Determination of Hydroperoxides and Structures by High-Performance Liquid Chromatography with Post-Column Detection with Diphenyl-1-Pyrenylphosphine

Toshiaki Ohshima[a,*], Anu Hopia[b], J. Bruce German[c], and Edwin N. Frankel[c]

[a]Department of Food Science and Technology, Tokyo University of Fisheries, Tokyo 108, Japan, [b]Department of Applied Chemistry and Microbiology, University of Helsinki, FIN-00014, Finland, and [c]Department of Food Science and Technology, University of California, Davis, California 95616

ABSTRACT: A high-performance liquid chromatographic method, using post-column detection with diphenyl-1-pyrenylphosphine (DPPP), was developed for the quantitative and qualitative determination of isomeric lipid hydroperoxides (OOH). The OOH eluted from a normal-phase column were passed through a photodiode array detector and then mixed with DPPP solution in a reaction coil heated at 80°C. DPPP oxide formed by the reaction with OOH was determined by monitoring the fluorescence intensity at 380 nm and excitation at 352 nm. The conjugated diene OOH (13-*cis,trans*- and 9-*cis,trans*-OOH) and nonconjugated OOH (12-*cis-trans*- and 10-*cis,trans*-OOH) from photosensitized oxidation of methyl linoleate were determined in a molar ratio of 31:29:19:21, respectively. However, only the two conjugated hydroperoxides were detected by ultraviolet absorption at 234 nm. Further applications were carried out for the determination of OOH of methyl oleate and methyl linolenate. This method proved to be useful for the determination of the OOH containing both conjugated and nonconjugated diene structures.

Lipids 31, 1091–1096 (1996).

A normal-phase column high-performance liquid chromatography (HPLC) method has been applied successfully to separate positional isomers of hydroperoxides (OOH) formed as primary oxidation products of fatty acid esters (1). Ultraviolet (UV) light absorption at 234 nm due to conjugated dienes has been used to detect separated OOH isomers. However, the UV measurement at 234 nm is not specific to OOH (1,2), nor is it suitable for determination of nonconjugated OOH (3). One solution to this problem involves HPLC separation of lipid OOH with post-column chemiluminescence detection based on luminol or isoluminol oxidation during a reaction of hydroperoxides and cytochrome C (4,5). Some applications to biological systems have also been reported (6–8). Generally, nonpolar solvent systems based on *n*-hexane are used in HPLC for separation of OOH positional isomers (1). However, a post-column reaction of luminol to luminol oxide progresses only under aqueous alkaline conditions (6). Therefore, the number and type of organic solvents used for mobile phase are relatively limited, as hydrophilic nonpolar solvent mixtures do not mix well with hydrophobic luminol reagents in a post-column reaction coil.

Diphenyl-1-pyrenylphosphine (DPPP) is a recently developed fluorescent reagent which reacts specifically with various lipid OOH to form DPPP oxide even though in the presence of lipid alcohol. The DPPP oxide is excited with UV light at 352 nm and gives the emission wavelength at 380 nm (9). This reaction progresses in many different types of solvents (9), and therefore not only a reversed-phase, but also a normal-phase HPLC separation mode are useful for the DPPP fluorescence post-column detection. A few applications of this system have already been reported for analyses of OOH of triacylglycerol, cholesterol, cholesterol esters, and phospholipids in biological systems (10–12). This study was aimed at applying the DPPP fluorescence detection HPLC system to the study of lipid hydroperoxide positional isomers and their structures.

In this paper, autoxidized and photosensitized-oxidized unsaturated fatty acid methyl esters (FAME) were used as model lipids. To evaluate the efficiency of the present methology, results were compared to previous studies based on gas chromatography/mass spectrometry (GC/MS) and HPLC with UV detection.

MATERIALS AND METHODS

Lipid standard. Methyl oleate, methyl linoleate, and methyl linolenate of >99% purity were purchased from Nu-Chek-Prep. Inc. (Elysian, MN). These esters were purified by pas-

*To whom correspondence should be addressed at Department of Food Science and Technology, Tokyo University of Fisheries, Konan 4, Minato-ku, Tokyo 108, Japan.

