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High-performance liquid chromatography and post-column derivatization with diphenyl-1-pyrenylphosphine for fluorimetric determination of triacylglycerol hydroperoxides

Kazuaki Akasaka, Setsu Ijichi, Kenji Watanabe, Hiroshi Ohrui and Hiroshi Meguro*

Department of Food Chemistry, Faculty of Agriculture, Tohoku University, Tsutsumidorui-Amamiyamachi, Aoba, Sendai 981 (Japan)

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ABSTRACT

Triacylglycerol monohydroperoxides (TG-mHPO) were selectively detected at the picomole levels after post-column reaction with diphenyl-1-pyrenylphosphine (DPPP). TG-mHPO were separated on two types of reserved-phase columns, an ODS column and a phenylated silica gel column, which were useful for determining TG-mHPO at their molecular species levels and their class levels, respectively. After the separation, DPPP solution was mixed with the eluent followed by reaction in a stainless-steel coil 20 m × 0.5 mm I.D. at 80°C, then the fluorescence intensity of DPPP oxide was measured ($\lambda_{ex.}$ 352 nm, $\lambda_{em.}$ 380 nm). Using these systems, TG-mHPO were determined in the range 2–1000 pmol. The relative standard deviations were 2.3–2.8%.

INTRODUCTION

Recently, lipid peroxidation has attracted much attention as one of the factors causing certain diseases and ageing [1–4]. In lipid peroxidation, hydroperoxides are produced both enzymatically and non-enzymatically. Several methods have been proposed for determining small amounts of hydroperoxides in foods and biological materials [5–8]. These methods, however, have some problems with reard to sensitivity, selectivity, complexity or interference from other components.

Recently, luminol and isoluminol chemiluminescence method have been reported as highly sensitive and selective high-performance liquid chromatographic HPLC post-column detection methods for lipid hydroperoxides [9,10]. An electrochemical detection system has also been used for this purpose [11]. These methods, however, have the drawback of unsuitability of organic solvents for HPLC separation.

Recently, we reported that a series of arylphosphines, prepared by replacing one phenyl group of triphenylphosphine with an aromatic fluorophore, have no fluorescence but their oxides possess strong fluorescence [12], diphenyl-1-pyrenylphosphine (DPPP) oxide giving the strongest fluorescence intensity. This reagent made it possible to determine lipid hydroperoxides selectively with high sensitivity. Some applications of the reagent to the determination of lipid hydroperoxides have been reported [13–15]. In this paper, we describe the application of the reagent to the HPLC post-column determination of triacylglycerol hydroperoxides.

EXPERIMENTAL

Chemicals

Diphenyl-1-pyrenylphosphine was synthesized as described previously [12]. Other reagents used were of special or super-special pure grade (Wako, Osaka, Japan). Trilinolein, trilinolenin and triolein were purchased from Sigma (St. Louis, MO, USA). Methylene blue, triphenylphosphine and solvents

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such as methanol, 1-butanol, acetone and chloroform were purchased from Wako and *tert.*-butylhydroxytoluene (BHT) from Tokyo Kasei Kogyo (Tokyo, Japan). The solvents for dissolving or extracting the samples contained 0.03% of BHT as an antioxidant.

Margarine, butter and mayonnaise products and salad oils were purchased locally. Vegetable oils were purchased from Nacalai Tesque (Kyoto, Japan).

Preparation of hydroperoxides and hydroxides

Trilinolein, trilinolenin and vegetable oils were autoxidized at room temperature in the dark for 12–72 h. Triolein was photooxidized in the presence of 0.1–0.3 m*M* methylene blue in ethanol. They were used after purification to triacylglycerol (TG) monohydroperoxides (mHPO) using silica gel column chromatography [16]. Their hydroperoxide contents were determined by spectrofluorimetry [13]. They were stored in a refrigerator at −25°C as a solution in chloroform–methanol (1:1).

