Highly Sensitive Flow Injection Analysis of Lipid Hydroperoxides in Foodstuffs

Kazuaki Akasaka, Tomoko Takamura, Hiroshi Ohrui, Hiroshi Meguro, and Kenichi Hashimoto*

Department of Applied Biological Chemistry, Faculty of Agriculture, Tohoku University, 1-1 Tsutsumidori-Amamiya-
machi, Aoba-ku, Sendai 981, Japan
* Food Research Laboratory, NOF Corporation, Food Products Div., Oji Plant, 18-11, Toshiba 4-chome, Kita-ku, Tokyo
114, Japan
Received March 19, 1996

Lipid hydroperoxides in oils and foods were measured by a flow injection analysis system with high sensitivity and selectivity. After sample injection, lipid hydroperoxides were reacted with diphenyl-1-pyrenylphosphine (DPPP) in a stainless steel coil, then the fluorescence intensity of DPPP oxide, that was produced by the reaction, was monitored. By this method, trilinolein hydroperoxide showed good linearity between 0.4 and 79 pmol and their detection limits were 0.2 pmol (signal-to-noise ratio = 3). The method made it possible to inject samples at 2-min intervals. There was a good agreement of the amounts of lipid hydroperoxides in oils and foods between by the batch method with DPPP and by the proposed method (coefficient of correlation: $r = 0.999; n = 21$; peroxide value = 0.09–167 meq/g). With this method, the calibration graph of trilinolein hydroperoxide was useful for all samples tested.

Key words: flow injection analysis; lipid hydroperoxide; diphenyl-1-pyrenylphosphine

Lipid hydroperoxides are primary products of enzymatic or non-enzymatic peroxidation of unsaturated fatty acid moieties, and they are gradually decomposed to secondary products such as aldehydes, alcohols, carboxylic acids, epoxides, and hydrocarbons. Since these lipid peroxides are not only toxic but also causes of off-flavor, it is necessary to control their production at very low levels in foodstuffs.

Some indexes had been proposed to estimate lipid peroxidation in foodstuffs such as the peroxide value ($P^V$), acid value ($A^V$), and carbonyl value ($C^V$). Among them, $P^V$ measured by iodometry was the most widely used as an official method. However, the iodometry had some problems such as low sensitivity and complicated and time-consuming procedures. Therefore some other detection methods such as colorimetry and potentiometry were tried. The colorimetric detection of $I_2$ was used in flow injection analysis (FIA) of lipid hydroperoxides. The FIA system not only made the analysis simple and rapid but also prevented the oxidation of $I_2$ during the reaction. However, the method required the removal of oxygen from the solvents completely and the sensitivity was at the nano-mole level. FIA methods with the luminol chemiluminescence were also proposed to measure lipid hydroperoxide. These methods required the removal of radical scavengers in samples because they quenched the luminol chemiluminescence. Some other methods such as colorimetry using methylene blue derivatives, ferrous oxidation-xylenol orange, and enzymatic methods were also proposed to measure lipid hydroperoxides.

We have reported a phosphine reagent, diphenyl-1-pyrenylphosphine (DPPP), as a highly sensitive and selective fluorescence reagent of lipid hydroperoxides. This reagent had no fluorescence, but its oxide, which was produced by oxidation with lipid hydroperoxides, had strong fluorescence. This reagent was used to measure lipid hydroperoxides by both batch and HPLC post-column methods. Here, we report a highly sensitive FIA of lipid hydroperoxides in foodstuffs using DPPP.

Materials and Methods

Chemicals. DPPP was prepared by the coupling reaction of 1-pyrenyl magnesium bromide and diphenylphosphoryl chloride under nitrogen atmosphere. Trilinolein and triolein were purchased from Sigma Chem. (St. Louis, U.S.A.). Vegetable oils, such as soybean oil, cottonseed oil, linseed oil, olive oil, sunflower oil, corn oil, sesame oil, and germ oil were purchased from Nacalai Tesque (Osaka, Japan). Edible oils, butters, margarines, and mayonnaises were purchased from markets in Sensai (Japan). Methanol was HPLC grade of Kanto Chem. (Tokyo, Japan), and other solvents used were purchased from Wako Pure Chem. (Osaka, Japan), *tert*-Butylhydroxytoluene (BHT), *meta*-cresol, *beta*-carotene, *tert*-butylhydroxyanisol (BHA), and propylgallate were also purchased from Wako Pure Chem. and were used as received.

Standard hydroperoxides of trilinolein, triolein, and some vegetable oils were prepared by autoxidation under oxygen atmosphere at 50°C followed by purification with silica gel column chromatography. The target fractions, which were detected on TLC by spraying a DPPP methanol solution, was collected and the solvent was evaporated. Then, the hydroperoxides were dissolved in methanol-1-butanol (1:1, v/v) and stored in the dark at −20°C.

