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Effects of Riboflavin and Fatty Acid Methyl Esters on Cholesterol Oxidation during Illumination

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The effect of riboflavin or fatty acid methyl esters on cholesterol photooxidation was studied. Samples containing cholesterol, either alone or in combination with riboflavin or fatty acid methyl esters, were illuminated at 25 °C in an incubator for 28 days. The various cholesterol oxidation products (COPs) and cholesterol were analyzed by gas chromatography−mass spectrometry (GC−MS), and riboflavin was determined by HPLC. Results showed that the presence of riboflavin or fatty acid methyl esters facilitated production of COPs and degradation of cholesterol, and the degradation fits a first-order model. The COPs formed during light storage included 7α-OH, 7β-OH, 7-keto, 3,5-cholestadien-7-one, 5,6α-EP, and 5,6β-EP. The addition of riboflavin caused formation of 3,5-cholestadien-7-one through dehydration of 7-keto, whereas in the presence of docosahexaenoic acid methyl ester, the formation of 5,6α-EP or 5,6β-EP was favored. Riboflavin was more effective for generation of COPs than fatty acid methyl esters.

KEYWORDS: Riboflavin; fatty acid methyl ester; cholesterol photooxidation; GC−MS

INTRODUCTION

Cholesterol, an important class of neutral lipids, is widely present in food products of animal origin. As an essential nutrient for maintaining normal physiological function, cholesterol often exists in free form or in ester linkage with long chain fatty acids in foods (*1*). However, because of the presence of one unsaturated double bond, cholesterol may undergo oxidation to form cholesterol oxidation products (COPs) when exposed to light or heat (*2, 3*). It has been well established that the consumption of COPs in excess may induce mutagenicity and carcinogenicity (*4, 5*). Thus, the variety and amount of COPs formed in model systems or food products during illumination or heating has to be explored.

The formation mechanism of COPs during heating has been well documented and illustrated (*3, 6, 7*). However, the cholesterol photooxidation was not often studied. In the presence of light the triplet oxygen could be converted to singlet oxygen and in turn react with cholesterol to form 5,6α-EP or 5,6β-EP (*8*). However, in the presence of some other components such as fatty acids, some more varieties of COPs were formed during light storage (*2*). For instance, several reports have shown that during illumination of egg powder or cheese powder, some COPs such as 5,6β-EP, 7-keto, triol, 7α-OH, and 7β-OH were formed (*2*). The presence of unsaturated fatty acids or photosensitizers such as chlorophyll have been reported to accelerate the cholesterol oxidation during heating or illumination (*9*−*11*). In a review dealing with photosensitized oxidation of lipids, Girotti (*8*) reported that both 7α-OOH and 7β-OOH were formed as reactive intermediates in the presence of triplet oxygen, whereas in the presence of singlet oxygen both 5α-OOH and 5β-OOH were formed. As chlorophyll is not commonly found in meat and egg products, the effect of some other photosensitizers, such as riboflavin, on cholesterol photooxidation has to be investigated. Also, the effect of riboflavin on the degradation of cholesterol during illumination remains unknown. Furthermore, it will be difficult to assess the formation mechanism of COPs during light storage in food systems because of possible interference by the presence of other components such as protein, carbohydrate, and fat. Using a model system to study the phenomenon of cholesterol photooxidation would make it easier to interpret results. Fatty acid methyl esters were selected instead of triglycerides because the former should be more susceptible to promoting cholesterol oxidation than the latter (*9*). The objectives of this study were to develop a model system for illumination and to determine the effect of riboflavin or fatty acid methyl esters on cholesterol photooxidation.

MATERIALS AND METHODS

Materials. Cholesterol and cholesterol oxidation products (COPs) standards, including cholestan-5α,6α-epoxy-3β-ol (5,6α-EP), cholestan-5β,6β-epoxy-3β-ol (5,6β-EP), 5-cholesten-3β-ol-6-one (6-keto), 5-cholesten-3β-ol-7-one (7-keto), cholestane-3β,5α,6β-triol (triol), 5-cholesten-3β,20α-diol (20-OH), 5-cholestane-3β,25-diol (25-OH), and internal standard 5α-cholestane were purchased from Sigma Chemical Co. (St. Louis, MO). 5-Cholesten-3β,7α-diol (7α-OH), 5-cholesten-3β,19-diol (19-OH), 5-cholesten-3β,7β-diol (7β-OH), and 3,5-cholestadien-7-one were obtained from Steraloids Co. (Wilton, NH). Fatty acid methyl ester standards, including stearic acid methyl ester, linoleic acid methyl ester, and *cis*-4,7,10,13,16,19-docosahexaenoic acid methyl ester were

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also obtained from Sigma. Riboflavin was from Merck Co. (Darmstadt, Germany). The derivatization reagent Sylon BTZ was from Supelco Co. (Bellefonte, PA). Solvents such as methanol, isopropyl alcohol, and pyridine were from Merck. Deionized water was obtained using a Milli-Q water purification system (Millipore Co., Bedford, MA). A HP-5MS capillary column (30 m × 0.25 mm i.d., 0.25 μm thickness, 5% diphenylpolysiloxan/95% dimethylpolysiloxan) used for separation of COPs and fatty acid methyl ester was from Agilent Technology Co. (Palo Alto, CA). A Vydac 201TP54 C18 column (250 × 4.6 mm i.d., 5 μm) used for separation of riboflavin was from Vydac Co. (Hesperia, CA).

