Analysis of Estolides in Technical Hydroxylated Fatty Acids from Plant Oils

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ABSTRACT: Gel permeation chromatography of hydroxylated fatty acids (HOFA), prepared from various plant oils by a novel technical process, showed the presence of considerable amounts of estolides formed by intermolecular esterification of the HOFA. Thin-layer chromatographic fractionation followed by gas chromatography of the fractions revealed that the nonpolar estolides contain predominantly saturated fatty acids esterified to *threo*-9,10-dihydroxy octadecanoic acid or dihydroxy tetrahydrofuran octadecanoic acids, e.g., 9,12-dihydroxy-10,13-epoxy octadecanoic acid and 10,13-dihydroxy-9,12-epoxy octadecanoic acid. The fractions of polar estolides consist mainly of intermolecular esters of the above dihydroxy fatty acids.

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An accompanying paper (1) has shown that hydroxylated fatty acids (HOFA) prepared by a novel technical process (2,3) from the oils of rapeseed, sunflower, linseed, and *Euphorbia lathyris* seed contain as major constituents *threo*-9,10-dihydroxy octadecanoic acid, derived from oleic acid, and positional and stereoisomers of dihydroxy tetrahydrofuran (THF) octadecanoic acids, e.g., 9,12-dihydroxy-10,13-epoxy octadecanoic acid and 10,13-dihydroxy-9,12-epoxy octadecanoic acid, derived from linoleic acid by intramolecular THF ring formation. The present communication reports the analysis of technical HOFA products mainly by gel permeation chromatography (GPC), i.e., size-exclusion chromatography. So far, GPC has been used for the analysis of low-molecular lipids only to a minor extent, but it has been shown to be a valuable method for the separation of polymerized lipids and lipoproteins (4). As early as 1973, dimeric, trimeric, and higher oligomeric triacylglycerols were determined by GPC in thermal-oxidatively treated oils (5,6). The application of spherical, pressure-stable gels that are resistant to organic solvents enabled the separation of organic compounds according to their molecular size by high-performance GPC. This method was used for the analysis of monomeric and polymeric glycerolipid derivatives in used deep-frying fats and autoxidized fats (7–9).

In the present study, technical HOFA and technical HOFA methyl esters were analyzed by high-performance GPC to obtain information on the molecular weight distribution of the reaction products, which are of commercial interest for the manufacture of technical products, e.g., polyurethanes and polyesters (10–12).

EXPERIMENTAL PROCEDURES

Chemicals. HOFA and HOFA methyl esters derived from low erucic acid rapeseed oil, conventional sunflower oil, linseed oil, and seed oil of *E. lathyris*, as well as dimeric and trimeric fatty acids, were technical products from Harburger Fettchemie Brinkman & Mergell (HOBUM) (Hamburg, Germany). Hydroxy fatty acid standards, i.e., ricinoleic acid as well as *threo*- and *erythro*-9,10-dihydroxy octadecanoic acids, were purchased from Sigma Chemie (Deisenhofen, Germany). Methyl esters of the standards were prepared according to Chalvardjian (13). Trimethylsilyl (TMSi) derivatives were prepared with *N,O-bis*-trimethylsilyl acetamide [(BSA) Machery-Nagel, Düren, Germany] as silylating reagent (14). THF [high-performance liquid chromatography (HPLC)-grade] was purchased from Zinsser Analytic (Frankfurt/M., Germany).

Analytical thin-layer chromatography (TLC). Samples of technical HOFA or HOFA methyl esters derived from various plant oils were separated on layers of silica gel H (E. Merck, Darmstadt, Germany) with hexane/diethyl ether/acetic acid (20:80:1, by vol) or hexane/diethyl ether (1:4, vol/vol), respectively. Oleic and ricinoleic acids as well as *threo*- and *erythro*-9,10-dihydroxy octadecanoic acids and methyl esters derived therefrom were used as standards. All lipid fractions on TLC plates were detected by charring with chromic–sulfuric acid spray and heating at 200°C.

Preparative TLC. Technical HOFA methyl esters were solubilized in dichloromethane/methanol (1:1, vol/vol), applied to layers of silica gel H (layer thickness 0.5 mm) and fractionated with hexane/diethyl ether (1:4, vol/vol). Lipid fractions were recovered from silica gel by elution with water-saturated diethyl ether.

Saponification of estolides. Nonpolar and polar estolides were saponified overnight under nitrogen with 5 mL of 90% ethanolic KOH (0.5 N) at room temperature. The reaction

mixture was acidified with 6 N HCl, diluted with 5 mL water and extracted three times with diethyl ether. The organic extract was washed with water till neutral, the solvent was evaporated and the fatty acids were dried in a stream of nitrogen.