Trilinolein hydroxides were prepared by reducing the hydroperoxides with triphenylphosphine in chloroform–methanol (1:1) at 0°C for more than 1 h and used without purification.

Separation and detection of hydroperoxides

Two systems were used to separate TG hydroperoxides. In the first system (system A), the analytical column used was TSK-gel ODS 80Tm (5 μm) (150 mm × 4.6 mm I.D.) (Tosoh, Tokyo, Japan; eluted with 1-butanol–methanol (10:90, v/v). In the other system (system B), a Develosil Ph-5 (5 μm) column (150 mm × 4.6 mm I.D.) (Nomura Chemical, Aichi, Japan) and methanol–water (95:5, v/v) eluent were used. The flow-rate of the eluent was 0.6 ml/min in both systems.

The post-column derivatization and the detection conditions were same in both systems. The HPLC eluent was monitored by UV detection at 210 or 235 nm prior to the post-column reaction with DPPP. The eluent was mixed with DPPP reagent [3 mg in 400 ml of acetone–methanol (1:3, v/v)], which was kept in an ice-bath in the dark. The flow-rate of the reagent solution was 0.3 ml/min and the mixture was passed through a 20 m × 0.5 mm I.D. reaction coil (stainless steel) at 80°C followed by another coil (0.5 m) in a water-bath at 20°C. Detection was performed by monitoring the fluorescence intensities at 380 nm with excitation at 352 nm. The mobile phase and the reagent solution were degassed by ultrasonic treatment under reduced pressure before use.

Sample preparation

Edible oils (200–400 mg) were diluted to 20 ml with chloroform and aliquots were used as samples. Commercially available mayonnaise, butter and margarine extracts were prepared in the following manner. To 0.3–1 g of the sample, 0.5 ml of water was added. Lipids were extracted from the mixture with two 1.5-ml portions of chloroform–methanol (2:1) followed by dilution to 5 ml with chloroform. Aliquots of 10 μl were injected into the HPLC system. The sample size could be minimized to less than 1 mg by reducing the scale of the procedure.

Equipment

The HPLC pump used was a CCPM multi-pump (Tosoh), which pumped both the mobile phase and the reagent solution with a single system. The reaction oven was an RE-8000 reactor. The detectors used were a UV-8000 spectrophotometer and an FS-8000 spectrofluorimeter (Tosoh). The data processor used was an SC-8010 (Tosoh) or a Chromatocoder 12 (System Instrument, Tokyo, Japan).

RESULTS AND DISCUSSION

The mechanism of the reaction of hydroperoxides with DPPP was described previously [12,13]. Hydroperoxides oxidized DPPP quantitatively to a strongly fluorescent oxide. The reaction proceeded in many organic solvents, such as methanol, ethanol, acetonitrile, ethyl acetate, chloroform, benzene and hexane, suggesting that all these solvents can be used as the mobile phase in HPLC. It was also possible to add water to the mobile phase up to 10%. The addition of larger amounts of water made DPPP insoluble in the reaction mixture. DPPP was less reactive to dialkyl peroxides, and not reactive to unoxidized fatty acids, hydroxy acids and their esters [13]. Therefore, they do not interfere in the determination of hydroperoxides.

Fig. 1 shows the chromatograms of a mixture of trilinolein and its monohydroperoxides (a,b) and a mixture of trilinolein and its hydroxide (c). Both unoxidized and hydroxylated trilinoleins gave

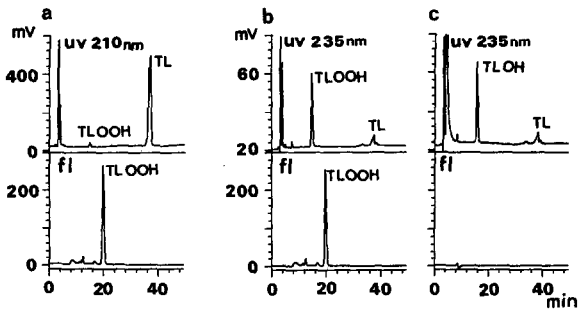

Fig. 1. HPLC of trilinolein and its hydroperoxy and hydroxy derivatives with system A. Sample: (a) and (b) trilinolein (TL, 50.0 nmol) + hydroperoxides (TL-OOH, 214 pmol); (c) TL (50.0 nmol) + hydroxide (TL-OH; 214 pmol).