Sample preparation procedure. Vegetable oils and edible oils (up to 500 mg) were filled up to 5ml with methanol-1-butanol (1:1, v/v). Butters, mayonnaises, and margarines (up to 100 mg) were suspended in 5 ml of the methanol-1-butanol solution and then they were vortexed for 1 min, and centrifuged at 3000 rpm below 10°C. After adequate dilution with methanol-1-butanol solution, a portion was used as a sample for FIA analysis without further processing.

Flow injection analysis.

System 1: The FIA system was shown in Fig. 1. The sample injected into the system (1 50μl) flowed with methanol-1-butanol (1:1, v/v) at the rate of 0.8 ml/min. After mixing a reagent solution (DPPP: 3 mg in 400 ml of methanol-1-butanol (1:1) which contained 200 mg of BHT) at 0.3 ml/min, the mixture was reacted in a stainless steel coil (0.5 mm i.d. x 30 m) at 80°C.

System 2: The sample was 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), which contained 200 mg of BHT) at 0.6 ml/min, the mixture was reacted in a stainless steel coil (0.5 mm i.d. x 50 m) at 80°C.

In both systems, after cooling in 0.5 ml of stainless coil (0.5 mm i.d.) in a water jacket, the fluorescent intensity at 380 nm (excitation at 352 nm)
Flow Injection Analysis of Lipid Hydroperoxides

Fig. 1. Flow Injection System.

Fig. 2. Typical FIA Signals.

Table 1. Effects of Antioxidation Agents on a Peak Area of Trilinolein Hydroperoxide

<table>
<thead>
<tr>
<th>Antioxidation agent</th>
<th>Amount of addition (ng/injection)</th>
<th>Relative peak area of trilinolein hydroperoxide</th>
</tr>
</thead>
<tbody>
<tr>
<td>BHT</td>
<td>20</td>
<td>105</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>108</td>
</tr>
<tr>
<td></td>
<td>2,000</td>
<td>102</td>
</tr>
<tr>
<td>BHA</td>
<td>149</td>
<td>101</td>
</tr>
<tr>
<td></td>
<td>1,400</td>
<td>102</td>
</tr>
<tr>
<td></td>
<td>14,000</td>
<td>98.0</td>
</tr>
<tr>
<td>a-Tocopherol</td>
<td>100</td>
<td>105</td>
</tr>
<tr>
<td></td>
<td>1,000</td>
<td>89.8</td>
</tr>
<tr>
<td></td>
<td>10,000</td>
<td>93.0</td>
</tr>
<tr>
<td>β-Carotene</td>
<td>19</td>
<td>90.8</td>
</tr>
<tr>
<td></td>
<td>190</td>
<td>90.8</td>
</tr>
<tr>
<td></td>
<td>1,900</td>
<td>87.1</td>
</tr>
<tr>
<td>Propyl galate</td>
<td>60</td>
<td>94.7</td>
</tr>
<tr>
<td></td>
<td>600</td>
<td>96.3</td>
</tr>
<tr>
<td></td>
<td>6,000</td>
<td>89.4</td>
</tr>
</tbody>
</table>

* The peak area of trilinolein hydroperoxide (43 pmol/injection) without addition of antioxidant agents was 100.

was monitored.

The amounts of hydroperoxides in all samples were calculated by using of the calibration graph of trilinolein hydroperoxide.

**Equipment.** Two HPLC pumps, CCPD and CPPM (Toosoh, Tokyo, Japan), were used to send a sample and a DPPP solution, respectively. A sample injector used was a Rheodyne type 7125 with a 100-μl loop. Stainless steel coils (0.5 mm i.d.) were used as all piping including a reaction coil. A reaction oven, a spectrofluorometer, and a data processor were used RE-8000, FS-8000, and SC-8010 (Toosoh), respectively.

**Measurement by batch method.** All samples were also measured by the batch method with DPPP described previously to compare with the proposed method.\(^{31}\)

**Results and Discussion**

We have reported some HPLC post-column systems with DPPP for the analysis of lipid hydroperoxides at their class or molecular levels.\(^ {23} - ^ {27}\) Therefore, we tried to build up an FIA system by eliminating a separation column from the typical HPLC post-column system shown as system 1.

Since this method measured hydroperoxides without any separation, we tested the effects of coexistence substances, such as antioxidation agents and solvents, on peak area. Although significant signals were observed by injection of some antioxidation agents, they had almost no influence on the peak area that was attributed to trilinolein hydroperoxides as shown in Table 1. The signals, which appeared upon injection of antioxidation agents, were not due to the antioxidation agents themselves but their oxidized products because the peaks detected by the fluorometry did not agree with the positions where the antioxidation agents should be eluted from a reversed phase column when they were analyzed by an HPLC-post-column system. It was also supported by the results that these peaks were reduced by treatment with NaBH₄ or triphenylphosphine (data not shown).

