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Isolation of lipid hydroperoxides by preparative thin-layer chromatography of autoxidized esters of polyunsaturated fatty acids

Hydroperoxides of polyunsaturated fatty acids are the principal products in the autoxidation of lipids in biological materials. Free-radical reactions are induced by the dissociation of lipid hydroperoxides[1]. Such free-radical reactions can result in polymerization of proteins[2] and damage to biological membranes and subcellular organelles[3,4].

Pure lipid hydroperoxides, if readily available, could be important compounds in investigations of free-radical reactions involving biological materials containing lipids. PRIVETT *et al.* have prepared hydroperoxides of polyunsaturated fatty acids by either lipoxidase-catalyzed or autoxidation reactions, followed by countercurrent solvent distribution[5]. FRANKEL *et al.* developed an improved method for concentrating the hydroperoxides by liquid partition chromatography[6]. Other methods in the past have included urea fractionation[7], low-temperature crystallization[8], and adsorption column chromatography[9]. The purpose of this paper is to report a relatively simple and fast method for the preparation of hydroperoxides, in purities as high as 95 %, of polyunsaturated fatty acid esters by means of preparative thin-layer chromatography, which is an improvement over the past methods.

Experimental

Hydroperoxides were prepared from esters of polyunsaturated fatty acids by autoxidation. The esters that were used were the following: methyl linoleate, methyl linolenate, ethyl arachidonate, methyl eicosapentaenoate, and ethyl esters of a fish oil fatty acid fraction obtained from menhaden (*Brevoortia tyrannus*). Individual crude samples of hydroperoxides were resolved by thin-layer chromatography.

Materials. Methyl linoleate was prepared from methyl esters of safflower oil and was purified by urea-inclusion compound fractionation, using an adaptation of the method of KEPPLER *et al.*[10]. The methanolysis method of GAUGLITZ AND LEHMAN[11] was used to prepare the esters from commercial safflower oil. A purity of 99.9 % methyl linoleate was found by a gas–liquid chromatographic (GLC) method similar to that previously published[12].

Methyl linolenate was prepared via a methanolysis reaction of linseed oil. The polyunsaturated methyl esters were concentrated by solvent crystallization and urea fractionation. The concentrate, which was analyzed by GLC, was found to contain 74.6 % methyl linolenate, 19.9 % methyl linoleate, and 5.5 % methyl oleate. This concentrate of methyl linolenate was used without further purification.

Ethyl arachidonate was obtained from Hoffmann-La Roche (Nutley, N.J.)* and was used without further purification. The sample was found to be 99.3 % pure by GLC analysis.

Methyl eicosapentaenoate was procured from Sigma Chemical Company (St. Louis, Mo.). The labeled assay was given as 90+% by the supplier. The ester was used without further purification.

* Company names are mentioned merely to simplify descriptions; no endorsement of commercial products is implied.

Ethyl esters of a menhaden oil fatty acid fraction were obtained from the U.S. Bureau of Commercial Fisheries Technological Laboratory (Seattle, Wash.). The esters were decolorized by adsorptive bleaching and were crudely fractionated by molecular distillation. This treatment removed the natural antioxidants and other non-glyceride compounds that might otherwise have been present in the oil[13,14]. The ethyl esters of the polyunsaturated fatty acids were analyzed by GLC. Table I presents the results of the GLC assay.

TABLE I

COMPOSITION OF FRACTIONATED ETHYL ESTERS OF MENHADEN (*Brevoortia tyrannus*) OIL FATTY ACIDS DETERMINED BY GAS–LIQUID CHROMATOGRAPHY

Fatty acid designation	*Percentage composition*	*Fatty acid designation*	*Percentage composition*
16:0	0.9	20:3	0.3
16:1	0.3	20:4ω6	2.6
18:0	3.3	22:1	0.5
18:1	7.5	20:4ω3	2.9
18:2ω6	0.5	20:5ω3	30.4
18:3ω3	1.4	24:1	3.0
18:4ω3	3.4	22:4ω3	2.5
20:1	2.6	22:5ω3	7.8
20:2	0.5	22:6ω3	29.6