Gas chromatography (GC) of TMSi derivatives of technical HOFA products. GC was carried out in a Hewlett-Packard HP-5890 Series II gas chromatograph. HOFA, technical methyl esters of HOFA, or saponified estolide fractions were silylated overnight at room temperature with BSA (14) and separated on an HP-1 capillary column (Hewlett-Packard, Waldbronn, Germany; 25 m × 0.32 mm i.d., 0.52 µm film thickness), with helium as carrier gas (column head pressure 120 kPa) and a temperature program of 4 min at 150°C initially, followed by linear programming from 150 to 250°C at a rate of 10°C/min; finally the temperature was held at 250°C for 16 min. The split ratio was 30:1, and the injector and thermal conductivity detector (TCD) temperature were both 270°C.

GPC. The chromatographic system consisted of a Kontron pump 325 (Eching, Germany), a Rheodyne 7125 injector with 20 µL sample loop (Cotati, CA), a Gilson model 131 refractive index detector (Villiers le Bel, France), and a Kontron data system 450-MT2. HOFA or methyl esters of HOFA were dissolved in THF at 5% (wt/vol). Separations were performed on two columns in series (7.5 × 300 mm, each) of PLgel (Polymer Laboratories, Shropshire, United Kingdom; particle size 5 µm, column 1: pore size 500 Å and column 2: pore size 100 Å) with a pre-column (7.0 × 50 mm). The columns were eluted at room temperature with THF at a flow rate of 0.75 mL/min.

RESULTS AND DISCUSSION

HOFA and methyl esters derived therefrom were technical products prepared from various plant oils, such as rapeseed, sunflower, and linseed oil as well as the seed oil of *E. lathyris* (2,3). HOFA or HOFA methyl esters were analyzed by GPC to determine the molecular weight distribution of the reaction products.

Figure 1 shows the gel permeation chromatogram of technical HOFA derived from rapeseed oil. At least five distinct peaks of different molecular weights were detected. About 45% of the sample consists of monomeric dihydroxy fatty acids and about 7% of saturated (nonhydroxylated) fatty acids. The remaining peaks were assigned to dimeric (28%), trimeric (13%), and tetrameric HOFA including traces of higher oligomers (7%). The identity of oligomeric fatty acids, very likely formed from dihydroxy fatty acids by intermolecular esterification with saturated (nonhydroxylated) fatty acids or other HOFA, was confirmed by comparison with the retention times of various standards (Fig. 2).

The results obtained with technical HOFA products from sunflower oil, *E. lathyris* oil, and linseed oil are given in Table 1. In these products, the proportion of monomeric HOFA varies between 40 and 45%, and that of oligomeric fatty acids between 48 and 56% (Table 1).

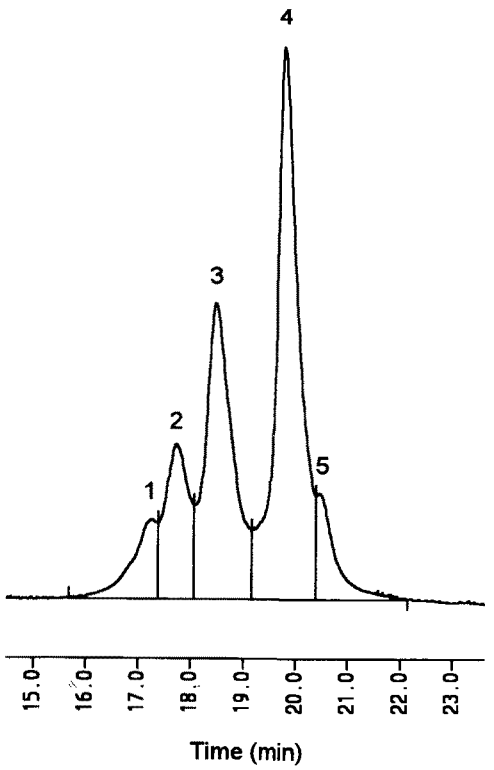

FIG. 1. Gel permeation chromatogram of technical hydroxylated fatty acids derived from rapeseed oil: (1) tetramers and higher oligomers, retention time (RT) = 17.3 min; (2) trimers, RT = 17.7 min; (3) dimers, RT = 18.5 min; (4) dihydroxy octadecanoic acids, RT = 19.8 min; (5) saturated fatty acids, RT = 20.4 min.

