JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Kinetic Model for Studying the Effect of Quercetin on Cholesterol Oxidation during Heating

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Inhibition of the heat-induced cholesterol oxidation at 150 °C by incorporation of quercetin was kinetically studied. Results showed that without quercetin, the cholesterol oxidation products (COPs) concentration increased with increasing heating time. A low amount (0.002%, w/w) of quercetin was effective in inhibiting the formation of COPs during the initial heating period (≤30 min) at 150 °C. However, after prolonged heating (30−120 min), a low antioxidant activity was observed because of the degradation of quercetin. When using nonlinear regression models for kinetic study of cholesterol oxidation in the absence of quercetin, the epoxidation showed the highest rate constant ($h^{-1} = 683.1$), followed by free radical chain reaction ($h^{-1} = 453.5$), reduction ($h^{-1} = 290.3$), dehydration ($h^{-1} = 155.5$), triol dehydrogenation ($h^{-1} = 5.35$), dehydrogenation ($h^{-1} = 0.68$), thermal degradation ($h^{-1} = 0.66$), and triol formation ($h^{-1} = 0.38$). However, in the presence of quercetin, the reaction rate constants (h^{-1}) for epoxidation (551.4), free radical chain reaction (111.7), and thermal degradation (0.28) were reduced greatly. The kinetic model developed in this study can be used to predict the inhibition of COPs by quercetin during the heating of cholesterol.

KEYWORDS: Cholesterol oxidation; quercetin; heating; kinetics; HPLC

INTRODUCTION

Cholesterol, an important biological compound that is widely distributed in egg and meat products (*1*−*3*), can undergo oxidation to form cholesterol oxidation products (COPs) during heating (*4*−*6*) or illumination (*7*). Many studies have demonstrated that the consumption of COPs in excess may have adverse effects on human health (*8*, *9*).

The formation mechanism of COPs during heating or illumination of cholesterol has been well illustrated (*7*, *10*). Just like lipid oxidation, a series of free radical chain reactions can occur initially during heating and lead to the formation of various COPs such as 7-ketocholesterol (7-keto), 7α-hydroxycholesterol (7α-OH), 7β-hydroxycholesterol (7β-OH), 5,6α-epoxycholesterol (5,6α-EP), and 5,6β-epoxycholesterol (5,6β-EP) (*5*). In view of the impact of COPs on human health, the inhibition of COPs formation by the addition of antioxidant is extremely important. Several studies have shown that the incorporation of antioxidants was effective in inhibiting cholesterol oxidation during high-temperature processing or storage (*11*, *12*). However, there is a paucity of data regarding the kinetic study of inhibition mechanism.

Flavonoids, a class of polyphenols, are mainly present in fruits and vegetables and have received considerable attention in recent years because of their vital roles in the prevention of chronic diseases such as coronary heart disease (*13*, *14*), which can be attributed to their high antioxidant activity (*15*, *16*). Rice-Evans et al. (*17*) demonstrated that naturally occurring flavonoids such as quercetin possessed 4 times higher antioxidant activity than vitamins C and E. Thus, it is imperative to learn about the effect of quercetin on the inhibition of COPs formation during heating. In this study we tried to use a heating system and develop a kinetic model for elucidating the inhibition mechanism of cholesterol oxidation by quercetin.

MATERIALS AND METHODS

Materials. Quercetin, lauryl alcohol, cholesterol, and several COPs standards, including 5,6α-EP, 5,6β-EP, 7-keto, and 5α-cholestane-3β,5,6β-triol (triol) were obtained from Sigma Chemical Co. (St. Louis, MO). Both 7β-OH and 7α-OH standards were from Steraloids Inc. (Wilton, NH). These standards were used without further purification. Paraffin oil was from Merck Co. (Darmstadt, Germany). The HPLC-grade solvents, ethanol, 2-propanol, and *n*-hexane, were from Mallinckrodt Co. (Paris, KY), whereas 1,2-dichloroethane was from Riedel-de Häen Co. (Barcelona, Spain). The NH_2 cartridges were from Unichrom Scientific Co. (Taipei, Taiwan).

Instrumentation. The HPLC instrument consists of a Jasco PU-980 pump (Tokyo, Japan), a Jasco 830 refractive index detector, and an SIC Chromatocoder 12 integrator (System Instruments Co., Tokyo, Japan). Two Lichrospher 100 CN columns (244 × 4.0 mm i.d. each) containing 5-μm particle were from Merck. CHEN-WIN computer software system (Shuen-Hua Co., Taipei, Taiwan) was used to process data.

Heating of Cholesterol. A mixture containing 400 μL of paraffin oil and 100 μL of lauryl alcohol was poured into a 100-mL round-bottom flask, and 100 mg of cholesterol standard as well as 0.002 mg (0.002% of cholesterol) quercetin (in 100 μL of ethanol) was added. Lauryl alcohol was used to dissolve quercetin, cholesterol, and COPs. In addition, lauryl alcohol has been reported to promote triol formation

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10.1021/jf052529r CCC: $33.50
Published on Web 01/21/2006

through alcoholysis (*18*). For the control treatment, only 100 mg of cholesterol standard was added to the mixture of 400 μL of paraffin oil and 100 μL of lauryl alcohol. The flask was placed in a paraffin oil bath, which was preheated for 5 min with nitrogen gas flushing into the flask at the same time, until the internal temperature of the flask reached 150 ± 1 °C. After that, the oxygen was pumped through, and heating times of 0, 5, 10, 30, 60, and 90 min were started for the control treatment. For the quercetin treatment, the heating time was extended to 120 min. After heating, the flask was inserted into dry ice to terminate the reaction, and each sample was subjected to HPLC analysis. Triplicate experiments were performed for each heating treatment, and the data were subjected to nonlinear regression analysis by SAS (*19*).

