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Formation of Cholesterol Oxidation Products in Marinated Foods during Heating

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The objectives of this study were to develop a gas chromatography−mass spectrometry (GC-MS) method to analyze the contents of cholesterol oxidation products (COPs) in marinated eggs, pork, and juice and to compare the effect of heating time and soy sauce or sugar on the formation of COPs. By using a silica cartridge for purification and GC-MS with selected ion monitoring for detection, seven COPs, including 7α-hydroxycholesterol, 7β-hydroxycholesterol, 5,6α-epoxycholesterol, 5,6β-epoxycholesterol, 5α-cholestane-3β, 5,6β-triol, 5-cholesten-3β-25-diol, and 7-ketocholesterol, as well as internal standard 5α-cholestane, were resolved within 16 min by using a HP-5MS capillary column. During marinating, the levels of most COPs followed an increasing trend with increasing heating time. However, a higher amount of COPs was generated for ground pork as compared to eggs. The incorporation of soy sauce or sugar (1 and 10%) was effective in inhibiting COPs formation, with the latter being more pronounced than the former in both marinated eggs and pork.

KEYWORDS: Cholesterol oxidation products; marinated eggs; marinated pork; GC-MS; Maillard browning reaction products

INTRODUCTION

Cholesterol oxidation products (COPs), formed from cholesterol during food processing or storage, can be widely distributed in cholesterol-rich products such as eggs and meat (*1*, *2*). It has been well established that in the presence of oxygen, light, and heat, cholesterol can be susceptible to COPs formation because of the presence of one unsaturated double bond (*3*, *4*). The mechanism of COPs formation has been reported to be similar to lipid oxidation, which involves free radical reaction and hydroperoxide formation as well as degradation (*3*, *4*).

Numerous reports have revealed the intake of COPs in excess may possess cytotoxic, mutagenic, and carcinogenic effects (*5*, *6*). Among the various COPs, 7-ketocholesterol (7-keto) was shown to be the most potent inhibitor of proliferation, while 5-cholesten-3β-25-diol (25-OH) was the potent inducer of apoptosis (*5*). Therefore, it is of great importance to learn about the variety and amount of COPs in cholesterol-rich food products.

Many methods have been developed to analyze the various COPs in food products (*2*, *7*, *8*). Raith et al. (*2*) developed a liquid chromatography−mass spectrometry (LC-MS) method to determine COPs in processed foods. However, only five COPs were separated within 20 min. Shan et al. (*7*) compared the advantages and disadvantages for analysis of COPs by thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC), and gas chromatography (GC) and concluded that GC provides better efficiency in terms of identification and quantitation than TLC or HPLC.

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Marinating, a traditional Chinese cooking method, is often conducted by immersing the food items in a flavor-rich fluid (marinated juice) containing different kinds of ingredients such as soy sauce and sugar and heating at about 100 °C for an extensive period of time (1 h or above) in a closed pan (*9*, *10*). Of the various food products, both eggs and ground pork have become the most popular food commodities in Taiwan (*9*, *10*). However, the content and variety of COPs present in these marinated food products remain unknown. The objectives of this study were to develop a GC-MS method for analysis of COPs contents in marinated eggs, pork, and juice and to compare the effect of heating time and the addition of sugar or soy sauce on the formation or inhibition of COPs.

MATERIALS AND METHODS

Materials. All samples of 40 kg of ground pork, 500 eggs, 1.6 L of soy sauce, and 3 kg of sugar (sucrose) were purchased from a local market in Taipei, Taiwan. Cholesterol and COPs standards, including 5,6α-epoxycholesterol (5,6α-EP), 5,6β-epoxycholesterol (5,6β-EP), 5α-cholestane-3β, 5,6β-triol (triol), 7-keto, 25-OH, and internal standard 5α-cholestane, were from Sigma (St. Louis, MO). The other COPs standards, 7α-hydroxycholesterol (7α-OH) and 7β-hydroxycholesterol (7β-OH), were from Steraloids Co. (Wilton, NH). The purities of all of these standards ranged from 95 to 99% and were used without further purification. The derivatization reagent Sylon BTZ was from Supelco Co. (Bellefonte, PA). Reagents, including zinc sulfate, cupric sulfate, citric acid, potassium iodide, sulfuric acid, starch, sodium thiosulfate, potassium hydroxide, and acetic acid were from Merck Co. (Darmstadt, Germany). Formaldehyde (37% solution) was from J. T. Baker Co. (Phillipsburg, NJ), and glacial acetic acid was from Nacalai Tesque Co. (Kyoto, Japan). Standard sodium hydroxide solution (0.1 N) was from Showa Co. (Kyoto, Japan). Anhydrous sodium sulfate and *meta-*

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phosphoric acid were from Riedel de Häen Co., and PICB (1-pentane sulfuric acid sodium salt) was from Sigma. A GC HP-5MS capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness, 5% diphenyl polysiloxane and 95% dimethyl polysiloxane) was used to separate the various COPs.

Instrumentation. The GC instrument (model 6890) equipped with a mass spectrometer (model 5973) was from Agilent Technologies (Palo Alto, CA). The freeze dryer (model FD 24) was from Chin-Ming Co. (Taipei, Taiwan). The rotary evaporator (model N-1) was from Eyela Co. (Tokyo, Japan). The high-speed centrifuge (Sorvall RC5C) was from Du Pont Co. (Wilmington, DE). The spectrophotometer (model E3021) was from Cecil Co. (Cambridge, England). The silica cartridge (sorbent weight, 500 mg; column volume, 6 mL) was from Agilent Technologies.

