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Development of phosphine reagents for the high-performance liquid chromatographic–fluorometric determination of lipid hydroperoxides

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Abstract

Phosphine reagents were designed and synthesized as a new type of fluorescent reagents for the determination of lipid hydroperoxides in foodstuff and biological materials. All phosphine reagents prepared had no fluorescence but their oxides, which were produced by the reaction of the phosphines with hydroperoxides, had strong fluorescence. Among the phosphine reagents prepared, diphenyl-1-pyrenylphosphine had the most suitable properties as a fluorescent reagent and was successfully applied to the determination of hydroperoxides by batch, flow injection and HPLC post-column methods.

Keywords: Fluorescence detection; Food analysis; Derivatization, LC; Detection, LC; Flow injection analysis; Lipid hydroperoxides; Diphenylpyrenyl phosphine; Phosphines; Organophosphorus compounds

1. Introduction

Unsaturated fatty acids and their esters are oxidized easily to hydroperoxides which cause deterioration of foods [1–3]. Recently, lipid hydroperoxides have attracted much attention as one of the risk factors of aging and some diseases such as cancer, atherogenesis and so on [4–7].

It had been difficult to determine lipid hydroperoxides in foodstuff and biological materials because of their trace amounts, instability and diversity. While iodometry [8] and its modified methods [9–11] have been most widely used for the determination of lipid hydroperoxides in foodstuff, they did not satisfy us with their sensitivity (detection limit; 10^{-6} mol order) and simplicity, especially for the analysis of biological materials. Although thiobarbituric acid methods [12,13] have been widely used to determine them in biological materials as a simple and sensitive method (detection limit; 10^{-10} mol order), they have some drawbacks in selectivity and quantitativity because some compounds other than hydroperoxides also give similar dye [14,15]. Several enzymatic methods had been proposed for the determination of total lipid hydroperoxides in biological materials with high selectivity and sensitivity [16–18]. Although the enzymatic method using cycloxygenase was the most sensitive method among them (detection limit; 10 pmol) [18], it was not easy to get the enzyme in hand. The colorimetric detection of I_3^- has been introduced in flow injection analysis (FIA) for lipid hydroperoxides [19]. Although the method has made it possible to determine lipid hydroperoxides of 30 samples within 60 min at the 10^{-10} mole level, the system is somewhat complicated and requires the complete removal of oxygen from solvents. FIA methods with the luminol chemiluminescence have also been reported to de-

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termine lipid hydroperoxides at the picomole level [20]. These methods require the removal of radical scavengers in the sample before analysis because they quench the luminol chemiluminescence. Therefore, the methods could be applied for the restricted purposes. Some HPLC methods have been reported to detect lipid hydroperoxides at their classes or molecular levels with UV detection at 235 nm of the conjugate diene system [21–23]. However, their sensitivity and selectivity did not satisfy us to determine hydroperoxides in foodstuff and biological materials, because their detection limit is at the sub-nmol level and all compounds with absorption at 235 nm give peaks by the methods. The determination of lipid hydroperoxides in foodstuff and biological materials at their classes or individual molecular species at the picomole level has been required. In this paper, we describe (1) design, synthesis and properties of phosphine reagents and (2) application of them to the determination of total hydroperoxides by both batch and flow injection methods, and of hydroperoxides at their classes or individual molecular species by HPLC.

2. Design, synthesis and properties of phosphine reagents [24,25]

Triphenylphosphine has been widely used to reduce quantitatively hydroperoxides to the corresponding alcohols under very mild conditions (Fig. 1) [26]. By the reaction, the triphenylphosphine was oxidized quantitatively to its phosphine oxide. Since the absorbance at 260 nm of the oxide is about 10 times stronger than that of the phosphine, it would be possible to determine hydroperoxides by the increase of the absorbance at 260 nm by the reaction. However, the method seemed to us unsatisfactory for the determination of lipid hydroperoxides in foodstuff and biological materials because of its low sensitivity.

Since it is well known that fluorometry is much more sensitive and selective than UV detection, we have prepared several triarylphosphines (Fig. 2) having fluorophore(s) instead of phenyl group(s) of triphenylphosphine and studied the change of fluorescence spectra by oxidation [25]. These phosphines were prepared by the Grignard reaction of aryl magnesium halide and chlorophosphines, and their oxides were prepared by oxidation of the phosphines with hydrogen peroxide (Fig. 3). To our great luck, all phosphines had no fluorescence but their oxides had very strong fluorescence. Therefore, only measuring the strength of fluorescence intensity of the oxides by the reaction is necessary for the determination of the hydroperoxides. This is one of the ideal properties of our fluorogenic reagents for the analysis of hydroperoxides.

The reaction of phosphine reagents with hydroperoxides proceeded in many kinds of solvents such as water–alcohol mixtures, alcohols, chloroform, ethyl acetate, benzene and *n*-hexane. No restriction of the solvents is another ideal property of our reagents. Fig. 2 shows the relative reactivities of phosphine reagents with *tert*.-butyl hydroperoxide and the relative fluorescence intensities of their oxides. Their reactivities were accelerated by decrease of the steric hindrance around the phosphorus atom and by an increase of electron density on the phosphorous atom. The phosphine oxides which had a 1-pyrenyl group showed stronger fluorescence than the others.

