JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Fluorometric and Fluorescent Image Analysis Methods for Determination of Lipid Hydroperoxides in Oil Models with 3-Perylene Diphenylphosphine (3-PeDPP)

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Fluorometric and fluorescent image analysis methods with 3-perylene diphenylphosphine (3-PeDPP) were developed to measure lipid hydroperoxides in refined tuna oil. The fluorescence intensity of 3-perylene diphenylphosphineoxide (3-PeDPPO, the corresponding peroxidation product of 3-PeDPP) has been used for assessing lipid hydroperoxides in refined tuna oil models, and the results are compared to those of a classical method using ferric−xylenol orange complex. There were good correlations between the lipid hydroperoxide contents determined by the 3-PeDPPO fluorescence and the xylenol orange methods ($R^2 = 0.984$). Moreover, the novel probe 3-PeDPP and the fluorescent image analysis enabled us to evaluate two-dimensionally the lipid hydroperoxide contents in tuna oil droplets. These results suggest that the fluorometric and fluorescent image analyses using 3-PeDPP would be suitable for batch and two-dimensional determinations of lipid hydroperoxides in oil and oil-containing materials.

KEYWORDS: 3-Perylene diphenylphosphine (3-PeDPP); lipid peroxidation; fluorescent probe; fluorescent image analysis

INTRODUCTION

Reaction of oxygen with unsaturated lipids produces a wide range of compounds with hydroperoxides as the initial product (*1*). The lipid hydroperoxides are susceptible to further oxidation or decomposition to secondary reaction products such as aldehydes, ketones, acids, and alcohols (*2*). Lipid peroxidation is a deteriorative process that has a significant commercial implication in terms of product quality. Lipid peroxidation is a major cause of fish oil deterioration during storage because of the high content of polyunsaturated fatty acids (*3*). There are various methods available for evaluating lipid peroxidation in foods. The methods are generally divided into two groups. The first group measures the primary oxidative changes, such as peroxide value (*4*) or conjugated dienes (*5*), and the other group determines the secondary changes that occur in the system, such as the generation of 2-thiobarbituric acid reactive substances (TBARS) (*6*). Specific compounds such as hexanal and secondary oxidation products are also measured by headspace gas chromatography (*7*). In recent years, the significantly advancing investigations have been to develop a high-performance liquid chromatographic method to separate the hydroperoxides, followed with postcolumn derivatization and fluorescence detection for improving the specificity and sensitivity over other available methods such as hydroperoxides of phosphatidylcholine, phosphatidylethanolamine, cholesteryl ester, and fish oil enriched mayonnaises (*8*−*12*). Because these methods need uniformity of oil samples, complicated sample preparation procedures, such as lipid extraction and homogenization for oil-containing materials, are necessary before determination. On the other hand, changes in the fluorescence spectrums of triarylphosphines with the fluorophore instead of the phenyl group of the triphenylphosphine with an oxidation reaction have been reported, such as diphenyl-1-pyrenylphosphine (DPPP) (*13, 14*). Although a direct image analysis without sample preparation procedures was carried out with DPPP oxide fluorescence (*15*), DPPP has the fluorescence excitation and emission wavelengths inside the UV region, causing lipid peroxidation by itself. The DPPP image analysis of lipid-containing fluorescent compounds such as tocopherols will also be interfered because of the overlapped fluorescence. The purpose of the present study is to develop new fluorometric and fluorescent image analysis methods using 3-perylene diphenylphosphine (3-PeDPP) with a longer excitation wavelength for determining lipid hydroperoxides in oil samples.