Extraction and Purification of COPs in Food Sample. A method similar to that used by Razzazi-Fazeli et al. (*1*) was modified for extraction and purification of COPs. A 1 g egg or 3 g of ground pork sample was mixed with 30 mL of chloroform−methanol (2:1, v/v) in a flask and shaken for 30 min, after which the mixture was filtered through filter paper with 0.5 g of anhydrous sodium sulfate to remove residual moisture. The filtrate was evaporated to dryness under vacuum and dissolved in 5 mL of hexane and filtered through a 0.45 μm membrane filter for purification. For extraction of COPs from marinated juice, a 30 mL sample was mixed with 30 mL of hexane and the mixture was shaken for 30 min, followed by sonication for 10 min to remove excessive air bubbles, collection of the upper layer (hexane layer), evaporation to dryness under vacuum, and dissolution in 5 mL of hexane for purification. The extract (5 mL) was poured into a silica cartridge, which was previously activated with 15 mL of hexane, followed by elution of impurities successively with a solvent system of 10 mL of hexane−diethyl ether (95:5, v/v), 25 mL of hexane−diethyl ether (90:10, v/v), and 15 mL of hexane−diethyl ether (80:20, v/v). Finally, the COPs were eluted with 5 mL of acetone. After evaporation to dryness under nitrogen, the residue was dissolved in 1 mL of pyridine and 50 μL was collected for derivatization prior to GC-MS analysis. A 50 μL sample was poured into a 250 μL vial insert, followed by addition of 10 μL of internal standard 5α-cholestane (5 ppm) and 50 μL of Sylon BTZ. Then, the mixture was left to stand in the dark for 1 h to allow complete derivatization, after which 1 μL of sample was collected for GC-MS analysis.

GC-MS Analysis of COPs. A HP-5 MS capillary column was used to separate seven COPs, including 7α-OH, 7β-OH, 5,6α-EP, 5,6β-EP, 7-keto, 25-OH, and triol, as well as internal standard 5α-cholestane within 16 min with helium as the carrier gas, a flow rate of 0.8 mL/min, and a split ratio at 5:1. The injector temperature was 280 °C, and the column temperature was programmed as follows: 220 °C in the beginning, increased to 270 °C at a rate of 15 °C/min, to 280 °C at 1 °C/min, maintained for 2 min, to 290 °C at 5 °C/min, maintained for 10 min. The interface temperature for GC-MS was 270 °C, with an electron multiplier voltage of 1360 V and an ionization energy of 70 V. Perfluorotributylamine was used for auto tune with *m*/*z* intensity at 69, 219, and 502. The selected ion monitoring (SIM) mode was used to detect COPs according to the elution order: (i) 0−13 min; *m*/*z* 217, 218, 372, 456, 457, and 458 for 5α-cholestane, 7α-OH and 7β-OH; (ii) 13−14 min; *m*/*z* 366, 384, 441, 442, 459, and 474 for 5,6α-EP and 5,6β-EP; (iii) 14−15.4 min; *m*/*z* 321, 403, 404, 456, and 457 for triol; (iv) 15.4−16 min; *m*/*z* 131, 367, 457, 472, 473, and 474 for 25-OH and 7-keto, of which 25-OH was mainly detected by the ion (*m*/*z* 131) and 7-keto was detected by the other five ions (**Table 1**). In addition, the various COPs were also identified by comparing retention times of unknown peaks with reference standards and cochromatography with added standards. Quantitation was carried out by using an internal standard 5α-cholestane. Seven concentrations of 50 μL of each COP standard (3,2, 0.9, 0.7, 0.6, 0.5, and 0.3 ppm for 5,6α-EP; 3, 0.8, 0.6, 0.5, 0.4, 0.3, and 0.2 ppm for 5,6β-EP; and 3, 0.8, 0.5, 0.4, 0.3, 0.1, and 0.05 ppm for the other COPs) were prepared and mixed with 10 μL of internal standard (0.5 ppm) and 50 μL of Sylon BTZ in a vial, and the mixture was standing in the dark at 25 °C for 1 h for derivatization. Then, 1 μL was collected and injected into GC. The calibration curve of each COP standard was obtained by plotting the concentration ratio against the area ratio, and the linear regression equations and correlation coefficients (r^2) were calculated. Eight concentrations (10, 20, 25, 30, 50, 200, 300, and 400 ppm) were also prepared for each COP standard, and the detection limits (DLs) were based on S/N ≥ 3, while quantitation limits (QLs) were based on S/N ≥ 10. Recovery was performed by adding two concentrations (40 and 60 ppm) of a mixture of COPs standards (1 mL) to sample for extraction, purification, and separation. After quantitation, the recovery of each COP was obtained based on the ratio of the amount of each COP standard after and before GC. The amounts of various COPs in food samples were calculated using a formula as described in a previous study (*11*).

Table 1. Ratio of Mass to Charge (*m*/*z*) Used for Detection of COPs by SIM Mode According to Elution Order

COPs	group[a]	*m*/*z*[b]
5α-cholestane	1	*217*, *218*, *372*, 456, 457, 458
7α-OH	1	217, 218, 372, *456*, *457*, *458*
7β-OH	1	217, 218, 372, *456*, *457*, *458*
5,6β-EP	2	*366*, *384*, 441, 442, *459*, *474*
5,6α-EP	2	366, 384, *441*, *442*, 459, 474
triol	3	*321*, *403*, *404*, *456*, *457*
25-OH	4	*131*, 367, 457, 472, 473, 474
7-keto	4	131, *367*, *457*, *472*, *473*, *474*

[a] Group 1, 0–13 min; group 2, 13–14 min; group 3, 14–15.4 min; and group 4, 15.4–22 min. [b] Values in italics represent the major ions used to detect COPs.