Instrumentation. A gas chromatograph equipped with a mass selective detector (model 5973) was from Agilent Technology Co. (Palo Alto, CA). The HPLC system consists of a DG-440 degasser (Phenomenex Co., Torrance, CA), a Rheodyne 7161 injector (Rheodyne Co., Rhonert Park, CA), a Jasco 821-FP fluorescence detector, and a Jasco PU-980 pump (Jasco Co., Tokyo, Japan). The spectrophotometer (PU-1580) was from Jasco Co. (Tokyo, Japan). The low-temperature incubator (TL 520R) was from Seng-Long Co. (Taipei, Taiwan). The light meter (TES-1330) was from Tai-Se Electronic Co. (Taipei, Taiwan).

Effect of Riboflavin on Cholesterol Photooxidation. A stock solution of 20 000 ppm cholesterol was prepared by dissolving 2.007 g cholesterol in 100 mL of pyridine, and 200 ppm riboflavin was prepared by dissolving 0.0502 g of riboflavin in 250 mL of water. Three different combinations were used for illumination: (1) 10000 ppm cholesterol, (2) a mixture of 10000 ppm cholesterol and 50 ppm riboflavin, and (3) a mixture of 10000 ppm cholesterol and 100 ppm riboflavin. Prior to illumination, each mixture was sonicated for 1 h and stored in the dark for 12 h to make riboflavin soluble in the solution. Then 0.4 mL was collected and poured into a 2-mL glass vial, after which the vial was sealed with an aluminum cap. A total of 60 vials were attached to a rotating disk with a diameter of 40 cm, which was hooked to the top of the incubator. Three 40-W fluorescent tubes with a length of 40 cm each were placed at the bottom of the incubator. The distance between sample vials and fluorescent tubes was about 50 cm, and the illumination intensity was 2000−3000 lux at 25 °C. The illumination time was 28 days. Two samples were collected for analyses of COPs, cholesterol, and riboflavin according to the following time intervals: 0, 1, 2, 3, 7, 14, 21, and 28 days. Likewise, the control treatment was performed using the same combinations and collection procedures shown above with the exception that all the vials were wrapped with aluminum foil and placed in an incubator for dark storage for 28 days.

Effect of Fatty Acid Methyl Esters on Cholesterol Photooxidation. One concentration (2000 ppm) each of stearic acid methyl ester, linoleic acid methyl ester, and docosahexaenoic acid methyl ester was prepared by dissolving 0.0409, 0.0404, and 0.0400 g in 20 mL in pyridine, respectively. Three combinations of cholesterol and fatty acid methyl esters were used for illumination treatment: (1) a mixture of 10000 ppm cholesterol and 1000 ppm stearic acid methyl ester; (2) a mixture of 10000 ppm cholesterol and 1000 ppm linoleic acid methyl ester; and (3) a mixture of 10000 ppm cholesterol and 1000 ppm docosahexaenoic acid methyl ester. A total of 60 vials were used and all the samples were illuminated and analyzed in the same way used as riboflavin samples as described above. Likewise, 60 vials were also stored in the dark for 28 days.

HPLC Analysis of Riboflavin. A modified HPLC method (*12*) was used to determine riboflavin content. A Vydac 201TP54 C18 column (250 × 4.6 mm i.d.) and a mobile phase of methanol/water (35:65, v/v) with fluorescence detection at 445 nm (excitation) and 525 nm (emission) were used. The injection volume was 20 μL and the flow rate was 1.25 mL/min. Ten concentrations (0, 10, 20, 30, 40, 50, 60, 70, 80. and 90 ppm) of riboflavin in pyridine were prepared and 20 μL was injected into HPLC for preparation of standard curve. Three analyses were performed for each concentration, and the curve was obtained by plotting the average concentration against the average area, and the correlation coefficient (r^2) for the standard curve was 0.9986. The amount of riboflavin was thus calculated from the regression equation of the standard curve.

GC−MS Analysis of Fatty Acid Methyl Esters and COPs. A 10-μL sample was mixed with 10 μL of Sylon BTZ in a 2-mL glass vial which was then sealed with an aluminum cap. All the vials were placed at 25 °C in the dark for 1 h for the derivatization reaction to proceed, after which 10 μL of internal standard 5α-cholestane (1000 ppm) was added for GC−MS analysis. The injection volume was 1 μL. An HP-5MS capillary column, a carrier gas of He with flow rate at 1 mL/min, and a split ratio of 30:1 were used. The injector temperature was 280 °C. The initial column temperature was 200 °C, then increased to 270 °C at a rate of 10 °C/min, and maintained for 23 min. The interface temperature for GC−MS was 270 °C, with an electron multipler voltage of 70 ev and ion voltage of 1360 V. The total ion mode was used for detection with a scanning range of 35−500 and rate at 2.94 scans/sec. Perfluorotributylamine was used for auto tune with a *m*/*z* intensity at 69, 219, and 502. The various COPs, fatty acid methyl esters, and cholesterol were identified by comparison of retention times of unknown peaks with reference standards and cochromatography with added standards. In addition, the NIST mass spectra database was used for confirmation of the identity of unknown COPs.

