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Formation and Inhibition of Cholesterol Oxidation Products in Tea-Leaf Eggs during Marinating

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The objectives of this study were to develop a GC-MS method for determination of cholesterol oxidation products (COPs) in tea-leaf eggs and study the formation and inhibition of COPs as affected by heating time and various ingredients in marinated juice. The various COPs in egg and juice samples were extracted by a solvent system of chloroform/methanol (2:1, v/v), followed by purification using a silica cartridge and GC-MS for subsequent separation and quantitation, with high recovery ranges from 85.9 to 98.3% and from 83.1−100.1% being obtained for egg and juice, respectively. 5α-Cholestane was shown to be an appropriate internal standard for quantitation. A total of five COPs, including 7-keto, 5,6 β-EP, 7α-OH, 7β-OH, and triol, were formed in tea-leaf eggs during marinating, but not in marinated juice. A peak level of total COPs (2272.2 ng/g) was generated in tea-leaf eggs after 24 h of heating, but reduced to 1068.2 ng/g in 48 h. Both the total phenolic and flavonoid compounds in tea-leaf eggs showed a time-dependent increase during marinating and so did the pH and browning index in tea-leaf eggs and juice. The incorporation of soy sauce or black tea leaf into juice was effective in inhibiting COPs formation in tea-leaf eggs, with the latter being more pronounced than the former. The formation of Maillard reaction products during marinating as well as the presence of total phenolic and total flavonoid in black tea leaf was mainly responsible for COPs reduction in tea-leaf eggs.

KEYWORDS: Antioxidant; black tea leaf; soy sauce

INTRODUCTION

Tea-leaf egg, a popular food commodity widely sold in convenience stores all over Taiwan, has received considerable attention in the past decade as it may pose a major threat to human health because of the presence of an abundant amount of cholesterol (*1*). Tea-leaf eggs are routinely produced by cooking fresh chicken eggs in boiled water for 30 min, followed by cracking the shell slightly and a second cooking in marinated juice containing water, soy sauce, sugar, salt, and tea leaf for an extended period of time to obtain tea-flavored eggs (*1*). The main function of tea leaf is to impart characteristic color and flavor to eggs during marinating. It was estimated that approximately 40 million tea-leaf eggs are consumed in Taiwan annually (*2*). However, tea-leaf eggs may be susceptible to the formation of cholesterol oxidation products (COPs) amid a long-time exposure to heat during marinating. Thus, it is imperative to learn about the variety and amount of COPs in tea-leaf eggs during cooking.

COPs, formed through oxidation of cholesterol containing one unsaturated double bond, can be widely distributed in cholesterol-rich food products, especially when exposed to drastic conditions of light, heat, or oxygen (*3*, *4*). It has been well documented that COPs may induce coronary heart disease through accumulation of low-density lipoprotein in blood vessels (*5*) and cause atherosclerosis through apoptosis of vascular smooth muscle cells (*6*). In view of the impact of COPs to human health, finding an appropriate way to prevent COP formation during cooking is extremely important.

Due to the presence of COPs in minor amounts in food products, the analysis of COPs has been a challenge. The extraction of COPs is often carried out by a combination of nonpolar and low-polar solvents such as hexane and ether (*7*), followed by saponification to remove neutral lipid, cholesterol ester, and water-soluble impurities (*8*), with cold saponification being shown to produce a higher recovery than hot saponification, but the former method is more time-consuming (*8*). Instead of saponification, solid-phase extraction is also employed to remove cholesterol ester and impurities (*7*, *9*). Both Sep-Pak C18 and silica gel cartridge used for COP purification have been shown to generate a high recovery as the extraction time was shortened substantially (*3*, *8*). For instance, with the silica gel cartridge a recovery ranging from 83.1 to 118.6% was found for six COPs in eggs, including 7α-OH, 7β-OH, triol, 7-keto, 5,6 α-EP, and 5,6β-EP (*3*). Following extraction and purification, the separation, identification, and quantitation of COPs are conducted by high-performance liquid chromatography (HPLC), coupled with ultraviolet (UV), refractive index (RI), or mass spectrometry (MS) detectors, and gas chromatography−mass spectrometry (GC-MS) (*3*, *10*−*12*). Each method has advantages and limitations. Both normal-phase and reversed-phase HPLC can be used for COP separation; however, the separation efficiency of the former was inferior to that of the latter (*13*). Moreover, a low sensitivity was found for HPLC with RI detection, whereas HPLC with UV detection can be interfered with by impurities to decrease quantitation accuracy (*11*). To overcome this drawback, HPLC-MS was

