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Development of Phosphine Reagents for Fluorometric Determination of Lipid Hydroperoxides

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Summary

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

Introduction

Unsaturated fatty acids and their esters are oxidized easily to hydroperoxides which cause lowering of food qualities because of off-flavour and toxities (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 (4-9).

It had been difficult to determine lipid hydroperoxides in foodstuff and biological materials because of their trace amounts, instablity and diversity. While iodometry (10) and the modified methods (11-13) were most widly used for the determination of lipid hydroperoxides in foodstuff, they did not satisfy us with their sensistivity and simplicity for the analysis of biological materials. Although thiobarbituric acid methods (14-16) were widly used to determine them in biological materials, they have some problems such as selectivity and quantitativity (17, 18). Some enzymatic methods had been proposed to determine total lipid hydroperoxides in biological materials with high selectivity and sensistivity (19-21). These methods also have problems such as simplicity and availability of the enzymes. Some HPLC methods have been reported to determine lipid hydroperoxides at their class or molecular levels with UV 235 nm

detection, conjugated diene system (22–24). However, they did not satisfy us with their sensitivity and selectivity. Under these situations, we tried to develop new type of fluorescence reagents for lipid hydroperoxides. Here, I describe (1) design, synthesis and properties of new reagents and (2) their applications to the determination of total hydroperoxides by a batch method, and (3) applications to HPLC post-column methods.

1. Design, synthesis and properties of phosphine reagents

Triphenylphosphine has been widly used to reduce lipid hydroperoxides to the corresponding alcohols under mild conditions (Fig. 1) (25). The strong reducing power of the phosphine is explained by its unstable three-coordinate structure which easily changes to more stable penta-corrdinate compound accompained with the extention of electron shell on phosphorous atom. By the reaction, the phosphine itself was oxidized quantitatively to the phosphine oxide. Since the

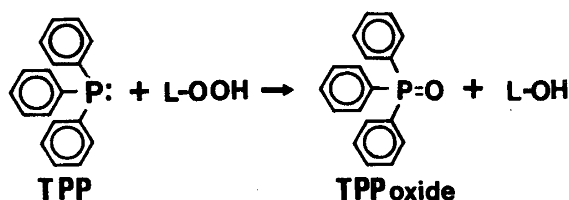
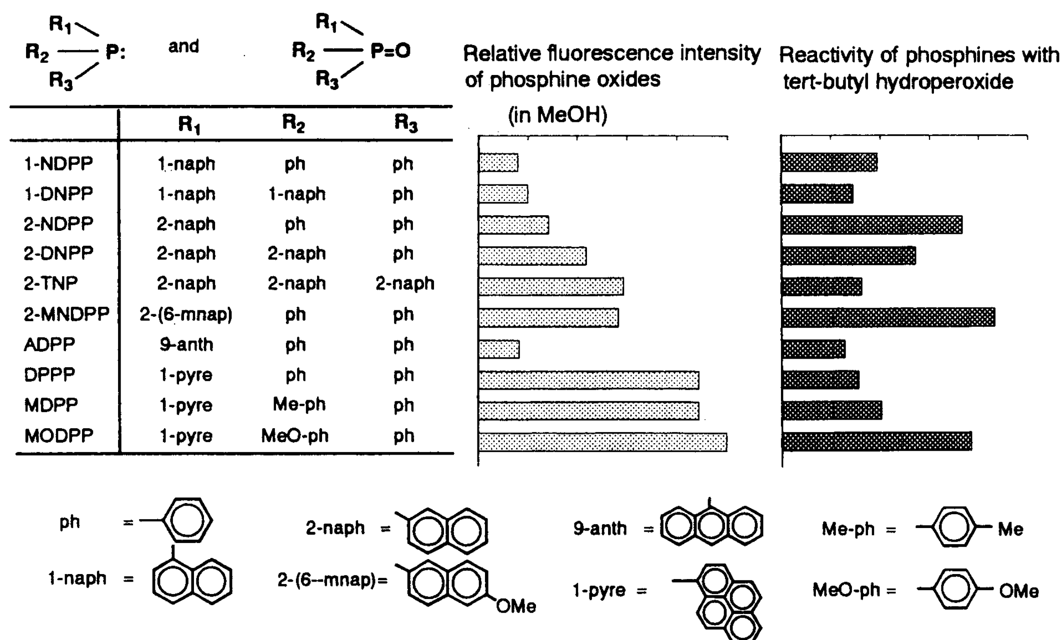


