Reinvestigation of the Polymethylene-Interrupted 18:2 and 20:2 Acids of *Ginkgo biloba* Seed Lipids

Robert L. Wolff[a,*], William W. Christie[b], and Anne M. Marpeau[c]

[a]ISTAB, Université Bordeaux 1, Talence, France, [b]Scottish Crop Research Institute, Invergowrie, Dundee, Scotland, and [c]Laboratoire de Chimie des Substances Végétales, Institut du Pin, Talence, France

ABSTRACT: The fatty acid composition of *Ginkgo biloba* seed lipids was reinvestigated with particular emphasis on the polymethylene-interrupted octadecadienoic and eicosadienoic acids. Analysis of the picolinyl esters and 4,4-dimethyloxazoline derivatives by capillary gas–liquid chromatography on a highly polar cyanopropyl polysiloxane stationary phase coupled with mass spectrometry revealed the presence of three such acids, with the structures 5,9-18:2, 5,11-18:2, and 5,11-20:2. This indicated that in *G. biloba* seeds, *cis*-vaccenic (11-18:1) acid may be a substrate for the Δ5-desaturase characteristic of gymnosperms. The 5,11-18:2 acid was not limited to *G.* biloba, as it may occur in a few other species. The 5,11-20:2 acid is a common component of the seed lipids from almost all gymnosperm species analyzed so far.

Paper no. J8630 in *JAOCS 76*, 273–277 (February 1999).

KEY WORDS: 4,4-Dimethyloxazoline derivatives, eicosadienoic acids, *Ginkgo biloba*, gymnosperms, mass spectrometry, octadecadienoic acids, picolinyl esters, seed lipids.

Gellerman and Schlenk (1,2) were apparently the first researchers to study the fatty acid composition of *Ginkgo biloba* seed and leaf lipids, about 35 yr ago. These authors established that the seed lipids contained some acids that were considered uncommon curiosities at that time, i.e., 5,11-18:2, 5,11,14-18:3, 5,11-20:2, and 5,11,14-20:3 acids (plus 5,11,14,17-20:4 acid in the leaf lipids). However, the chemical methods used to establish these structures were rather complicated and led sometimes to ambiguous results. For example, these authors hesitated on the structure 5,11-18:2, which was later reported as 5,9- *or* 5,11-18:2 acid (3). Takagi and Itabashi (4) analyzed the same material by capillary gas-liquid chromatography (GLC) and could confirm some of the results of Schlenk and Gellerman (1–3). However, they established that the uncommon octadecatrienoic acid had the structure 5,9,12-18:3 instead of 5,11,14-18:3, and they additionally found that two polymethylene-interrupted octadecadienoic acids were simultaneously present, and for which they proposed the structures 5,9- and 5,11-18:2 acids on the basis of their equivalent chain lengths. Kim *et al*. (5) identified the same Δ5-olefinic acids as Takagi and Itabashi (4), except that they were unable to characterize the 5,11-18:2 acid and they only tentatively identified the 5,11-20:2 acid. More recently, Hierro *et al*. (6) reinvestigated the fatty acid composition of *G. biloba* seed lipids by mass spectrometry (MS) of their picolinyl esters, and they essentially found the same Δ5-unsaturated polymethylene-interrupted fatty acids (Δ5-UPIFA) as the former authors, but they failed to characterize the 5,11-18:2 acid and did not report the presence of 5,11-20:2 acid.