MATERIALS AND METHODS

Chemicals. 3-Perylene diphenylphosphine (3-PeDPP) was prepared from 3-bromoperylene and triphenylphosphine synthesized and identified by previous descriptions (*16, 17*) according to the method of Akasaka et al. (*13*). Boron trifluoride in methanol (14%) and polyoxyethylene (*20*) sorbitan monolaurate (tween 20) were purchased from Wako Pure Chemical Industries (Osaka, Japan). Xylenol orange (3,3′-bis[*N*,*N*-bis(carboxymethyl)-aminomethyl]-*o*-cresolsulfonphthalein di-

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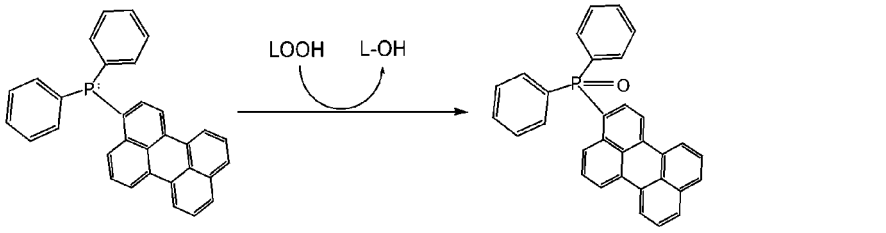
10.1021/jf050611g CCC: $30.25
Published on Web 08/19/2005



3-Perylene diphenylphosphine (3-PeDPP) (non-fluorescent compound)

3-Perylene diphenylphosphine oxide (3-PeDPPO) (fluorescent compound with λex 440 nm, λem 470 nm)

Figure 1. Reaction of 3-PeDPP with hydroperoxides.

sodium salt) was purchased from Dojindo (Kumamoto, Japan). Ferrous ammonium sulfate and sulfuric acid (95%) were purchased from Kokusan Chemical (Tokyo, Japan). Standard compounds of α-, β-, δ-, and γ-tocopherol were obtained from Eisai Co., Ltd (Tokyo, Japan). Cumene hydroperoxide (CuOOH, 80%) was purchased from Sigma-Aldrich Japan (Tokyo, Japan). 2, 6-Di-*tert*-butyl-*p*-cresol (BHT) was purchased from Tokyo Kasei Kogyo Co., Ltd (Tokyo, Japan). Other organic solvents were of analytical grade and used without further purification.

Oil Samples. Refined tuna oil (kindly given by Dr. Wanchai Worawattanamateekul, Department of Fisheries Products, Bangkok, Thailand) was produced from high-quality tuna oil (food grade) under nitrogen injection and together with natural tocopherol added. The refined tuna oil was kept at −85 °C until use. The tuna oil was placed on a silica gel 60 column (Spherical, 40−50 μm, Kanto Chemical Co. Inc., Tokyo, Japan) and eluted with *n*-hexane, followed by 7% (v/v) diethyl ether in *n*-hexane to remove tocopherols. The resulting tocopherol-free tuna oil (refined tuna oil) was kept at −85 °C until use (initial lipid hydroperoxide level: 1.25 ± 0.1 μmol/g of oil).

Detection of Lipid Hydroperoxides. *Detection of Lipid Hydroperoxides with 3-PeDPP by a Spectrofluorometer*. Oil samples were prepared by dissolving 5.0−10.0 mg of each oil in 25 mL of chloroform/methanol (1:1, v/v). Subsequently, 2.9 mL of these oil solutions was mixed with 0.1 mL of 3-PeDPP methanol solution at the final concentration of 2.5 μM in a glass reaction vessel (Reacti-vial, Pierce, Rockford, IL). The vial was tightly capped and kept at room temperature for 30 min in the dark. For detection of lipid hydroperoxides during autoxidation, 2.9 mL of oil solution was mixed with 0.1 mL of 3-PeDPP methanol solution at the final concentration of 75 μM. The fluorescence intensity of 3-PeDPPO was measured using a spectrofluorometer, model RF-1500 (Shimadzu, Kyoto, Japan), of which the excitation and emission wavelengths were 440 and 470 nm, respectively.