Effect of Marinating Time on COPs Formation. The standard formula of 3 kg unmarinated juice was prepared by mixing 300 g of soy sauce (10%), 30 g of sugar (1%), and 2670 g of water (89%), and the mixture was poured into a 4 L sauce pan and preheated to 100 °C. Prior to marinating, eggs were heated in boiling water for 30 min and shelled. The 1 kg ground pork or 10 shelled eggs were poured into the sauce pan separately and marinated for 4, 6, 8, 12, and 24 h for the former and 4, 6, 8, 10, 12, and 24 h for the latter. Duplicate experiments were carried out, and a total of 24 treatments were used, in which raw ground pork and shelled eggs were used as control treatments. After the desired heating time shown above was reached, a sample of 50 g of ground pork, 50 g of egg, or 250 mL of juice were collected separately. Before extraction, both ground pork and eggs were freeze-dried and ground into fine material, and a sample of 3 g of ground pork, 1 g of egg, or 30 mL of juice was collected separately for subsequent GC-MS analysis.

Effect of Juice Composition on COPs Formation. Five combinations of 3 kg of unmarinated juice were prepared as follows: (i) soy sauce (300 g, 10%) and water (2700 g, 90%); (ii) sugar (30 g, 1%) and water (2970 g, 99%); (iii) soy sauce (30 g, 1%) and water (2970 g, 99%); (iv) sugar (300 g, 10%) and water (2700 g, 90%); and (v) water (3000 g, 100%). Each juice was poured into a sauce pan preheated to 100 °C. Likewise, 1 kg of ground pork or 10 shelled eggs was poured into the pan separately and heated for 24 h for the former and 10 h for the latter. Duplicate experiments were performed, and a total of 20 heating treatments were used. The sample preparation procedure prior to analysis was the same as described above.

Analysis of Residual Amino Nitrogen. The residual amino nitrogen was analyzed using a method as described by Lee and Lai (*12*). Briefly, a 3 g sample of egg or pork was mixed with 30 mL of deionized water and the mixture was shaken for 30 min and left to stand for 30 min, the mixture was filtered by suction, and the filtrate was collected. The residue was dissolved in 30 mL of deionized water and filtered again. The filtrates were combined and diluted to 60 mL with deionized water. For marinated juice, a 25 mL sample was collected and directly analyzed. A 25 mL sample solution of egg, pork, or juice was poured into a flask separately and mixed with 20 mL of formaldehyde solution and 20 mL of deionized water. Meanwhile, another 25 mL sample solution was also poured into a flask containing 40 mL of deionized water, which was used as a blank solution. A few drops of indicator phenolphthalein (0.5%) was added to each flask and titrated with 0.05 N NaOH until a red color appeared. The amount of residual amino nitrogen was calculated using a formula as described by Lee and Lai (*12*).

Analysis of Reducing Sugar. A method based on Luff-Schoorl was used to analyze reducing sugar in food samples (*13*). Briefly, a 10 g sample of egg or pork was mixed with 50 mL of deionized water, shaken for 30 min, and centrifuged for 20 min (6000*g*). The supernatant was collected, and the residue was repeatedly extracted. Both supernatants were pooled for reducing sugar analysis. For marinated juice, a 25 mL sample was collected and analyzed directly for reducing sugar. A 25 mL sample solution of egg, pork, or juice was poured into a 100 mL volumetric flask separately and mixed with 50 mL of deionized water, 5 mL of Carrez solution I, and 5 mL of Carrez solution II. After it was shaken thoroughly, the solution was diluted to 100 mL with deionized water and left to stand for 10 min for filtration. A 25 mL filtrate was poured into a flask containing 25 mL of Loffœche solution and 1 g of boiling stone, which was heated until boiling and maintained for 10 miin. After the mixture was cooled to room temperature, a 10 mL potassium iodide solution (30%) was added, followed by gradual addition of 25 mL of sulfuric acid (25%) and 2 mL of starch solution (1%), and then, the mixture was titrated with 0.1 N sodium thiosulfate until a yellow color appeared. For a blank test, the same procedure was used with the exception that 25 mL of deionized water was used instead of a sample solution. The amount of reducing sugar was calculated based on a formula and a table as described by Kruger and Bielig (*13*).

RESULTS AND DISCUSSION

Analysis of COPs by GC-MS. On the basis of a previous report by Hu and Chen (*11*), they used a HP-5MS capillary column and developed a temperature programming condition to resolve eight COPs, including 7α-OH, 7β-OH, 5,6α-EP, 5,6β-EP, 3,5-cholestadien-7-one, triol, 6-keto, and 7-keto, within 25 min. However, the separation time is lengthy. Larkeson et al. (*14*) pointed out that the major COPs present in food samples include 7-keto, 7α-OH, 7β-OH, 5,6α-EP, 5,6β-EP, triol, and 25-OH. Because in our pre-experiment, no 6-keto and 3,5-cholestadien-7-one were detected in marinated food samples, only seven COPs, i.e., 7α-OH, 7β-OH, 5,6α-EP, 5,6β-EP, triol, 25-OH, and 7-keto, were investigated in this study. After numerous studies, the most appropriate temperature programming condition by using a HP-5MS capillary column was found to be 220 °C in the beginning, raised to 270 °C at a rate of 15 °C/min, to 280 °C at 1 °C/min, maintained for 2 min, raised to 290 °C at 5 °C/min, and maintained for 10 min. Seven COPs and the internal standard 5α-cholestane were separated within 16 min, with the retention times being 7.55 min for 5α-cholestane, 10.87 min for 7α-OH, 12.65 min for 7β-OH, 13.34 min for 5,6β-EP, 13.56 min for 5,6α-EP, 14.91 min for triol, 15.85 min for 25-OH, and 15.99 min for 7-keto (**Figure 1**). Because of possible interferences of COPs identification by the presence of impurities in food samples and enhancement of sensitivity, the SIM mode was used for detection. The ratio of mass to charge (*m*/z) used for detection of COPs is shown in **Table 1**, and the various COPs were detected by dividing into four groups according to elution order.