Abbreviations: DPPP, diphenyl-1-pyrenylphosphine; FAME, fatty acid methyl ester; GC/MS, gas chromatography/mass spectrometry; HPLC, high-performance liquid chromatography; OOH, hydroperoxide; TMS, trimethylsilyl; UV, ultraviolet.

sage through a Sep-Pak silica cartridge (Waters Associates, Milford, MA) just before use (13).

Chemicals. DPPP was kindly donated by the Tosoh Company (Tokyo, Japan). Methylene blue trihydrate and $NaBH_4$ (Wako Pure Chemicals, Osaka, Japan) were of reagent grade. *n*-Hexane of HPLC grade was used after glass-distillation. Diethyl ether of reagent grade was purified by a combination of the first washing with ferrous sulfate–sulfuric acid solution to remove peroxide impurities followed by distillation in glass. Methanol, 1-butanol, and acetone (all HPLC grade) were used as purchased.

Autoxidation of lipids. One hundred mg of FAME were dissolved in *n*-hexane and placed into a glass test tube (10 mm × 13 mm). *n*-Hexane was flushed out with a nitrogen stream. Autoxidation of FAME was carried out in air at 35°C in the dark. The autoxidized FAME was dissolved in *n*-hexane and separated by HPLC.

Photosensitized oxidation of lipids. The oxidation of FAME was sensitized with methylene blue (1 mg/g ester) in a methanol solution with continuous gentle stirring in a jacketed flask cooled to 3°C. The solution in the flask was exposed to a 1000-watt tungsten light source through a 3-cm layer of water to filter out infrared radiation (14). Methanol was evaporated under a nitrogen stream in the dark and the residual photosensitized-oxidized lipid was dissolved in 25% diethyl ether in *n*-hexane. The solution was passed through a Sep-Pak silica cartridge to remove the photosensitizer. The eluent was made up to 10 mL with *n*-hexane and 20 µL solution was injected to the HPLC column.

The HPLC system. A schematic of the HPLC system used is shown in Figure 1. The mobile phase passed through an HPLC column with an HPLC pump (model 1050; Hewlett-Packard, Boise, ID). The eluant from the column was then passed through a diode array detector (HP 1040A) and subsequently to a stainless-steel reaction coil (0.5 mm i.d. × 10 m). At the inlet of the reaction coil, a DPPP reagent solution was pumped (HPLC pump, model 45; Waters Associates) and mixed with the eluent in a T-connector. The reaction coil was immersed in a water bath heated at 80°C. The eluent from the coil was cooled to room temperature by passing through a stainless steel coil (0.5 mm i.d. × 5 m), immersed in a water tank, and led to a fluorescence photospectrometer (model 650-40; Perkin Elmer, Norwark, CO). The intensity of the fluorescence signal was integrated with a chromatographic integrator (Hewlett-Packard model 3390A).

Separation and detection of OOH. A normal-phase column (Supelcosil LC-Si, 2.1 mm i.d. × 250 mm, 5 µm; Supelco, Bellefonte, PA) was used to separate positional isomers of fatty ester OOH. A mixture of 500 mL of *n*-hexane and 34 mL of diethyl ether was pumped at a rate of 0.6 mL/min. The DPPP solution (3 mg in a mixture of 200 mL 1-butanol and 200 mL methanol) was pumped at a flow rate of 0.6 mL/min.

Lipid OOH eluted from the column were passed through the dioide array detector and the absorption due to conjugated dienes was monitored at 234 nm (1). The fluorescence intensity of the DPPP oxides formed by reacting lipid hydroperoxides and DPPP was monitored at 380 nm with excitation at 352 nm (9).

