Gas–Liquid Chromatography–Mass Spectrometry of the 4,4-Dimethyloxazoline Derivatives of Δ5-Unsaturated Polymethylene-Interrupted Fatty Acids from Conifer Seed Oils

Olivier Berdeaux[a] and Robert L. Wolff[b,*]

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

ABSTRACT: The fatty acids from the seed oils of three Conifer species (one Pinaceae, *Pinus pinaster,* and two Cupressaceae, *Chamaecyparis lawsoniana* and *Biota orientalis*) have been analyzed as their 4,4-dimethyloxazoline (DMOX) derivatives by gas–liquid chromatography coupled with mass spectrometry. The structures of six Δ5-unsaturated polymethylene-interrupted fatty acids (Δ5-UPIFA) were established, confirming previous studies in which they were identified by their equivalent chainlengths (ECL) and by comparison with related authentic standards. These acids were: *cis*-5,*cis*-9 18:2, *cis*-5,*cis*-9,*cis*-12 18:3 *(P. pinaster)*, *cis*-5,*cis*-9,*cis*-12,*cis*-15 18:4 *(C. lawsoniana)*, *cis*-5,*cis*-11 20:2, *cis*-5,*cis*-11,*cis*-14 20:3 (all species), *cis*-5,*cis*-11,*cis*-14,*cis*-17 20:4 *(B. orientalis)* acids. In addition, *cis*-9 18:1, *cis*-9,*cis*-12 18:2 (all species) and *cis*-9,*cis*-12,*cis*-15 18:3 (Cupressaceae) acids, together with their elongation products [*cis*-11 20:1, *cis*-11,*cis*-14 20:2 (all species) and *cis*-11,*cis*-14,*cis*-17 20:3 *(B. orientalis)* acids] were also identified. In the mass spectra, DMOX derivatives of all Δ5-UPIFA showed an intense peak at *m/z* 153, which is a diagnostic ion of fatty acid derivatives with a Δ5-ethylenic bond. Other double bonds were localized by ion pairs that differed by 12 atomic mass units. The present study fully justifies the use of ECL to identify Δ5-UPIFA in Conifer seed oils, in which they are ordinary components.

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KEY WORDS: Conifer seed oil, Δ5-ethylenic bond, 4,4-dimethyloxazoline derivatives, equivalent chainlengths, gas–liquid chromatography, mass spectrometry.

Δ5-Unsaturated polymethylene-interrupted fatty acids (Δ5-UPIFA) have been identified in various plant species, most often in their *cis* form, but they appear to be characteristic components of Conifer seed oils (1–3), and probably more generally of Gymnosperm seed oils (1). Depending on the species, Conifer seed oils may contain several components of a series of six Δ5-UPIFA. The profile of these acids may even be used as a chemotaxonomic means to distinguish between the main families (for example, Pinaceae vs. Cupressaceae) (3).

All Conifer seed oils contain *cis*-9 18:1 and *cis*-9,*cis*-12 18:2 acids, together with low amounts of their elongation products, *cis*-11 20:1 and *cis*-11,*cis*-14 20:2 acids (1–3). They also contain the Δ5-desaturation products of the two latter acids, namely *cis*-5,*cis*-11 20:2 and *cis*-5,*cis*-11,*cis*-14 20:3 acids (1–3). They may contain the direct Δ5-desaturation product of *cis*-9 18:1 acid (*cis*-5,*cis*-9 18:2 acid) as a main component (some Taxaceae) and that of *cis*-9,*cis*-12 18:2 acid (*cis*-5,*cis*-9,*cis*-12 18:3 acid) in high amounts (most Pinaceae) (1–3). If the seed oils are rich in *cis*-9,*cis*-12,*cis*-15 18:3 acid (Cupressaceae and Taxodiaceae), they also contain its elongation product, *cis*-11,*cis*-14,*cis*-17 20:3 acid, and the Δ5-desaturation products of both acids (*cis*-5,*cis*-9,*cis*-12,*cis*-15 18:4—not systematically and generally in minute amounts—and *cis*-5,*cis*-11,*cis*-14,*cis*-17 20:4 acids) (1,3).