Diphenyl-1-pyrenylphosphine (DPPP) was judged as the most suitable reagent for the determination of

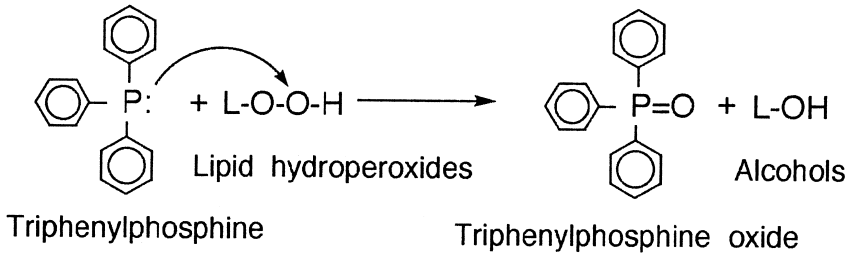
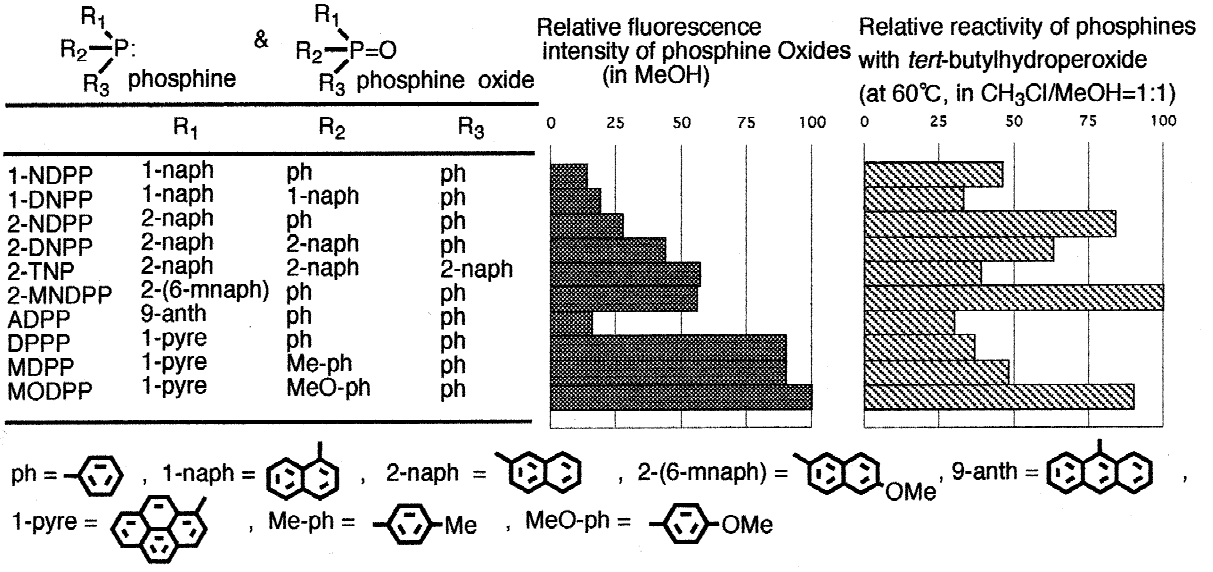


Fig. 1. Reaction of triphenylphosphine with hydroperoxides.



	R_1	R_2	R_3
1-NDPP	1-naph	ph	ph
1-DNPP	1-naph	1-naph	ph
2-NDPP	2-naph	ph	ph
2-DNPP	2-naph	2-naph	ph
2-TNP	2-naph	2-naph	2-naph
2-MNDPP	2-(6-mnaph)	ph	ph
ADPP	9-anth	ph	ph
DPPP	1-pyre	ph	ph
MDPP	1-pyre	Me-ph	ph
MODPP	1-pyre	MeO-ph	ph

Fig. 2. Structures and reactivity of phosphine reagents and relative fluorescence intensities of their oxides.

lipid hydroperoxides because of its sensitivity, reactivity, stability against air oxidation, and easy preparation. This reagent did not give its oxide by the reaction with unoxidized lipids such as triacylglycerols, cholesterol, cholesterol esters, phosphatidylcholines, phosphatidylethanol amines and free fatty

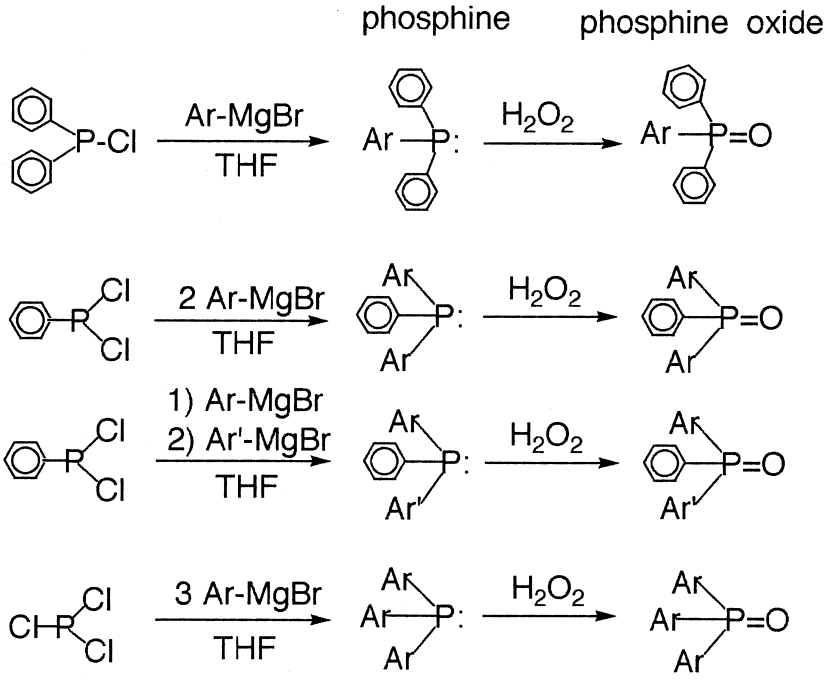


Fig. 3. Synthesis of phosphine reagents and their oxides.

acids, hydroxy lipids, alkyl halides and antioxidation agents such as butylhydroxytoluene (BHT), butylhydroxyanisol, tocopherols, β-carotene, propylgaleate.