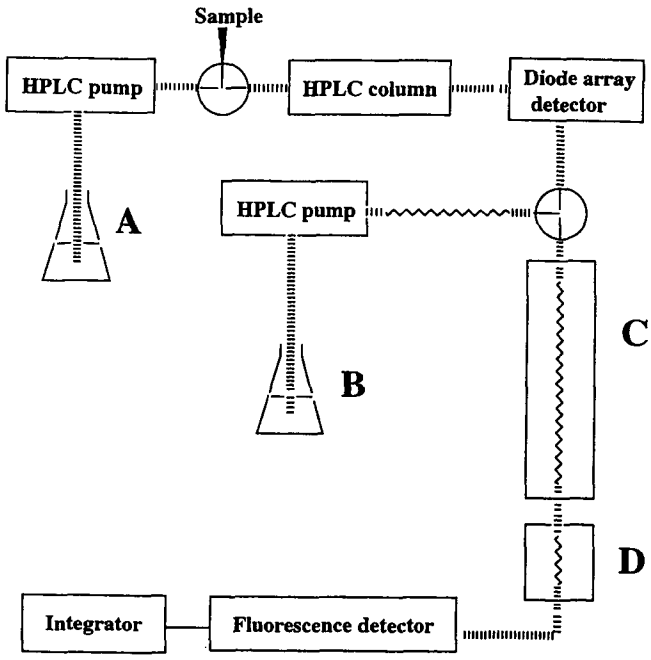

FIG. 1. Diagram of the diphenyl-1-pyrenylphosphine (DPPP)-fluorescence detection high-performance liquid chromatography (HPLC) system. A, HPLC pump for a mobile phase; B, HPLC pump for the DPPP solution; C, reaction coil; D, cooling coil.

GC/MS. OOH positional isomers separated by the HPLC column and monitored with the diode array detector were fractionated before mixing with the DPPP solution by disconnecting the flow tubing at the inlet of the T-connector behind the column. Butylated hydroxyanisole (200 ppm) was added to suppress formation of secondary oxidation products. The hydroperoxides were reduced to the corresponding hydroxy esters with $NaBH_4$ (15), followed by hydrogenation to avoid rearrangement of hydroperoxides, and subsequently converted to trimethylsilyl (TMS) ether derivatives by adding hexamethyldisilazane and trimethylchlorosilane (14).

The TMS derivatives were separated with a gas chromatography (model GC 17A; Shimadzu, Kyoto, Japan) equipped with a Supelcowax-10 fused silica open-tubular column (0.25 mm i.d. × 25 m, 0.25 µm; Supelco Japan Ltd., Tokyo, Japan).

The outlet of the column was connected directly to an electron impact ion source of a quadrupole mass spectrometer (Shimadzu model QP 5000). The column oven temperature was programmed from 150 to 220°C at 1°C/min. Injection port temperature was 250°C. Helium was used as carrier gas. Mass spectra were acquired using 3 KV accelerating energy, 70 eV electron beam energy, and a source temperature of 260°C by using a computer system (Shimadzu Class-5000).

RESULTS

Separation of positional isomers of methyl linoleate OOH. Photosensitized-oxidized methyl linoleate showed four peaks

by HPLC with post-column DPPP fluorescence detection (Fig. 2A). However, only two peaks were shown by UV at 234 nm (Fig. 2B). MS (data not shown) confirmed the identity of the positional isomers as follows: 13-*cis,trans*-, 12-*cis,trans*-, 9-*cis,trans*-, and 10-*cis,trans*-hydroperoxy methyl linoleates based on the literatures (15,16).

Typical chromatograms of autoxidized methyl linoleate OOH detected by fluorescence at 380 nm and absorption at 234 nm (Fig. 2, C and D). Four peaks, including methyl linoleate 13-*cis,trans*-, 13-*trans,trans*-, 9-*cis,trans*-, and 9-*trans,trans*-hydroperoxy isomers, were identified by MS of the corresponding TMS derivatives of hydroperoxides separated by HPLC (data not shown).

Separation of other fatty acid methyl ester OOH. Peak assignment was confirmed for every peak component fractionated by HPLC (data not shown). Although methyl 10-*cis,trans*- and 9-*cis,trans*-hydroperoxy monoene hydroperoxides could not be detected by UV absorption at 234 nm (Fig. 3B), the isomers were detected by DPPP-fluorescence (Fig. 3A). For photosensitized-oxidized methyl linolenate, the peaks due to 13-*cis,trans*-, partially separated 16-*cis,trans*- and 12-*cis,trans*-, 15-*cis,trans*-, 9-*cis,trans*-, and 10-*cis,trans*-methyl linolenate OOH were readily measured by DPPP-fluorescence detection (Fig. 3C). The nonconjugated hydroperoxide isomers, 15-*cis,trans*- and 10-*cis,trans*-methyl linolenate hydroperoxy, were not detected by UV absorption at 234 nm (Fig. 3D).