Autoxidation procedures to prepare lipid hydroperoxides. Crude mixtures of methyl linoleate hydroperoxides were prepared by periodic shaking of a stoppered flask containing methyl linoleate (4 g) and an atmosphere of pure oxygen, first at 2–4° for a period of 2½ months and finally at room temperature for a period of 148 h. During the total period, oxygen was reintroduced into the flask five times. At the end of the first 2½ months, the peroxide value of the sample was 18.8 mequiv./kg; at the end of the total period, the peroxide value was 1,700 ± 10 mequiv./kg (27.8 % of theoretical value for methyl linoleate monohydroperoxides).

A procedure that was followed for methyl linolenate, ethyl arachidonate, methyl eicosapentaenoate, and the menhaden-oil ethyl ester fraction was simpler and faster than that for methyl linoleate. This procedure involved simply placing the esters into individual Erlenmeyer flasks (50 ml size) and periodically agitating in air at room temperature until the peroxide levels reached the range of 8–25 % of the theoretical values for monohydroperoxide esters. Specifically, the final peroxide value found for each ester sample was as follows: methyl linolenate, 448 mequiv./kg; ethyl arachidonate, 1,360 mequiv./kg; methyl eicosapentaenoate, 1,280 mequiv./kg; and menhaden oil ethyl esters, 1,030 mequiv./kg.

Peroxide values. Peroxide values were determined by iodometry. The Wheeler method as modified by SWERN[15] was adapted to the determinations.

Gas–liquid chromatography. A Varian Aerograph Series 1520 gas chromatograph with a hydrogen-flame detector was used for GLC analyses. The GLC column was composed of a 179 cm by 1/8 in. O.D. stainless steel tube containing 4.0 % (w/w)

diethylene glycol succinate polyester on 90–100 mesh Anakrom SD (Analabs, Inc.). Analyses were performed after the column was conditioned overnight at 190°, with nitrogen being used as the carrier gas and flowing at the rate of 20 ml/min.

For the menhaden oil esters, conditions for temperature programming were as follows: initial column temperature 140°; program 140° to 160° at 10°/min; hold at 160° for 12 min; program 160° to 187° at 30°/min; and hold at 187°. Nitrogen carrier flow was about 10 ml/min; hydrogen flow and air flow to the detector was 25 and 200 ml/min, respectively. Electrometer range setting was at 10^{-11} A/mV. A 0–1 mV recorder was used. The volume of the sample was 0.04 μl.

Preparative thin-layer chromatography. Weighed samples (0.34–0.63 g) of autoxidized methyl linoleate were streaked on 2 mm layers of Silica Gel F_{254} (20 × 20 cm plates; E. Merck AG, Darmstadt) at a distance of 1.5 cm along one edge and about 0.5 cm from each side of the plates. The chromatoplates were then developed for a distance of 17–18 cm with a solution of petroleum ether (b.p. 30–60°)–diethyl ether (60:40, v/v). The hydroperoxide band was detected by viewing the developed chromatoplates under short-wavelength ultraviolet light. The hydroperoxides were revealed as a dark band on the phosphor-containing Silica Gel F_{254} plates. A reference chromatoplate, on which methyl linoleate and reduced methyl linoleate hydroperoxides (via $NaBH_4$ reduction) were spotted, was analyzed simultaneously with a preparative chromatoplate to aid in identification of band components.

The separated isomeric hydroperoxides of methyl linoleate were recovered by scraping the hydroperoxides from their corresponding region of the plates and extracting them with peroxide-free diethyl ether. The ether was removed by evaporating it in a stream of nitrogen at room temperature.

The autoxidized products of methyl linolenate, ethyl arachidonate, methyl eicosapentaenoate, and ethyl esters of the menhaden oil fatty acid fraction were separated by preparative TLC in the same manner as that for products of methyl linoleate oxidation. The respective hydroperoxides were likewise recovered from preparative chromatoplates.