Detection of Lipid Hydroperoxides with the Xylenol Orange Method. The lipid hydroperoxides in oil were analyzed according to the method of Nourooz-Zadeh et al. (*18*). Oil samples were prepared for assay by dissolving 10 mg of oil in 0.1 mL of *n*-propanol. An aliquot of the oil solution (0.1 mL) was then mixed with FOX2 reagents, 0.9 mL, in a centrifuge tube. This mixture was incubated at room temperature for 30 min and was centrifuged at 1500 *g* for 3 min. The supernatant was used for measurement of absorbance at 560 nm with a UV−vis spectrophotometer, model 160A (Shimadzu, Kyoto, Japan).

Determination of Oxygen Absorption in the Vial Headspace during Autoxidation. Oxygen absorption was determined as described previously (*19*). In brief, 50 mg samples were accurately weighed into glass vials of 68.7 mL volume, which were sealed with Teflon-lined septa. After sealing, 0.1 mL portions of air in the headspaces of the vials were collected with a gastight microsyringe and analyzed on a Shimadzu gas chromatograph, model GC3BT, equipped with a glass column (3 mm i.d. × 1.7 m) packed with molecular sieve 5A (80−100 mesh, Nihon Chromato Co. Ltd., Tokyo, Japan) and a thermal conductivity detector. The ratio between N_2 and O_2 in the headspace air was determined at the proper intervals.

Determination of Tocopherol Isomer Contents. Tocopherol isomer contents in oil were determined by reverse-phase high-performance liquid chromatography according to the method of Xu et al. (*20*). An oil sample (100 mg) was dissolved in 1 mL of *n*-propanol. The tocopherol isomers were separated in an HPLC system equipped with a Mightysil RP-18 GP column (250 × 4.6 mm i.d., 3 μm, Kanto Chemical Co. Inc., Tokyo, Japan) and detected with a fluorescence detector (Shimadzu RF-10AXL) with excitation and emission wavelengths of 290 and 330 nm, respectively. The mobile phase was methanol/acetonitrile/dichloromethane (25:22:3, v/v/v) with a flow rate of 1 mL/min.

Determination of Fatty Acid Composition. Accurately weighed oil (ca 50 mg) was saponified by 1 M NaOH in methanol and subsequently methylated with 14% boron trifluoride in methanol to obtain free fatty acid methyl esters (*21*). The resulting fatty acid methyl esters were analyzed with a Shimadzu GC 14B gas chromatograph, equipped with a SUPELCOWAX-10 fused silica open tubular capillary column (0.25 mm i.d. × 30 m, 0.25 μm film thickness, Supelco, Tokyo, Japan) and a flame ionization detector. The column oven was held at 150 °C for 1 min and raised to 240 °C at the rate of 1 °C/min. Helium was used as a carrier gas with a column inlet pressure of 2 kg/cm^2. The fatty acid methyl esters were identified with reference standard GLC-68A from Nu Check Prep (Elysian, NW, USA). Peak areas of fatty acid methyl esters were normalized as a percentage of total methyl esters.

Fluorescent Image Analysis of Lipid Hydroperoxides in Oil Droplets. Droplet samples of refined tuna oil were prepared for fluorescent image analysis by suspending the refined tuna oil (300 μL) in distilled water (700 μL) containing 0.1% of tween 20 as an emulsifier at the ratio of 3:7 (v/v). The solution was vigorously mixed on a vortex mixer for 10 min until oil droplets were formed. These oil droplets (50 μL) were mixed with a 3-PeDPP methanol solution at the final concentration of 50 μM. The 3-PeDPP-labeled oil droplets were kept at room temperature for 30 min in a dark area. The fluorescent images of the lipid hydroperoxides formation in the oil droplets were obtained with an Olympus IX70 inverted microscope (Olympus, Tokyo, Japan) equipped with a UApo 40 lens and U-MNBV cube filter unit (420−440 nm excitation, 475 nm emission). Digital images were collected with an Olympus model C-3040 Camedia digital camera (Olympus, Tokyo, Japan). The 3-PeDPP-labeled oil droplets (10 μL) on glass coverslips were examined under the fluorescent microscope to determine the fluorescence intensity of 3-PeDPPO generated through reactions between 3-PeDPP and lipid hydroperoxide in the oil droplets. The fluorescence intensity was analyzed as an averaged 8-bit density value for each droplet by ImageJ software (National Institutes of Health, Bethesda, MD).