Quantitative analyses. Tables 1, 2, and 3 summarize peak area ratios of OOH of methyl oleate, linoleate, and linolenate photosensitized-oxidized to different peroxide values. Previously reported results show that the ratios of isomeric OOH formed from photosensitized-oxidized methyl esters were 47.7–51:52.3–49 for methyl oleate (14, 15,17). For 13-*cis,trans*-, 12-*cis,trans*-, 9-*cis,trans*-, and 10-*cis,trans*-hydroperoxides, the previously reported ratios were 34.5–35.0, 17.0–17.0, 31.9–32.0, 16.7–17.0 (14,15).

Reproducibility of the determination of hydroperoxides by this HPLC system was evaluated (Table 4). For the hydroperoxides from methyl oleate and from methyl linoleate, good reproducibility in determination of peak areas was obtained. However, for the methyl linolenate OOH, the peak areas of 10-*cis,trans*-hydroperoxy methyl linolenate were not reproducible due to miss integration of peak area. The use of peak height for quantification may resolve this problem.

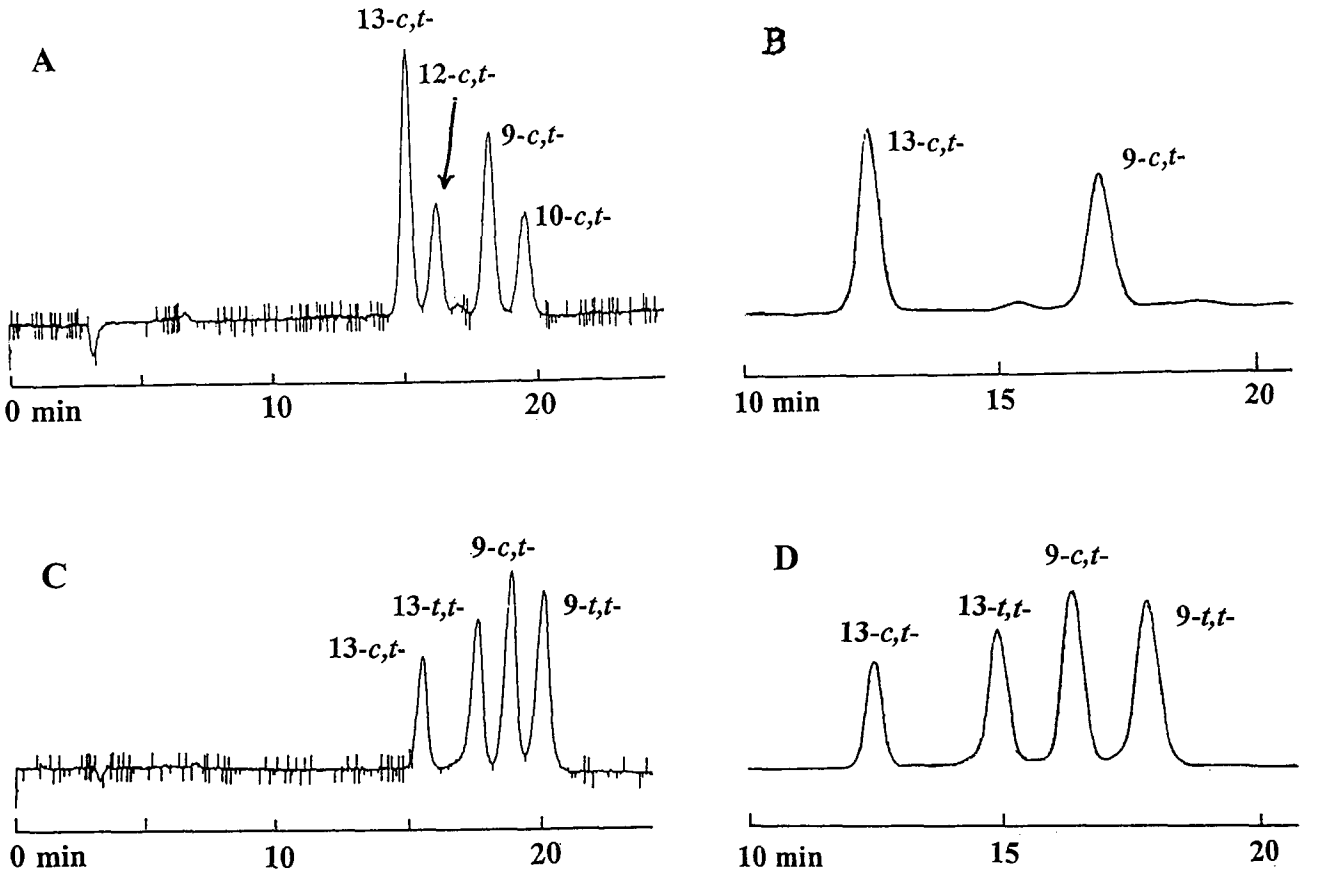

FIG. 2. Typical chromatograms of methyl linoleate isomeric hydroperoxides. Photosensitized-oxidized methyl linoleate detected by the DPPP-fluorescence detection (A) and by ultraviolet (UV) absorption at 234 nm (B). Autoxidized methyl linoleate detected by the DPPP-fluorescence (C) and by UV light absorption at 234 nm (D). See Figure 1 for abbreviations; *c,t-, cis,trans-*.