3. Determination of total hydroperoxides by batch method with DPPP [27–29]

At first, to show the basic property of DPPP as an analytical reagent, the determination of lipid hydroperoxides by the batch method was described. Lipid hydroperoxides were determined by the increase of fluorescence intensity of DPPP oxide (emission at 380 nm, excitation at 352 nm) after the reaction, which was performed by mixing 50 μl of the reagent solution (1 mg/10 ml in chloroform) and 100 μl of a sample solution, tightly capping a screw cap, and standing it at 60°C for 60 min in the dark. Here, to prevent the oxidation of lipids during the reaction, BHT was added to the reaction mixture as an antioxidation agent. After the reaction, it was cooled in an ice bath. Then, 3.0 ml methanol was added to it followed by measurement of its fluorescence intensity emission at 380 nm (excitation at 352 nm). The self-degradation of lipid hydroperoxides was negligible during the reaction. Here, BHT gave no influence on the determination. With the very simple procedure described above, 0.1–7.0 nmol levels of lipid hydroperoxides were determined with good reproducibility (relative standard deviation, RSD=1.8%, 0.7 *n* eq./tube, $n=7$). The peroxide value (PV) determined by the present method gave good agreements with those by the iodometry (Fig. 4, $r=0.9995$, $n=41$). For the determination of PVs of some food samples such as butters, margarine and mayonnaises, the present method required only suspending them in $CHCl_3$–MeOH as a sample preparation procedure, and 100 μl of the aliquot was used for analysis without any further procedures.

4. Determination of total hydroperoxides by flow injection analysis (FIA) with DPPP [30,31]

DPPP was also useful to the on-line detection system in an FIA and an HPLC with post-column system (Fig. 5). The FIA system used here was built up by elimination of the separation part (d) from the post-column HPLC system shown in Fig. 5. This system showed us the basic performance of DPPP as a reaction reagent for the on-line detection system. In this system, the sample was injected into the system (1–50 μl) flowed with methanol–1-butanol (1:1, v/v) at the rate of 0.7 ml/min. After mixing a reagent solution [DPPP; 12 mg in 400 ml of methanol–1-butanol (1:1, v/v), which contained 200 mg of BHT] at 0.6 ml/min, the mixture was reacted in a stainless steel coil (50 m×0.5 mm I.D.) at 80°C.

With this system, we have tested the effect of coexisting substances, such as antioxidation agents and solvents, on peak area of trilinolein hydroperoxide. Although significant signals were observed by injection of some antioxidation agent solutions themselves due to the oxidized antioxidants, they had almost no influence on the peak area that was attributed to trilinolein hydroperoxide as shown in Table 1. To show that these signals were not attributed to antioxidation agents themselves but their oxidized products, they were analyzed by a HPLC post-column system with a reversed-phase column (see below). The peaks detected by the fluorometer did not agree with the positions where the antioxidation agents should be eluted from a reversed-phase column. It was also supported by the results that these peaks were reduced or disappeared by treatment with triphenyl phosphine or $NaBH_4$. This is another ideal property of the reagent because antioxidants such as BHT affect on the reaction of chemiluminescence determination of hydroperoxides with isoluminol and luminol [20]. The quenching of the chemiluminescence with luminol or isoluminol by antioxidants was applied to the measurement of antioxidation activity of radical scavengers [32,33].

The injection of a halogenated solvent, such as chloroform and dichloromethane, gave a faint positive signal while almost no signal was observed upon injection of methanol–1-butanol (1:1). A negative peak was observed upon injection of BHT solution probably due to its partially suppressing effect of baseline level. The peak could be eliminated by addition of BHT to the reagent solution, which suppressed the whole baseline level without influence on the signal area of lipid hydroperoxides.

The peak area almost agreed with each other (relative standard deviation; RSD=3.7%) by injec-