In unpublished experiments, the ambiguity concerning the existence of 5,9-18:2 and/or 5,11-18:2 acids could be explained by their behavior during capillary GLC. With a polyethylene-glycol stationary phase (DB-Wax; J&W Scientific, Folsom, CA), similar to that used by Hierro *et al.* (6) (CP-Wax 52 CB, Chrompack, Middelburg, The Netherlands) and Kim *et al.* (5) (Carbowax 20M; Hewlett-Packard, Avondale, PA) for their GLC–MS or GLC studies, the 5,9-18:2 acid was well-resolved from other adjoining peaks (11-18:1 and 9,12-18:2 acids), but it showed a shoulder on the descending edge of the peak (results not shown). On the other hand, with a cyanopropyl polysiloxane stationary phase (CP-Sil 88, Chrompack), two peaks with base-line resolution were apparent, one of which could be identified as 5,9-18:2 acid by its equivalent chainlength (ECL), and the other was unknown but could correspond to the 5,11-18:2 acid. In the present study, another cyanopropyl polysiloxane stationary phase (BPX 70; SGE, Melbourne, Australia) was used to establish the structures of the two octadecadienoic acids by GLC–MS of their picolinyl ester and 4,4-dimethyloxazoline (DMOX) derivatives. Our results show unambiguously that both the 5,9- and 5,11-18:2 acids coexist in *G. biloba* seed lipids, demonstrating that 11-18:1 acid may be a substrate of the Δ5-desaturase in some gymnosperms. We also confirm the presence of 5,11-20:2 acid, a component present in the seed lipids of all gymnosperm species analyzed so far.

EXPERIMENTAL PROCEDURES

Seeds. Ginkgo biloba seeds, collected in Italy, were purchased from the Versepuy Society (Le Puy-en-Velay, France). The extraction of lipids from dehulled seeds (66 g) and the preparation of fatty acid methyl esters (FAME) were performed as described in detail elsewhere for other gymnosperm seeds (7).

Analytical GLC. FAME were analyzed in a Carlo Erba 4130 chromatograph (Carlo Erba, Milano, Italy) equipped

*To whom correspondence should be addressed at ISTAB, Laboratoire de Lipochimie Alimentaire, Université Bordeaux 1, Avenue des Facultés, 33405 Talence Cedex, France. E-mail: r.wolff@istab.u-bordeaux.fr

with a DB Wax column (30 m × 0.32 mm i.d., 0.5 μm film; J &W Scientific). The oven temperature was 190°C, and the inlet pressure of the carrier gas (helium) was 140 kPa (flow rate, 3 mL/min). Alternately, a CPSil 88 column (50 m × 0.25 mm i.d., 0.2 μm film; Chrompack) was operated at 160°C with helium at 100 kPa (flow rate, 0.8 mL/min). The injector (split mode) and the flame-ionization detector were maintained at 250°C. Quantitative data were calculated by an SP 4290 integrator (Spectra Physics, San Jose, CA). Identification of fatty acids was made *via* their ECL as described previously (8).

GLC–MS. Methyl esters were hydrolyzed to the free fatty acids (9) before conversion to the picolinyl ester derivatives as described by Balazy and Nies (10). Alternately, DMOX derivatives were prepared directly from FAME by the method of Fay and Richli (11). The derivatives were submitted to GLC–MS, with a Hewlett-Packard 5890 Series II Plus gas chromatograph attached to an HP model 5989 MS apparatus. The latter was used in the electron impact mode at 70 eV with a source temperature of 250°C. The chromatograph was fitted with on-column injection, and equipped with a capillary column of fused silica coated with BPX-70 (50 m × 0.22 mm i.d.,