Extraction and Purification of Cholesterol and COPs. A modified method based on that of Nourooz-Zadeh (*20*) was used to extract and purify COPs. The heated mixture was added to 20 mL of hexane/2-propanol (3:2, v/v) and shaken vigorously for 3 min, after which the solution was centrifuged at 26000*g* for 5 min. The upper layer was collected and poured into a centrifuged tube, and 12 mL of distilled water was added. The solution was centrifuged again, and the upper phase was also collected and evaporated to dryness at 35 °C. The residue was dissolved in 1 mL of hexane/1,2-dichloroethane (1:1, v/v) and poured into a NH_2 cartridge. In the beginning, 5 mL of hexane was added to the cartridge to remove impurities such as hydrocarbon, cholesterol ester, and triglyceride. Then 25 mL of hexane/1,2-dichloroethane/2-propanol (50:30:15, v/v/v) was added to elute cholesterol and COPs. The solution was evaporated to dryness at 35 °C, dissolved in hexane/2-propanol (95:5, v/v), and filtered through a 0.2-μm membrane filter for HPLC analysis. For recovery study, a fixed concentration of various COPs standards was added to the sample for extraction and purification. After HPLC analysis, the recovery of each COP was obtained on the basis of the ratio of the amount of each COP standard after and before HPLC.

TLC Analysis of COPs. *Preparation of Wurster Dye.* The Wurster dye was prepared according to a method of Smith and Hill (*21*). One gram of *N*,*N*-dimethyl-*p*-phenylenediamine dihydrochloride was dissolved in 100 mL of 50% methanol solution (in water), which was shaken, and 1 mL of glacial acetic acid was added. Then the mixture was poured into a glass vial and stored at −20 °C for use.

Separation of COPs by TLC. Initially the plate coated with silica gel was placed in a glass tank lined with a filter paper (20 × 20 cm) and equilibrated for 30 min with 200 mL of benzene/ethyl acetate (60:40, v/v) (*22*) before separation. A 10-μL volume of sample extract was spotted on the glass plate with a micropipet, and the solvent front was allowed to run for a distance of ∼18 cm at room temperature. Then the plate was dried in an oven at 110 °C for 10 min and sprayed with 50% H_2SO_4, and the color development of each spot (COPs) under UV radiation at 254 nm was recorded. Both 7α-hydroperoxycholesterol (7α-OOH) and 7β-hydroperoxycholesterol (7β-OOH) bands were identified and quantified on the basis of the Wurster dye method as shown in a previous study (*7*).

HPLC Analyses. A mobile phase of hexane/2-propanol (95:5, v/v) and two LiChrospher 100 CN columns with a flow rate at 1.0 mL/min and RI detection (sensitivity = 16×10^{-5} RIU) were used to separate cholesterol and various COPs. Two columns connected in series were used to enhance the resolution power of COPs. Identification was carried out by comparing retention times of unknown peaks with standards and cochromatography with added standards. Because of the absence of a suitable internal standard, each COP was quantified using an external calibration method. Seven concentrations of cholesterol (500, 1000, 2500, 5000, 18000, 25000, and 50000 μg/mL) and each COP (50, 100, 200, 500, 1000, 2500, and 5000 μg/mL) used to prepare the standard calibration curves were injected onto the HPLC separately, and the curve for cholesterol and each COP standard was obtained by plotting concentration against area. The regression equations and correlation coefficients (r^2) were calculated using a CHEN-WIN computer software system, and a high r^2 value (>0.99) was found for cholesterol and various COPs standards. Each COP was quantified using a method as described by Chien et al. (*5*). The eluates of 7α-OOH and 7β-OOH were collected separately and then quantified using the Wurster dye method (*7*). As described above, recovery was also performed by adding standards of cholesterol and various COPs to samples for extraction and HPLC analysis, and a high recovery of 97−100% was achieved.

Kinetic Analyses of COPs. The concentration changes of cholesterol and various COPs during heating of cholesterol in the presence or absence of quercetin were analyzed statistically by using a nonlinear regression procedure of SAS (*19*). All of the rate constants for each reaction were estimated by using the least-squares method with a nonlinear regression procedure−Marquardt iterative approach until the convergence of the parameters was best fitted. The precision of the parameters of the kinetic equations was also evaluated.

Statistical Analyses. Analyses of variance and comparisons among the residual amounts of cholesterol at different heating time intervals were conducted using the SAS system (*19*). After a preliminary *F* test, the differences among the residual cholesterol were determined by Duncan's multiple-range test at a 5% probability level.

RESULTS AND DISCUSSION

It has been well established that cholesterol is relatively stable in solid form and is prone to undergo autoxidation in liquid form (*23*, *24*), especially when the temperature reaches its melting point (148.5 °C) or higher (*5*). At a temperature of 200−300 °C, the thermal degradation of cholesterol has been reported to dominate, instead of autoxidation (*25*). After various trials, the most suitable temperature for studying the inhibition kinetics of cholesterol oxidation by quercetin was found to be 150 °C. Also, this temperature is similar to that used for low-temperature frying of foods. Thus, the heating temperature of 150 °C was selected in this study to minimize the loss of cholesterol due to thermal degradation and accelerate the formation of COPs at the same time.