FIG. 1. Reaction of triphenylphosphine with hydroperoxides



	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-mnap)	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 reactivities of phoshine reagents and relative fluorescence intensities of their oxides

absorbance at 260 nm of the oxide was about 10 times stronger than that of the phosphine, it was possible to determine hydroperoxides by the increase of absorbance at 260 nm after the reaction. Since the method seemed to me not usefull for the determination of lipid hydroperoxides in foodstuff and biological materials because of the low sensitivity and selectivity, it promped us to design new type of reagents which will satisfy me with all respects.

Since the extention of electron shell on phosphorous atom made us to expect useful spectral change, especially on fluorescence spectrum, we designed aromatic phosphine reagents which had one or more aromatic fluorophore(s) (Fig. 2). Phosphines 1-NDPP, ADPP and DPPP were prepared by replacement of one phenyl group of triphenylphosphine with each fluorophore (26). All phosphine reagents and their oxides were also prepared by the coupling reaction of phosphoryl chloride and the corresponding Grignard reagents of aromatic fluorophores (Fig. 3) (27). To me great luck, all phosphines synthesized had no fluorescences but their oxides had very strong fluorescences. This is one of the ideal properties as a fluorescence reagent.

The reaction of phosphine reagents with hydroperoxides proceeded in second-order nucleophilic reaction manner with highly selectivity. Many kinds of solvents such as alchohols, chloroform, ethyl acetate, benzene and hexane were useful as the reaction solvent. Figure 2 also shows the reactivities of phosphine reagents with tert-butyl hydroperoxide and the relative fluorescence intensities of their oxides. Their reactivities were accelated by decrease of the steric hinderan-

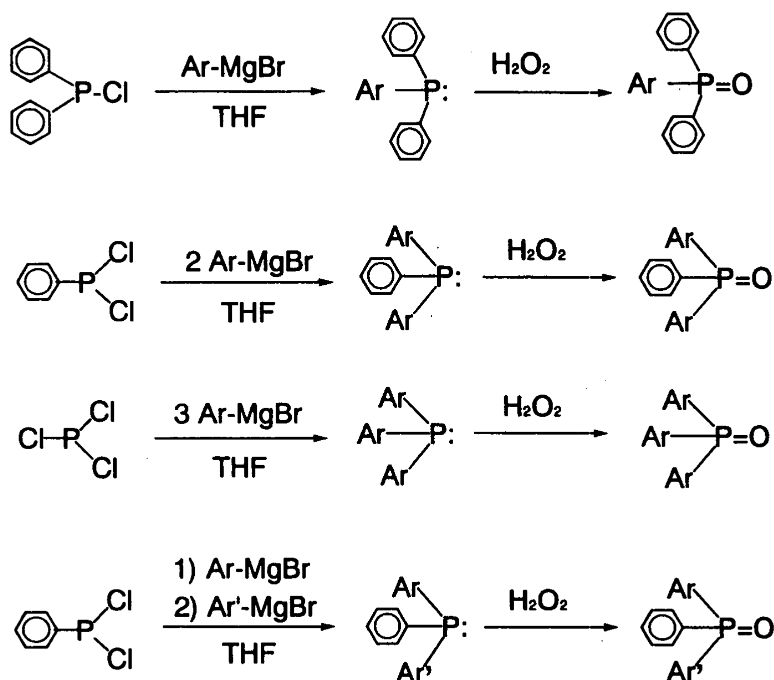


FIG. 3. Synthesis of Phosphine reagents and their oxides

ce around phosphorous atom and by increase of electron density on phosphorous atom. The phosphine oxides which had a pyrenyl group showed stronger fluorescences than the others.

Diphenyl-1-pyrenylphospine (DPPP) was judged as the most suitable reagent for the determination of lipid hydroperoxides because of its sensitivity, reactivity and easy preparation.