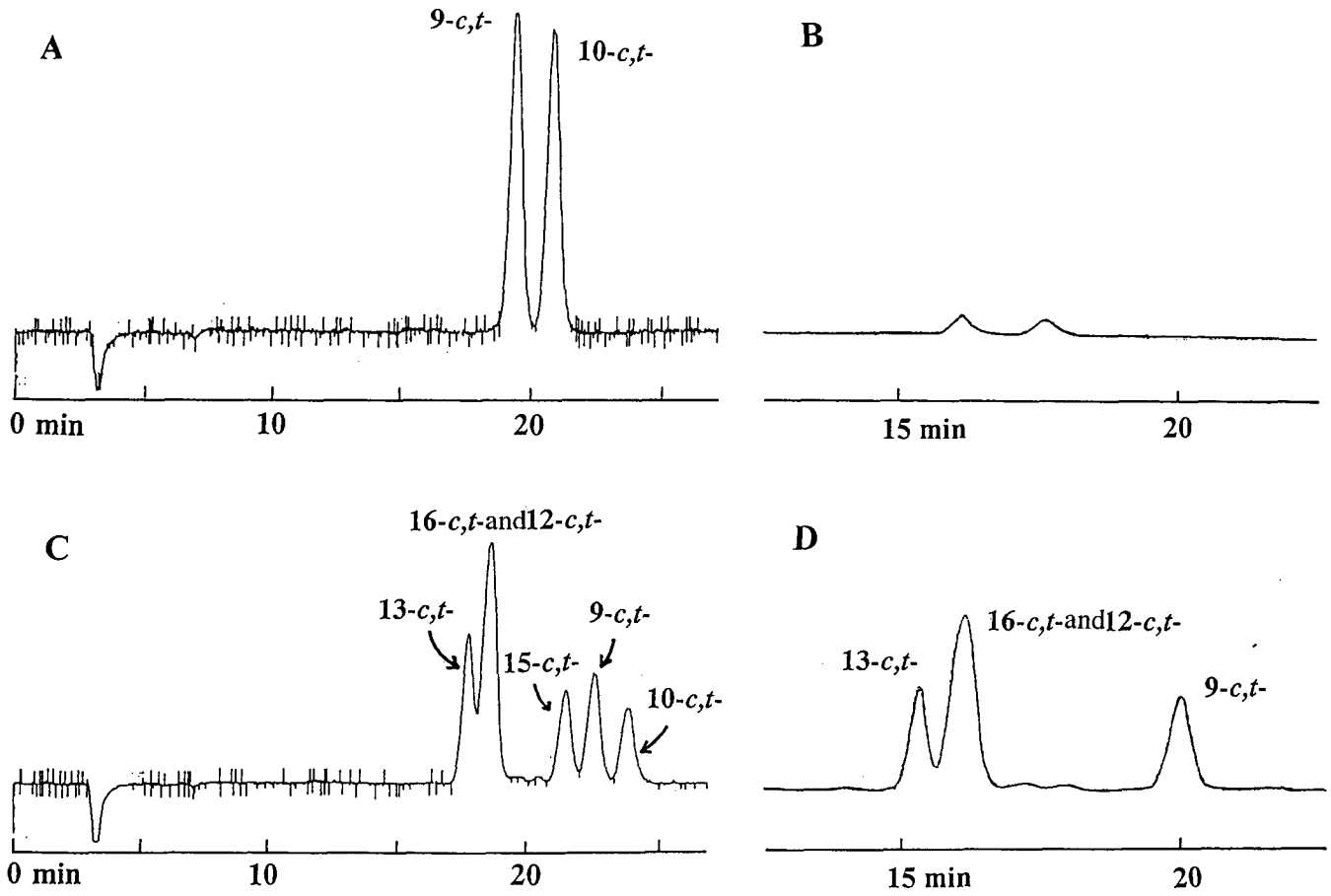

FIG. 3. Typical chromatograms of methyl oleate and methyl linolenate isomeric hydroperoxides. A and C, photosensitized-oxidized methyl oleate and methyl linolenate hydroperoxides, respectively, detected by DPPP-fluorescence; B and D, detected by absorption at 234 nm. See Figures 1 and 2 for abbreviations.

DISCUSSION

This study showed that the DPPP fluorescence detection coupled with HPLC is a simple and accurate method to determine the distribution of OOH isomers of methyl oleate, linoleate, and linolenate. Autoxidation of methyl linoleate produced four conjugated positional isomers of monohydroperoxides, i.e., 13-*cis,trans*-OOH, 13-*trans,trans*-OOH, 9-*cis,trans*-OOH, and 9-*trans,trans*-OOH, which can be analyzed by UV light absorption (1).

However, the nonconjugated singlet oxygen oxidation products of methyl linoleate, 12-*cis,trans*-OOH and 10-*cis,trans*-OOH, are not detectable by UV light absorption. These nonconjugated hydroperoxides are detectable by the DPPP fluorescence detection system, because DPPP is directly oxidized by hydroperoxides to form a fluorescent DPPP oxide. However, in the chemiluminescence detection, several kinds of oxygen radicals and excited oxygens oxidize luminol (5). In the chemiluminescence detection, nonconjugated OOH isomers of methyl linoleate and methyl linolenate were detectable in HPLC, however, separation between certain isomers was not completed (18). Nonlipid components such as sugar, amino acids and ascorbic acid contaminated into a methanol-aqueous layer in a stage of lipid extraction interfere with the accurate analysis of hydroperoxides by causing quenching of the chemiluminescence (5).