Due to the poor solubility of the mixture of quercetin, cholesterol, and COPs in the oil, a solvent system of lauryl alcohol/paraffin oil (1:4, v/v) was used instead. After several preliminary studies, it was observed that the incorporation of 0.002 mg (0.002%, w/w) of quercetin was adequate to inhibit the formation of COPs for 100 mg of cholesterol. No COPs were detected for the 2-h heating period when a higher amount of quercetin (>0.01 mg) was used. An average loss of 2.1% was found for cholesterol when preheated alone from 25 to 150 °C with nitrogen flushing before oxygen pumping, whereas a slightly higher loss (3.1%) was shown for cholesterol heated together with quercetin. This result should be due to thermal degradation of cholesterol under anaerobic condition, and the presence of quercetin failed to protect cholesterol from thermal degradation during the preheating period. In other words, quercetin may be effective only in inhibiting cholesterol oxidation. Also, there is no significant difference in cholesterol loss for the presence and absence of quercetin. Therefore, for kinetic study of each COP formation, initial levels of 96.9 and 97.9 mg of cholesterol were used for studying the heating treatments with and without quercetin, respectively (**Table 1**).

Formation of COPs. Figure 1 shows the major reaction pathways and rate constants of cholesterol oxidation and degradation based on our previous study (*6*). In the presence of lauryl alcohol, 5,6α-EP or 5,6β-EP may undergo alcoholysis to form triol (*6*, *18*), which can be further dehydrogenated to cholestan-3β,5α,6-one (*26*).

Figures 2 and **3** show the HPLC chromatograms of COPs formed during heating at 150 °C in the absence and presence of quercetin, respectively. A total of eight major COPs, including 5,6α-EP, 5,6β-EP, 7-keto, 7α-OH, 7β-OH, 7α-OOH, 7β-OOH, and triol were adequately resolved within 40 min, with the elution order being cholesterol, 5.6α-EP, 5.6β-EP, 7-keto, 7α-OH, 7β-OH, 7α-OOH, 7β-OOH, and triol. No COPs were formed at 0 min of heating, indicating that cholesterol oxidation did

Table 1. Inhibitory Effect of Quercetin on Percentage Changes of Cholesterol[a] during Heating at 150 °C

heating time (min)	cholesterol[b] (%) control[c]	% remaining	quercetin[c]	% remaining
0	97.9 ± 0.6Aa	100.0	96.9 ± 0.3Aa	100.0
5	93.0 ± 1.0Ba	95.0	95.6 ± 0.2Ab	98.7
10	86.6 ± 0.3Ca	88.4	93.9 ± 0.1Bb	97.0
30	63.0 ± 1.0Da	64.3	85.9 ± 0.1Cb	88.7
60	48.8 ± 0.6Ea	49.8	78.5 ± 0.3Db	81.0
90	35.5 ± 0.2Fa	36.2	69.3 ± 0.4Eb	71.6
120	–[d]	–	53.0 ± 2.1F	54.7

[a] Values are expressed as percentage relative to cholesterol content at heating time = 0 min. [b] Cholesterol (100 mg) was dissolved in 500 μL of lauryl alcohol/paraffin oil (1:4, v/v) solvent in the absence (control) or presence of 0.002 mg of quercetin (quercetin). [c] Mean and standard deviation of triplicate determinations; entries bearing different letters of A–F (a–b) in the same column (row) are significantly different ($p < 0.05$). [d] Not detected.

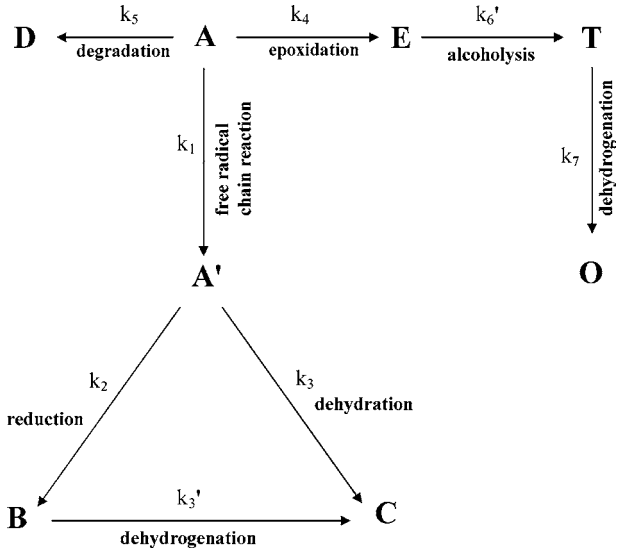

Figure 1. Major autoxidation and degradation pathways of cholesterol. All compounds were solubilized in the lauryl alcohol/paraffin (1:4, v/v) solvent: cholesterol (A), 7-hydroperoxycholesterol (A′), 7-hydroxycholesterol (B), 7-ketocholesterol (C), 5,6-epoxycholesterol (E), degraded products (D), cholestan-3β,5α,6β-triol (T), cholestan-3β,5α,6-one (O). k_{1-5}, k_3', k_6', and k_7 are the corresponding rate constants. The scheme is based on a study by Chien et al. (*6*).