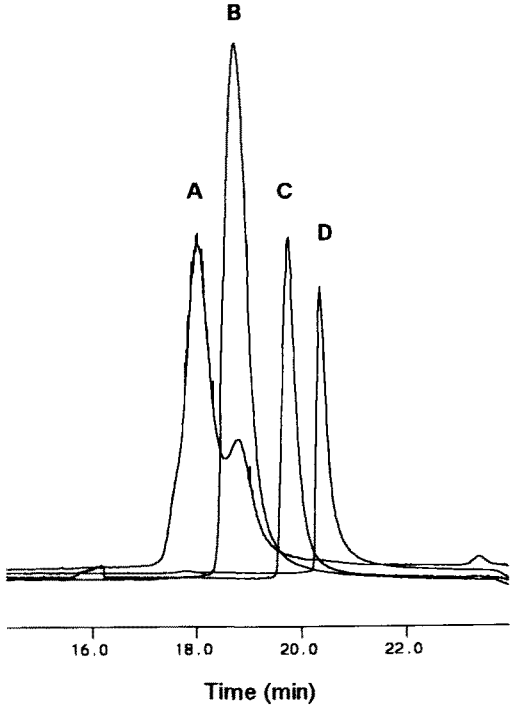

FIG. 2. Chromatogram overlay plot of various standard fatty acids separated by gel permeation chromatography: (A) trimeric fatty acids (with minor part of dimeric fatty acids), RT = 18.0 min; (B) dimeric fatty acids, RT = 18.6 min; (C) *threo*-9,10-dihydroxy octadecanoic acid, RT = 19.8 min; (D) stearic acid, RT = 20.4 min. See Figure 1 for abbreviation.

TABLE 1
Percentage of Saturated Fatty Acids and HOFA Monomers and Oligomers Derived from Various Seed Oils as Analyzed by GPC

		HOFA				
Source oil	Saturated FA	Monomeric	Dimeric	Trimeric	Tetrameric[a]	Total oligomers
Rapeseed	7	45	28	13	7	48
Sunflower	5	45	28	15	7	50
Euphorbia lathyris	4	40	29	15	12	56
Linseed	6	41	30	16	7	53
After additional saponification:						
Rapeseed	9	79	12	—	—	12
Sunflower	6	82	12	—	—	12

[a]Including traces of higher oligomers. FA, fatty acids; HOFA, hydroxylated fatty acids; GPC, gel permeation chromatography.

Plotting the logarithm of the calculated molecular weights of HOFA oligomers against their retention times showed a linear relationship between the *threo*-9,10-dihydroxy octadecanoic acid standard and the technical oligomers derived by intermolecular esterification (Fig. 3).

When the HOFA product derived from rapeseed oil was saponified in the technical process, the proportion of oligomers was reduced from about 48 to 12% (Table 1); the remaining oligomeric compounds may consist predominantly of dimer acids that are attached by C-C bonds (12,15). Finally, 79% of the sample consisted of monomeric HOFA (Table 1).

Similar results were obtained when the HOFA product from sunflower oil was saponified in the technical process (Table 1). After the hydroxylation process, the product consists of about 45% monomeric hydroxy fatty acids and 50% oligomers. Subsequent saponification of this product leads to about 82% HOFA monomers and only 12% nonsaponifiable dimeric fatty acids (Table 1).

Methylation of the HOFA products from rapeseed, sunflower, *E. lathyris*, and linseed oil in the technical process slightly reduced the proportion of oligomers to about 44–49% (Fig. 4) as compared to 48–56% in the fatty acids (Fig. 1) and correspondingly increased the proportion of monomers.

Technical HOFA methyl esters from *E. lathyris* seed oil and sunflower oil were separated by preparative TLC into

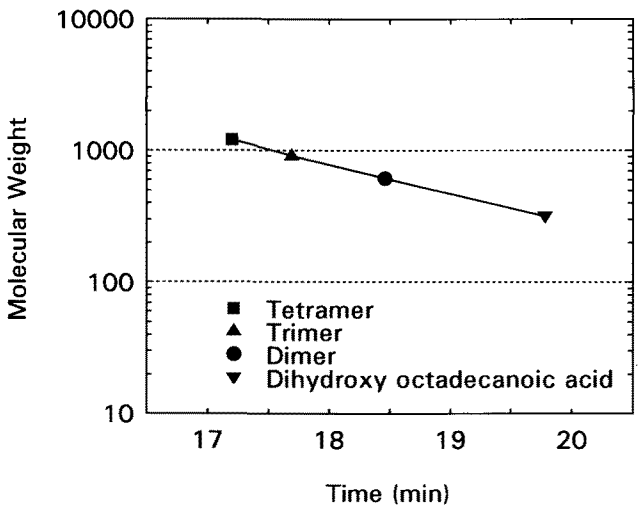

FIG. 3. Semi-log plot of calculated molecular weights of *threo*-9,10-dihydroxy octadecanoic acid as well as di-, tri- and tetramers of technical hydroxylated fatty acids against retention time in gel permeation chromatography.