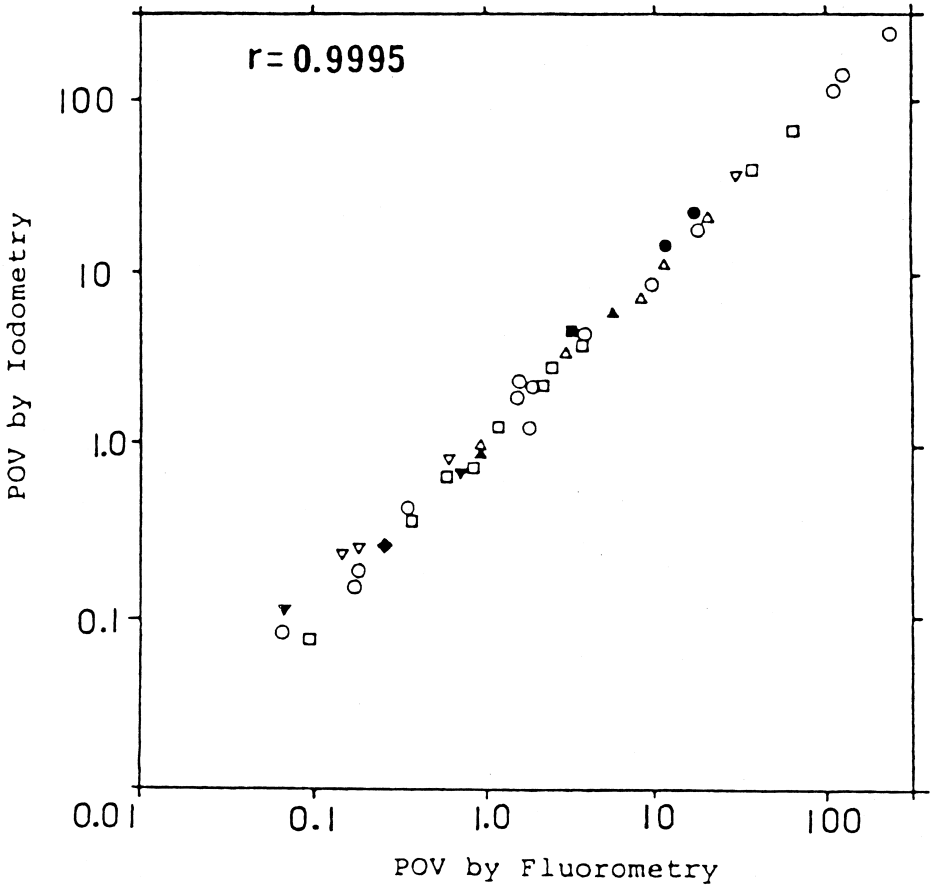


Fig. 4. Relationship between peroxide values determined by the fluorometry with DPPP and the iodometry. The samples were vegetable oils (○), free fatty acids (●), a wheat oil (■), salada oils (△), and extracts from butters (▼), margarines (□) and mayonnaise (♦) with chloroform–methanol (2:1) with phase separation, evaporation and resolving procedures, and the suspension of butters (▲) and margarines (▽) in chloroform–methanol (2:1) without further treatments.

tion of the same amount of hydroperoxides (101 pmol/injection) even though the sample size was changed from 10 to 50 μl. This system allowed us to inject samples at 2-min intervals. By the system, it was possible to measure 2–201 pmol of trilinolein hydroperoxides (coefficient of correlation; $r=0.9969$). The relative standard deviations of peak areas were 3.2% (20 pmol, $n=8$) and 1.7% (201 pmol, $n=8$), and the detection limit was 0.2 pmol (signal-to-noise ratio; $S/N=3$). There were good accordances between the results measured by the batch method and those by this system for 37 samples that were vegetable oils, edible oils and margarines ($r=0.9981$, PV=0.16–172, the calibration curve of trilinolein hydroperoxide was used for all samples to calibrate their amounts). These results showed us that DPPP had high potential as an analytical reagent for the on-line post-column detection system.

5. Application of DPPP method to HPLC post-column system

5.1. Basic system

In order to study the mechanism and effects of lipid peroxidation in foodstuff and biological materials, it has been necessary to obtain information (species, amounts, stability and so on) about the