Most of these "uncommon" (uncommon in laboratories, not in nature) fatty acids have been structurally characterized by one or several analytical techniques, such as gas–liquid chromatography (GLC) alone or coupled with mass spectrometry (MS) of appropriate derivatives, silver-ion thin-layer chromatography, ^{1}H- and ^{13}C-nuclear magnetic resonnance (NMR) spectroscopy, and chemical localization of the ethylenic bonds (frequently ozonolysis) (1–7). In two recent studies (2,3), the individual Δ5-UPIFA in the seed oils of thirty two different Conifer species have been quantitated after their identification by comparison of their equivalent chainlengths (ECL) with those calculated with ECL of related authentic standards by GLC on one (2) or two (3) capillary columns with different polarities, and appropriate calculations (2). The overall content of Δ5-UPIFA in the seeds analyzed in one of these studies (2) was later confirmed by ^{13}C-NMR spectroscopy (7), but this technique did not give access to individual Δ5-UPIFA.

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

Consequently, the identification of individual Δ5-UPIFA remains to be justified *via* their ECL, though good agreements were obtained between experimental ECL and calculated ECL (2,3). These agreements can hardly be attributed to chance, but we felt that it was necessary to establish the structures of indivividual Δ5-UPIFA through another analytical means to definitively sustain our previous identifications, exclusively based on ECL (2,3).

For this purpose, we have analyzed by GLC–MS the 4,4-dimethyloxazoline (DMOX) derivatives of fatty acids prepared from the seed oils of *Pinus pinaster* (Pinaceae; a source of *cis*-5,*cis*-9 18:2, *cis*-5,*cis*-9,*cis*-12 18:3 and *cis*-5,*cis*-11,*cis*-14 20:3 acids), *Chamaecyparis lawsoniana* (Cupressaceae; a source of *cis*-5,*cis*-9,*cis*-12,*cis*-15 18:4 acid), and *Biota orientalis* (Cupressaceae; a source of *cis*-11,*cis*-14,*cis*-17 20:3 and *cis*-5,*cis*-11,*cis*-14,*cis*-17 20:4 acids). Data obtained in the present study confirm point by point results, previously established by comparison of experimental and calculated ECL (2,3), and fully support the conclusions of these studies. Moreover, they also emphasize the usefulness of ECL to predict the structures of Δ5-UPIFA, at least in Conifer seed oils, where these acids are ordinary components.

EXPERIMENTAL PROCEDURES

Seeds. Conifer seeds were obtained from the D'a Noste Society (Vendays-Montalivet, France), the French National Office for Forests (O.N.F.; Supt, France), and the Vilmorin Society (La Ménitré, France).

Oil extraction and fatty acid methyl ester (FAME) preparation. The oils were extracted from crushed, undehulled seeds, according to Folch *et al.* (8) as described previously (2,3). FAME were prepared according to Morrison and Smith (9) as detailed previously (2,3).

DMOX derivative preparation. FAME were converted into DMOX derivatives by heating with 2-amino-2-methylpropanol in a sealed vial at 170°C for 18 h (10,11). The cooled reaction mixture was dissolved in dichloromethane (3 mL) and washed twice with 1 mL of distilled water. After drying of the organic phase, the solvent was removed under a stream of N_2, and the sample was dissolved in hexane prior to further analyses.

GLC–MS of DMOX derivatives. An HP 5890 gas-chromatograph (Hewlett-Packard, Palo Alto, CA) coupled with a 5970 quadrupole mass-selective detector (Hewlett-Packard), was used to separate and to identify the DMOX derivatives. Two fused-silica capillary columns were employed: a BPX 70 column (50 m × 0.33 mm i.d., 0.25 μm film; SGE, Melbourne, Australia) and a DB Wax column (30 m × 0.25 mm i.d., 0.25 μm film; J&W Scientific, Folsom, CA). Helium was used as a carrier gas with a linear velocity of 35 cm/s. The oven was programmed from 60 to 170°C at 20°C/min. Splitless injection was used with the injection port maintained at 250°C. Mass spectra were generated by electronic impact at 70 eV.

RESULTS AND DISCUSSION

In the present study, we have established the mass spectra of the DMOX derivatives of all fatty acids occurring in *P. pinaster*, *C. lawsoniana*, and *B. orientalis* seed oils. The results for unsaturated acids are summarized in Table 1. Only the diagnostic fragments that allow localization of the double-bond positions are presented. As an illustration, Figure 1 shows the mass spectrum and the fragmentation pattern of the DMOX derivative of *cis*-5,*cis*-11,*cis*-14,*cis*-17 20:4 acid. We limit our discussion to this acid and consider the other fatty acids in general terms.