peaks with UV monitoring, but no peak with spectrofluorimetry (7). On the other hand, the trilinolein-mHPO gave peaks with both UV and spectrofluorimetric detection. These results demonstrate that spectrofluorimetric detection is specific to hydroperoxides. The inclusion of a UV detector prior to the post-column reaction may be not essential for this system.

Fig. 2 shows the effect of the flow-rate of DPPP solution on the peak height of trilinolein-mHPO. The peak was higher at lower flow-rates, but the peak height variations was largest at 0.2 ml/min because it was difficult to maintain a constant flow-rate. This effect was clearer in system B than A. Fig. 3 and Table I show the effects of reaction temperature and reaction coil length on the peak height, respectively. The peaks became higher with increase in the reaction temperature and with the use of a

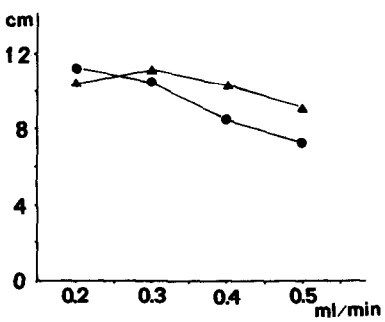

Fig. 2. Effect of flow-rate of the reagent solution on the peak height of trilinolein-mHPO (107 pmol) in (▲) system A and (●) system B.

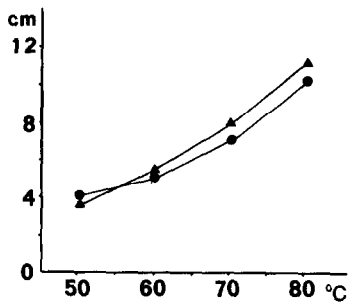

Fig. 3. Effect of reaction temperature on the peak height of trilinolein-mHPO (107 pmol) in (▲) system A and (●) system B.

longer coil without raising the baseline noise levels. The peaks were about 25% higher in a stainless-steel coil than in a PTFE coil. This may be attributed to the difference in their thermal conductivities. Therefore, the reaction was conducted in a 20-m stainless-steel coil at 80°C in our systems.

Table II shows the effect of the concentration of the reagent solution. The peaks became higher with increase in the reagent concentration. However, the signal-to-noise ratio was not improved with increase in concentration because of the increase in the baseline noise level. We selected the 3 mg per 400 ml solution as the reagent solution. Although the peak height decreased on addition of acetone to the reagent solution, acetone–methanol (1:3) was used as the solvent in our systems because of the improvement in the stability against oxidation of DPPP by the oxidant in the solution. Methanol would be less useful as a solvent for the reagent solution because the baseline noise level was doubled after keeping the reagent solution for 1 day at −25°C. Therefore, it was neccesary to prepare the

TABLE I

EFFECT OF REACTION COIL LENGTH ON THE PEAK HEIGHT OF TRILINOLEIN-mHPO (107 pmol)

Coil	Length (m) (× 0.5 mm I.D.)	Peak height (cm)	
		System A	System B
PTFE	10	5.40	3.90
Stainless steel	4	3.75	2.08
	10	6.47	5.20
	20	11.39	10.40

TABLE II

EFFECT OF CONCENTRATION OF THE REAGENT SOLUTION ON THE PEAK HEIGHT OF TRILINOLEIN-mHPO (107 pmol)

System	Parameter	Concentration of DPPP (mg per 400 ml)		
		1.5	3.0	6.0
A	Peak height (cm)	5.41	11.20	19.36
	Relative noise level	1.0	1.4	2.0
B	Peak height (cm)	5.43	10.48	14.50
	Relative noise level	1.0	1.2	1.6

solution just before use when methanol was used as the solvent. With the use of methanol, the peak was 20–35% higher than with acetone–methanol (1:3). The fluorescence intensity of DPPP oxide was only 5% stronger in methanol than in acetone–methanol (1:9). The decrease in the peak height in acetone–methanol was partly attributed to this effect, but the main reason is the lower reactivity of DPPP with hydroperoxides in the solvent system.