Quantification of Fatty Acid Methyl Esters and COPs. Fatty acid methyl esters and COPs were quantified using an internal standard. Ten concentrations (0−1000 ppm) of each fatty acid methyl ester in pyridine, and one concentration (1000 ppm) of 5α-cholestane was mixed. Three analyses were carried out, and the standard curve of each fatty acid methyl ester was prepared by plotting concentration ratio (fatty acid standard vs internal standard) against area ratio (fatty acid vs internal standard). The correlation coefficients (r^2) for methyl esters of stearic acid, linoleic acid, and docosahexanoic acid were 0.9938, 0.9867, and 0.9854, respectively. The amount of each fatty acid methyl ester was obtained using the following formula:

$$\text{amount (ppm) of fatty acid methyl ester} = \frac{\text{conc (ppm) of IS} \times \text{area of fatty acid methyl ester}}{\text{area of IS} \times \text{RF}}$$

where RF (response factor) = slope of the regression line and IS = internal standard.

The quantification of COPs and cholesterol were the same as described above, with the exception that 10 concentrations (0, 50, 100, 200, 300, 400, 500, 1000, 1500, and 2000 ppm) were used for preparation of standard curves.

Determination of Degradation Rate Constant of Cholesterol. The degradation rate constant (day^{-1}) of cholesterol during illumination was obtained using the following formula:

$$K = -\ln(CA/CA_0)/t$$

where CA is the concentration of cholesterol after illumination, CA_0 is the initial concentration of cholesterol, and *t* is illumination time (day).

The correlation coefficient (r^2) was calculated from the plot of the logarithm of the cholesterol concentration versus time. The degradation rate constants (day^{-1}) of riboflavin or fatty acid methyl ester were obtained using the same formula as described above.

Statistical Analysis of Data. All the data were subjected to analysis of variance and Duncan's multiple range test ($\alpha = 0.5$) using SAS (*13*). The data for regression equations were obtained using Microsoft Excel 2000 (9.0.4402 SR1). The HPLC data were processed using a Borwin-PDA version 1.5, and the GC−MS data were processed using an Enhanced Chemstation G1701BA version B1.0.

RESULTS AND DISCUSSION

Development of a Model System for Illumination. The model system for cholesterol photooxidation was originally developed by Kulig and Smith (*14*), who conducted an experiment by illuminating a mixture of cholesterol and hematoporphyrin with a 20 W fluorescent tube for 24 h in the presence of oxygen. Since then several authors have used a similar system for studying cholesterol photooxidation (*9*). However, in our study riboflavin was used instead of hematoporphyrin because