RESULTS AND DISCUSSION

Reaction of 3-PeDPP with Hydroperoxides. The structures of 3-PeDPP and 3-PeDPPO are shown in **Figure 1**. The 3-PeDPP is a nonfluorescent compound. The reduction of hydroperoxides with 3-PeDPP and formation of 3-PeDPPO, the corresponding oxidation product of 3-PeDPP, resulted in the exhibition of intense fluorescence with an excitation wavelength of 440 nm and an emission wavelength of 470 nm (*16, 17*). This increase in fluorescence intensity is believed to be due to decreases of the steric hindrance around the phosphorus atom and an increase of electron density on the phosphorus atom (*9, 11*).

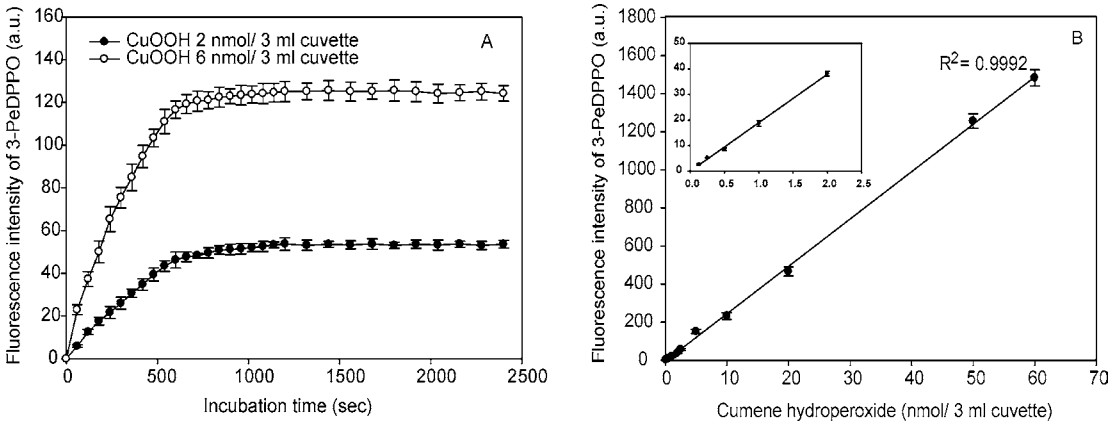


Figure 2. (A) Time course analysis of 3-PeDPPO formation in the presence of different concentrations of cumene hydroperoxide. The fluorescence intensity of 3-PeDPPO was measured continuously after the addition of CuOOH at λ_{ex} of 440 nm and λ_{em} of 470 nm with a spectrofluorometer ($n = 3$). (B) Dose-dependencies of 3-PeDPPO formation with CuOOH ($n = 3$). An inset shows the dose-dependency with CuOOH concentrations lower than 2 nmol.

The 3-PeDPP (final concentration of 2.5 μM) was oxidized in the presence of cumen hydroperoxide (CuOOH) of 2 and 6 nmol in a 3 ml cuvette at room temperature in a homogeneous solution (chloroform/methanol 1:1 v/v) under stirring and darkness. The fluorescence intensity increased exponentially for 10 min with 3-PeDPPO formation, was stable within 20 min, and did not decrease until 40 min (**Figure 2A**).

Performance of the 3-PeDPP Assays. The fluoresecence intensity of 3-PeDPPO in the homogeneous solution (chloroform/methanol, 1:1, v/v) increased with increasing concentration of CuOOH (**Figure 2B**). This finding indicated that the fluorescence intensity increased proportionally with concentrations of hydroperoxide produced in homogeneous solutions and that 3-PeDPPO would be suitable for measurement of lipid hydroperoxide in homogeneous solution. The detection limit of hydroperoxides with 3-PeDPPO fluorescence calculated according to the equation of Miller and Miller (*22*) under this experimental condition was 0.111 nmol of CuOOH in a 3ml cuvette, and the application range was 0.1−60.0 nmol of CuOOH in a 3 ml cuvette with a good correlation ($R^2 = 0.9992$).