In system A, the ODS column, one of the most popular reversed-phase columns, was used for the separation of TG-mHPO. As shown in Fig. 1, trilinolein-mHPO were detected as a single peak by spectrofluorimetry and determined in the range 2.1–1000 pmol (r = 0.999). The relative standard deviation of the peak area was 2.3% (107 pmol, n = 6).

Trilinolein-mHPO were not composed of a single molecular species but a mixture of some positional isomers which had a hydroperoxy group at different positions. However, these isomers were hardly separated by this system. On the other hand, as shown in Fig. 4, several peaks were observed in a mixture of TG-mHPO which were prepared by autoxidation of vegetable oils and subsequently purified by silica gel column chromatography. The peaks between 14 and 35 min should be the TG-mHPO composed of various fatty acids with different partition numbers (PN) (Partition numbers are defined by the equation PN = TC − 2DB, where TC and DB are the numbers of total carbon atoms and double bonds of fatty acids of which the TG is composed, respectively; for TG, there is a linear relationship between PN and log t_R, where t_R is the retention time). As shown in Fig. 5, six model TG-mHPO showed linear relationships between PN and log t_R as for TG [17]. This means that the TG-mHPO species could be roughly predicted from their retention times and the fatty acid compositions in the same way as for TG.

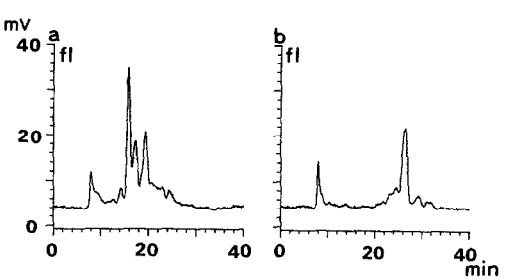

Fig. 4. HPLC of TG-mHPO in vegetable oils with system A. Sample: (a) TG-mHPO in linseed oil (296 pmol); (b) TG-mHPO in olive oil (166 pmol).

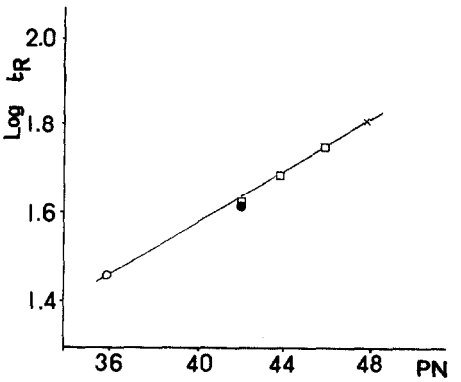

Fig. 5. Relationship between partition number of TG-mHPO and log t_R. The HPLC conditions used were the same as in system A except for the reaction coil length (10 m). Sample: trilinolenin-mHPO (PN = 36, ○), trilinolein-mHPO (PN = 42, ●), triolein-mHPO (PN = 48, ×) and TG-mHPO (PN = 42, 44 and 46, □) prepared from TG after separation at each PN on an ODS column.