not occur with nitrogen flushing prior to heating to 150 °C (**Figures 2** and **3**). Without quercetin, the COPs were formed gradually during the initial heating period (≤30 min) and increased sharply thereafter (**Figure 2**). Also, the amount of COPs followed an increased trend for the increase of heating time. The level of 7-OOH (7α-OOH and 7β-OOH) accounted for 0.00037% of cholesterol after 10 min of heating and then reached a plateau (1.2% of cholesterol) in 90 min (**Figures 2** and **4**). Meanwhile, the degradation of 7-OOH proceeded quickly as soon as it was formed from cholesterol, which in turn led to the formation of 7-OH and 7-keto through reduction and dehydration, respectively. On the basis of the initial amount of cholesterol, the percentages of 7-OH and 7-keto formed were 4.2 and 3.8%, respectively, over a 90-min heating period (**Figure 4**). On the other hand, with quercetin, an extremely low amount of COPs was formed during the 30-min heating period and increased drastically after 60 min (**Figure 3**), probably because of quercetin degradation and its ability to

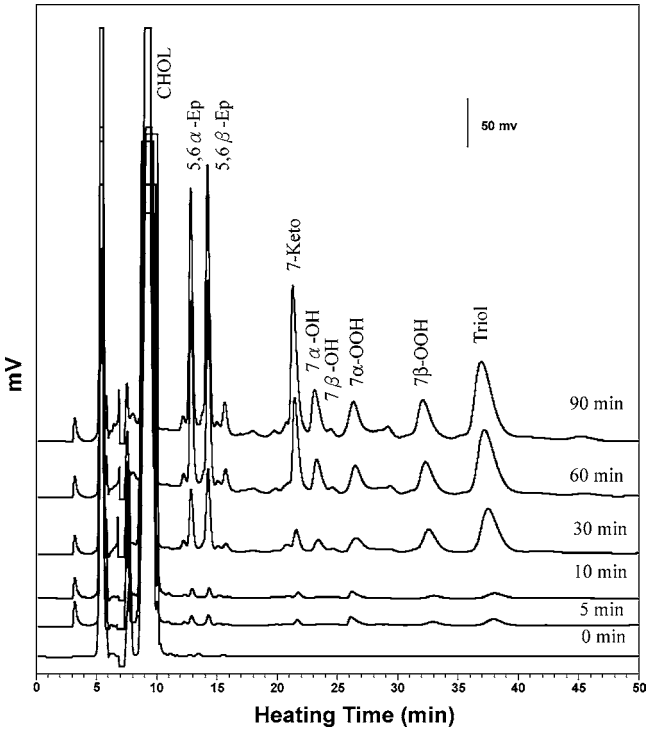

Figure 2. HPLC chromatograms of COPs formation during heating of cholesterol alone at 150 °C.

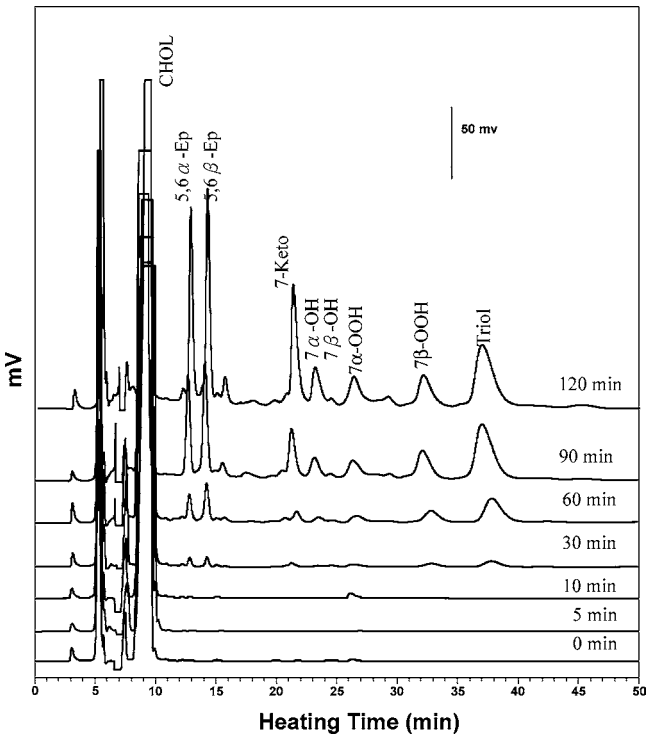

Figure 3. HPLC chromatograms of COPs formation in the presence of quercetin (0.002%, w/w) during heating of cholesterol at 150 °C.

react with peroxy radicals, which are responsible for the process of radical oxidation. From **Figure 4**, two important points, including the autocatalytic nature of the cholesterol oxidation and the induction period (~90 min) caused by the addition of quercetin, appeared to be evident. The former is probably caused by the thermal decomposition of hydroperoxides with the formation of free radicals, which accelerate oxidation, whereas the latter is caused by quercetin to donate a hydrogen atom to