The effect of increasing refined tuna oil content on the fluorescence intensity in the 3-PeDPP assay (final concentration of 2.5 μM) was examined. This assay method successfully gave a linear correlation between oil contents up to 2 mg (data not shown) and the fluorescence intensity of 3-PeDPPO. A final oil content of 0.5−1.0 mg was, therefore, adopted in the present experiments to prevent saturation in the fluorescence intensity by overproduction of lipid hydroperoxides in the following assays.

Chemical methods based on redox reactions such as oxidation of ferrous ions in an acidic medium containing the dye xylenol orange have become the classical methods for determination of lipid hydroperoxides (*23*, *24*). These methods have been reported to have high sensitivity, being comparable to iodometric assay (*25*). There was a linear correlation between lipid hydroperoxide contents in refined tuna oil determined by 3-PeDPPO fluorescence and the ferric−xylenol orange complex absorbance at 560 nm. The linear regression for the relationship between lipid hydroperoxides determined by the ferric−xylenol orange complex and the fluorescence intensity of 3-PeDPPO at the excitation wavelength of 440 nm and emission wavelength of 470 nm attained 0.984 in the determination coefficient (R^2) (**Figure 3**). For the 3-PeDPPO-fluorescence method 1.0 mg of the lipid was enough for the assay, but the xylenol orange method needed 10−300 mg of sample as a minimal amount

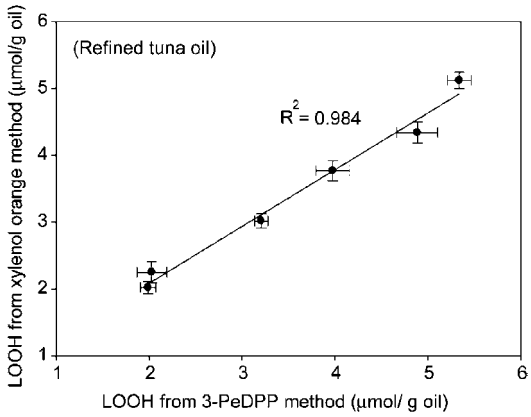


Figure 3. Relationship between lipid hydroperoxides determined by the xylenol orange method and the fluorescent probe 3-PeDPP method in the refined tuna oil model ($n = 3$).

(*26*). Moreover, the xylenol orange method also requires knowledge of the nature of the hydroperoxides present in the sample and careful control of the conditions used. This is because the apparent molar absorption coefficient depends on many variables such as the amount of sample, solvent used, and source of xylenol (*26*). From these results it is supposed that the 3-PeDPPO-fluorescence method is more sensitive and simple than the xylenol orange method for the determination of lipid hydroperoxides in oil models.

Changes in Oxygen Absorption, Lipid Hydroperoxides, and Tocopherol Contents of Refined Tuna Oils during Autoxidation. The refined tuna oil was used as the model of autoxidation. The marine oil is rich in polyunsaturated fatty acids (PUFA), especially those of the ω-3 family, such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), and these PUFA are highly sensitive to peroxidation (*3*). The relationships between the oxygen absorption in the headspace oxygen content of the refined tuna oil and the refined tuna oil supplemented with 30 μg of α-tocopherol/g of oil during incubation at 40 °C are shown in **Figure 4A**. Oxygen disappearance in the headspace is directly related to lipid peroxidation of the refined tuna oil because of oxygen reaction with oils to produce peroxy radicals and hydroperoxides (*27*). The oxidation is also influenced by antioxidants, such as α-tocopherol, and the fatty acid composition of the oils (*28*). As expected, in the refined tuna oil rapid oxygen absorption occurred in the initial autoxidation stage and was stable after