The injection of a halogenated solvent, such as chloroform and dichloromethane, gave a faint signal while almost no signal was observed upon injection of methanol-1-butanol (1:1). While a negative peak was observed upon injection of the BHT solution, the peak could be eliminated by addition of BHT to the reagent solution without influence on the signal of lipid hydroperoxides.

The peak area almost agreed with each other (R.S.D. = 3.7%) by injection of the same amount of hydroperoxides

(101 pmol/injection) even though the sample size was changed from 10 to 50 μl. This meant that it was allowed to inject from 10 to 50 μl of a sample. This system allowed us to inject samples at 2-min intervals.

By the system (system 1), it was possible to measure 2-201 pmol of trilinolein hydroperoxides \( (r = 0.9968, \text{Fig. 2}) \). 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 2.0 pmol \( (\text{signal-to-noise ratio}; S/N = 3) \). There were good accuracies between the results measured by the batch method and those by this system for 37 samples that were vegetable oils, edible oils, and margarines \( (r = 0.9981, \text{PV} = 0.16-172, \text{Fig. 3A}) \). Here, the calibration curve of trilinolein hydroperoxide was used for all samples to calculate their amounts. However, butters gave 1.5-2.5 times higher values measured by the FIA method than these by the batch method \( (\text{Fig. 4A}) \). Since the detection reaction is not completed usually in flow detection system, the
Fig. 3. Relationships between Hydroperoxides in Oils and Foodstuffs Measured by Batch Method and FIA Method.

Fig. 4. Relative Peak Areas of Hydroperoxides in Oils and a Butter.

A; system 1

B; system 2

Fig. 5. Ratio of Fluorescence Intensities of a Butter Extract and Trilinolein Hydroperoxide.

A butter extract and trilinolein hydroperoxide were reacted with DPPP at 60°C, respectively, and their fluorescence intensities were measured at 10-min intervals. Here, the samples used had the same fluorescence intensity at 60 min by the batch method.

differences of reactivities between analytes with the reagent give significant influences to the result. The higher values of a butter sample might be attributed to the higher reactivity of components in the butter with DPPP. The HPLC analysis of butter extracts showed that the hydroperoxides detected were not triacylglycerol (TG) hydroperoxides but more polar ones such as decomposed products of TG hydroperoxides. Figure 5 shows the fluorescence intensity ratios of trilinolein hydroperoxides to a butter extract measured by the batch method. The ratio had higher value at the initial stage, and it gradually decreased and reached plato at 10 min. This meant that the components in the butter were more reactive with DPPP than trilinolein hydroperoxide. To overcome this problem, we tried to complete the reaction as much as possible by using a longer reaction coil and a higher concentration of reagent solution and by increasing the rate of the reagent solution mixed to a sample flow. By using the modified conditions (system 2), there were good agreements between relative peak areas of lipid hydroperoxides as shown in Fig. 4B. Here, the hydroperoxide contents of samples were measured by the batch method. Although hydroperoxides of olate gave somewhat lower values, they were minor components in foodstuffs because of the much lower reactivity of olate to peroxidation than polyenoic acid derivatives. This meant that the proposed method made it possible to measure total hydroperoxides in oils and foodstuffs including butters. There were good accordancess between the results measured by the batch method and the modified system for 21 samples that were vegetable oils, edible oils, margarines, and butters ($r = 0.999, PV = 0.09 - 167, \text{Fig. 3B}$). The calibration graph of trilinolein hydroperoxides showed a good linearity between 0.4 and 79 pmol ($r = 0.9998$). The relative standard deviations ($n = 8$) were 3.8% (4.9 pmol) and 1.9% (40 pmol), and the detection limit was 0.2 pmol ($S/N = 3$). The improvement in sensitivity was due to the longer reaction time and higher reagent concentration of system 2 than those of system 1. This system also allows us to inject samples at 2-min intervals.

Here, the lipids were extracted from foods, such as butters, margarines, and mayonnaises, with methanol–I-butanol (1:1) because the extract gave the same result of the
methanol–chloroform (1:1) extract in which most lipid should be extracted. The recoveries were compiled in Table II. This also supported that most lipids were extracted into methanol–1-butanol solution.

System 2 made it possible to measure lipid hydroperoxides in oils and foods with high sensitivity and speed. System 1 was more economical than system 2 in their hardware and the reagent consumption, and it was also useful for the analysis of oil samples. The proposed methods made it possible to measured trace lipid hydroperoxides in food-stuff with a very simple procedure without using halogenated solvents. These methods should be useful to measure lipid hydroperoxides in biological materials as well as foodstuffs.

References
4) Official and Tentative Methods of the Japan Oil Chemists’s Soc., 2.4.12.2–94.
5) Official and Tentative Methods of the Japan Oil Chemists’s Soc., 2.4.1–72.