Extraction and Purification of COPs. Initially, a method developed by Nourooz-Zadeh (*15*) was used to extract and purify COPs from marinated foods. However, a low recovery (34%) was found for triol when using a NH_2 cartridge for purification. On the basis of the reports by several authors (*1*, *8*, *16*), they demonstrated that a silica cartridge could provide a better purification efficiency than a NH_2 cartridge. Thus, in our study, a silica cartridge was used instead. A silica cartridge was prewet with 15 mL of hexane for activation, after which a 5 mL sample extract was poured into the cartridge and three solvent systems as described above were added in order to remove impurities. Then, the various COPs were eluted with 5 mL of acetone, and the eluate was evaporated to dryness under nitrogen and dissolved in 1 mL of pyridine for derivatization and GC-MS analysis. A high recovery was found for 7α-OH, 7β-OH, triol, and 7-keto, which equaled to 100.5, 99.5, 98.5, and 118.6% for eggs, respectively, and 92.1, 94.4, 98.8, and 108% for pork. However, both 5,6α-EP and 5,6β-EP showed a lower recovery, which amounted to 85.5 and 83.1% for eggs and 74.4 and 81.4% for pork, respectively, which may be due to instability of the epoxy-containing COPs during heating (*3*, *4*). For marinated pork juice, a high recovery was observed for only 7α-OH (102.2%) and 7β-OH (101.6%), with the other five COPs showing low recovery (<84.4%). No recovery was performed for marinated egg juice since no COPs were detected. The r^2 values for all seven COPs were higher than 0.99, and the linear equations were as follows: 7α-OH ($y = 1.1177x + 0.0539$), 7β-OH ($y = 2.0489x + 0.0444$), 5,6β-EP ($y = 18.766x + 0.0381$), 5,6α-EP ($y = 31.063x + 0.359$), triol ($y = 2.5066x + 0.0585$), 25-OH ($y = 3.1997x - 0.0257$), and 7-keto ($y = 7.8981x - 0.0117$). The DLs of COPs standards based on S/N ≥ 3 for 7α-OH, 7β-OH, 5,6α-EP, 5,6β-EP, triol, 25-OH, and 7-keto were 20, 25, 200, 300, 30, 50, and 50 ppb, respectively, while the QLs based on S/N ≥ 10 were 66, 83, 660, 990, 99, 165, and 165 ppb. Nevertheless, a study by Guardiola et al. (*16*) reported that both DL and QL cannot be determined by COPs standards alone. Instead, some other factors such as sample weight, extraction, purification, and derivatization steps should also be taken into consideration. In our experiment, the extraction, purification, and derivatization steps are the same for all of the food samples, and the only difference is sample weight, i.e., 30 g for marinated juice, 3 g for pork, and 1 g for egg. Therefore, both DL and QL for marinated juice and pork were adjusted to be lower than COPs standards by 30- and 3-fold, respectively, with marinated eggs remaining the same (**Table 2**).

Effects of Heating Time on the COPs Contents in Marinated Foods. Table 3 shows the effect of heating time on the COPs contents in marinated eggs and pork as well as pork juice. Prior to marinating, shelled eggs were found to contain three COPs, 7-keto (1254 ng/g), 7α-OH (1240 ng/g), and 7β-OH (944 ng/g). In general, the levels of most COPs followed an increasing trend with increasing heating time, and triol was not formed until 4 h. After extensive heating for 24 h, an increase by 809, 762, and 217 ng/g was observed for 7α-OH, 7β-OH, and triol, respectively. The formation of triol is probably due to hydrolysis of 5,6α-EP or 5,6β-EP under acidic conditions (*3*, *4*), as evidenced by a drop of pH from 7.53 to 6.13 in eggs during marinating. This result may also explain why both 5,6α-EP and 5,6β-EP were not detected during heating. For 7-keto, it showed a decreased tendency over a 12 h heating period, followed by a sharp increase thereafter. Theoretically, 7-keto can be formed through dehydration of cholesterol hydroperoxide, which can also be reduced to form 7α-OH or 7β-OH, however (*3*, *4*). Thus, the decrease of 7-keto is probably due to the reduction reaction proceeding faster than the dehydration reaction during the initial heating period, while a reversed trend occurred after prolonged heating for 24 h. The total amount of COPs was raised to 5550 ng/g after 24 h of heating, which was 1.61 times higher than the control treatment. Nevertheless, no COPs were detected in marinated egg juice, indicating the leaching of COPs from solid eggs to juice would be difficult.

The effect of heating time on the COPs contents in marinated ground pork is also shown in **Table 3**. Raw ground pork was found to contain four COPs, i.e., 7-keto (198 ng/g), 7β-OH (118.1 ng/g), 25-OH (97.2 ng/g), and 7α-OH (76.9 ng/g). As