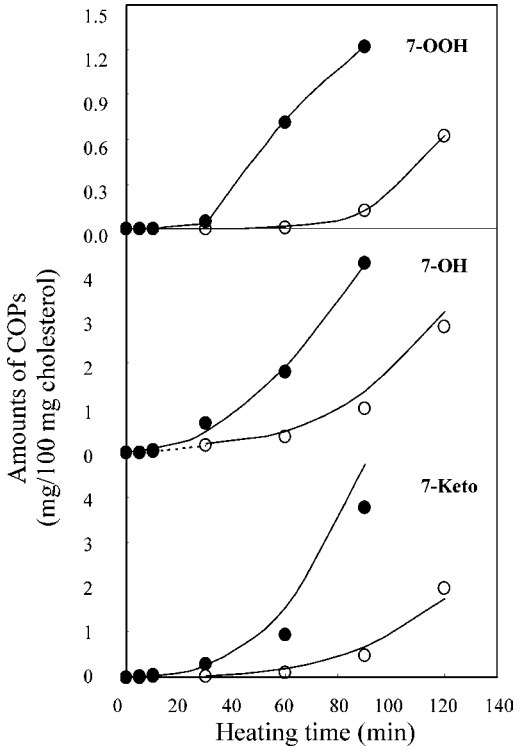

Figure 4. Inhibitory effect of quercetin on major COPs formed in the C-7 oxidation route during heating of cholesterol at 150 °C: ●, mean of experimental data for cholesterol only; ○, mean of experimental data for cholesterol in the presence of quercetin; —, best fitting line. Triplicate experiments were performed.

stabilize peroxy radicals. This phenomenon proved that the addition of 0.002% (w/w) quercetin should exert a strong antioxidant effect on cholesterol oxidation during the initial heating period at 150 °C.

Compared to the control treatment, the levels of 7-OOH were 6.6 and 4.5 times lower for the heating times 0 and 10 min after quercetin addition, respectively, demonstrating that quercetin may possess a strong antioxidant activity against cholesterol peroxidation. Similarly, the amounts of 7-OH and 7-keto were correspondingly decreased by 4.3- and 7.9-fold, respectively, when compared to the control treatment after 90 min of heating. This phenomenon may be due to the slow formation of 7-OOH during heating of cholesterol together with quercetin. However, after prolonged heating for 120 min, the contents of 7-OOH, 7-OH, and 7-keto increased substantially, probably because of the thermal degradation or free radical scavenging capacity of quercetin.

The epoxidation of cholesterol also occurred along with the formation of 7-OOH during heating of cholesterol without quercetin, and the levels of 5,6-epoxides (5,6α-EP and 5,6β-EP) showed a tendency to increase (**Figures 2** and **5**). A small content of 5,6-epoxides was induced initially, which then rose to 1.8 and 12.2% of cholesterol, respectively, after 30 and 90 min of heating (**Figure 5**). The formation curve of 5,6-epoxides was similar to that of 7-OOH, implying that the cholesterol epoxidation may proceed quickly as soon as 7-OOH is generated (*22*, *27*). The amount of 5,6β-EP formed from 7-OOH was higher than that from 5,6α-EP (**Figure 2**), demonstrating a greater stability for the β-form as compared to the α-form (*27*). Conversely, with quercetin, the formation of 5,6-epoxides was greatly reduced by 6.2-fold after 30 min of heating (**Figures 3** and **5**). However, after heating for 60 min, a large increase was shown for 5,6-epoxides, which may be attributed to rapid

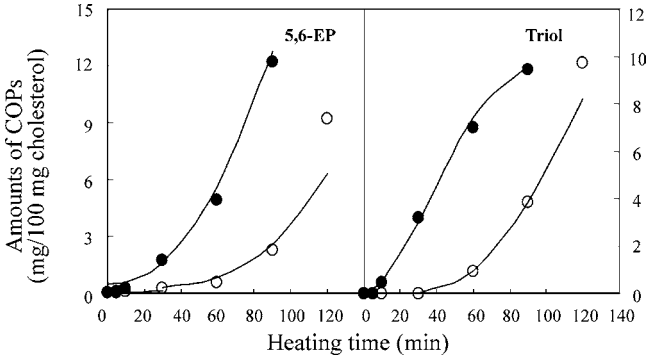

Figure 5. Inhibitory effect of quercetin on 5,6-EP and triol formation during heating of cholesterol at 150 °C: ●, mean of experimental data for cholesterol only; ○, mean of experimental data for cholesterol in the presence of quercetin. —, best fitting line. Triplicate experiments were performed.

formation of 7-OOH. Nevertheless, the amount of 5,6-epoxides produced after 90 min of heating was 5.3 times lower than for the control treatment (**Figure 5**).

The formation of triol was also observed in this study (**Figures 2**, **3**, and **5**). Theoretically, triol can be formed from 5,6-epoxides in an aqueous solution through acidic hydrolysis (*10*). In our experiment, triol was probably formed through alcoholysis because lauryl alcohol was used instead of water (*6*, *18*). In the absence of quercetin, a significant amount of triol was formed after 30 min of heating, and then a sharp increase (9.7%) followed after 90 min (**Figure 5**). In contrast, with quercetin, an insignificant quantity of triol was found during the initial heating period, followed by a gradual increase to 9.8% after extensive heating to 120 min. Despite that, the level of triol formed was lower than for the control treatment under the same heating time.

By comparison of the results shown above, it can be concluded that the total amount of COPs was greatly reduced in the presence of quercetin. Approximately 0.5% COPs of cholesterol was formed after 30 min of heating, which was lower than for the control treatment (5.9%) by 11.8-fold. However, the level of COPs further increased to 7.7% in 90 min and attained a peak (24.4%) in 120 min, with the former being lower than for the control treatment by 4-fold. As explained before, this is probably because of drastic degradation of quercetin when it is heated beyond 60 min. It was also shown that the amount of cholesterol reduced during heating was not equal to that of COPs formed, which may arise from the phenomenon that, in addition to oxidation, the thermal degradation of cholesterol may also proceed simultaneously.