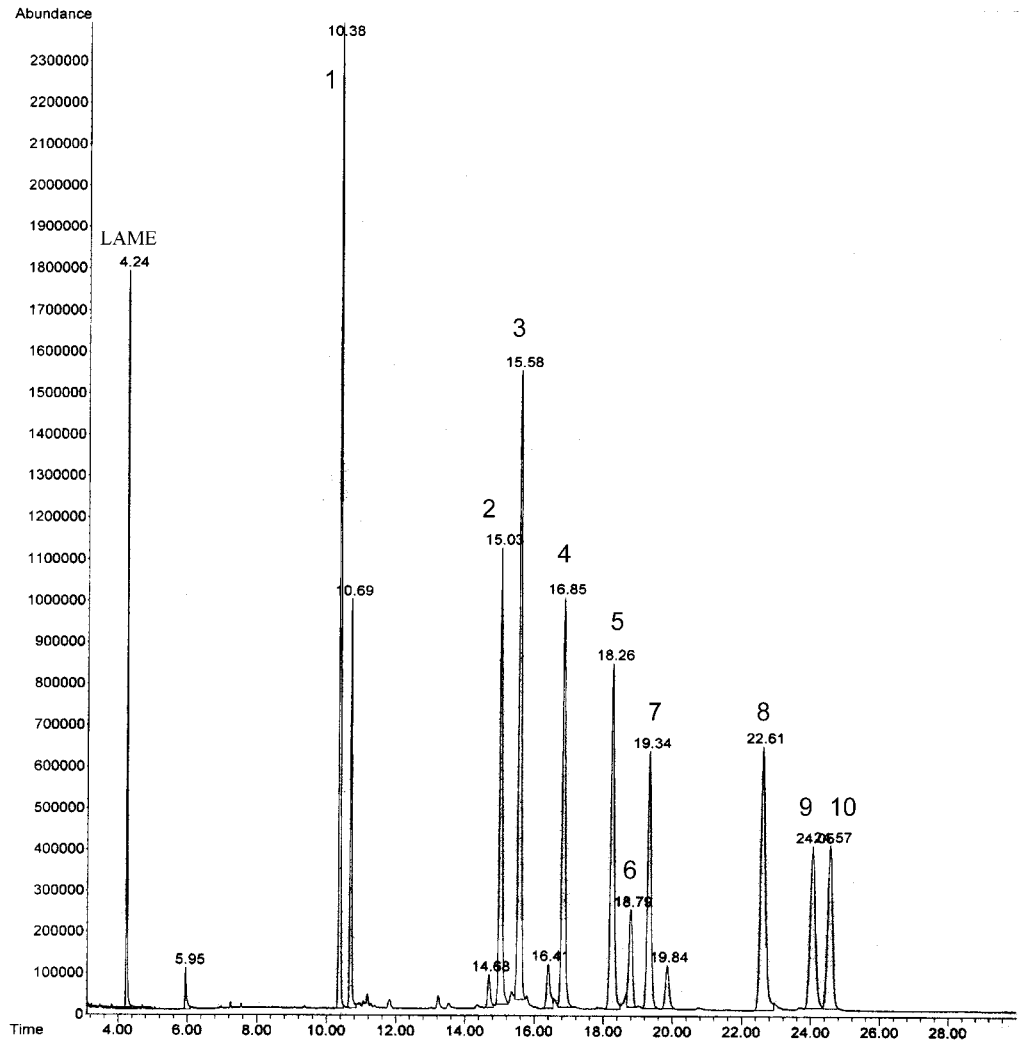

Figure 1. GC–MS–scanning chromatogram of trimethylsilylated cholesterol and 8 COPs standards plus one internal standard and LAME. Chromatographic conditions described in text. Peaks: 1, 5α-cholestane (internal standard); 2, 7α-OH; 3, cholesterol; 4, 3,5-cholestadien-7-one; 5, 7β-OH; 6, 5,6β-EP; 7, 5,6α-EP; 8, triol; 9, 6-keto; 10, 7-keto; LAME, linoleic acid methyl ester.

the former occurs more often in egg yolk or muscle foods together with cholesterol. Furthermore, to study the mechanism of cholesterol photooxidation in the presence of photosensitizer or fatty acid methyl esters would be easier to accomplish than in real food systems because of the complex nature of the latter. In our system a rotating disk with 60 vials was placed on the top, while three fluorescent tubes were at the bottom, of an incubator. This design allowed all samples in the bottoms of the vials to be exposed to light directly and with the same light intensity. The concentrations of riboflavin were fixed at 0, 50, and 100 ppm, and those of cholesterol and fatty acid methyl esters were fixed at 10000 and 1000 ppm, respectively. In general, the concentration of cholesterol in egg yolks or muscle foods is about 1000 times higher than that of riboflavin (*15*). A high cholesterol concentration (10000 ppm) was selected to enhance formation and detection of COPs during illumination.

Analysis of COPs and Fatty Acid Methyl Esters by GC−MS. The separation of COPs has been previously achieved by TLC, HPLC, or GC−MS (*16*−*18*). Of the various methods, GC−MS provided a fast separation and identification technique with high sensitivity (*19*). Thus, we developed a GC−MS method for separation of COPs. By using a HP-5MS column and a temperature programming method, eight COPs plus cholesterol and internal standard 5α-cholestane were resolved within 25 min. **Figure 1** shows the GC−MS−scanning chromatogram of linoleic acid methyl ester, cholesterol, 8 COPs, and 5α-cholestane. Only one fatty acid methyl ester was shown on the chromatogram, because a mixture containing only one fatty acid methyl ester and cholesterol was illuminated at a time. The retention times for stearic acid methyl ester, linoleic acid methyl ester, and docosahexaenoic acid methyl ester were 4.39, 4.24, and 6.68 min, respectively, whereas those of 5α-cholestane, 7α-OH, cholesterol, 3,5-cholestandien-7-one, 7β-OH, 5,6β-EP, 5,6α-EP, triol, 6-keto, and 7-keto were 10.38, 15.03, 15.58, 16.85, 18.26, 18.79, 19.34, 22.61, 24.06, and 24.57 min, respectively. The side-chain products such as 20-OH and 25-OH were not found during cholesterol oxidation in this study. 5α-cholestane was used as an internal standard instead of 19-OH because a large peak response occurred for the former. **Table 1** shows the match quality of unknown peaks with mass spectra of COPs standards. "Match quality" is a value representing the probability that the unknown is correctly identified. A high match quality (>90%) was found for 6 COPs, including 7α-OH, 7β-OH, 5,6α-EP, 5,6β-EP, 7-keto, and 3,5-cholestadien-7-one.

Analysis of Riboflavin. By using a Vydac 201TP54 C18 column (250 × 4.6 mm i.d.) and a mobile phase of methanol/water (35:65, v/v) with a flow rate at 1.25 mL/min, the riboflavin peak was eluted at 5.25 min (**Figure 2**) with detection at 445 nm (excitation) and 525 nm (emission).

Table 1. Match Quality of Unknown COPs with Mass Spectra of COPs Standards

	m/z	match quality with COPs standard spectra[b]
7α-OH	*456*, (546)[a]	99%
7β-OH	*456*, (546)	91%
5,6α-EP	366, *384*, 456, (474)	99%
5,6β-EP	247. 281, 356, *384*, 445 459, (474)	95%
7-keto	367, 382, 457, *(472)*	96%
3,5-cholestadien-7-one	161,*174*,187, (382)	99%

[a] Values in parentheses represent the molecular ion, and values in italics represent the base ion. [b] "Match quality" is a value representing the probability that unknown is correctly identified.