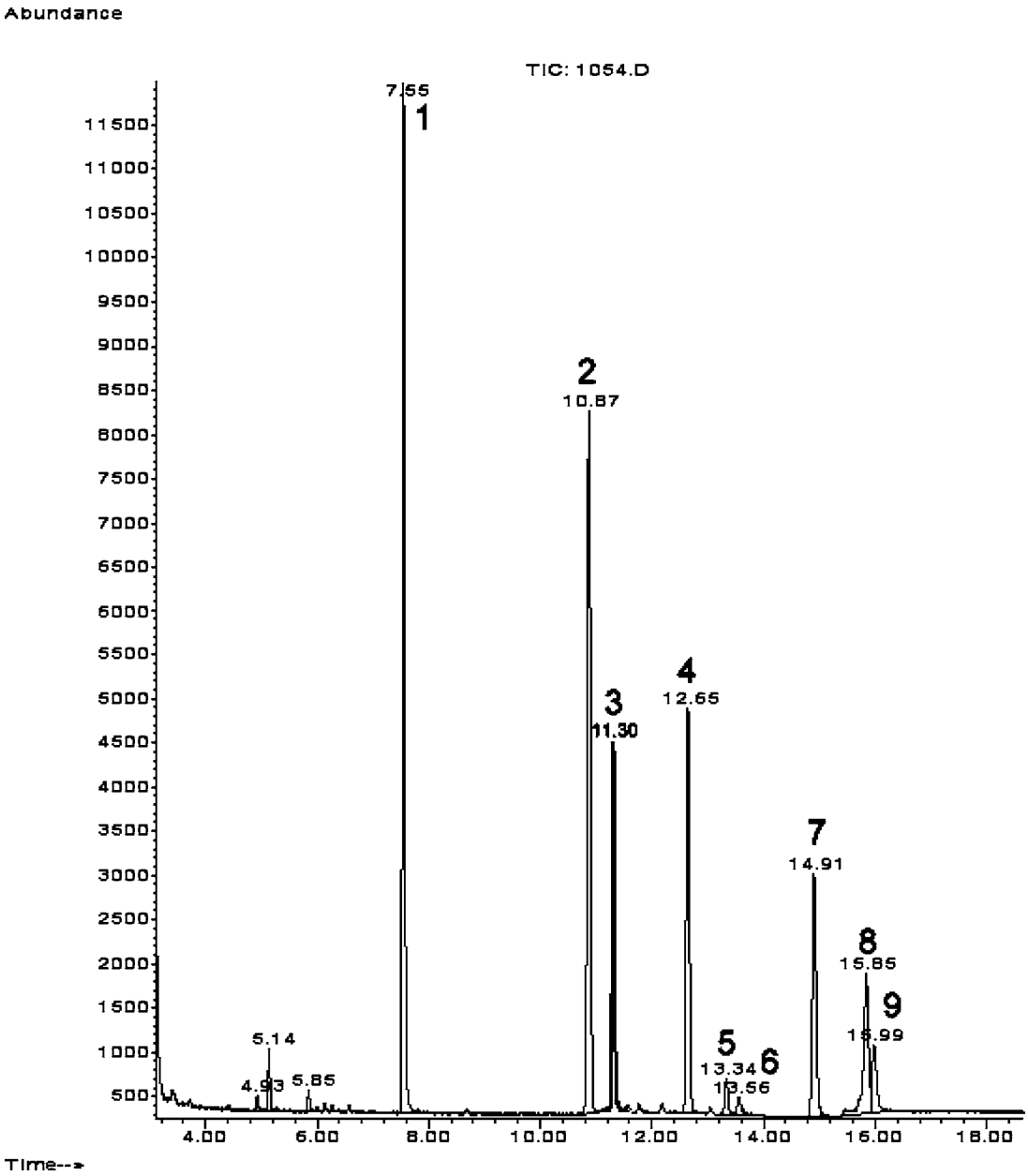

Figure 1. GC-MS chromatogram of seven COPs standards plus internal standard 5α-cholestane detected by SIM mode. Peaks: 1, 5α-cholestane; 2, 7α-OH; 3, cholesterol; 4, 7β-OH; 5, 5,6β-EP; 6, 5,6α-EP; 7, triol; 8, 25-OH; and 9, 7-keto. Chromatographic conditions are described in the text.

Table 2. DL and QL Relative to the Whole Method for Marinated Eggs, Pork, and Juice

sample	parameter	7α-OH	7β-OH	5,6β-EP	5,6α-EP	Triol	25-OH	7-keto
marinated egg	DL	20.0[a]	25.0	200.0	300.0	30.0	50.0	50.0
	QL	66.0	83.0	660.0	990.0	99.0	165.0	165.0
marinated pork	DL	6.7	8.3	66.7	100.0	10.0	16.7	16.7
	QL	22.1	27.4	220.1	330.0	33.0	55.1	55.1
marinated pork juice	DL	0.7	0.8	6.7	10.0	1.0	1.7	1.7
	QL	2.3	2.6	22.1	33.0	3.3	5.6	5.6

[a] Results expressed as ppb in marinated samples.

compared to shelled eggs (3438 ng/g), the total amount of COPs (490 ng/g) in raw ground pork was much lower, which can be attributed to the presence of a higher level of cholesterol in eggs and heating treatment for eggs before marinating. In most instances, the contents of 7α-OH, 7β-OH, 25-OH, and 7-keto increased with increasing heating time, and a pronounced increase by 70.1, 157.9, 64.8, and 75 ng/g was found 24 h after heating, respectively. Another COP, triol, did not form until a heating time of 4 h was reached, and a slight change was followed afterward, implying that triol may also undergo degradation after prolonged heating. Just like marinated eggs, the formation of triol during the initial heating period should be due to hydrolysis of 5,6α-EP or 5,6β-EP under acidic conditions, as shown by a pH drop from 6.38 to 5.53. However, both 5,6α-EP and 5,6β-EP remained undetected over a 24 h heating period. An inconsistent change occurred for 7α-OH and

Table 3. Effect of Heating Time on the Contents (ng/g) of COPs in Marinated Eggs and Pork as Well as Pork Juice[a]