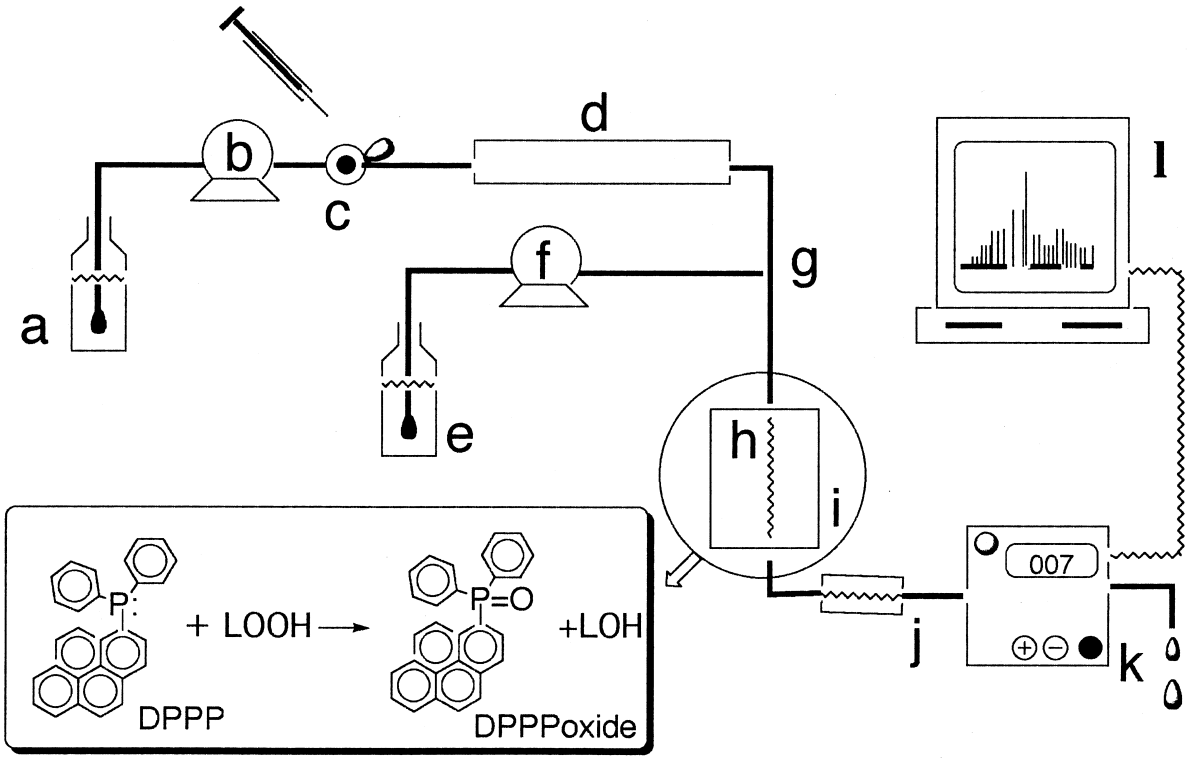


Fig. 5. System diagram of HPLC (and FIA) system. (a) mobile phase solvent, (b) pump, (c) sample injector, (d) separation column, (e) reagent solution, (f) pump, (g) mixing port, (h) reaction coil, (i) reaction oven, (j) cooling coil with water jacket, (k) fluorescence detector, (l) data processor.

Table 1
Effects of antioxidants on a peak area of trilinolein hydroperoxide

Antioxidants	Amount of addition (ng/injection)	Relative peak area of trilinolein hydroperoxide[a]
BT	20	105
	200	108
	2000	102
BHA	140	101
	1400	102
	14 000	98.0
	100	105
α-Tocopherol	1000	89.8
	10 000	93.0
	19	90.8
β-Carotene	190	90.8
	1900	87.1
	60	94.7
	600	96.3
Propyl galate	6000	89.4

[a] The peak area of trilinolein hydroperoxide (43 nmol/injection) without addition of antioxidants was 100.

hydroperoxides formed. Therefore, we tried to determine lipid hydroperoxides in such samples at their class or individual molecular levels. Since HPLC was one of the most useful tools to separate trace compounds under mild conditions, we designed an HPLC post-column system with DPPP. The basic system was built up by introduction of separation part (d) into the FIA system described above (Fig. 5) with some modification of conditions such as mobile phase solvent, concentration of reagent solution, flow-rates of mobile phase and the reagent solution and so on. Here, basically the reaction coil used was a stainless steel coil (0.5 mm I.D.) with 20 m length to avoid the peak broadening and the reaction was performed at 80°C.

Fig. 6 shows a typical chromatogram of trilinolein (TL) hydroperoxide and its related compounds [34]. Here, separation was performed by an ODS column eluted with 1-butanol–methanol (1:9, v/v), and the

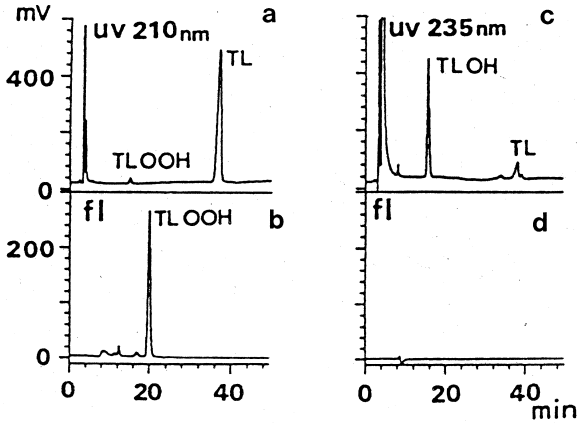


Fig. 6. Typical chromatograms of trilinolein (TL; 50 nmol), trilinolein hydroxide (TL-OH; 214 pmol) and trilinolein hydroperoxide (TL-OOH; 214 pmol) on an ODS column.

eluent from the column was monitored by a UV detector followed by post-column detection with DPPP. Therefore, there was about 6 min lag time on the peaks detected by fluorometry. By the system, TL hydroperoxide gave a peak by the fluorometric detection at 20 min (Fig. 6b). The unoxidized TL, which was detected at 36 min by UV at 210 nm (Fig. 6a), gave no peak by the fluorometric detection (Fig. 6b), while it should elute at 42 min on the system. The hydroxy compounds (TL-OH), which were detected at 14 min by UV at 235 nm (Fig. 6c), also gave no peak by fluorometric detection (Fig. 6d), while it should elute at 20 min. This suggests that the excess amounts of unoxidized and hydroxy lipids gave no influence on the fluorometric detection method with DPPP. Here, 1–2 pmol levels of the hydroperoxide were detected with high selectivity and reproducibility.

Since the post-column reaction proceeded in many kinds of organic solvents, this system allowed us to use a wider range of solvents, from aqueous alcohols to *n*-hexane, as a mobile phase solution. This made it possible to use many types of separation modes such as reversed-phase and normal-phase. It was also possible to use gradient elution mode. Some applications of the method are compiled in Table 2.

5.2. Determination of triacylglycerol (TG) hydroperoxides on reversed-phase columns [34]

Since TG in oils contained various acyl moieties, the TG hydroperoxides were separated into several peaks depending on their fatty acid components on an ODS column (Fig. 7a and b). There was a linear relationship between PN and log t_R. Here PN was defined by the equation PN=TC-2DB, where TC and DB were the number of total carbon atoms and double bonds of acyl moiety of which the TG monohydroperoxide was composed, respectively, and t_R was its retention time.