In system B, a phenylated silica gel column was used as an analytical column. The separation patterns of TG-mHPO with this column were different from those with the ODS column. The difference would be due to the different characteristics of phenyl and alkyl groups. Fig. 6 shows typical chromatograms of TG-mHPO. The peaks of TG-mHPO in vegetable oils were broader and, in some instances, showed a small difference (less than 1

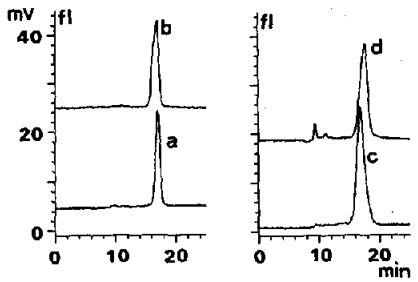

Fig. 6. HPLC of TG-mHPO with system B. Sample:(a) trilinolein-mHPO (45.5 pmol), (b) trilinolenin-mHPO (55.0 pmol), (c) TG-mHPO in linseed oil (100 pmol), (d) TG-mHPO in olive oil (82.8 pmol).

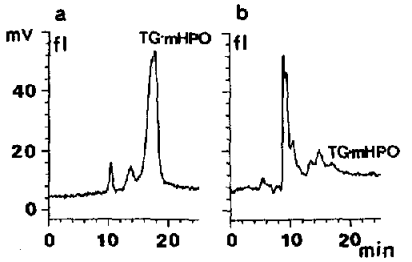

Fig. 7. HPLC of hydroperoxides in a salad oil (a, TH = 153 pmol) and a margarine extract (b, TH = 69.0 pmol) with system B.

min) between their retention times. This suggests that TG-mHPO which had different PN did not have exactly the same retention time, but almost. In practice, TG-mHPO gave a single peak regardless of their fatty acid compositions. This allowed us to determine TG-mHPO at the class level. Table III shows the peak-area ratio of trilinolein and vegetable oil-mHPO. Although there was a *ca.* 10% variation between them, it was almost possible to use the calibration graph of trilinolein-mHPO for TG-mHPO in vegetable oils. The ratio for triolein-mHPO was smaller than the others, which could be attributed to the lower reactivity of triolein-mHPO with DPPP.

Using the proposed method, TG-mHPO was determined in the range 2–300 pmol. The relative standard deviation of the peak area of trilinolein-mHPO was 2.8% (45.5 pmol, $n = 6$).

Fig. 7 shows the chromatograms of a salad oil and an extract from a food sample. Before TG-mHPO several peaks were detected. Judging from their retention times, they were probably the bis- or trishydroperoxides of TG or lower molecular weight hydroperoxides such as hydroperoxides of free fatty acids, mono- or diacylglycerols and degradation products of TG-mHPO. Total hydroperoxides (TH) were determined from the total peak area using the calibration graph of trilinolein-mHPO. They agreed with the results obtained by the batch method ($r = 0.997, n = 10$) [18]. Recoveries of added TG-HPO from the vegetable oils and the foods were 95–102% ($n = 6$). Although the composition of the injection solvent seemed unsuitable for a reversed-phase system, there was no problem with injections of up to 10 μl.

TABLE III

RELATIVE PEAK AREA OF TG-mHPO WITH SYSTEM B

Lipid hydroperoxide	Relative peak area
Trilinolein-mHPO	1.00
Trilinolenin-mHPO	1.00
Triolein-mHPO	0.71
Safflower oil-mHPO	1.10
Corn oil-mHPO	1.10
Cottonseed oil-mHPO	1.04
Soybean oil-mHPO	0.94
Linseed oil-mHPO	1.01
Olive oil-mHPO	0.94
Peanut oil-mHPO	0.88
Rapeseed oil-mHPO	1.04
Sesame oil-mHPO	1.07

CONCLUSIONS

Lipid hydroperoxides were detected at picomole levels with high selectivity using the proposed post-column derivatization system. The use of the phenylated column made it possible to determine TG-mHPO at their class levels and the total hydroperoxides in vegetable oils and some food extracts. The method should be useful for studying the initial stage of lipid peroxidation and for checking food quality based on its high sensitivity, selectivity and peak separation patterns. Moreover, the method should be useful for determining TG hydroperoxide in biological materials.

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