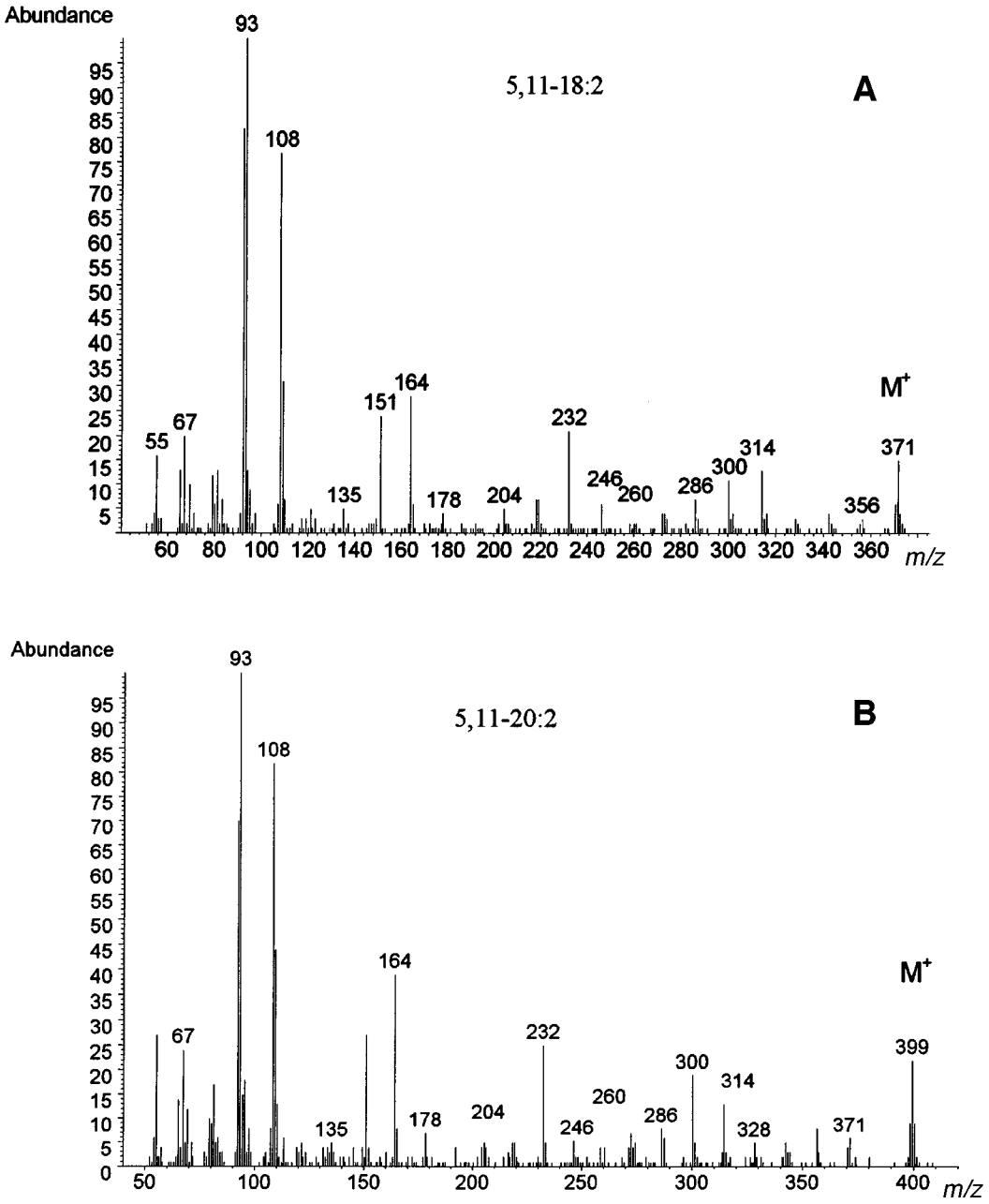

FIG. 1. Mass spectra of the picolinyl esters of 5,11-octadecadienoate (A) and 5,11-eicosadienoate (B) from *Ginkgo biloba* seed lipids.

0.25 μm film; SGE). After holding the temperature at 80°C for 3 min, the column was temperature-programmed at 20°C/min to 160°C, then at 2°C/min to 260°C, where it was held for 5 min. Helium at 1 mL/min was the carrier gas.