2. Determination of total hydroperoxides by batch method with DPPP (28-30)

Lipid hydroperoxides were determined by the increase of fluorescence intensity of DPPP oxide (at 380 nm, excitation at 352 nm) after the reaction. As shown in Fig. 4, the reactions of DPPP with lipid hydroperoxides completed within 60 min at 60°C. Here, to prevent oxidation of lipids during the reaction, butyl hydroxy toluene (BHT) was added to the reaction mixture. The degrada-

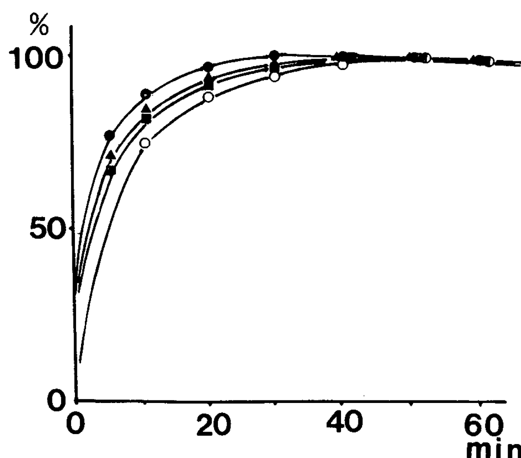


FIG. 4. Reaction time course of DPPP with hydroperoxides at 60°C
The samples were hydroperoxides of trilinolein (▼), triolein (●), linseed oil (△), cottonseed oil (▲), and soybean oil (○).

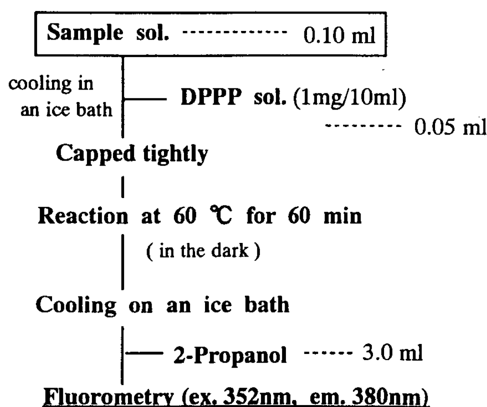


FIG. 5. Determination procedure of hydroperoxides with DPPP by batch method

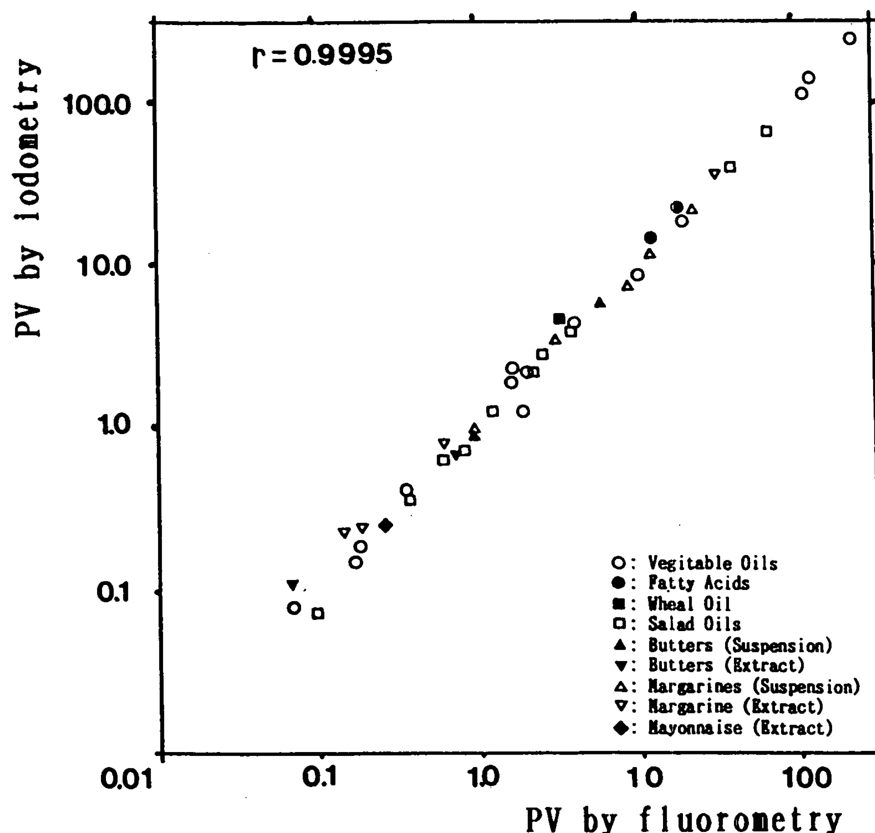


FIG. 6. Relationship between peroxide values determined by the fluorometry with DPPP and the iodometry

tion of lipid hydroperoxides was negligible during the reaction. Here, BHT gave no influence for the determination. With very simple procedure (Fig. 5), 0.1~7 nmol levels of lipid hydroperoxides were determined with good reproducibility. The peroxide values determined by the present method showed good agreements with those by the iodometry (Fig. 6). For the determination of peroxide values of some food samples such as butters, margarins and mayonnaises, the present method required only suspending them in chloroform/methanol as a sample preparation prodedure.