Thermal Degradation of Cholesterol during Heating. **Table 1** shows the percentage changes of cholesterol during heating at 150 °C for varied lengths of time in the absence or presence of quercetin. A significant percentage difference occurred between the control and quercetin treatments for all heating times. Without quercetin, cholesterol decreased by 35.7% after 30 min of heating, and greater losses by 50.2 and 63.8% were shown after 60 and 90 min of heating, respectively (**Table 1**). This level was lower than that in a previous study by Chien et al. (*5*), who reported that only ∼33.3% cholesterol remained after heating a cholesterol thin film at 150 °C for 30 min. Osada et al. (*4*) also found that the residual percentage was 40% after 150 °C heating of cholesterol for 24 h. This difference may be due to the variety of solvents used to dissolve cholesterol and the pumping rate of oxygen into the heating system. On the contrary, with quercetin, the degradation of

cholesterol proceeded slowly during the initial heating period, which can be accounted for by 19.0% cholesterol loss after 60 min of heating. However, the loss further increased to 45.3% thereafter, which was much lower than the for control treatment. This result demonstrated that quercetin exhibits a strong antioxidant activity against COPs formation. Several studies have also proved that flavonoids such as quercetin can inhibit free radical chain reaction by donating hydrogen atoms to stabilize free radicals (*28*, *29*).

Kinetic Studies on Thermal Degradation and Oxidation of Cholesterol. As both α- and *β*-forms of COPs possess similar reactivity toward oxidation, only five major COPs, including 7-OOH (7α- and 7*β*-OOH), 7-OH (7α- and 7*β*-OH), 7-keto, 5,6-EP (5,6α- and 5,6*β*-EP), and triol, as well as cholesterol were selected for kinetic study. The initial heating period (≤30 min) was considered as a lag period because quercetin showed a high antioxidant activity. Thus, the concentration changes of various COPs during heating of cholesterol together with quercetin for 30 min were subjected to kinetic study by using the nonlinear regression models. The COPs formation during the heating period of 30−120 min was not kinetically analyzed because a low antioxidant activity of quercetin should follow a reaction rate model similar to that of the control treatment.

According to a paper by Chien et al. (*6*), the reactions for the oxidative pathway can be divided into two major routes of C-7 oxidation and epoxidation (**Figure 1**). The oxidation reaction involved the attack of free radicals on cholesterol to result in the formation of 7-OOH, which belongs to the second-order, and the rate equation for 7-OOH formation (A → A′) was listed in the equation, as described in a previous study (*5*). It has been established that cholesterol oxidation would be unlikely to proceed unless there is sufficient 7-OOH accumulated in the reaction system (*5*). Besides, 7-OOH can be further degraded to 7-OH or 7-keto through the first-order reaction (*5*, *30*, *31*). The rate equation for 7-OOH formation was thus modified as

$$\frac{d[A']}{dt} = k_1\left(1 - \frac{[A']}{[A']_{max}}\right)[A'] - k_2[A'] - k_3[A'] = m_1[A'] - \frac{k_1}{[A']_{max}}[A']^2 \quad (1)$$

where $[A']_{max}$ is the maximum attainable concentration of 7-OOH, [A′] is the percentage concentration of 7-OOH at *t* min of heating time, and k_1, k_2, and k_3 are the reaction rate constants (h^{-1}).

In addition, the rate equations of C-7 oxidation routes B and C were written as in a previous study (*5*). Because both cholesterol and 7-OOH were involved in the epoxidation reaction (*32*), the rate equation for 5,6-EP formation was also shown as in a previous study (*5*). Both 7-OOH and 5,6-EP formation as well as thermal degradation of cholesterol may lead to a substantial decrease of cholesterol concentration. Thus, the overall decreasing rate of cholesterol could be regarded as the sum of the free radical chain reaction, epoxidation, and thermal degradation and can be written as

$$-\frac{d[A]}{dt} = k_1[A][A'] + k_4[A][A'] + k_5[A] = k_{14}[A][A'] + k_5[A] \quad (2)$$

where [A] is the percentage concentration of cholesterol at *t* min of heating time and k_4, k_5, and k_{14} are the reaction rate constants (h^{-1}).

For the control treatment, the concentration ratio of 7-OOH to cholesterol for the heating time of 30 min was 1/3500. However, for the quercetin treatment, the concentration of 7-OOH formed after 30 min of heating was much lower than that of cholesterol (30000[A′] ≅ [A]). Thus, the first term (k_{14}[A][A′]) on the right of eq 2 is relatively small as compared to the second term (k_5[A]). Furthermore, percentages of 2.8 and 0.3% of 5,6-EP were formed on the basis of cholesterol after 30 min of heating, respectively, with and without quercetin. Therefore, cholesterol degradation can be regarded as the main reaction to proceed during heating of cholesterol. Equation 2 was thus simplified as $-d[A]/dt = k_5[A]$, and the integration of the above equation gave

$$[A] = [A_0]\, e^{-k_5 t} \quad (3)$$

where $[A_0]$ is the percentage concentration of cholesterol at 0 min of heating time.