It is well accepted that oxidation by singlet oxygen takes place at both ends of the C=C bond (17). Therefore, photosensitized oxidation of methyl oleate forms only two isomers, i.e., 9-*cis,trans*-OOH and 10-*cis,trans*-OOH (13–15,17). The ratios of 9-*cis,trans*-OOH to 10-*cis,trans*-OOH reported little variation; 50:50 by GC/MS (17), 47.7:52.3 by GC/MS (14), and 51:49 by HPLC (13).

A number of previous studies on measurement of lipid hydroperoxide have been carried out by GC/MS. Derivatizations of OOH, including reduction and hydrogenation of hydroperoxy group to the corresponding hydroxy group and subsequent conversion to TMS ether group, are required before GC/MS analysis (14,19–21). These derivatizations are usually time-consuming and can possibly form artifacts during treatment of a sample OOH as well as unsaturated lipids. In contrast, the DPPP fluorescence detection system requires no derivatization of the sample.

It was also possible to determine monohydroperoxides at

TABLE 1
Relative Ratios of Photosensitized-Oxidized Methyl Oleate Hydroperoxide Positional Isomers (%)

Method	PV[a]	9-*c,t*-[b]	10-*c,t*-[b]	References
HPLC/DPPP[c]	36	50.0	50.0	
	38	47.5	52.5	
	92	51.3	48.7	
	98	50.3	49.7	
	171	50.5	49.5	
	179	52.0	48.0	
HPLC[d]	1727	47.7	52.3	13
GC/MS[e]	1727	51	49	14
GC/MS[e]	—	50	50	17

[a]Peroxide value (meq/kg).
[b]*c,t-, cis,trans-*.
[c]DPPP, diphenyl-1-pyrenylphosphine.
[d]Separated corresponding hydroxystearate and detected by ultraviolet at 212 nm. HPLC, high-performance liquid chromatography.
[e]Analyzed corresponding trimethylsilylated hydroxystearate. GC/MS, gas chromatography/mass spectrometry.

only picomole levels by this DPPP system. High specificity and sensitivity to hydroperoxides may be useful for kinetic studies on oxidation of polyunsaturated fatty acids in a biological membrane systems including effects of antioxidant compounds.