On the other hand, it was also possible to determine TG hydroperoxides at their class levels by using a phenylated silica gel column eluted with methanol–water (95:5, v/v). Since the column has

Table 2
Determination of hydroperoxides by HPLC post-column methods with DPPP

Hydroperoxides	Column	Solvent	Application sample	Ref.
TG hydroperoxides	ODS	MeOH–1-butanol	Oils and foods	[34]
			Human plasma	[37]
	Ph	MeOH–water	Oils and foods	[34]
TG & CE hydroperoxides	Silica gel	*n*-Hexane–1-butanol (gradient elution)	Oils	[35]
			Human plasma	[36,37]
PC hydroperoxides	Silica gel	MeOH–$CHCl_3$–water–1-butanol	Human plasma	[29,39–41]
			Bovine serum	[40]
PC and PE hydroperoxides	ODS+aminopropyl+ ODS	*n*-Hexane–1-butanol–MeOH	Human plasma	[42]
PC, TG and CE hydroperoxides	Silica-gel & ODS	*n*-Hexane–1-butanol–MeOH (column switching)	Human plasma	[45]
	ODS	MeOH–water–2-propanol (gradient elution)		[46]

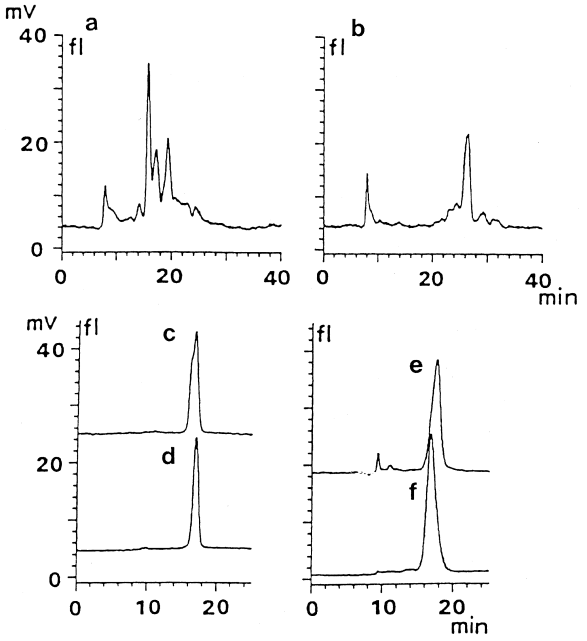


Fig. 7. Typical chromatograms of triacylglycerol hydroperoxides on an ODS (a,b) column and a phenylated silica gel column (c,d,e,f). (a) linseed oil, (b) olive oil, (c) triolein, (d) trilinolein, (e) linseed oil, (f) olive oil.

low selectivity to the number of carbon atoms and double bonds of acyl moieties, all TG hydroperoxides have almost the same retention time, between 16 and 18 min. As shown in Fig. 7e and f, hydroperoxides of some vegetable oils gave almost a single peak, although somewhat broad due to the variation of acyl moieties, and they were determined in the range of 2–300 pmol with good reproducibility (RSD=2.8%, 45.5 pmol, $n=6$). Although there was about 10% variation between the peak area ratio of TL hydroperoxides and the TG hydroperoxides in nine kinds of vegetable oils, it was almost possible to use the calibration graph of trilinolein hydroperoxides for the determination of TG hydroperoxides in oils. However, it was difficult to determine hydroperoxides of TG and cholesterol esters (CE) simultaneously at their class levels because CE hydroperoxides had almost the same retention time of TG hydroperoxides on a phenylated silica gel column.

5.3. Determination of TG and CE hydroperoxides on a normal-phase column [35–37]

The simultaneous determination of TG and CE hydroperoxides was performed by a normal-phase silica gel column with a gradient elution with *n*-hexane (solvent A) and *n*-hexane–1-butanol (20:1, v/v, solvent B). With this system, hydroperoxides of TGs, such as trilinolein, triolein, trilinolenin, olive oil, soybean oil and linseed oil, gave almost the same retention time at 12–14 min (Fig. 8, peak c), and hydroperoxides of cholesteryl linoleate, oleate and arachidonate gave a peak at 10–11 min (Fig. 8, peak b). These hydroperoxides were determined in the range 5–1000 pmol with 2 pmol of detection limits. It was possible to apply this system for the determination of them in human plasma with a slight modification, the addition of BHT to DPPP solution to eliminate some negative peaks. By this method, no TG hydroperoxides and 24.5±9.6 n*M* ($n=15$) CE hydroperoxides were detected in fresh human plasma (Fig. 8, peak a). Moreover, CE hydroperoxides were determined in high density lipoprotein (HDL; 6.8±2.6 n*M*) and combined low and very low

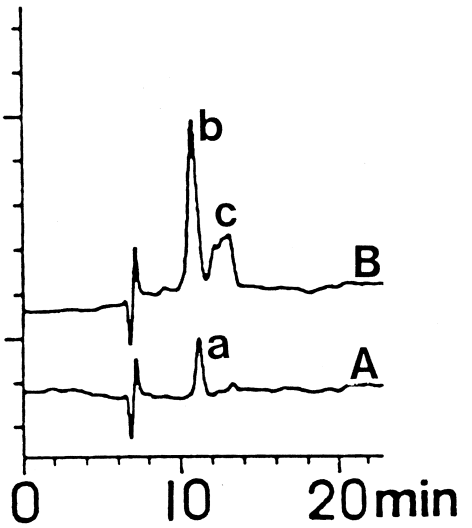


Fig. 8. Typical chromatograms of human plasma extract. (A) Fresh human plasma extract, (B) an extract from fresh human plasma to which standard CE and TG hydroperoxides were spiked.