RESULTS AND DISCUSSION

The picolinyl esters and DMOX derivatives of fatty acids prepared from *G. biloba* seeds were examined by GLC–MS, using a polar BPX-70 column for the separation. The spectra of the picolinyl esters are illustrated in Figure 1. The mass spectrum of picolinyl 5,11-octadecadienoate had a molecular ion at *m/z* = 371, while that for 5,11-eicosadienoate had the molecular ion at 28 amu higher at *m/z* = 399. In both spectra, gaps of 26 amu from *m/z* = 178 to 204 and from 260 to 286 confirmed the presence of double bonds in positions 5 and 11, respectively. With the latter, a gap of 40 amu for cleavage between carbon atoms 10 and 12, i.e., from *m/z* = 246 to 286, is easier to locate for identification purposes. The prominent ions at *m/z* = 300 and 314 also are a guide to the presence of the ethylenic bond in position 11 (12). An abundant ion at *m/z* = 232 is characteristic for a double bond in position 5 (13). On either side of the double bonds, there are regular series of ions 14 amu apart, confirming that no further double bonds are present. The spectra are best compared with the published spectrum of picolinyl 5,12-octadecadienoate, which is very similar except that the gap of 26 amu for the double bond in position 12 is shifted 14 amu to between *m/z* = 274 and 300 (14). The spectra of the DMOX derivatives supported these assignments (results not shown).

Our results show unambiguously that the 5,9- and 5,11-18:2 acids coexist in *G. biloba* seed lipids and confirm the tentative identification of the latter by Takagi and Itabashi (4), based on calculated ECL. Moreover, the structure 5,11-20:2, initially established by chemical means (1,2), and later by GLC and comparison with a reference specimen obtained from sea urchins (4), is now definitively established in *G. biloba.*

In the present study, the 5,9- and 5,11-18:2 acids accounted for 2.0 and 1.2%, respectively, of total fatty acids, which is in fairly good agreement with the level of the fatty acid reported as 5,9-18:2 acid (certainly, in retrospect, a mixture of 5,9- and 5,11-18:2 acids) by Kim *et al.* (5) and Hierro *et al.* (6) (2.8 and 2.7%, respectively). Takagi and Itabashi (4) found 1% of 5,9-18:2 acid and 1.8% of 5,11-18:2 acid in neutral lipids. The corresponding values in polar lipids were 1 and 0.9%, respectively.

According to Takagi and Itabashi (4), the 5,11-18:2 isomer was not limited to *G. biloba* seeds, as it also occurs in *Cycas revoluta* and *Podocarpus macrophylla* seeds, and in trace amounts in *Ephedra sinica* stalks. The 5,11-20:2 acid occurs in the seed lipids of all gymnosperm species analyzed so far (4,7,15–19), at levels always less than 1%. Probably because this acid is always low, and sometimes present in trace amounts only, some authors did not mention it for a few species (mainly Pinaceae) (20–22), though other authors mentioned its presence in the same species (4,7,15–19). In one study, the 5,11-20:2 acid was tentatively identified as a 13-20:1 acid, with a question mark (21). In fact, this monoene, which is the elongation product of *cis*-vaccenic acid, was detected only in *G. biloba* (4–6) and *E. campylopoda* (23) seed lipids among about 170 species of gymnosperm analyzed. A confusion between 13-20:1 and 5,11-20:2 acids is explained by the fact that the two acids elute very close to each other, with a poor resolution when analyzed by GLC on polyethylene glycol stationary phases (5). On the other hand, they cannot be confused when using a cyanopropyl polysiloxane stationary phase, where they emerge with elution times differing by several minutes (results not shown).

A metabolic pathway for the synthesis of common and Δ5-unsaturated fatty acids was proposed initially by Itabashi and Takagi (24), who analyzed the fatty acid composition of *Taxus cuspidata* (Japanese yew) seed, aril, and leaf lipids. Later, Wolff *et al.* (7) proposed a similar scheme based on the analysis of the seed fatty acids from 28 species belonging to the families Pinaceae, Taxaceae, Sciadopityaceae, Taxodiaceae, and Cupressaceae. Both groups of researchers postulated the existence of a Δ5-desaturase that would use 9-18:1, 9,12-18:2, 9,12,15-18:3, and their elongation products, 11-20:1, 11,14-20:2, and 11,14,17-20:3 acids as substrates.