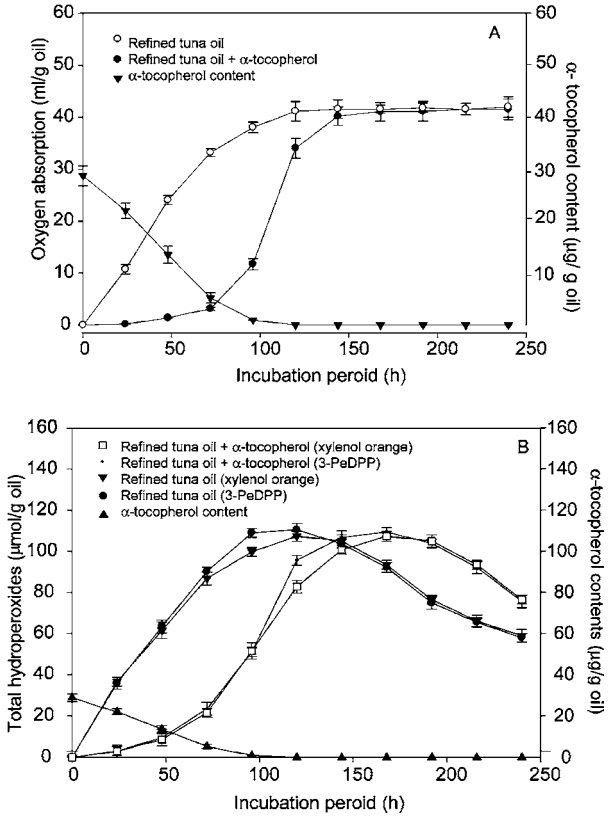


Figure 4. (A) Oxygen absorption curve of the refined tuna oil (○), the refined tuna oil + α-tocopherol (30 μg of α-tocopherol/g of oil) (●), and α-tocopherol content (▼) during autoxidation at 40 °C. (B) Total lipid hydroperoxide contents of the refined tuna oil + α-tocopherol (30 μg of α-tocopherol/g of oil) from xylenol orange (□), the refined tuna oil + α-tocopherol (30 μg of α-tocopherol/g of oil) from 3-PeDPP (•), the refined tuna oil from xylenol orange (▼), the refined tuna oil from 3-PeDPP (●), and α-tocopherol content (▲) during autoxidation at 40 °C. Results are given as μmol of cumene hydroperoxide equiv/g of oil. Values are mean ± SD ($n = 3$).

120 h, whereas oxygen absorption of the refined tuna oil supplemented with 30 μg of α-tocopherol/g of oil occurred from 72 h and was stable after 168 h of incubation at 40 °C.

As described in **Table 1**, total tocopherol contents of the refined tuna oil before stripping by column chromatography were 41.8 ± 1.0 μg/g of oil. α-Tocopherol was the main antioxidant present in tuna oil, while after stripping no tocopherol was detected in the refined tuna oil. In a model of the refined tuna oil supplemented with 30 μg of α-tocopherol/g of oil, until 96 h the α-tocopherol in the refined tuna oil decreased rapidly from 29.7 ±1.0 μg/g of oil to 3.5 ± 0.3 μg/g of oil and then almost disappeared after 120 h of incubation. When the tocopherols disappeared, oxygen absorption for refined tuna oil supplemented with tocopherol was accelerated (**Figure 4A**) from the propagation period until 168 h of incubation. The decreasing tocopherol content during lipid peroxidation was also well-consistent with the description by Niki (*29*).

The kinetic profiles of lipid hydroperoxides formation in both refined tuna oils were investigated by the 3-PeDPPO fluorescence and the xylenol orange methods during autoxidation at 40 °C. Total lipid hydroperoxides in the refined tuna oil determined by the 3-PeDPPO fluorescence and xylenol orange methods increased rapidly during 120 h incubation periods, and the maximal values of total lipid hydroperoxides were 110.5 ± 3.1 and 107.3 ± 3.7 μmol/g of oil after 120 h, respectively (**Figure 4B**). On the other hand, the total lipid hydroperoxide