The mass spectrum of the DMOX derivative of *cis*-5,*cis*-11,*cis*-14,*cis*-17 20:4 acid showed intense ions at *m/z* 113 and 126, which are characteristic of DMOX derivatives of fatty acids (10–12). The molecular ion appeared at *m/z* 357. The Δ5-ethylenic bond was localized by the intense peak at *m/z* 153. This ion is a diagnostic ion in the mass spectra of DMOX derivatives of fatty acids with their first double bond in position 5 (10,12). In DMOX derivatives of mono- or polyunsaturated fatty acids, a mass interval of 12 atomic mass units (amu) instead of the regular 14 amu between two neighboring homologous mass fragments containing n − 1 and n carbon atoms of the original acid moiety, indicates a double bond between n and n + 1 in the chain (12). Thus, the Δ11, Δ14, and Δ17 unsaturations were clearly located by ion pairs of *m/z* 222

TABLE 1
Characteristic Ions in the Mass Spectra of 4,4-Dimethyloxazoline Derivatives (DMOX) of Unsaturated Fatty Acids Present in Conifer Seed Oils and Their Structures

Fatty acid[a] DMOX	M^+ *m/z* (intensity, %)	Fragment *m/z* (intensity, %)
9-18:1	335 (8.0)	182 (19.6); 196 (4.2); 208 (4.0)
5,9-18:2	333 (2.0)	153 (8.2); 194 (1.5); 206 (2.7)
9,12-18:2	333 (10.9)	196 (5.0); 208 (2.2); 236 (10.5); 248 (3.8)
5,9,12-18:3	331 (2.3)	153 (5.4); 194 (1.1); 206 (1.4); 234 (1.0); 246 (1.5)
9,12,15-18:3	331 (25.6)	196 (5.9); 208 (3.2); 236 (11.8); 248 (5.1); 276 (14.0); 288 (6.9)
5,9,12,15-18:4	329 (8.9)	153 (9.1); 194 (1.2); 206 (3.5); 234 (1.7); 246 (3.7); 274 (5.2); 286 (1.2)
11-20:1	363 (13.6)	210 (11.9); 224 (2.3); 236 (4.0); 264 (11.3)
5,11-20:1	361 (31.7)	153 (7.1); 180 (11.0); 208 (3.8); 222 (2.8); 234 (1.3); 262 (4.7)
11,14-20:2	361 (31.7)	224 (6.9); 236 (11.0); 264 (10.6); 276 (8.9)
5,11,14-20:3	359 (1.7)	153 (9.8); 222 (1.3); 234 (1.1); 262 (1.4); 274 (1.1); 302 (4.5)
11,14,17-20:3	359 (18.0)	224 (2.2); 236 (1.2); 264 (7.0); 276 (3.0); 304 (5.1); 316 (1.9)
5,11,14,17-20:4	357 (6.5)	153 (15.2); 222 (2.4); 234 (4.0); 262 (3.8); 274 (3.7); 302 (4.1); 314 (2.2)

[a]All ethylenic bonds in the *cis* configuration.