	time (h)						
COPs	0	4	6	8	10	12	24
				marinated eggs			
7α-OH	1240 ± 46[b] d	1650 ± 8 bc	1391 ± 71 cd	1596 ± 90 c	1675 ± 113 bc	1906 ± 196 ab	2049 ± 196 a
7β-OH	944 ± 44 d	972 ± 173 cd	1382 ± 222 abc	1289 ± 225 bcd	1306 ± 60 abcd	1396 ± 221 ab	1706 ± 50 a
triol	ND	137 ± 38 bc	121 ± 17 bc	119 ± 20 c	140 ± 14 bc	175 ± 32 ab	217 ± 5 a
25-OH	ND	ND	ND	ND	ND	ND	ND
7-keto	1254 ± 35 b	1195 ± 140 bc	921 ± 164 cd	934 ± 73 cd	866 ± 20 d	833 ± 179 d	1578 ± 99 a
total	3438 ± 125 c	3954 ± 359 bc	3815 ± 474 bc	3938 ± 408 bc	3987 ± 207 bc	4310 ± 628 b	5550 ± 350 a
				pork			
7α-OH	76.9 ± 1.8 g	122 ± 13 ef	107 ± 7 f	115 ± 19 f		123 ± 16 ef	147 ± 7 e
7β-OH	118.1 ± 0.8 g	194 ± 27 f	155 ± 19 fg	185 ± 23 f		216 ± 41 ef	276 ± 28 e
triol	ND	35.6 ± 0.4 f	34.0 ± 0.6 g	34.9 ± 0.5 fg		35.1 ± 0.2 f	38.2 ± 0.1 e
25-OH	97.2 ± 5.3 h	118 ± 5 g	136 ± 3 f	124 ± 5 g		139.6 ± 0.9 f	162 ± 7 e
7-keto	198 ± 13 fg	227 ± 7.5 ef	234 ± 4 ef	161 ± 35 g		226 ± 39 ef	273 ± 13 e
total	490 ± 21 g	697 ± 53 f	666 ± 34 f	620 ± 83 fg		740 ± 97 f	896 ± 55 e
				pork juice			
7α-OH		5.6 ± 1.0 j	6.2 ± 1.1 j	6.9 ± 0.4 j		11.3 ± 1.8 i	13.5 ± 0.5 i
7β-OH		7.6 ± 1.2 k	10.3 ± 1.3 k	9.9 ± 0.1 k		16.6 ± 1.0 j	20.8 ± 1.4 i
triol		ND	ND	ND		ND	ND
25-OH		8.0 ± 1.1 j	6.9 ± 0.6 j	7.8 ± 1.4 j		12.8 ± 0.5 i	14.4 ± 1.4 i
7-keto		13.6 ± 1.1 l	17.0 ± 0.5 k	17.7 ± 1.2 k		23.4 ± 1.3 j	29.1 ± 1.2 i
total		34.8 ± 4.4 l	40.4 ± 3.5 kl	42.3 ± 3.1 k		64.1 ± 4.6 j	77.8 ± 4.5 i

[a] Values within a row with different letters are significantly different ($p < 0.05$). ND, not detected. Blank cell, not analyzed. [b] Mean of duplicate analyses ± standard deviation.

7β-OH during heating, which may be explained by both dehydrogenation and degradation reactions proceeding simultaneously (*3*, *4*). The total amount of COPs rose by 406 ng/g 24 h after heating, which was 1.83 times higher than the control treatment, revealing that cholesterol oxidation could proceed faster in ground pork than in eggs during marinating. This phenomenon may be accounted for by the presence of solid form for eggs, in which the yolk was covered by egg white and should limit its exposure to air. Conversely, the surface exposure to air was much larger for ground pork, which should favor promotion of cholesterol oxidation. In comparison with marinated pork, a much lower amount of COPs was formed in pork juice 24 h after heating, which equaled 13.5, 20.8, 14.4, and 29.1 ng/g for 7α-OH, 7β-OH, 25-OH, and 7-keto, respectively (**Table 3**). On the contrary, the other COPs, 5,6α-EP, 5,6β-EP, and triol, were not detected probably because of dilution effects.

Effect of Soy Sauce on the COPs Formation in Marinated Foods. Table 4 shows the effect of soy sauce and sugar on the formation of COPs in marinated eggs after heating for 10 h. The heating time of 10 h was selected mainly because during extensive marinating of eggs in the presence of 1% soy sauce or 1% sugar, moisture may penetrate into eggs to cause them to burst. However, this phenomenon was not observed for the treatment containing 10% soy sauce or 10% sugar. The total amount of COPs was raised from 3438 to 6383 ng/g, an increase by 2945 ng/g 10 h after heating for the control treatment containing only water, whereas an increase of 549 ng/g was shown for the treatment containing 10% soy sauce, 1% sugar, and 89% water (standard formula A), implying that the addition of soy sauce or sugar may inhibit COPs formation. Likewise, in the presence of 1% soy sauce and 99% water, the contents of triol (161 ng/g) and 7-keto (2121 ng/g) dropped by 105 and 265 ng/g, respectively, when compared to the control treatment. A greater loss by 612, 337, and 603 ng/g also occurred for 7α-OH, 7β-OH, and 7-keto, respectively, for the treatment containing 10% soy sauce and 90% water (**Table 4**). This outcome further demonstrated that the incorporation of a high level (10%) of soy sauce was more effective in retarding COPs formation, which may be due to the formation of Maillard browning reaction products (MRPs) during heating. Several reports have revealed that the formation of MRPs may possess antioxidant activity toward lipid oxidation (*17*−*19*). In addition, caramelization caused by sugar degradation during heating may also be responsible for the browning color. To further prove the formation of MRPs in our experiment, the absorbance measured at 420 nm was used as a browning index. A higher browning index (2.738) was shown for the treatment containing 10% soy sauce than that containing 1% soy sauce (0.295) (**Table 5**). Similarly, in marinated ground pork (**Table 4**), with 1% soy sauce, the levels of 7α-OH (251 ng/g), 7β-OH (529 ng/g), 7-keto (673 ng/g), and triol (58.9 ng/g) declined by 114, 267, 123, and 16.1 ng/g, respectively, as compared to the control treatment, and a greater loss by 269, 593, 560, and 39.3 ng/g was shown for 10% soy sauce. In addition, no 5,6α-EP or 5,6β-EP was detected. Marinated pork juice exhibited the same tendency (**Table 4**): A decrease by 61.7, 79.5, 84.9, 41.7, and 0.5 ng/g was observed for 7α-OH, 7β-OH, 7-keto, 5,6β-OH, and triol, respectively, in the presence of 1% soy sauce; however, for 10% soy sauce, a sharp decline by 80.2, 119.3, 35.3, and 115.9 ng/g for 7α-OH, 7β-OH, 25-OH, and 7-keto was found. Also, no triol, 5,6α-EP, and 5,6β-EP were detected. Moreover, the browning index in marinated pork was higher for 10% soy sauce (3.968) than for 1% soy sauce (0.474) (**Table 5**), demonstrating that a larger amount of MRPs produced during heating could provide a better inhibition effect in cholesterol oxidation. Nevertheless, the presence of some other functional components such as isoflavone in soy sauce in retarding cholesterol oxidation cannot be ignored.