Equation 1 is in the form of the Bernoulli equation ($n = 2$). By transformation of a variable, [A′], eq 4 was obtained by integration

$$[A'] = \left[\frac{k_1}{m_1[A']_{max}} + \left(\frac{1}{[A']_0} - \frac{k_1}{m_1[A']_{max}}\right) e^{-m_1 t}\right]^{-1} \quad (4)$$

where $[A'_0]$ is the percentage concentration of 7-OOH at 0 min of heating time and $m_1 = k_1 - k_2 - k_3$. For the epoxidation reaction, the formation of 5,6-EP follows second order and is dependent upon the concentrations of both cholesterol and 7-OOH (*5*, *32*). Thus, the rate equation for both 5,6-EP formation and degradation could be given as

$$\frac{d[E]}{dt} = k_4[A][A'] - k'_6[E] \quad (5)$$

where [E] is the percentage concentration of 5,6-EP at *t* min of heating time and k_4 and k'_6 are the reaction rate constants (h^{-1}).

As lauryl alcohol is used instead of water in the solvent system, alcoholysis of 5,6-EP is most likely to occur in our study for the reaction (E → T) in **Figure 1** according to March (*18*). Also, triol may be further dehydrogenated to form cholestan-3*β*,5α,6-one (T → O) (*26*). As a result, the rate equation of triol formation can be written as

$$\frac{d[T]}{dt} = k_6[L_0][E] - k_8[T] = k'_6[E] - k_7[T] \quad (6)$$

where [T] is the percentage concentration of triol at *t* min of heating time, $[L_0]$ is the percentage concentration of lauryl alcohol at time 0, and k_6, k_7, and k_8 are the reaction rate constants (h^{-1}).

Integration after substitution of eqs 3 and 4 into eq 5 gave eq 7 as follows:

$$[E] = [E_0]\, e^{-k'_6 t} + k_4[A_0]\, e^{-k'_6 t}\int_0^t \frac{e^{[k'_6 - k_5]s}}{\frac{m_2}{m_1} + \left[\frac{1}{[A'_0]} - \frac{m_2}{m_1}\right] e^{-m_1 s}}\, ds \quad (7)$$

Likewise, eq 8 was obtained by integration after substitution of eq 7 into eq 6

Table 2. Rate Constants of Cholesterol Autoxidation and Degradation

rate equation[b]	reaction	control[a]		quercetin[a]	
		k (h^{-1})	r^2	k (h^{-1})	r^2
chol $\xrightarrow{k_1}$ 7-OOH	free radical chain reaction	453.5 ± 0.0	1.00	111.7 ± 0.0	1.00
chol $\xrightarrow{k_4}$ 5,6-EP	epoxidation	683.1 ± 58.1	0.99	551.4 ± 47.4	0.88
chol $\xrightarrow{k_5}$ degradation	degradation	0.66 ± 0.02	0.99	0.28 ± 0.02	0.96
7-OOH $\xrightarrow{k_2}$ 7-OH	reduction	290.3 ± 0.0	1.00	76.8 ± 0.0	1.00
7-OOH $\xrightarrow{k_3}$ 7-keto	dehydration	155.5 ± 0.0	1.00	31.0 ± 0.0	1.00
7-OH $\xrightarrow{k_{3'}}$ 7-keto	dehydrogenation	0.68 ± 0.10	0.99	0.11 ± 0.02	0.99
5,6-EP $\xrightarrow{k_{6'}}$ triol	alcoholysis	0.38 ± 0.07	0.99	1.61 ± 0.06	1.00
triol $\xrightarrow{k_7}$ O	dehydrogenation	5.35 ± 1.00	0.64	0.0[c]	

[a] Control, cholesterol oxidation during heating ($0 < t < 90$ min). Quercetin, cholesterol oxidation in the presence of quercetin during heating ($30 < t < 120$ min). [b] Chol, cholesterol; 7-OOH, 7-hydroperoxycholesterol; 7-OH, 7-hydroxycholesterol; 7-keto, 7-ketocholesterol; 5,6-EP, 5,6-epoxycholesterol; triol, 5α-cholestan-3β,5α,6β-triol; O, cholesten-3β,5α-diol-6-one; k_1–k_5, k_3', k_6', and k_7 are rate constants. [c] No reaction product was detected.

$$[T] = k'_6 k_4 [A_0]\, e^{-k_8 t} \int_0^t e^{(k_8 - k'_6)v} \left(\int_0^v \frac{e^{[k'_6 - k_5]s}}{\frac{m_2}{m_1} + \left[\frac{1}{[A'_0]} - \frac{m_2}{m_1}\right] e^{-m_1 s}} ds \right) dv \quad (8)$$

where $[E_0]$ is the percentage concentration of 5,6-EP at 0 min of heating time and s, t, and v are time.

