | 22.9 | — | 2.6 | — | 1.5 | — | 5.3 | 15.4 | — | 3.1 |
| | TAG | 3.9 | 2.5 | 9.1 | — | — | 30.5 | — | 19.9 | — | 1.1 | — | 3.0 | 8.4 | 1.8 | 19.8 |
| *Juniperus* | DAG | 3.9 | 2.4 | 11.2 | — | — | 34.7 | — | 19.0 | — | 1.0 | — | 2.8 | 7.0 | 1.6 | 16.1 |
| *communis* | *sn*-2 | 1.3 | 0.7 | 5.8 | — | — | 15.8 | — | 5.5 | — | 0.2 | — | 0.8 | 1.0 | 0.4 | 1.7 |
| | *sn*-1,3 | 2.6 | 1.8 | 3.3 | — | — | 14.7 | — | 14.4 | — | 0.9 | — | 2.2 | 7.4 | 1.4 | 18.1 |
| | TAG | 7.3 | 4.9 | 37.4 | — | — | 44.3 | 0.5 | 1.0 | 0.8 | 1.0 | — | 0.6 | 2.2 | — | — |
| *P. pinea* | DAG | 5.6 | 3.6 | 38.3 | — | — | 47.3 | 0.4 | 0.9 | 0.6 | 0.8 | — | 0.5 | 1.8 | — | — |
| | *sn*-2 | 0.2 | — | 13.6 | — | — | 18.7 | 0.1 | 0.3 | 0.1 | 0.1 | — | — | 0.2 | — | — |
| | *sn*-1,3 | 7.1 | 4.9 | 23.8 | — | — | 25.6 | 0.4 | 0.7 | 0.7 | 0.8 | — | 0.6 | 2.0 | — | — |
| | TAG | 5.6 | 3.0 | 28.4 | — | 2.6 | 41.6 | 16.0 | — | — | 1.6 | — | — | 1.2 | — | — |
| *P. koraiensis* | DAG | 3.7 | 2.3 | 30.4 | — | 2.2 | 45.4 | 12.7 | — | — | 1.8 | — | — | 1.7 | — | — |
| | *sn*-2 | 0.1 | 0.1 | 12.2 | — | 0.3 | 18.9 | 1.0 | — | — | 0.8 | — | — | 1.0 | — | — |
| | *sn*-1,3 | 5.5 | 2.9 | 16.2 | — | 2.3 | 22.7 | 15.0 | — | — | 0.8 | — | — | 0.2 | — | — |
| | TAG | 5.0 | 3.4 | 27.8 | — | 1.0 | 42.3 | 7.9 | 1.4 | — | 1.3 | 1.1 | 1.1 | 7.7 | — | — |
| *P. pinaster* | DAG | 3.8 | 2.8 | 29.0 | — | 0.8 | 47.0 | 6.0 | 1.3 | — | 1.2 | 0.9 | 1.0 | 6.2 | — | — |
| | *sn*-2 | 0.1 | 0.3 | 10.8 | — | — | 20.3 | 0.1 | 0.4 | — | 0.2 | 0.2 | 0.3 | 0.5 | — | — |
| | *sn*-1,3 | 4.9 | 3.1 | 17.0 | — | 1.0 | 22.0 | 7.8 | 1.0 | — | 1.1 | 0.9 | 0.8 | 7.2 | — | — |

[a]TAG, purified triacylglycerols; DAG, mixture of 1,2- and 2,3-diacylglycerols generated by ethylmagnesium chloride.
[b]Fatty acid compositions for TAG and DAG are relative to the total in each fraction, and those for the *sn*-2 and *sn*-1,3 positions are relative to total fatty acids esterified to the three positions. In all instances, values are the means from two experiments. Data for components in amounts less than 0.5% in TAG are not reported.
[c]When the 11-18:1 acid is not reported, the 9-18:1 acid includes this isomer.

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