Effect of Sugar on the COPs Formation in Marinated Foods. The addition of 1% sugar to marinated eggs resulted in a decrease of 495, 294, 119, and 779 ng/g for 7α-OH, 7β-OH, triol, and 7-keto, respectively, while a larger decline by 1039, 308, 135, and 1384 ng/g was shown for 10% sugar. Both 25-OH and 5,6α-EP or 5,6β-EP were not detected (**Table 4**). This result clearly indicated, just like soy sauce, that sugar may also be effective in inhibiting cholesterol oxidation. Also, the higher the level of sugar, the greater the inhibition effect of cholesterol oxidation. Similar to soy sauce, a higher browning index (0.453)

Table 4. Effects of Soy Sauce or Sucrose on the Formation of COPs (ng/g) in Marinated Eggs and Pork as Well as Pork Juice after Heating for 10 or 24 h[a]

	ingredient						
COPs	100% water	1% soy sauce	1% sucrose	10% soy sauce	10% sucrose	standard formula A	standard formula B
				marinated eggs			
7α-OH	2143 ± 76[b] b	2548 ± 24 a	1648 ± 167 c	1531 ± 15 c	1104 ± 86 d	1675 ± 113 c	NA
7β-OH	1417 ± 164 a	1284 ± 159 ab	1123 ± 16 b	1080 ± 16 b	1109 ± 22 b	1306 ± 60 ab	NA
5,6β-EP	ND	ND	ND	ND	ND	ND	NA
5,6α-EP	ND	ND	ND	ND	ND	ND	NA
triol	266 ± 16 a	161 ± 13 bc	147 ± 16 bc	176 ± 12 b	131 ± 12 c	140 ± 14 bc	NA
25-OH	171 ± 15 a	ND	ND	ND	ND	ND	NA
7-keto	2386 ± 56 a	2121 ± 127 b	1607 ± 108 c	1783 ± 39 c	1002 ± 30 d	866 ± 20 d	NA
total	6383 ± 327 a	6114 ± 323 a	4525 ± 307 b	4570 ± 82 b	3346 ± 150 d	3987 ± 207 c	
				pork			
7α-OH	365 ± 38 e	251 ± 28 f	312 ± 32 ef	96 ± 16 g	120 ± 16 g	NA	147 ± 7 g
7β-OH	796 ± 69 e	529 ± 48 f	607 ± 77 f	203 ± 26 g	214 ± 33 g	NA	276 ± 28 g
5,6β-EP	ND	ND	ND	ND	ND	NA	ND
5,6α-EP	ND	ND	ND	ND	ND	NA	ND
triol	75 ± 13 e	58.9 ± 4.2 efg	63 ± 15 ef	35.7 ± 2.0 h	50.4 ± 5.0 fgh	NA	38.2 ± 0.1 gh
25-OH	194 ± 24 ef	210 ± 66 ef	209 ± 34 ef	253 ± 21 e	99 ± 15 g	NA	162 ± 7 fg
7-keto	796 ± 78 e	673 ± 48 f	627 ± 44 f	236 ± 52 g	159 ± 27 g	NA	273 ± 13 g
total	2226 ± 222 e	1722 ± 194 f	1818 ± 202 f	824 ± 117 g	642 ± 96 g	NA	896 ± 55 g
				pork juice			
7α-OH	96 ± 13 i	34.3 ± 5.9 j	26.7 ± 4.1 jk	15.8 ± 2.1 k	22.8 ± 3.7 jk	NA	13.5 ± 0.5 k
7β-OH	140 ± 18 i	60.5 ± 3.8 j	46.2 ± 3.0 jk	20.7 ± 3.4 l	36.9 ± 2.0 kl	NA	20.8 ± 1.4 l
5,6β-EP	41.7 ± 6.4 i	ND	ND	ND	ND	NA	ND
5,6α-EP	37.7 ± 6.4 i	40.9 ± 1.0 i	ND	ND	ND	NA	ND
triol	7.6 ± 1.7 i	7.1 ± 0.4 ij	3.8 ± 0.8 j	ND	5.6 ± 2.9 ij	NA	ND
25-OH	47.6 ± 5.7 j	60.9 ± 6.7 i	26.0 ± 3.8 k	12.3 ± 2.7 l	20.3 ± 4.4 kl	NA	14.4 ± 1.4 l
7-keto	148 ± 10 i	63.1 ± 4.7 j	50.0 ± 3.4 j	32.1 ± 5.4 k	49.8 ± 5.2 j	NA	29.1 ± 1.2 k
total	519 ± 61 i	267 ± 23 j	153 ± 15 k	81 ± 14 lm	135 ± 18 kl	NA	77.8 ± 4.5 m

[a] Values within a row with different letters are significantly different ($p < 0.05$). Standard formula A, 10% soy sauce and 1% sucrose marinated together for 10 h; standard formula B, 10% soy sauce and 1% sucrose marinated together for 24 h. ND, not detected; NA, not applicable. [b] Mean of duplicate analyses ± standard deviation.