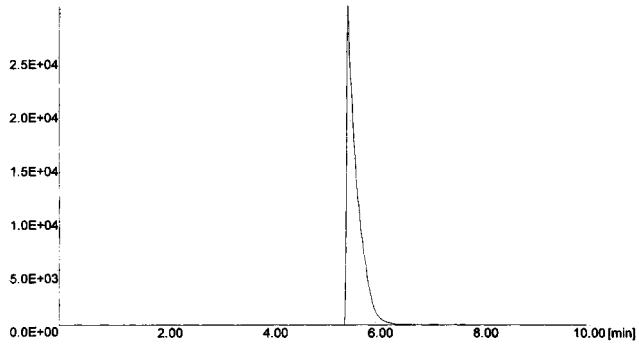

Figure 2. HPLC chromatogram of riboflavin standard. Chromatographic conditions described in text. Peak: riboflavin.

Effect of Illumination and Dark Storage on Riboflavin or Fatty Acid Methyl Esters. The residual amounts (ppm) and degradation rate constants (day^{-1}) of riboflavin or fatty acid methyl esters in the presence of cholesterol during light or dark storage at 25 °C for 28 days are shown in **Table 2**. No riboflavin was detected after exposure to light for 1 day. However, the yellow color still appeared, indicating the presence of degradation products such as lumiflavin and lumichrome (*20*). In contrast, no significant change was found for riboflavin during dark storage for 28 days. This result revealed that light could have a destructive effect on riboflavin. Nevertheless, in real food systems, the stability of riboflavin may be greatly enhanced. For instance, a 14% loss was observed in skim milk after illumination for 24 h (*21*). Apparently the presence of some other components in foods such as protein, carbohydrate, and fat may offer protection for riboflavin. For fatty acid methyl esters, they all followed a decreased order for each increasing illumination day. Docosahexaenoic acid methyl ester was the most susceptible to light degradation, followed by linoleic acid methyl ester and stearic acid methyl ester. A complete destruction of docosahexaenoic acid methyl ester occurred after light storage for 14 days. This result further demonstrated that the higher the degree of unsaturation of fatty acids, the faster the degradation rate during illumination (*9*). On the contrary, only slight loss occurred for all three fatty acid methyl esters during dark storage, as shown by a smaller degradation rate constant when compared to that for light storage.

Effect of Illumination and Dark Storage on Cholesterol. The residual amounts (ppm) and degradation rate constants (day^{-1}) of cholesterol in the presence of riboflavin or fatty acid methyl esters during light storage at 25 °C for 28 days are shown in **Table 3**. For all the treatments during light storage, the amount of cholesterol was found to decrease with increasing illumination time, and the degradation was found to fit a first-order model because a high linear correlation ($r^2 > 0.90$) occurred for the plot of the logarithm of the concentration of cholesterol versus time. The degradation of cholesterol was found to proceed faster with riboflavin than with fatty acid methyl esters. Theoretically, in the presence of riboflavin the triplet oxygen could be converted to singlet oxygen through energy absorption and transfer, which in turn reacted with the unsaturated double bond of cholesterol to facilitate degradation (*8*). Surprisingly, stearic acid methyl ester resulted in a higher loss of cholesterol when compared to that of linoleic acid methyl ester or docosahexaenoic acid methyl ester. Previous studies have shown that during heating the presence of unsaturated fatty acids should promote cholesterol oxidation more readily than the presence of saturated fatty acids (*9, 11*). Our result suggested that the fast destruction of linoleic acid methyl ester and docosahexaenoic acid methyl ester during illumination may fail to induce cholesterol degradation. It is also possible that both linoleic acid methyl ester and docosahexaenoic acid methyl ester could absorb a portion of light and thus the cholesterol degradation was not so pronounced when compared to that of stearic acid methyl ester. Moreover, in the presence of light cholesterol photooxidation may proceed faster than cholesterol degradation.

In contrast to the result shown above, a slight loss of cholesterol was observed for all the treatments during dark storage. In a similar report, Sander et al. (*22*) found that no COPs were detected when egg powder was stored at 4 °C in the dark for 2 months. Pie et al. (*17*) also reported that only a trace amount of COPs was present in meat after dark storage for 3 months.

Table 2. Residual Amount (ppm) and Degradation Rate Constant (day^{-1}) of Riboflavin (RF) or Fatty Acid Methyl Esters in the Presence of Cholesterol during Light or Dark Storage at 25 °C for 28 Days[a]