3. *Application for HPLC post-column system (30-38)*

To study on the mechanism and effects of lipid peroxidation in foodstuff and biological materials, it was neccesary to obtain informations about the hydroperoxides formed (species, amounts, stability, etc.). 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 design a HPLC system equipped with a post-column detection system with DPPP (Fig. 7). The reagent solution was mixed to the eluent from

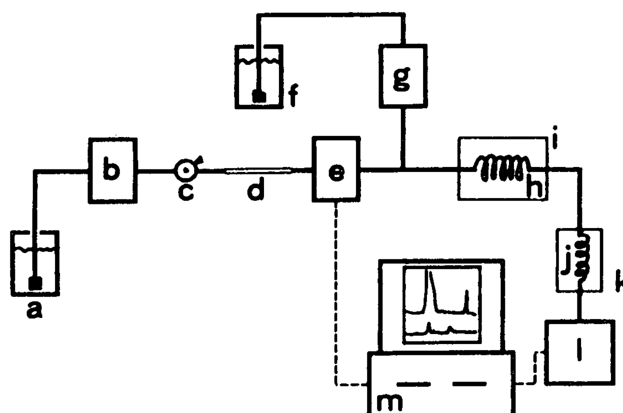


FIG. 7. HPLC system diagram
a : mobile phase solvent, b : pump, c : injector, d : separation column, e : UV detector, f : reagent (DPPP) solution, g : pump, h : reaction coil, i : reaction oven, j : cooling coil, k : water jacket, l : fluorometere, m : deta processor

a separation column, and the mixture was reacted by passing through a stainless steel coil at 80°C. After cooling, the fluorescence intensity was monitored by fluorometer.

By the system, 1～2 pmol levels of hydroperoxides were detected with high selectivity and reproducibility, and unoxidized and hydroxy compounds gave no influence for the determination (Fig. 8). Since the postcolumn reaction proceed-