Results and discussion

In the preparative TLC separations, the lipid hydroperoxides (LOOH) were resolved at a lower R_F value than the R_F value for the non-autoxidized lipid. For instance, in the case of ethyl arachidonate hydroperoxides, LOOH (20:4), the chromatoplate produced an R_F value range of 0.68 to 0.87 for the unreacted ester and an R_F value range of 0.38–0.58 for LOOH (20:4). Multiple developments of the chromatoplates were found to improve the resolutions on plates that had high load levels, *e.g.* 0.63 g.

The recovery of the various lipid hydroperoxides ranged in quantities from 21 mg to 52 mg, depending on the amounts streaked on the plates and the initial concentrations in the autoxidized samples. Recoveries from two chromatoplates of 22.2 wt.% and 23.6 wt.% of concentrated methyl linoleate hydroperoxides, which, when combined, resulted in a product that had a peroxide value of 5,750 $\pm$ 60 mequiv./kg. Based on a theoretical value of 6,125 mequiv./kg, the methyl linoleate hydroperoxides were 94 mole % pure.

Table II gives the analytical values for the recovered lipid hydroperoxides. The peroxide values indicate a high degree of concentration of the hydroperoxides.

No attempt was made to resolve *cis-trans* and *trans-trans* conjugated dienoate isomers, which are formed as the products of autoxidation. The work of PRIVETT and coworkers[16] aptly demonstrated the fact that low temperature (*e.g.* 0°) autoxidations result in lower ratios of *trans-trans* to *cis-trans* conjugated dienes than will be obtained at room temperature or higher (*e.g.* 24°). Control of isomeric ratios is possible, therefore, by maintaining the autoxidation temperature in the optimum range for the desired isomer.

TABLE II

ANALYTICAL VALUES OF LIPID HYDROPEROXIDES ISOLATED BY PREPARATIVE THIN-LAYER CHROMATOGRAPHY

Hydroperoxide		*Peroxide value*		$E^{1\%}_{1\,cm}$ *(isooctane)*		
Ester source	*Symbol*	*Found (mequiv./kg)*	*Theoretical*[a] *(mequiv./kg)*	*At 232–235 mμ*	*At 268 mμ*	*At 300 mμ*
Methyl linoleate	LOOH(18:2)	5,750	6,125	626 (766)[b]	29.6	8.3
Methyl linolenate	LOOH(18:3)	6,320	6,165	721 (728)[b]	63.9	4.0
Ethyl arachidonate	LOOH(20:4)	5,225	5,487	551	46	5.3
Methyl eicosapentaenoate	LOOH(20:5)	5,410	5,739	574	34.9	5.3
Menhaden oil ethyl esters	LOOH(PUFA)	4,710	5,150[c]	561	45.4	6.2

[a] Based on 100% ester monohydroperoxide.
[b] Theoretical value in parentheses based on published data for hydroperoxides with peroxide values of 6,100 mequiv./kg[17].
[c] Calculation based on GLC analysis (*cf.* Table I).

The hydroperoxides of the polyunsaturated fatty acid esters were found to be quite stable when diluted in ethanol and stored at 2–4° under nitrogen. The stability was determined spectrophotometrically by periodically measuring absorptions at 232–235 mμ and observing possible changes in $E^{1\%}_{1\,cm}$ values. For example, on three consecutive days, a $1.27 \cdot 10^{-2}$ molar solution of methyl eicosapentaenoate hydroperoxides was tested for loss of hydroperoxides by measuring $E^{1\%}_{1\,cm}$ in 98.0% (v/v) ethanol. During these days, the following $E^{1\%}_{1\,cm}$ (235 mμ) values were obtained: (1) 534, 561; (2) 533, 563; and (3) 538. These values are reasonably close to the value of $E^{1\%}_{1\,cm}$ 574, which was measured in isooctane at the beginning of the experimentation (*cf.* Table II).

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Food Science Pioneer Research Laboratory, E. H. GRUGER, JR.
Bureau of Commercial Fisheries, Seattle, Wash. 98102 (*U.S.A.*)

Department of Food Science and Technology, A. L. TAPPEL
University of California, Davis, Calif. 95616 (*U.S.A.*)

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