treat-ment[b]	day 0	1	2	3	7	14	21	28	rate constant (day^{-1})
I (L)	48.06aB	ND	ND	ND	ND	ND	ND	ND	– (RF)
II (L)	97.01aA	ND	ND	ND	ND	ND	ND	ND	– (RF)
III (L)	963.12aC	914.78bC	914.20bC	909.81bD	855.61cD	866.56cE	839.82cE	848.01cE	0.0038 (18:0)
IV (L)	1030.29aC	978.58bC	981.22bC	968.06bD	842.75cD	461.77dD	202.75eD	102.26fD	0.0831 (18:2)
V (L)	831.95aC	819.14aC	789.44bC	532.51cC	176.63dC	0.00eA	0.00eA	0.00eA	0.4996 (22:6)
I (D)	48.06aB	47.06aA	46.01aA	44.94aA	46.96aA	45.21aB	45.69aB	44.83aB	–(RF)
II (D)	97.01aA	99.12aB	97.58aB	97.82aB	96.21aB	100.68aC	99.54aC	96.74aC	–(RF)
III (D)	1011.28aC	1001.07aC	1007.00aC	1000.91aD	989.66aD	928.06bE	926.70bE	913.45bE	0.0040 (18:0)
IV (D)	953.97aC	956.56aC	957.16aC	954.34aD	879.78bD	921.86aE	916.64aE	893.84bE	0.0022 (18:2)
V (D)	915.14aC	905.75aC	907.05aC	853.43aD	847.55aD	902.72aE	916.53aE	890.59aE	0.0118 (22:6)

[a] Results are reported as average of duplicate analyses. ND: not detected. "-": not calculated. Results bearing different lower-case letters, a–f, in the same row are significantly different ($P < 0.05$). Results bearing different upper-case letters, A–E, in the same column are significantly different ($P < 0.05$). [b] I: Cholesterol 10000 ppm + riboflavin 50 ppm. II: Cholesterol 10000 ppm + riboflavin 100 ppm. III: Cholesterol 10000 ppm + 18:0 1000 ppm. IV: Cholesterol 10000 ppm + 18:2 1000 ppm. V: Cholesterol 10000 ppm + 22:6 1000 ppm. (L): Light storage. (D): Dark storage.

Table 3. Residual Amount (ppm) and Degradation Rate Constant (day^{-1}) of Cholesterol in the Presence of Riboflavin or Fatty Acid Methyl Esters during Light Storage at 25 °C for 28 Days[a]

treatment[b]	time (day) 0	1	2	3	7	14	21	28	rate constant (day^{-1})
VI ((L)	10336.86aA	10316.76aA	10303.25aA	9763.07bA	9790.16bA	9084.62cA	7360.93dA	6229.40eA	0.0175 ($R^2 = 0.9459$)
III ((L)	9946.27aA	9558.40abB	9318.71bB	9757.16abA	8556.17cB	7296.05dB	5234.47eB	3956.88fB	0.0322 ($R^2 = 0.9729$)
IV ((L)	10331.71aA	10149.70aA	10128.96aA	9678.58abA	9411.13bA	8604.36cC	7557.11dA	6648.46eA	0.0152 ($R^2 = 0.9901$)
V ((L)	9573.05aA	9500.47aB	9590.91aB	9485.86aA	9350.96aA	7203.79bB	6724.57bC	5604.49cC	0.0198 (R^2=0.9638)
I ((L)	9444.18aA	7504.66bC	7312.01bC	6185.65cB	5220.39dC	2547.79eD	2501.70eD	1291.24fD	0.0655 ($R^2 = 0.9628$)
II ((L)	9063.33aA	7562.09bC	6990.64cC	5640.00dC	4664.43eD	2183.65fD	2027.23fD	958.48gD	0.0747 ($R^2 = 0.9693$)
VI ((D)	10336.86cA	10552.56bA	11138.10aA	11191.44aA	10970.93abA	10972.68abA	10747.39abcE	10751.96abF	–
III ((D)	9946.27abA	9637.80abB	10195.45aA	10306.13aA	9898.91abA	9121.40cA	9562.98bcE	9864.62abE	–
VI ((D)	10331.71abA	10572.05aA	10550.83aA	10466.96abA	10642.10aA	10496.48abA	10031.71bE	10107.85abF	–
V ((D)	9573.05cA	10480.13bA	11145.39aA	10747.71abA	10237.73bA	10448.94bA	10322.47bE	10031.63bF	–
I ((D)	9444.18abA	9950.07aA	8892.84bcB	9367.04abA	9745.66aA	8435.36C	9044.30bCF	8726.17bG	–
II ((D)	9063.33bA	9062.31bB	8238.48cB	10221.63aA	9251.08bA	9562.33bA	8222.23cG	8812.37bG	–

[a] Results are average of duplicate analyses. "–": not calculated. Results bearing different lower-case letters, a–g, in the same row are significantly different ($P < 0.05$). Results bearing different upper-case letters, A–G, in the same column are significantly different ($P < 0.05$). Values represent the time period (0∼28 days) during which the degradation rate constant was calculated. [b] VI: Cholesterol 10000 ppm. III: Cholesterol 10000 ppm + 18:0 1000 ppm. IV: Cholesterol 10000 ppm + 18:2 1000 ppm. V: Cholesterol 10000 ppm + 22:6 1000 ppm. I: Cholesterol 10000 ppm + riboflavin 50 ppm. II: Cholesterol 10000 ppm + riboflavin 100 ppm. (L): light storage. (D): dark storage.