ACKNOWLEDGMENTS

The fund for Research Encouragement in Celebration of the Centennial Anniversary of the Founding of the Tokyo University of Fisheries to T.O. is acknowledged.

REFERENCES

1. Chan, H.W.S., and Levett, G. (1977) Autoxidation of Methyl Linoleate. Separation and Analysis of Isomeric Mixtures of Methyl Linoleate Hydroperoxides and Methyl Hydroxylinoleates, *Lipids 12,* 99–104.
2. Neff, W.E., Frankel, E.N., and Weisleder, D. (1981) High Pressure Liquid Chromatography of Autoxidized Lipids: II. Hydroperoxy-Cyclic Peroxides and Other Secondary Products from Methyl Linolenate, *Lipids 16,* 439–448.
3. Coxon, D.T., Price, K.R., and Chan, H.W.S. (1981) Formation, Isolation and Structure Determination of Methyl Linolenate Diperoxides, *Chem. Phys. Lipids 28,* 365–378.
4. Yamamoto, Y., Brodsky, M.H., Baker, J.C., and Ames, B.N. (1987) Detection and Characterization of Lipid Hydroperoxides at Picomole Levels by High-Performance Liquid Chromatography, *Anal. Chem. 160,* 7–13.
5. Miyazawa, T., Yasuda, K., and Fujimoto, K. (1987) Chemiluminescence High Performance Liquid Chromatography of Phosphatidylcholine Hydroperoxide, *Anal. Lett. 20,* 915–925.
6. Yamamoto, Y., and Niki, E. (1989) Presence of Cholesteryl Ester Hydroperoxide in Human Blood Plasma, *Biochem. Biophys. Res. Commun. 165,* 989–993.
7. Miyazawa, T. (1989) Determination of Phospholipid Hydroperoxides in Human Blood Plasma by a Chemiluminescence-HPLC Assay, *Free Rad. Biol. Med. 7,* 209–217.
8. Miyazawa, T., Suzuki, T., Fujimoto, K., and Yasuda, K. (1992) Chemiluminescent Simultaneous Determination of Phosphatidylcholine Hydroperoxide and Phosphatidylethanolamine Hydroperoxide in the Liver and Brain of the Rat, *J. Lipid Res. 33,* 1051–1059.

TABLE 2
Relative Ratios of Photosensitized-Oxidized Methyl Linoleate Hydroperoxide Positional Isomers (%)[a]

Method	PV	13-*c,t*-	12-*c,t*-	9-*c,t*,-	10-*c,t*,-	References
HPLC/DPPP	106	36.2	15.1	33.3	15.5	
	107	34.2	12.8	36.0	17.0	
	163	33.5	17.5	31.5	17.6	
	164	30.3	16.6	33.5	19.5	
	283	30.5	18.5	30.7	20.2	
	274	29.2	17.8	32.4	20.8	
	357	30.8	19.3	29.5	20.4	
	340	29.1	18.3	31.2	21.4	
HPLC[b]	1124	35	17	32	17	13
HPLC[c]	—	31.1	20.7	33.3	14.7	18
GC/MS[d]	1124	34.5	17.0	31.9	16.7	14

[a]See Table 1 for abbreviations.
[b]Separated corresponding hydroxystearate and detected by ultraviolet at 212 nm.
[c]Separated corresponding hydroperoxide and detected by chemiluminescence.
[d]Analyzed corresponding trimethylsilylated hydroxystearate.

TABLE 3
Relative Ratios of Photosensitized-Oxidized Methyl Linolenate Hydroperoxide Positional Isomers (%)[a]

Methods	PV	13-*c,t*-	16-*c,t*- and 12-*c,t*-	15-*c,t*-	9-*c,t*-	10-*c,t*-	Refs.
HPLC/DPPP	78	17.7	41.7	8.2	18.2	14.2	
	85	18.5	40.2	11.6	16.1	13.5	
	284	18.2	38.4	13.4	17.0	13.0	
	286	18.3	39.4	13.2	16.3	13.9	
	634	17.5	38.5	13.8	16.9	13.3	
	644	17.5	38.9	13.5	17.8	12.3	
HPLC[b]	1566	15	40	12	20	13	13
GC/MS[c]	1566	14.0	37.2	13.4	22.7	12.7	14

[a]See Table 1 for abbreviations.
[b]Separated corresponding hydroxystearate and detected by ultraviolet at 212 nm.
[c]Analyzed corresponding trimethylsilylated hydroxystearate.