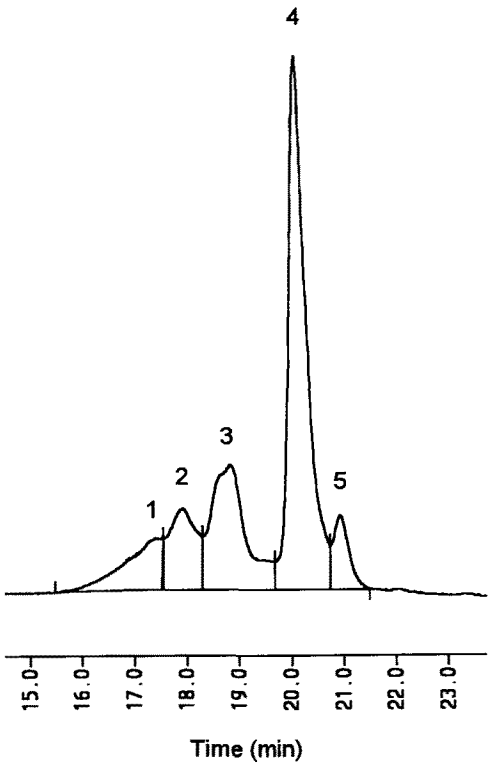

FIG. 4. Gel permeation chromatogram of technical product of methyl esters of hydroxylated fatty acids derived from rapeseed oil: (1) tetramers and higher oligomers, (2) trimers, (3) dimers, (4) methyl dihydroxy octadecanoates, (5) methyl esters of saturated fatty acids.

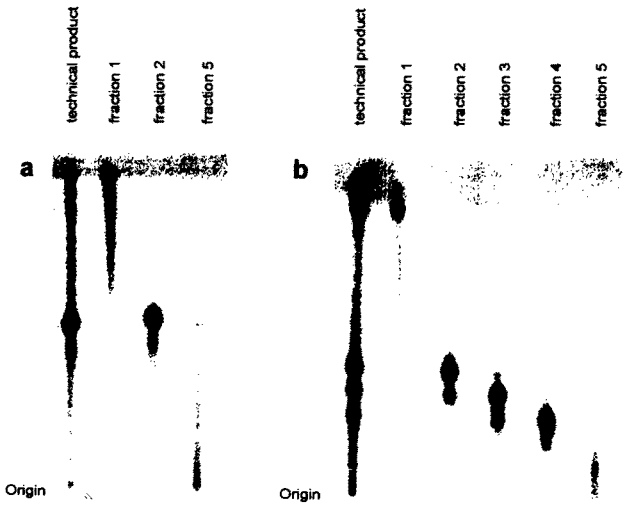

FIG. 5. Thin-layer chromatograms of fractions of technical hydroxylated fatty acid methyl esters from (a) *Euphorbia lathyris* oil and (b) sunflower oil, isolated by preparative thin-layer chromatography.

lipid fractions of various polarities (Fig. 5) as described in the accompanying paper (1). Fractionation of hydroxylated *E. lathyris* seed oil is shown in Figure 5a. The technical product from *E. lathyris* oil contains as major component *threo*-9,10-dihydroxy octadecanoic acid derived from oleic acid, which was present at a level of 84% in the original seed oil, while the proportion of dihydroxy THF derivatives, derived from 4% linoleic acid in the seed oil, is negligible (1). The fractions 1, 2, and 5 were isolated by preparative TLC, their purity was checked by TLC (Fig. 5a), and their composition was determined by GPC.

Because of incomplete TLC separation for HOFA methyl esters from sunflower oil, minor proportions of the adjacent fractions were present, in particular in fractions 2 and 3 (Fig. 5b).

To elucidate the molecular weight distribution of the various fractions, they were analyzed by GPC. This analysis of technical HOFA methyl ester fractions from *E. lathyris* seed oil, isolated by preparative TLC, revealed that in fractions 1 and 5 the oligomeric fatty acids occur in proportions of 74 and 57%, respectively, while fraction 2 contained only 20% oligomers but 78% monomeric HOFA and saturated fatty acids (Table 2).