Table 4. Amounts (ppm) of COPs Formed in the Presence of Cholesterol or in Combination with Riboflavin or Fatty Acid Methyl Esters during Light Storage at 25 °C for 28 Days[a]

COPs	treatment	day 0	1	2	3	7	14	21	28
7α-OH	VI	0.00aA	0.00aA	0.00aA	0.00aA	0.00aA	5.38bA	21.68cA	71.69dB
	III	0.00aA	0.00aA	3.35bB	5.29bB	6.03bB	6.33bA	8.20bA	17.26cA
	IV	0.00aA	0.00aA	0.00aA	1.21bB	3.54bB	5.84bA	12.82dA	63.09eB
	V	0.00aA	0.00aA	0.00aA	0.00aA	6.28bB	46.60cB	84.57dB	86.94dC
	I	0.00aA	0.00aA	12.73bC	32.94cC	57.55dC	100.99eC	109.70eC	98.91eC
	II	0.00aA	3.02bB	28.08cD	64.23dD	104.09eD	138.11fD	125.38fD	137.84fD
7β-OH	VI	0.00aA	0.00aA	0.00aA	0.00aA	1.73bA	16.58cA	42.17dC	75.28eD
	III	0.00aA	0.00aA	0.00aA	0.00aA	2.46bA	12.54cA	17.55cA	39.62dA
	IV	0.00aA	0.00aA	0.00aA	0.00aA	1.87bA	15.05cA	29.27dB	77.39eD
	V	0.00aA	0.00aA	1.88bB	1.90bB	20.28cB	62.90dD	64.25dD	75.00eD
	I	0.00aA	0.00aA	22.00bC	31.44bcC	35.62cC	36.95bB	33.26bB	46.26dB
	II	0.00aA	3.50bB	29.07cD	40.45dD	45.39dD	48.75dC	45.54dC	48.79dC
7-keto	VI	0.00aA	0.00aA	270.30bA	265.13bA	287.03bA	514.89cA	963.11dB	1554.52eE
	III	0.00aA	0.00aA	264.19bA	364.33cB	335.52cB	762.13dC	1035.98eC	1365.97fC
	IV	0.00aA	0.00aA	284.11bA	262.67bA	312.50bAB	570.80cB	904.29dA	1493.56eD
	V	0.00aA	0.00aA	273.42bA	268.32bA	409.97cC	781.12dC	1144.22eD	1199.88fA
	I	0.00aA	276.64bB	500.44cB	849.26dC	1051.34eD	1218.02fD	1722.18hE	1300.91gB
	II	0.00aA	350.47bC	675.46cC	1121.08dD	1512.50eE	1639.05fE	1941.34hF	1800.89gF
5,6α-EP	VI	0.00aA	0.00aA	102.52bAB	80.92bA	79.29bA	113.36bA	154.30cA	192.48dA
	III	0.00aA	0.00aA	94.78bAB	93.38bAB	81.75bA	169.42cB	223.88dB	338.61eD
	IV	0.00aA	0.00aA	74.85bA	92.28bAB	78.79bA	83.89bA	137.82cA	193.22dA
	V	0.00aA	0.00aA	69.19bA	101.33cAB	140.54dC	227.25eC	281.16fC	346.36gD
	I	0.00aA	96.08bB	100.00bAB	116.51bcB	141.10cC	240.98dC	269.36dC	307.63eC
	II	0.00aA	88.63bB	91.60bAB	99.47bAB	106.30bAB	185.43cB	245.68dbC	233.75dB
5,6β-EP	VI	0.00aA	0.00aA	419.33bB	408.11bA	417.56bA	506.22cA	723.10dA	1079.92eA
	III	0.00aA	0.00aA	408.01bAB	417.83bA	478.18cB	784.74dB	1123.57eB	1740.39fD
	IV	0.00aA	0.00aA	377.96bA	408.11bCA	424.10cA	521.34dA	693.69eA	1135.55fB
	V	0.00aA	0.00aA	459.35bC	456.56bA	757.52cD	1097.75dD	1627.67eE	1917.36fE
	I	0.00aA	408.11bB	477.98cCD	602.38dB	709.38eC	939.57fC	1178.46hC	1085.14gA
	II	0.00aA	431.76bB	517.09cD	676.47dC	866.98eE	1148.75fE	1328.46gD	1346.14gC
3,5-cholestadien-7-one	VI	0.00aA	0.00aA	0.00aA	0.00aA	0.00aA	0.00aA	0.00aA	0.00aA
	III	0.00aA	0.00aA	0.00aA	0.00aA	0.00aA	220.85bB	223.39bB	231.55bB
	IV	0.00aA	0.00aA	0.00aA	0.00aA	0.00aA	0.00aA	0.00aA	0.00aA
	V	0.00aA	0.00aA	0.00aA	0.00aA	0.00aA	0.00aA	0.00aA	0.00aA
	I	0.00aA	0.00aA	0.00aA	0.00aA	246.84bB	464.10cC	439.95cC	705.39dD
	II	0.00aA	0.00aA	0.00aA	204.73bB	265.47cB	498.55dC	467.02dC	632.59eC
total	VI	0.00a	0.00a	792.15b	754.16b	785.61b	1156.43c	1904.36d	2973.89e
	III	0.00a	0.00a	770.33b	880.83b	903.94c	1956.01d	2632.57e	3733.40f
	IV	0.00a	0.00a	736.92b	764.27b	820.80b	1196.92c	1777.89d	2962.81e
	V	0.00a	0.00a	803.84b	828.11b	1334.59c	2215.62d	3201.87e	3625.54f
	I	0.00a	780.83b	1113.15c	1632.53d	2241.83e	3000.61f	3752.91g	3544.24fg
	II	0.00a	877.38b	1341.30c	2206.43d	2900.73e	3658.64f	4153.42g	4200.00g