Among the above equations, both eqs 3 and 4 are in the forms of exact integration. As the exact integrations for eqs 7 and 8 are not possible, we can only get a better approximation to the integral term in the equation by using a quadratic polynomial of Taylor's series. For example, eq 7 can be expressed as follows:

$$[E] = e^{-k'_6 t}\left\{[E]_0 + k_4[A]_0[A']_0\left(t + \frac{1}{2}(m_1 - [A']_0 m_2 + k_6' - k_5)t^2 + \frac{1}{6}(m_1^2 - 3[A']_0 m_1 m_2 + 2[A']_0^2 m_2^2 + 2k_6' m_1 - 2[A']_0 k_6' m_2 - 2k_5 m_1 + 2[A']_0 k_5 m_2 + k_6'^2 - 2k_5 k_6' + k_5^2)t^3\right)\right\} \quad (9)$$

The experimental data were thus analyzed in sequence and fitted best to the corresponding nonlinear regression models as shown above. The data for computing k_5 in eq 3 should be the residual cholesterol as the loss of cholesterol can be attributed mainly to thermal degradation. Therefore, k_5 was recalculated by adding the total amount of COPs to the residual cholesterol, which was found to be 0.66 ± 0.02 h^{-1} ($r^2 = 0.99$) without quercetin and 0.28 ± 0.02 h^{-1} ($r^2 = 0.96$) with quercetin (**Table 2**). The rate constants in eq 4 and in the quadratic polynomial series for the eq 7 (i.e., eqs 9 and 8) as well as the others for 7-OH and 7-keto were estimated by using the least-squares method with a nonlinear Marquardt iterative approach until the convergences of the best-fitted parameters were met. The rate constants of the reaction pathways of cholesterol degradation and oxidation during heating at 150 °C with or without quercetin are shown in **Table 2**. With the exception of the dehydrogenation of triol ($r^2 = 0.64$), the r^2 values of all the reactions were >0.99 in the absence of quercetin and >0.88 in the presence of quercetin. Each COP was further plotted on the basis of the corresponding rate equation and the rate constants which are also shown in **Table 2**. With quercetin, all of the curves fit well with the data points (**Figures 4** and **5**), except for both 5,6-EP and triol when heated for 2 h (**Figure 5**).

For the various oxidation reactions, the correlation coefficients of the free radical chain reaction and epoxidation were 1.00 and 0.99 in the absence of quercetin and 1.00 and 0.88 in the presence of quercetin, respectively. This result implied that both reactions for the formation of 7-OOH and 5,6-epoxide from cholesterol fit well the second order. A high correlation coefficient was also observed for the reduction and dehydration reactions of 7-OOH (**Table 2**), indicating that the reactions for the formation of 7-OH and 7-keto fit the first order. Although the formation of triol by alcoholysis of 5,6-EP for the quercetin treatment was completely inhibited, the low correlation coefficient (0.64) for the control treatment revealed that the reaction of T → O (**Figure 1**) should be reassessed for the first-order model to better estimate the rate constant of k_7. The above results also suggested that despite the presence or absence of quercetin, the rate equations could successfully estimate the thermal degradation of cholesterol and predict the concentration changes of COPs during the entire heating period.

It was also shown that the k_4 value is very large with or without quercetin, suggesting that the 5,6-EP concentration increased sharply as soon as 7-OOH was formed during the free radical chain reaction. It may be postulated that most 7-OOH was converted to 5,6-EP in the beginning during heating, and thus the 7-OOH concentrations were maintained at a very low level (0.03% for the control and 0.003% for the quercetin treatment) over a 30-min heating period. Both are significantly lower than the initial maximum concentrations of 7-OOH, which amounted to 2.1 and 1.0% of total cholesterol in the absence and presence of quercetin, respectively ($[A'_{max}]$ in eqs 1 and 4). However, in the heating system, it is difficult for 7-OOH to maintain this level because it can undergo further degradation to form 7-OH or 7-keto via the C-7 route and 5,6-EP via the epoxidation route. Therefore, the actual maximum level of 7-OOH can be increased to only about 1.3% without quercetin and 0.7% with quercetin after 90 min of heating (**Figure 4**). By comparing the rate constants (h^{-1}) of the major reaction pathways of cholesterol oxidation, a similar trend can be found in the order $k_4 > k_1 > k_2 > k_3 > k_7$ for both the control and quercetin treatments. All of the rate constants (h^{-1}) are much higher than k_5, k_6', and k_3', which are approximately in the same magnitude. However, the rate constants of COPs formation with quercetin were 0.2−5.0 times lower than those without quer-

cetin, with the exception of k_6' in the alcoholysis reaction (1.61 > 0.38). Theoretically, quercetin may play an important role in opening the highly strained three-membered ring of 5,6-EP. Quercetin contains two ortho-hydroxyl groups and should be able to release a proton (H^+) in the heating system (*33*), and the protonated 5,6-EP may in turn undergo alcoholysis in an alcoholic solvent through antihydroxylation, which involved S_N2 or S_N1 attack (*18*).

Compared to the control treatment, all of the rate constants with quercetin treatment decreased substantially, especially for the free radical chain reaction (k_1) by 4.1-fold. A similar result was reported by Nielsen et al. (*34*), who studied cholesterol oxidation in a heterogeneous system initiated by water-soluble radicals. In **Figures 4** and **5**, the curves fit well with the experimental data based on the oxidation of cholesterol during heating. These results suggested that the kinetic models developed in this study can be used to predict the concentration changes of cholesterol and COPs during heating of cholesterol with or without quercetin. In conclusion, the major reaction pathways during the heating of cholesterol in the presence of oxygen include the thermal degradation of cholesterol and the formation of various COPs. With or without quercetin, the correlation coefficients (r^2) for all of the reactions ranged from 0.64 to 1.00 by nonlinear regression analyses. The reactions for 7-OOH and 5,6-EP formations fit the second order, whereas all of the other reactions fit the first order.

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Received for review October 12, 2005. Revised manuscript received December 13, 2005. Accepted December 19, 2005. This study was supported by a grant (NSC-91-2313-B-030-006) from the National Science Council, Taiwan.

JF052529R