Gel permeation chromatograms of fractions 1 and 5 of technical HOFA methyl esters derived from sunflower oil and isolated by preparative TLC (Fig. 5b) show high proportions of oligomers (49 and 64%, respectively), while in fractions 2–4 the various monomeric fatty acids predominate (63–75%; Table 2). Similar results were obtained when TLC fractions from hydroxylated rapeseed or linseed oil were analyzed.

The oligomeric HOFA methyl esters derived from *E. lathyris* and sunflower oil that predominate in fractions 1 and 5 (Table 2) were saponified to cleave the ester bonds. The resulting products were converted to the TMSi derivatives and analyzed by GC to determine the composition of fatty acids and HOFA in the nonpolar and polar estolide fractions. Estolides, obtained by dimerization of octadecadienoic acids, are totally saponifiable, and they could be characterized by their saponification products, which were stearic acid and hydroxyoctadecanoic acids (16).

The gas chromatogram of the saponified and silylated TLC fraction 1 of technical HOFA methyl esters derived from *E. lathyris* oil (Fig. 5a) showed high proportions of palmitic and stearic acids (60%) and a relatively small proportion of *threo*-9,10-dihydroxy octadecanoic acid (31%). In contrast, the derivatives of fraction 5 contained 88% *threo*-9,10-dihydroxy octadecanoic acid and only 6% saturated fatty acids (Table 3). Dihydroxy THF octadecanoic acid derivatives, derived

TABLE 2
Percentage of Saturated Fatty Acids and HOFA Monomers and Oligomers of Various Fractions of Technical HOFA Methyl Esters from *Euphorbia lathyris* and Sunflower Oil, Isolated by Preparative TLC and Analyzed by GPC

		HOFA				
Fraction	Saturated FA	Monomeric	Dimeric	Trimeric	Tetrameric[a]	Total oligomers
E. lathyris:						
Total	3	49	17	13	18	48
1	12	12	20	23	31	74
2	19	59	11	5	4	20
5	8	33	18	14	25	57
Sunflower:						
Total	5	50	24	12	9	45
1	25	26	29	13	7	49
2	15	60	15	6	4	25
3	14	57	19	6	4	29
4	13	50	23	8	6	37
5	10	26	33	18	13	64

[a]Including traces of higher oligomers. TLC, thin-layer chromatography. See Table 1 for other abbreviations.

TABLE 3
Gas Chromatographic Analysis of TMSi Derivatives of Various Fatty Acids Obtained *via* Saponification of Estolide Fractions Isolated by Preparative TLC from Technical HOFA Methyl Esters from *Euphorbia lathyris* and Sunflower Oil[a]

Fraction	Palmitic acid	Stearic acid	*threo*-9,10-Dihydroxy octadecanoic acid	Dihydroxy THF octadecanoic acids
E. lathyris:				
Total	11	3	85	trace
1	48	12	31	—
5	5	1	88	trace
Sunflower:				
Total	20	17	19	30
1	60	25	2	2
5	3	3	11	78

[a]TMSi; trimethylsilyl; THF, tetrahydrofuran. See Tables 1 and 2 for other abbreviations.

from 4% linoleic acid in the seed oil, are detected only in trace amounts in HOFA from *E. lathyris* seed oil (Table 3).

After saponification of the nonpolar TLC fraction 1 of technical HOFA methyl esters derived from sunflower oil (Fig. 5b), saturated fatty acids predominate (85%), while HOFA are detected only in low proportions (4%; Table 3). Because fraction 1 already contained nonesterified saturated fatty acids before saponification of estolides (Fig. 5b), the proportion of saturated fatty acids participating in estolide formation is obviously overestimated in this fraction. In contrast, *threo*-9,10-dihydroxy octadecanoic acid and dihydroxy THF octadecanoic acids are the main components (89%) after saponification of the polar TLC fraction 5, while the proportion of saturated fatty acids is drastically reduced (6%; Table 3).

GC analysis of silylated TLC fractions 1 and 5 of technical HOFA methyl esters derived from rapeseed and linseed oil resembles the results obtained from hydroxylated sunflower oil. Similar estolides are formed from dihydroxy THF hydrocarbons and saturated or unsaturated fatty acids in the brown alga *Notheia anomala* (17,18).

The results of this study show that technical HOFA products derived from various seed oils contain up to 56% estolides, which have potential use in lubricants, cosmetic, and ink formulations and in plasticizers (12). Most of the oligomeric compounds can be cleaved by alkaline hydrolysis, yielding around 80% monomeric dihydroxy fatty acids, which are useful starting materials for various technical products.

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