[a] Results are reported as average of duplicate analyses. Results bearing different lower-case letters, a–g, in the same row are significantly different for each COPs ($P < 0.05$). Results bearing different upper-case letters, A–F, in the same column are significantly different for each COPs ($P < 0.05$). [b] VI: Cholesterol 10000 ppm. III: Cholesterol 10000 ppm + 18:0 1000 ppm. IV: Cholesterol 10000 ppm + 18:2 1000 ppm. V: Cholesterol 10000 ppm + 22:6 1000 ppm. I: Cholesterol 10000 ppm + riboflavin 50 ppm. II: Cholesterol 10000 ppm + riboflavin 100 ppm.

Formation of COPs during Light Storage. Table 4 shows the amount of COPs formed in a model system containing cholesterol alone or in combination with riboflavin or fatty acid methyl esters. Five COPs (7α-OH, 7β-OH, 7-keto, 5,6α-EP, and 5,6β-EP) were present for all the treatments. Of the various COPs, the greatest amounts were found for 7-keto and 5,6β-EP. The formation of 7-keto is probably due to dehydration of cholesterol hydroperoxide such as 7-OOH or dehydrogenation of 7α-OH or 7β-OH (*3*). Theoretically, the large production of 5-OOH is expected because of reaction of singlet oxygen with unsaturated double bond of cholesterol during light storage (*8*, *14*). However, the 5-OOH could converted to the more stable 7-OOH (*7*). Likewise, the formation of 5,6α-EP or 5,6β-EP is probably due to cholesterol through 7-OOH (*3*), and the level for the latter was much higher than that for the former. It has been well established that 5,6β-EP exhibited a higher stability than 5,6α-EP (*23*). Compared to the other COPs, both 7α-OH and 7β-OH were present in small amounts and were probably formed through reduction of 7α-OOH and 7β-OOH, respectively (*3*). No triol was detected because triol could be formed only through hydrolysis from 5,6α-EP or 5,6β-EP under acidic conditions (*7*). Similarly, no side-chain products of cholesterol were detected. According to a report by Smith (*23*), the side-chain products of cholesterol would be more readily formed when cholesterol in crystalline state was heated or illuminated. In our system the cholesterol solution was exposed to light instead and could account for this difference. Interestingly, 3,5-cholestadien-7-one was detected only in systems containing cholesterol and stearic acid methyl ester or riboflavin. Several studies showed that 3,5-cholestadien-7-one was formed from 7-keto under basic conditions (*7*, *23*). Our result suggested that the presence of riboflavin could facilitate formation of 3,5-cholestadien-7-one through dehydration of 7-keto by light energy. The absence of 3,5-cholestadien-7-one in both systems of cholesterol and linoleic acid methyl ester or docosahexaenoic acid methyl ester is probably because of absorption of a portion of light by the unsaturated fatty acid methyl esters.

When cholesterol was illuminated alone, 7α-OH was not formed until 14 days and then reached a plateau after 28 days. The highest level of 7α-OH occurred in a system containing cholesterol and riboflavin, indicating that riboflavin possessed the ability to facilitate 7α-OH formation. Similar phenomenon also applied to 7β-OH, however, the level was lower than that of 7α-OH. This result seemed to be contradictory to a report by Smith (*23*), who pointed out that 7β-OH should be more susceptible to formation than 7α-OH. In a later study, several authors demonstrated that under drastic oxidation conditions, 7α-OH was more readily formed than 7β-OH (*24*). In our study, the addition of riboflavin may provide an environment for promoting photooxidation, and thus the formation of 7α-OH was favored. Unsaturated fatty acid methyl esters were found to induce formation of 7α-OH and 7β-OH more easily than saturated fatty acid methyl esters.

For most treatments, the level of each COPs was found to increase with increasing illumination time. Most COPs were present only after illumination for 1 day, and 5,6β-EP was the most susceptible to formation, followed by 7-keto, 5,6α-EP, 7β-OH, 7α-OH, and 3,5-cholestadien-7-one. In addition to 7α-OH, the largest amount of 7-keto and 3,5-cholestadien-7-one was found in the presence of riboflavin, whereas in the presence of docosahexaenoic acid methyl ester, the greatest amount of 5,6β-EP and 5,6α-EP occurred. This result revealed that both riboflavin and docosahexaenoic acid methyl ester could accelerate COPs formation, however, the former may provide a more pronounced effect because of complete degradation or absorption of light of the latter during illumination. Although riboflavin was completely degradated after light storage for 1 day, the formation of yellow degradation products such as lumiflavin, lumichrome, formylmethioflavin, and carboxylmethioflavin may possess the ability to promote cholesterol oxidation (*20*). The same trend was shown by the total amount of COPs. A system of cholesterol and stearic acid methyl ester generated a higher amount of COPs than that of linoleic acid methyl ester or docosahexaenoic acid methyl ester, whereas the largest level was found for cholesterol and riboflavin.

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