TABLE 4
Standard Deviations of the Isomeric Hydroperoxide Analyses by HPLC/DPPP[a]

Isomeric hydroperoxides	Relative ratio (%)[b]	SD[c]
Methyl oleate/OOH, PV = 92		
9-*c,t*-	50.5 ± 0.89	2.1
10,*c,t*-	49.5 ± 0.89	
Methyl linoleate-OOH, PV = 123		
13-*c,t*-	30.8 ± 0.70	0.5
12-*c,t*-	19.4 ± 0.18	
9-*c,t*-	29.3 ± 0.59	
10-*c,t*-	20.6 ± 0.26	

[a]See Tables 1 and 3 for abbreviations. OOH, hydroperoxide.
[b]Mean ± SD (n = 5).
[c]Standard deviation on HPLC by analysis of variation (Ref. 13).

9. Akaska, K., Suzuki, T., Ohrui, H., and Meguro, H. (1987) Study on Aromatic Phosphines for Novel Fluorometry of Hydroperoxides (II)—The Determination of Lipid Hydroperoxides with Diphenyl-1-Pyrenylphosphine, *Anal. Lett. 20,* 797–907.
10. Akasaka, K., Ohrui, H., and Meguro, H. (1993) Determination of Triacylglycerol and Cholesterol Ester Hydroperoxides in Human Plasma by High-Performance Liquid Chromatography with Fluorometric Postcolumn Detection, *J. Chromatogr. 617,* 205–211.
11. Akasaka, K., Ohrui, H., and Meguro, H. (1994) Measurement of Cholesterol Hydroperoxides of High and Combined Low and Very Low Lipoprotein in Human Plasma, *Biosci. Biotech. Biochem. 58,* 396–399.
12. Akasaka, K., Ohata, A., Ohrui, H., and Meguro, H. (1995) Automatic Determination of Hydroperoxides of Phosphatidylcholine and Phosphatidylethanolamine in Human Plasma, *J. Chromatogr. 665B,* 37–43.
13. Neff, W.E., and Frankel, E.N. (1980) Quantitative Analyses of Hydroxystearate Isomers from Hydroperoxides by High Pressure Liquid Chromatography of Autoxidized and Photosensitized-Oxidized Fatty Esters, *Lipids 15,* 587–590.
14. Frankel, E.N., Neff, W.E., and Bessler, T.R. (1979) Analysis of Autoxidized Fats by Gas Chromatography-Mass Spectrometry: V. Photosensitized Oxidation, *Lipids 14,* 961–967.
15. Chan, H.W.S., and Levett, G. (1977) Oxidation of Methyl Oleate: Separation of Isomeric Hydroperoxyoctadecenoates and Methyl Hydroxystearates by High Performance Liquid Chromatography, *Chem. Ind. (London),* 692–693.
16. Frankel, E.N. (1985) Chemistry of Free Radical and Singlet Oxidation of Lipids, *Prog. Lipid Res. 23,* 197–221.
17. Terao, J., and Matsushita, S. (1977) Geometric Isomers of Monohydroperoxides Formed by Autoxidation of Methyl Linoleate, *Agric. Biol. Chem. 41,* 2401–2405.
18. Kondo, Y., Kawai, Y., Miyazawa, T., and Mizutani, J. (1993) Chemiluminescence-HPLC Analysis of Fatty Acid Hydroperoxide Isomers, *Biosci. Biotech. Biochem. 57,* 1575–1576.
19. Toyoda, I., Terao, J., and Matsushita, S. (1982) Hydroperoxides Formed by Ferrous Ion-Catalyzed Oxidation of Methyl Linolenate, *Lipids 17,* 84–90.
20. Yamauchi, R., Yamada, T., Kato, K., and Ueno, Y. (1983) Monohydroperoxides Formed by Autoxidation and Photosensitized Oxidation of Methyl Eicosapentaenoate, *Agric. Biol. Chem. 47,* 2897–2902.
21. VanRollins, M., and Murphy, R.C. (1984) Autoxidation of Docosahexaenoic Acid: Analysis of Ten Isomers of Hydroxydocosahexaenoate, *J. Lipid Res. 25,* 507–517.

[Received May 21, 1996, and in revised form July 18, 1996; Revision accepted July 31, 1996]