density lipoproteins (LDL+VLDL; 20.0±5.6 n*M*) by using a normal-phase column system. Here, human plasma lipoprotein ($n=10$) were fractionated from 2 ml of plasma with dextran sulfate and Mg^{2+} to each fraction. The method was also useful to follow the increase and decrease of CE hydroperoxides in each lipoprotein fraction by the incubation of fresh plasma spiked with dilinoleoyl phosphatidylcholine (PC) hydroperoxides. At the early stage, CE hydroperoxides increased more rapidly in HDL than in LDL+VLDL. Then they reached a maximum and decreased gradually upon continued incubation. Frei et al. also reported the increase of CE hydroperoxides in human plasma upon incubation with PC hydroperoxides [38]. They speculated on a mechanism in which lecithin-cholesterol acyltransferase (LCAT) might catalyze the transacylation of an acyl group with a hydroperoxide from PC to free cholesterol in plasma, however they did not show further evidence. This rapid increase of CE hydroperoxides in HDL suggested strongly that the transacylation was catalyzed by LCAT. This was supported by incubation tests of plasma with dilinoleoyl PC and dioleoyl PC hydroperoxides. Although, molecular species of CE hydroperoxides could not be determined by the normal-phase column system, they were separated on an ODS column depending on their fatty acid components. By the incubation of plasma with PC hydroperoxides, CE hydroperoxides which had the corresponding acyl moiety to the spiked PC hydroperoxides was observed. The contribution of LCAT to this reaction was also clearly supported by the fact that no increase of CE hydroperoxides was observed by using plasma, which was heated at 58°C for 30 min before incubation to inactivate LCAT, instead of fresh plasma.

5.4. Determination of PC hydroperoxides [29,39–41]

Since phospholipids, especially PC, were the important components of lipoprotein and biological membranes, it had been strongly required to determine them in biological materials. Therefore, we tried to determine PC hydroperoxides, which comprised 50–70% of the total phospholipids in human plasma. PC hydroperoxides were separated on a silica gel column eluted with chloroform–1-butanol–methanol–water (50:50:200:15, v/v). By this system, PC hydroperoxides were determined at the range of 1–650 pmol and its relative standard deviation was 1.8% (108 pmol, $n=7$) and 3.4% (21.6 pmol, $n=7$). PC hydroperoxides were determined as 30–75 n*M* in fresh human plasma ($n=13$), and 9–100 n*M* ($n=67$) in bovine serum. The PC hydroperoxide in bovine serum altered seasonally, higher in winter than summer, and the degree was larger in younger ones than in elder ones. While Miyazawa et al. also reported the presence of PC hydroperoxide in human plasma by using a chemiluminescence HPLC method with isoluminol [42,43], Yamamoto et al. reported its absence by using a chemiluminescence HPLC method with luminol [44]. These methods involve extraction of lipids with organic solvents and the evaporation of the solvents. The procedures were not only tedious but also have the possibility of the artificial formation or degradation of hydroperoxides. To make it clear that PC hydroperoxide was present in human plasma or not, we designed an automatic method for its determination. After sample plasma was deproteinized with a 4-fold volume of methanol and centrifugation, the supernatant (2–8 ml) was directly injected into an HPLC system without further treatment. Here, the system allowed us to inject samples up to 2 ml at once and up to 8 ml as a total volume by four times the injection. Hydroperoxides were concentrated on an ODS column by eluting with 80% methanol in water. Then, the hydroperoxides were eluted by changing a mobile phase solvent (*n*-hexane–1-butanol–methanol–water, 3:10:40:3) and only the target fraction was introduced into two analytical columns, a silica gel column and an aminopropylsilica gel column which were connected in series, through a column switching device. By the system, both PC and phosphatidylethanolamine (PE) hydroperoxides were determined at picomole levels within 30 min (Fig. 9A). By using only a silica gel column as an analytical column, PC hydroperoxides were determined within 20 min (Fig. 9C), and the samples could be injected into the system at 15-min intervals. With the system, 4–24 n*M* of PC hydroperoxides were detected in

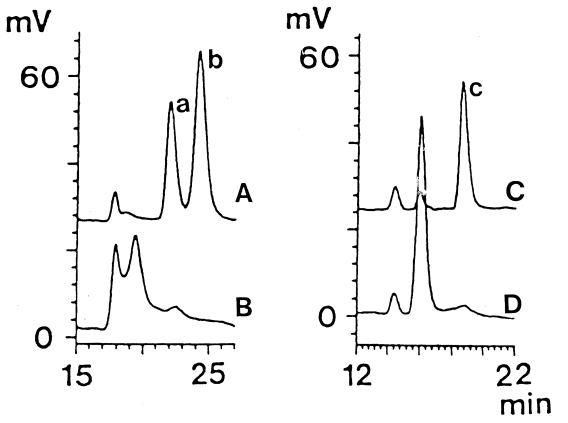


Fig. 9. Chromatograms of phospholipid hydroperoxides. (A,B): aminopropyl column + silica gel column system, (C,D): a silica gel column system. Peaks a and c=standard PC hydroperoxide and peak b=standard PE hydroperoxide. Deproteinated plasma samples were injected twice (2 ml/injection, total injection volume: 4 ml) for chromatograms B and D.

fresh human plasma ($n=8$) while PE hydroperoxides were not detected (Fig. 9B and D). These values should be most reliable because these systems made the sample preparation procedures minimized by using an on-line sample preparation system. These systems should be useful tools for routine analysis of PC hydroperoxides in human plasma.