Table 5. Changes of Maillard Browning Reaction Index in Marinated Eggs and Pork When Heated with Soy Sauce or Sugar[a]

	Maillard browing reaction index[b]		
ingredient	marinated egg	marinated pork	control treatment[c]
100% water	0.053 ± 0.014[d] e	0.152 ± 0.002 d	
1% soy sauce plus 99% water	0.345 ± 0.002 c	0.474 ± 0.028 c	0.295 ± 0.003 b
10% soy sauce plus 90% water	3.074 ± 0.039 a	3.968 ± 0.166 a	2.738 ± 0.022 a
1% sugar plus 99% water	0.132 ± 0.006 d	0.287 ± 0.005 cd	<0.005 c
10% sugar plus 90% water	0.453 ± 0.003 b	0.823 ± 0.023 b	<0.005 c

[a] Values within a column with different letters are significantly different ($p < 0.05$). Blank cell, used as blank solution. [b] Absorbance measured at 420 nm. [c] Treatment without marinating. [d] Mean of duplicate analyses ± standard deviation.

Table 6. Changes of Free Amino Acid in Marinated Egg and Pork When Heated with Soy Sauce or Sugar[a]

	free amino acid (ppm)[b]	
ingredient	marinated egg	marinated pork
100% water	54.5 ± 2.5b	240.3 ± 1.7c
1% soy sauce plus 99% water	65.7 ± 3.3b	308.4 ± 1.7b
10% soy sauce plus 90% water	721.4 ± 12.4a	847.7 ± 5.0a
1% sugar plus 99% water	34.0 ± 1.7c	197.0 ± 0.0d
10% sugar plus 90% water	9.5 ± 1.5d	204.0 ± 14.9d

[a] Values within a column with different letters are significantly different ($p < 0.05$). [b] Mean of duplicate analyses ± standard deviation.

was shown for 10% sugar than for 1% sugar (0.132) (**Table 5**). In marinated pork containing 1% sugar, the levels of 7α-OH, 7β-OH, triol, and 7-keto were lowered by 53, 189, 12, and 169 ng/g, respectively, as compared to the control treatment (**Table 4**). For 10% sugar, a marked loss by 245, 582, 24.6, 95, and 637 ng/g was attained for 7α-OH, 7β-OH, triol, 25-OH, and 7-keto, respectively. Likewise, a higher browning index was found for 10% sugar (0.823) than for 1% sugar (0.287) in marinated pork (**Table 5**), implying that the former was more effective in inhibiting cholesterol oxidation.

By comparison, the incorporation of sugar to marinated pork or eggs provided a better inhibition effect in cholesterol oxidation than soy sauce. Nevertheless, the effect of sugar on COPs formation in marinated pork juice remains inconsistent. For instance, with 1% sugar in pork juice, the contents of 7α-OH, 7β-OH, triol, 25-OH, and 7-keto decreased by 69.3, 93.8, 3.8, 21.6, and 98 ng/g, respectively, when compared to the control treatment. However, with sugar levels raised to 10%, the COPs formation only showed a slight change, probably because ground pork contained a higher amount of fat, which should make cholesterol oxidation proceed faster in pork juice, even in the presence of a high level of sugar.

To further investigate the role of MRPs in inhibiting cholesterol oxidation, both amino acid and reducing sugar contents in marinated eggs and pork were determined. **Table 6** shows the changes of free amino acid in marinated eggs and pork when heated with sugar or soy sauce. A lower level of free amino acid was found for 10% sugar than for 1% sugar in marinated eggs, which may be due to hydrolysis of the former to produce more reducing sugar during heating, which in turn reacts with amino acid to generate a higher amount of MRPs. For the treatment containing 1 or 10% soy sauce, the discussion on change of amino acid may be ignored since soy sauce itself contains a relatively high amount of amino acid. Surprisingly,

Table 7. Changes of Reducing Sugar Content in Marinated Egg and Pork When Heated with Soy Sauce or Sugar[a]

ingredient	reducing sugar (g/L)[b]	
	marinated egg	marinated pork
100 water	ND	ND
1% soy sauce plus 99% water	0.41 ± 0.01 d	0.37 ± 0.03 d
10% soy sauce plus 90% water	5.28 ± 0.03 a	3.69 ± 0.06 b
1% sugar plus 99% water	1.23 ± 0.00 c	0.80 ± 0.01 c
10% sugar plus 90% water	1.33 ± 0.06 b	13.97 ± 0.00 a

[a] Values within a column with different letters are significantly different ($p < 0.05$); ND, not detected. [b] Mean of duplicate analyses ± standard deviation.

a reversed trend was found for marinated pork; that is, there is no significant difference in amino acid content for the treatments containing 1 and 10% sugar. In contrast, a much higher level of amino acid was shown for marinated pork than for marinated eggs, which may be caused by the difference in heating time, i.e., 24 h for the former and 10 h for the latter. More amino acids may be released after 24 h of heating of pork to react with reducing sugar for MRPs formation.

Table 7 shows the changes of reducing sugar in marinated eggs and pork in the presence of soy sauce or sugar. A different trend was found when compared to amino acid changes in **Table 6**. In marinated pork, a higher amount of reducing sugar (13.97 g/L) was formed for 10% sugar than for 1% sugar (0.8 g/L). However, in marinated eggs, a less amount of reducing sugar (1.33 g/L) was formed for 10% sugar, which was slightly higher than 1% sugar (1.23 g/L). As explained above, this difference may be caused by a longer heating time (24 h) for pork to produce more reducing sugars and amino acids for MRPs formation. In contrast, a lower level of MRPs was generated for solid eggs, which should be less liable to form amino acids and reducing sugars.

By comparison of the results shown above, three COPs, 7α-OH, 7β-OH, and 7-keto, were detected in shelled eggs, while four COPs, 7α-OH, 7β-OH, 25-OH, and 7-keto, were detected in raw ground pork. During marinating, one more COP, triol, was produced for both eggs and pork. Sugar was more effective in inhibiting COPs formation in marinated eggs and pork than soy sauce, whereas soy sauce was more effective in marinated pork juice. Marinated pork was more susceptible to COPs formation than marinated eggs during heating. No COPs were detected in marinated egg juice. The amounts of most COPs increased with increasing time for both marinated eggs and pork. Further research is necessary to characterize MRPs and to study the effect of antioxidants on the COPs formation in marinated foods.

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