Table 1. Fatty Acid Composition of the Refined Tuna Oil and Tocopherol Contents before and after Silica Gel Chromatography ($n = 3$)

fatty acid	(% of total fatty acids)
myristic acid (14:0)	3.42 ± 0.02
palmitic acid (16:0)	1.99 ± 0.14
stearic acid (18:0)	5.99 ± 0.36
arachidic acid (20:0)	tr[a]
behenic acid (22:0)	tr[a]
lignoceric acid (24:0)	tr[a]
palmitoleic acid (16:1, ω-9)	5.13 ± 0.98
oleic acid (18:1, ω-9)	18.94 ± 0.84
linoleic acid (18:2, ω-6)	6.26 ± 0.32
linolenic acid (18:3, ω-3)	0.50 ± 0.02
EPA (20:5 ω-3)	6.81 ± 0.52
heneicosenoic acid (21:1)	tr[a]
DHA (22:6, ω-3)	26.63 ± 0.92
unknown	6.37 ± 0.73

tocopherol	tocopherol content (μg/g of oil)
Before Purification	
α-tocopherol	28.16 ± 2.34
β- + γ-tocopherol	8.57 ± 1.98
δ-tocopherol	5.12 ± 0.97
After Purification	
α-tocopherol	
β- + γ-tocopherol	
δ-tocopherol	

[a] tr, <0.01%.

formation in the refined tuna oil supplemented with 30 μg of α-tocopherol/g of oil occurred slowly until 72 h of incubation because of the residual α-tocopherol, followed by the propagation period until 168 h of incubation. The rate of accumulation of lipid hydroperoxides achieved a maximal value (109.5 ± 2.1 μmol/g of oil from 3-PeDPPO fluorescence and 106.8 ± 5.0 μmol/g of oil from the xylenol orange method) at 168 h. The values of oxygen absorption at this stage were 41.0 ± 1.8 and 41.6 ± 1.9 mL/g of the refined tuna oil and of the refined tuna oil supplemented with 30 μg of α-tocopherol/g of oil, respectively. The maximal levels were obtained when the rate of hydroperoxide decomposition became equal to the rate of their formations, as reported previously (*30*, *31*). After the rate of hydroperoxide decomposition exceeded the rate of their production, the total lipid hydroperoxides decreased. The critical balance points were estimated as about 120 and 168 h for the refined tuna oil and the refined tuna oil supplemented with α-tocopherol, respectively, while the oxygen absorption was stable after these points (**Figure 4A**).

3-PeDPPO Fluorescent Image Analysis of Lipid Hydroperoxides in Refined Tuna Oil Droplets during Autoxidation. Fluorescent image analysis is a selective and sensitive method that offers several advantages: easy and accurate identification of structures, quantification at low amounts, high analysis speed, and rapid sample preparation and determination (*32*). The digital image analysis techniques are generally used to uncover information of images. The information is brought out by transforming the digital image or by directly extracting data values from the image. We carried out the fluorescent image analysis for evaluation of lipid hydroperoxide formation in the refined tuna oil droplets during autoxidation under fluorescent microscopic observation. Lipid hydroperoxide levels were calculated from 3-PeDPPO fluorescent digital images in refined tuna oil droplets by an image analysis software (ImageJ) and compared to lipid hydroperoxide levels estimated by the ferric−