5.5. Simultaneous determination of PC, TG and CE hydroperoxides [45,46]

It had been very difficult to determine lipid hydroperoxides simultaneously because of their wide range of polarity. We developed a simultaneous determination method of hydroperoxides of PC, TG and CE by using a column switching technique. The system has two silica gel columns (one for the class separation and another for the analytical separation of PC hydroperoxide), an ODS column for the separation of TG and CE hydroperoxides, two column switching valves, and one DPPP post-column detection unit. In the system, at first a sample was separated into a combined TG and CE hydroperoxides fraction and a PC hydroperoxides fraction on a short silica gel column. Each fraction was introduced into an ODS column and another silica gel column by a column switching device, respectively. At the initial stage, the eluate from an analytical silica gel column was monitored by a post-column detection system to determine the PC hydroperoxides. Then the eluate from an ODS column was introduced into the detection system through another switching device to determine TG and CE hydroperoxides. With this system, the hydroperoxides were determined at picomole levels within 32 min. Their relative standard deviations of the peak areas were between 1.6 and 3.1% (45–62 pmol, $n=6$). The method was successfully applied to determine lipid hydroperoxides in human plasma. The separation on two analytical columns were performed at the same time independently by the same solvent system, thus saving time and sample. This column switching technique for the simultaneous determination of lipid hydroperoxides was applied to the chemiluminescence HPLC [47].

The simultaneous determination using a gradient elution mode was also developed. Here, we applied two strategies to minimize the baseline drift caused by the continuous change of solvent composition. One was to minimize the change of solvent composition in the reaction solution by increasing the ratio of the DPPP solution to the eluate. The other was to increase the reaction rate by increasing the polarity of the reaction solution by addition of water to the reagent solution, which was effective for this purpose by increasing the peak height relative to the baseline drift. With the system, eight kinds of hydroperoxides (hydroperoxides of linoleic acid, oleic acid, PC dilinoleoyl, PC dioleoyl, trilinolein, triolein, cholesteryl linileate and cholesteryl oleate) were individually determined within 45 min at picomole levels with good reproducibilities (RSD= 2.4–6.5%, 12.5 pmol, $n=5$). This system was successfully downsized to semi-micro size by using a 2.0 mm I.D. column and a 30 m length stainless steel coil (0.25 mm I.D.). In Fig. 10, typical chromatograms by the semi-micro system are shown. Although the baseline drift due to the gradient elution was observed (Fig. 10c), eight hydroperoxides were separated within 40 min (Fig. 10a and b) and each of them could be detected at several picomole level with less than 10% of RSD at 10 pmol injection ($n=5$). The system made it possible to save the amounts of reagents and the solvents by needing less

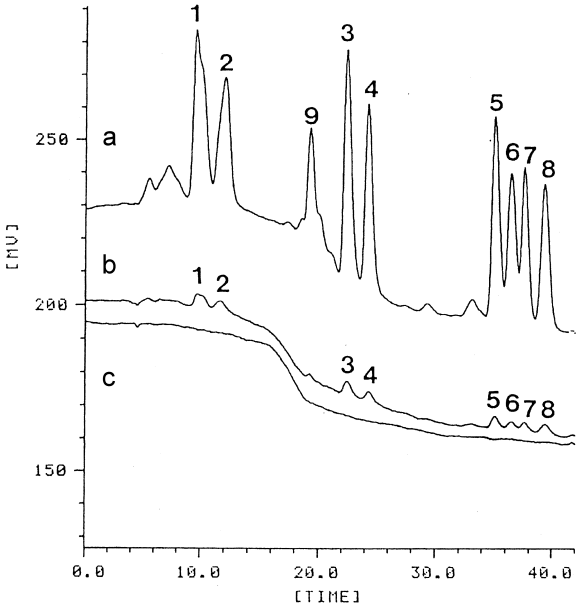


Fig. 10. Typical chromatograms for lipid hydroperoxides by the semi-micro system. The chromatograms show hydroperoxides (a; 50 pmol each, and c; 5 pmol each) and baseline (c). The peaks are hydroperoxides of linoleic acid (1), oleic acid (2), PC dilinoleoyl (3), PC dioleoyl (4), trilinolein (5), cholesteryl linoleate (6), cholesteryl oleate (7) and triolein (8). Peak 9 is dihydroperoxides of PC dilinoleoyl.

than 1/10 and 1/3, respectively, of the amounts needed for the conventional-size system without reducing sensitivity and reproduciblity.

Another HPLC method by using gradient elution was reported for the determination of lipid hydroperoxides with post-column detection by a ferrous/xylenol orange reagent [48]. The determination rage of the method was 40–2000 pmol.

The determination methods with DPPP were as highly sensitive and selective as chemiluminescence methods and allowed us to use many kinds of separation techniques without influence by coexistent antioxidants such as BHT and α-tocopherol.

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