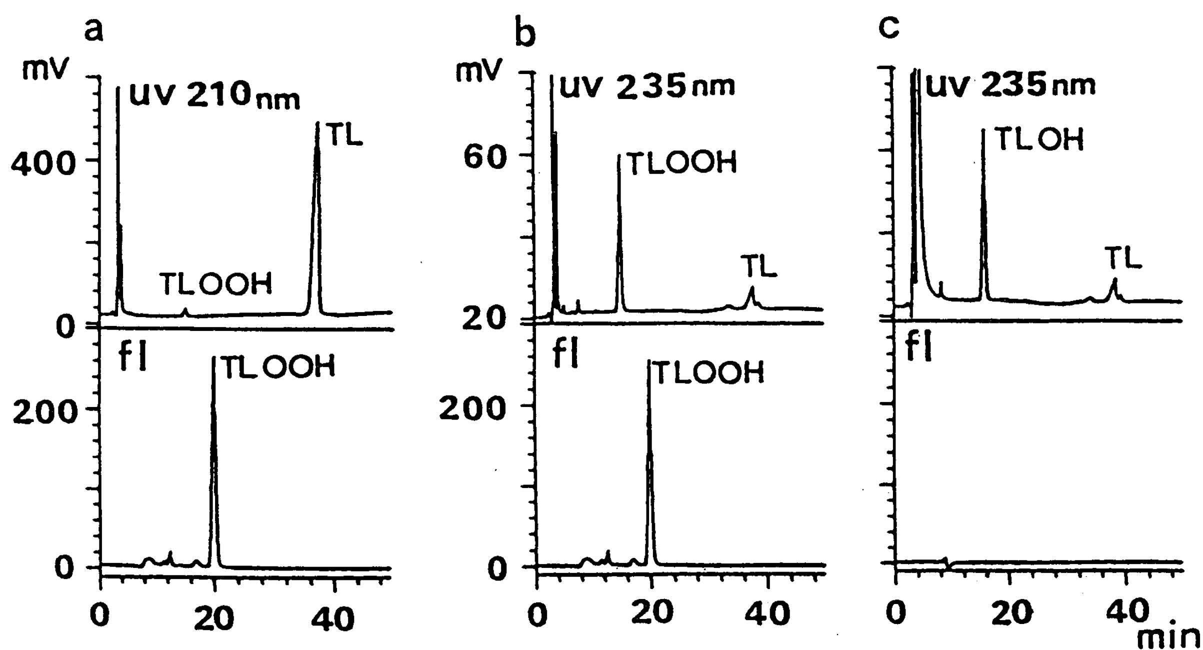


FIG. 8. Chromatograms of TL (trilinolein ; 50 nmol), TL hydroperoxide (TLOOH ; 214 pmol), and TL hydroxide (TLOH ; 214 pmol) Detection : uv 210 nm or 235 nm & fluorometry with DPPP (fl)

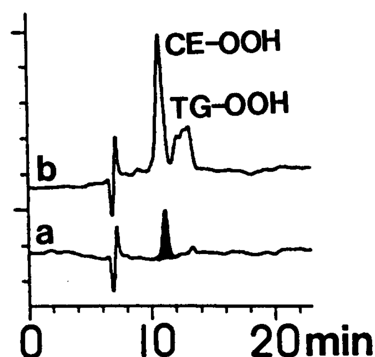


FIG. 9. Typical chromatograms of TG and CE hydroperoxides
a) human plasma extract, b) hydroperoxides of trilinolein (TG-OOH) & cholesteryl linolenate

TABLE 1 *Determination of hyfroperoxides by HPLC post-column methods*

	hydroperoxides	column	solvent	applied sample	ref.
1	PC-OOH	silica-gel	MeOH/$CHCl_2$/H_2O	human plasma	30-32
				bovine serum	32
2	TG-OOH	ODS	MeOH/1-BuOH	oils and foods	33
		ph	MeOH/H_2O	oils and foods	33
3	TG- & CE-OOH	silica-gel	Hexane/1-BuOH	oils	34
			(gradient elution)	human plasma	35
4	PC-, TG-,	silica gel	Hexane/1-BuOH/MeOH/	human plasma	36
	& CE-OOH	+ODS	H_2O (column switching)	LDL, HDL	37
5	PC- & PE-OOH	ODS+NH_2+	Hexane/1-BuOH/MeOH/	human plasma	38
		Silica-gel	H_2O (column switching)		

PC : phosphatidylcholine, PE : phosphatidylethanolamine, TG : triacylglycerol, CE : cholesterol ester

ed in many kinds of organic solvents, this system allowed us to use wide range of solvents as a mobile phase solution from aqueous alchohols to hexane. This made possible to use many types of separation modes such as reversed phase and normal phase. It was also possible to use gradient elution mode (Fig. 9). Some applications of the method were complied in Table 1. These systems made it possible to determine hydroperoxides of phospholipids, triacylglycerols (TG) and cholesterolesters (CE) in foodstuff and biological materials. The combination of two separation systems by using column switching technique made it possible to determine hydroperoxides of phosphatidylcholine, TG and CE simultaneously (37). The typical chromatograms were shown in Fig. 10. The use of an automatic sample preparation system made direct injection of deproteinzed plasma

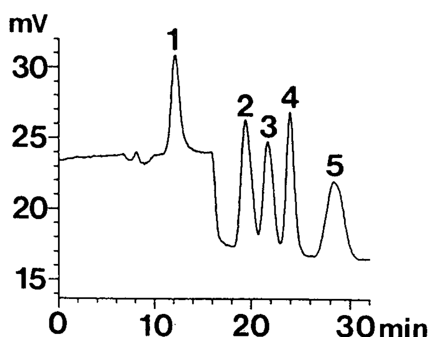


FIG. 10. Typical chromatogram of hydroperoxides of phosphatidyleholine (1), trilinolein (2), cholesteryl linolenate (3), cholestryl olate (4), and triolein (5)

possible. By using this system, phosphatidylcholine and phosphatidylethanolamine hydroperoxides were determined after only deprotenization of a plasma sample with 4-fold of methanol (38).

This detection method was as highly sensitive and selective as chemiluminescence methods (39, 40) and allowed us to use many kinds of separation techniques without influence by coexistent compound such as radical trapping agents.

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