In the light of the present study, which essentially confirms the tentative identifications by Takagi and Itabashi (4), we can add some precisions to the formerly proposed biosynthesis of Δ5-UPIFA in gymnosperm seeds. The level of 9-16:1 acid in *G. biloba* seed lipids is exceptionally high among gymnosperms, reaching *ca.* 3.1–3.4% of total fatty acids (4–6). Apparently, this monoenoic acid is a good substrate of the elongase that leads to the formation of *cis*-vaccenic (11-18:1) acid, in the range 17–22%, which is higher than the level of oleic acid (4-6). This is also exceptional among gymnosperms. Both octadecenoic acids are elongated to 11-20:1 and 13-20:1 acids, but to a low extent only; each eicosenoic acid represents less than 0.7% of the total fatty acids. Of these, only the 11-20:1 isomer is apparently substrate of the Δ5-desaturase.

A 5,11-18:2 acid was reported in *E. campylopoda* seed oil at a level of 2.0% of total fatty acids (23). Because the content of 11-18:1 acid is fairly high in this species (6.2%), the presence of 5,11-18:2 acid appears theoretically possible, but it is also probable that this acid may be a mixture of 5,9- and 5,11-18:2 acids, as shown in the present study for *G. biloba* seeds. This deserves reinvestigation. In two major studies, Takagi and Itabashi (4) and Wolff *et al.* (7) observed that gymnosperm seed lipids always contained several members of the Δ5-UPIFA series, a situation that also occurred in conifer leaf lipids (25). However, Vickery *et al.* (26), who analyzed some Australian and Tasmanian species of the families Cupressaceae, Podocarpaceae, Taxodiaceae, and Zamiaceae, only mentioned the 5,11,14,17-20:4 acid as a Δ5-UPIFA in seed and leaf lipids. Here too, reinvestigations are needed.

ACKNOWLEDGMENT

This paper is published as part of a program funded by the Scottish Office Agriculture, Environment and Fisheries Dept.

