A Simple Fluorometry of Hydroperoxides in Oils and Foods

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A fluorometry of hydroperoxides in oil and food samples was developed using diphenyl-1-pyrenylphosphine (DPPP) with a sample size of less than 20 mg by a batch method. The sensitivity was more than 10,000 times that of the conventional iodometry. A good accordance was obtained between peroxide values (POV) measured this way and by iodometry (r = 0.9995, n = 41, POV = 0.04–240).

Lipid peroxidation has received much attention because of its toxicities, bitter tastes and off-flavors.1–3 To prevent lipid peroxidation of foods, some efforts have been made such as packaging, adding antioxidation reagents, and removing oxygen. Lipid peroxidation in vivo is also speculated to relate to some diseases or aging.4-7 Therefore, it is necessary to measure lipid hydroperoxides, the key primary products in the sequences, with high sensitivity and selectivity.

The iodometries are the most common methods to measure lipid peroxides.8,9) The methods, however, are not satisfactory in simplicity and sensitivity. Some colorimetries were proposed to improve their defects.10–12) Hara et al. reported a measurement of peroxide value (POV) with a small amount of sample by a combination of iodometry and potentiometry.13,14) The thiobarbituric acid (TBA) methods were most widely used to study lipid peroxidation in biological materials.15,16) Some enzymatic methods such as glutathion peroxidase, peroxigenase and cyclooxygenase methods have also been proposed.17–19) Recently, luminal and isoluminal chemiluminescence methods were reported as highly sensitive and selective HPLC methods.20,21) However, these methods have some problems such as selectivity, simplicity, or interference of coexistent substances.

Recently, we reported a new fluorescent reagent, diphenyl-1-pyrenylphosphine (DPPP), for the measurement of hydroperoxides with high sensitivity and selectivity.22,23) DPPP itself is almost not fluorescent but its oxidized product, DPPP oxide, has strong fluorescence. It was successfully used to measure phosphatidylcholine hydroperoxides in plasma and serum by a flow system.24,25) In this paper, we report some applications of DPPP to the measurement of hydroperoxides in vegetable and edible oils and foods by a batch method.

Materials and Methods

Chemicals. Diphenyl-1-pyrenylphosphine (DPPP) was prepared from 1-bromopropyne and triphenylphosphine as described previously.22) Triolein, trilinolein, and phosphatidylcholine (from egg yolk) were purchased from Sigma Chem. Co. (St. Louis, U.S.A.). Vegetable oils were purchased from Nakalai Chem. Co. (Kyoto, Japan) and Kanto Chem. Co. (Tokyo, Japan). Edible oils, butters, margarines, and mayonnaises were purchased from a market. Other reagents used were super pure or analytical grade from Wako Pure Chem. Co. (Osaka, Japan).

Apparatus. A spectrofluorometer used was FP-550A (Japan Spectroscopic Co. Ltd., Tokyo, Japan) with a pump unit, model FP-2050. An isothermal incubator used was Toyof TCI-1 (Toyo Kagaku Sangyog Co., Tokyo, Japan).

Sample preparation of oils and foods. A 0.2–1000 mg sample was weighed exactly and filled up to 5 ml with chloroform (containing 10 mg/ml of butylhydroxytoluene: BHT)-methanol (2:1, v/v) (procedure I). A 100 µl sample was used for analysis without further procedures.

Food samples such as butters, margarines, and mayonnaises were also extracted by the following manner (procedure II). The 200–600 mg sample was exactly weighed and added of 0.5 ml of saturated aqueous sodium chloride solution, 0.5 ml of methanol, and 2 ml of BHT chloroform solution (10 mg/ml). Then the mixture was vigorously shaken and centrifuged at 3000 rpm for 5 min. The lower layer was collected, 2 ml of the chloroform was added to the upper layer and it was extracted again. The two extracts were combined and filled up to 5 ml with chloroform-methanol (2:1, v/v). A part of it was used for the analysis and 3 ml of the sample was evaporated under reduced pressure. The residual extract was completely dried with a vacuum pump and then weighed. These procedures were done under cooling on an ice bath, shaded from a strong light irradiation.

Measurement procedure. To a test tube (13 mm x 100 mm) with a screw cap, 100 µl of sample solution and 50 µl of DPPP chloroform solution (1 mg/10 ml) were added under cooling in an ice bath. This was copped tightly and left for 60 min at 60°C in the dark. Then it was cooled in an ice bath and 3 ml of 2-propanol was added before the measurement. Its fluorescence intensity at 380 nm was measured (excitation wavelength at 352 nm).