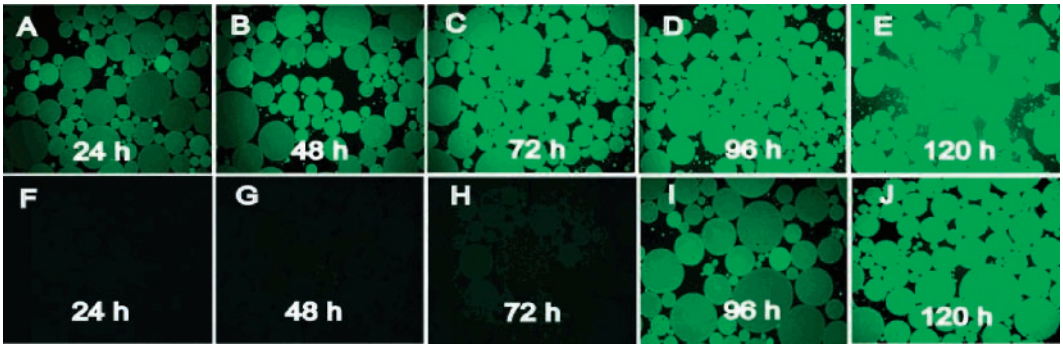


Figure 5. 3-PeDPPO fluorescent images in refined tuna oil droplets. A–E show fluorescent images of refined tuna oil droplets and F–J show fluorescent images of refined tuna oil + α-tocopherol (30 μg of α-tocopherol/g of oil) droplets incubated at 40 °C from 24 to 120 h.

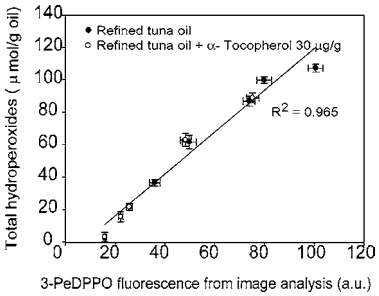


Figure 6. Relationship between the lipid hydroperoxide levels in refined tuna oil and refined tuna oil + α-tocopherol (30 μg of α-tocopherol/g of oil) droplets determined by 3-PeDPPO fluorescent image analysis for each droplet and the ferric–xylenol orange method for batch samples during autoxidation at 40 °C. Data are represented as mean ± SD. The vertical SD bars represent the standard deviation of the data obtained by the ferric–xylenol orange method ($n = 3$). The horizontal SD bars are the standard deviation of the 3-PeDPPO fluorescence in each lipid droplet ($n = 10$–20 for each image field).

xylenol orange method. The digital images of 3-PeDPPO fluorescence in both refined tuna oil droplets during autoxidation (24−120 h) are shown in **Figure 5**. The strong fluorescence of 3-PeDPPO was observed at the refined tuna oil droplets after 24 h of incubation at 40 °C in the absence of α-tocopherol (35.9 ± 2.1 to 104.6 ± 2.9 AU). But a lower fluorescence intensity of 3-PeDPPO was obtained in tuna oil droplets supplemented with 30 μg of α-tocopherol/g of oil (15.8 ± 0.7 to 75.3 ± 2.5 AU). Image analysis data also showed a good correlation with the data of lipid hydroperoxides by the ferric−xylenol orange method ($R^2 = 0.965$, **Figure 6**). Moreover, the oil droplet volume of about 10.0 μL is enough for the determination of lipid hydroperoxides by 3-PeDPPO fluorescence coupled with fluorescent image analysis. Thus, 3-PeDPP and image analysis enabled us to estimate two-dimensionally the lipid hydroperoxide levels in oil.

Conclusion. On the basis of the study of refined tuna oil models, the new fluorescence probe (3-PeDPP) appears capable of dynamically monitoring the oxidative stability (autoxidation) of tuna oil. These model studies show that the fluorescence intensity of 3-PeDPPO provides us with the quantitative analysis of the intermediate hydroperoxides in a good correlation with the lipid peroxidation extents determined by the ferric−xylenol orange method and oxygen absorption. The 3-PeDPPO fluorescence coupled with fluorescent image analysis is a new tool suitable for evaluation of the lipid hydroperoxide level in oil and a potential tool for evaluation of lipid hydroperoxides in oil-in-water emulsions and aqueous colloidal systems.

ABBREVIATIONS USED

3-PeDPP, 3-perylene diphenylphosphine; 3-PeDPPO, 3-perylene diphenylphosphine oxide; CuOOH, cumene hydroperoxide; LOOH, lipid hydroperoxides.

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Received for review March 18, 2005. Revised manuscript received June 23, 2005. Accepted July 14, 2005. This work was supported in part by a Grant-in-Aid from the Japanese Ministry of Education, Science and Culture and by the Asahi Breweries Foundation.

JF050611G