REFERENCES

1. Gellerman, J.L., and H. Schlenk, A Group of Fatty Acids with Tetramethylene Interruption in the Double Bond System, *Experientia 19*:522–-523 (1963).
2. Schlenk, H., and J.L. Gellerman, Arachidonic, 5,11,14,17-Eicosatetraenoic and Related Acids in Plants-Identification of Unsaturated Fatty Acids, *J. Am. Oil Chem. Soc. 42*:504–511 (1965).
3. Gellerman, J.L., and H. Schlenk, Preparation of ^{14}C-Labeled Fatty and Anacardic Acids from *Ginkgo biloba*, *Lipids 4*:484–487 (1969).
4. 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).
5. Kim, S.-J., K.-H. Lee, Y.-S. Kim, and Y.-G. Joh, Studies on the Presence of All-*cis*-Δ5,11,14-C20:3 Fatty Acid in the Seeds of Gingko, *J. Korean Oil Chem. Soc. 10*:57–65 (1993).
6. Hierro, M.T.G., G. Robertson, W.W. Christie, and Y.G. Joh, The Fatty Acid Composition of the Seeds of *Ginkgo biloba*, *J. Am. Oil Chem. Soc. 73*:575–579 (1996).
7. Wolff, R.L., L.G. Deluc, and A.M. Marpeau, Conifer Seeds: Oil Content and Fatty Acid Composition, *Ibid. 73*:765–771 (1996).
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, *Ibid.73*:1323–1326 (1996).
9. Christie, W.W., *Gas Chromatography and Lipids*, Oily Press, Dundee, Scotland, 1989, pp. 64–84.
10. Balazy, M., and A.S. Nies, Characterization of Epoxides of Polyunsaturated Fatty Acids by Mass Spectrometry *via* Pyridinylmethyl Esters, *Biomed. Environ. Mass Spectrom. 18*:328–336 (1989).
11. Fay, L., and U. Richli, Location of Double Bonds in Polyunsaturated Fatty Acids by GC–MS After 4,4-Dimethyloxazoline Derivatization, *J. Chromatogr. 541*:89–98 (1991).
12. Harvey, D.J., Mass Spectrometry of Picolinyl and Other Nitrogen-Containing Derivatives of Lipids, *Advances in Lipid Methodology–One*, edited by W.W. Christie, The Oily Press, Dundee, 1992, pp. 19–80.
13. Christie, W.W., E.Y. Brechany, and R.T. Holman, Mass Spectra of the Picolinyl Esters of Isomeric Mono- and Dienoic Fatty Acids, *Lipids 22*:224–228 (1987).
14. Christie, W.W., E.Y. Brechany, F.D. Gunstone, M.S.F. Lie Ken Jie, and R.T. Holman, Mass Spectra of the Picolinyl Ester Derivatives of Some Non-Methylene-Interrupted Octadecadienoic Acids*, Ibid. 22*:664–666 (1987).
15. Wolff, R.L., B. Comps, L.G. Deluc, and A.M. Marpeau, Fatty Acids of the Seeds from Pine Species of the *Ponderosa-Banksiana* and *Halepensis* Sections. The Peculiar Taxonomic Position of *Pinus pinaster, J. Am. Oil Chem. Soc. 74*:45–50 (1997).
16. Wolff, R.L., L.G. Deluc, A.M. Marpeau, and B. Comps, Chemotaxonomic Differentiation of Conifer Families and Genera Based on the Seed Oil Fatty Acid Compositions: Multivariate Analyses, *Trees 11*:57–65 (1997).
17. Wolff, R.L., B. Comps, A.M. Marpeau, and L.G. Deluc, Taxonomy of *Pinus* Species Based on the Seed Oil Fatty Acid Compositions, *Ibid 11*:113–118 (1997).
18. Imbs, A.B., and L.Q. Pham, Fatty Acids and Triacylglycerols in Seeds of Pinaceae Species, *Phytochemistry 42*:1051–1053 (1996).
19. Wolff, R.L., and C.C. Bayard, Fatty Acid Composition of Some Pine Seed Oils, *J. Am. Oil Chem. Soc. 72*:1043–1046 (1995).
20. Seher, A., M. Krohn, and Y.S. Ko, Zusammensetzung Einiger Koreanischer Samenöle, *Fette Seifen Anstrichm. 79*:203–206 (1977).
21. Kim, S.J., G.S. Kim, M.O. Yi, and Y.G. Joh, Fatty Acid Composition of Some Seed Oils from the Pinaceae Family, *J. Korean Oil Chem. Soc. 9*:149–156 (1992)
22. Hu, Z.L., X.P. Li, and H. Bao, Distribution of Fatty Acids from the *Pinus* Seed Oils and the Chemotaxonomic Survey, *J. Plant Res. Environ. (Zhiwu Ziyuan Yu Huanjing) 1*:15–18 (1992).
23. Kleiman, R., G.F. Spencer, F.R. Earle, and I.A. Wolff, Fatty Acid Composition of *Ephedra campylopoda* Seed Oil, *Chem. Ind.:*1326–1327 (1967).
24. Itabashi, Y., and T. Takagi, *Cis*-5-Olefinic Nonmethylene-Interrupted Fatty Acids in Lipids of Seeds, Arils and Leaves of Japanese Yew, *Yukagaku 31*:574–579 (1982).
25. Jamieson, G.R., and E.H. Reid, The Leaf Lipids of Some Conifer Species, *Phytochemistry 11*:269–275 (1972).
26. Vickery, J.R., F.B. Whitfield, G.L. Ford, and B.H. Kennett, The Fatty Acid Composition of Gymnospermae Seed and Leaf Oils, *J. Am. Oil Chem. Soc. 61*:573–575 (1984).

[Received September 2, 1997; accepted January 9, 1999]