The concentrations of hydroperoxides were obtained as those of DPPP oxide. This was done by measuring standard DPPP oxide solution that was prepared by the reaction of a known amount of DPPP and an excess of hydroperoxides without purification.

Iodometric measurement procedure. POV was also estimated by the iodometry of the Japan Oil Chemists' Society Standard Method for Analysis of Fats and Oils (2.4.12–71).

Results and Discussion

The principle was previously described.21) Practically non-fluorescent DPPP was oxidized by hydroperoxides to produce strongly fluorescent DPPP oxide quantitatively. Therefore, the fluorescence intensity of DPPP oxide directly shows the amounts of hydroperoxides.

Figure 1 shows the fluorescence developments of the reaction of DPPP with some lipid hydroperoxides. The reaction patterns of hydroperoxides of vegetable oils was similar to those of trilinolein hydroperoxides. Those hydroperoxides reacted within 40 min at 60°C. The reaction of trilein hydroperoxides was the slowest, but it was completed within 60 min.

This method was also applicable for the phospholipids hydroperoxides. Some food samples contain large amounts.
of phospholipids. The reaction of DPPP with phosphatidylcholine hydroperoxides proceeded within 40 min at 60°C. More than 95% of the hydroperoxides reacted with DPPP at 20 min. The decreases of the hydroperoxides were about 8% and 17% after incubation without DPPP at 60°C for 30 min and 60 min, respectively. This means that most hydroperoxides reacted before they decomposed to unreactive compounds.

In chloroform solution, DPPP was gradually oxidized and the background fluorescence was increased under strong light. The reagent solution and the reaction solution should be kept from a strong light, especially UV. The crystalline reagent was stable for more than 2 years and the chloroform solution could be used for more than one week if it was stored in the dark below −10°C.

In our previous report, 3 ml of methanol was added before the measurement. However, some oil samples were insoluble in methanol due to the poor solubilities of triacylglycerols. So 2-propanol was used instead of methanol.

The calibration curves of hydroperoxides in trilinolein, trilinolenin, some vegetable oils and phosphatidylcholine showed good linearities in the range of 0.1 to 7.3 nano equivalent (n eq.) in the reaction solutions. The coefficient of variations of trilinolein hydroperoxides were 1.4% (n = 5, 7.3 n eq./tube) and 1.8% (n = 5, 0.7 n eq./tube).

Figure 2 shows the relationships between the sample size and fluorescence intensities. In each case, a linear relationship was obtained. These results show that the reaction is not interfered with by coexistent substances in a reaction mixture, mainly unoxidized triacylglycerols, phospholipids, and antioxidation agents, within 20−30 mg of a sample in a tube. This means that the samples with more than 0.02 of POV can be measured by this method. An adequate amount of hydroperoxides to be measured was 1−5 n eq. in a tube.

Margarines, butters, and mayonnaises were analyzed after the lipid was extracted by procedure II. The amounts of the extracts also showed good linear relationships to their fluorescence intensities. The recoveries of hydroperoxides from foods samples were 93.8−103.4% (n = 4). On the test, butters and margarines (POV = 0.05−0.29) were used as samples and the hydroperoxides prepared from linseed oil were added to them as a standard hydroperoxide. The amount of extracted oil was checked by weighing a 3 ml sample of the extract after complete removal of the solvent.

In this method, procedures I and II were used for oil samples and fat and food samples, respectively. However, procedure I was also applicable for the latter samples. Insoluble components did not influence the measurement. By procedure I, 92.2−99.4% of standard hydroperoxides were recovered from the food samples, and a good accordance was obtained between the values by procedures.
I and II (coefficient of correlation; $r = 0.9996, n = 9$) as shown in Fig. 3.

To compare this method with a conventional method, the same samples were analyzed by iodometry at the same time. The relationship between POVs measured by the two methods is shown in Fig. 4. In food samples, the values were expressed as the value per wet weight of a sample instead of lipids by procedure I. A good accordance between the two methods was obtained ($r = 0.9995, n = 41$). The iodometry required a large amount of sample. For example, in an oil with a POV of one, iodometry need 10 g of the sample, while this fluorometry needs several mg. This method is simple and several samples could be analyzed at the same time by a single person within 100 min.

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