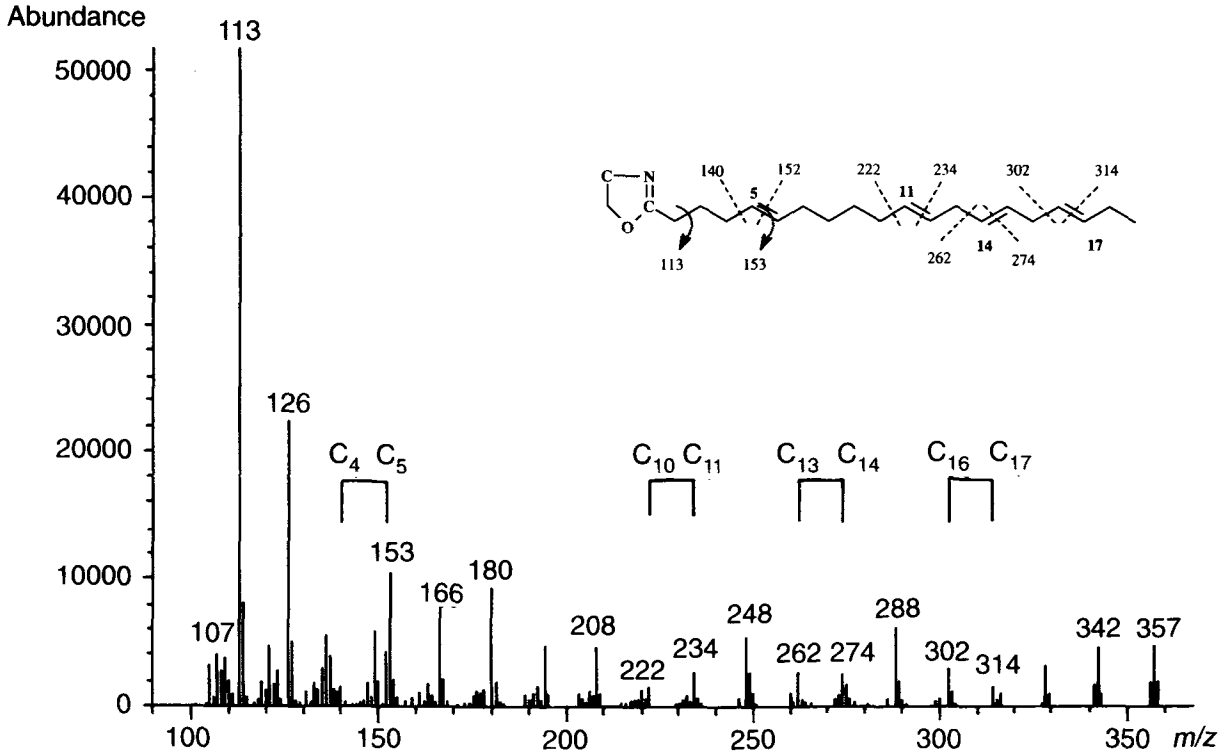

FIG. 1. Mass spectrum and fragmentation pattern of the 4,4-dimethyloxazoline derivative of *cis*-5,*cis*-11,*cis*-14,*cis*-17 20:4 acid.

(C_{10}) and 234 (C_{11}), *m/z* 262 (C_{13}) and 274 (C_{14}), and *m/z* 302 (C_{16}) and 314 (C_{17}).

Based on the same principles, all other unsaturated fatty acids, including Δ5-UPIFA, could be identified. Clearly, our results sustain previous identifications by GLC and arithmetic calculation of ECL by using appropriate standards (2,3). ECL values given in Table 2 can thus be usefully employed to identify Δ5-UPIFA without other complementary analytical technique. We also confirm the existence of the *cis*-5,*cis*-9,*cis*-12,*cis*-15 18:4 acid in some Conifer seed oils, an acid that was only tentatively identified until now (1,3). This acid is generally a minor component in Cupressaceae seeds, except for those of *C. lawsoniana*, where it reaches *ca.* 2% of total fatty acids. On the other hand, this acid is a widespread component in Conifer needle lipids (13). Confirmation of the identities of Δ5-UPIFA also supports the biochemical pathways for

TABLE 2
Experimental and Calculated Equivalent Chainlengths of Δ5-Unsaturated Polymethylene-Interrupted Fatty Acids Found in Conifer Seed Oils on Three Different Capillary Columns[a]

		CP Sil 88 (Ref. 2)		DB Wax (Ref. 3)		Silar 5CP (Ref. 1)	
Chainlength	Double-bond position	Exp.	Calc.	Exp.	Calc.	Exp.	Calc.
C_{18}	5	18.44		18.16		18.11	
	9	18.67		18.23		18.24	
	5,9	19.10	19.11	18.43	18.39	18.37	18.35
	9,12	19.57		18.69		18.69	
	5,9,12	20.02	20.00	18.91	18.89	18.82	18.82
	9,12,15	20.62		19.37		19.24	
	5,9,12,15	21.09	21.07	19.59	19.59	19.37	19.37
C_{20}	5	20.40		20.12		20.10	
	11	20.59		20.21		20.21	
	5,11	21.02	20.99	20.37	20.33	20.32	20.31
	11,14	21.50		20.69		20.66	
	5,11,14	21.94	21.93	20.83	20.85	20.77	20.77
	11,14,17	22.52		21.33		21.21	
	5,11,14,17	22.97	22.97	21.49	21.47	21.33	21.32

[a]Exp., experimental values; Calc., calculated values. Values calculated according to Reference 2.

their synthesis as recently proposed (3). In Conifer seeds, the Δ5-desaturase(s) may introduce a Δ5-ethylenic bond in *cis*-9 18:1, *cis*-9,*cis*-12 18:2, and *cis*-9,*cis*-12,*cis*-15 18:3 acids, and also in the elongation products of these acids, *cis*-11 20:1, *cis*-11,*cis*-14 20:2 and *cis*-11,*cis*-14,*cis*-17 20:3 acids. These possibilities result in the potential formation of six different Δ5-UPIFA, three of which have 18 carbon atoms and the three others 20 carbon atoms. In *Ginkgo biloba* seeds, which is a Gymnosperm but not a Conifer, the *cis*-11 18:1 acid, a major component in the oil, also seems to be a substrate for the desaturase, leading to the formation of *cis*-5,*cis*-11 18:2 acid (1) in addition to *cis*-5,*cis*-9 18:2 acid.

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