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used frequently in recent years to separate and quantify COPs, with the detection limit being down to 15−30 ppb (*13*). Compared to HPLC-MS, GC-MS with selected ion monitoring (SIM) detection provided a higher sensitivity and a better separation power in resolving geometrical isomers of COPs, but this method required a lengthy derivatization step (*3*). Nonetheless, GC-MS was often adopted for COP analysis in food products (*3*). The objectives of this study were to develop an analytical method for the determination of COPs in tea-leaf eggs by employing a silica cartridge for purification and a GC-MS technique for quantitation. In addition, the possible inhibition of COP formation by incorporating various ingredients into juice during marinating of tea-leaf eggs was investigated.

MATERIALS AND METHODS

Materials. A total of 500 eggs, 16 L of soy sauce, and 1.5 kg of black tea leaves were purchased from a local store in Taipei, Taiwan. Cholesterol and COP standards, including cholestan-5α,6α-epoxy-3β-ol (5,6α-EP), cholestan-5β,6β-epoxy-3β-ol (5,6β-EP), 5-cholesten-3β-ol-7-one (7-keto), cholestane-3β,5α, 6β-triol (triol), 5-cholestene-3β-25-diol (25-OH), and 5α-cholestane (internal standard), were procured from Sigma (St. Louis, MO). 5-Cholestene-3β,7α-diol (7α-OH) and 5-cholestene-3β,7β-diol (7β-OH) were obtained from Steraloids (Wilton, NH). The purities of all the standards ranged from 95 to 99%, and they were used without further purification. The derivatization reagent containing BSA, TMCS, and TMSI (3:2:3) was from Supelco (Bellefonte, PA).

The HPLC grade solvents including methanol, acetone, ethyl acetate, diethyl ether, *n*-hexane, and chloroform were from Mallinckrodt (Paris, KY). Pyridine was from J. T. Baker (Phillipsburg, NJ). Deionized water was made using a Milli-Q purification system from Millipore Co. (Bedford, MA). Anhydrous sodium sulfate, sodium hydroxide, and sodium carbonate were from Riedal-de Haën Co. (Barcelona, Spain). Gallic acid and catechin standards were from Sigma. Folin−Ciocalteu reagent was from Merck (Darmstadt, Germany). Sodium nitrite and aluminum chloride were from Nacalai Tesque (Kyoto, Japan). An HP-5MS capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness, 5% diphenylpolysiloxane, 95% dimethypolysiloxane) was from Agilent Technologies (Palo Alto, CA).

Instrumentation. The gas chromatograph (model 6890) equipped with a mass spectrometer (model 5973) was from Agilent Technologies. The freeze-dryer (FD24) was from Chuan-Hua Co (Taipei, Taiwan). The rotary evaporator (N-1) was from Eyela (Tokyo, Japan). The spectrophotometer (CE3021) was from Cecil Co. (Cambridge, U.K.). The high-speed centrifuge (Sorvall RC5C) was from DuPont (Wilmington, DE).

Effect of Heating Time on COP Formation in Tea-Leaf Eggs and Marinated Juice. A total of 100 raw eggs were divided into 10 groups with 10 each. Initially, each eggshell was broken slightly, and 10 eggs were poured into an electric cooker containing 3 kg of fresh marinated juice, composed of 300 g of soy sauce (10%), 30 g of black tea leaf (1%), and 2670 g of water (89%). This is the standard formula widely used in convenience stores of Taiwan for marinating tea-leaf eggs. The black tea leaf was packed in a small cloth bag to prevent overheating during cooking. The cooker was heated for about 40 min until the internal temperature of juice reached 100 °C to boil eggs, followed by marinating hard-boiled eggs at 70 °C for 0, 12, 24, 36, and 48 h. The cooker was covered with a lid to prevent moisture loss during heating and replenished with fresh marinated juice every hour to maintain a constant level at 3000 g. This is the standard preparation method for making tea-leaf eggs in convenience stores of Taiwan. The black tea leaf was removed after 1 h of marinating to prevent the formation of an astringent flavor in the eggs. Duplicate experiments were carried out for each heating temperature for a total of 10 treatments. After marinating, 6 eggs and 250 mL of marinated juice were collected from each group separately for COP analysis. Prior to analysis, 6 eggs were shelled, freeze-dried, and ground into powder, after which 1 g was collected for COP analysis.

Effect of Marinated Juice Composition on COP Formation in Tea-Leaf Eggs and Marinated Juice. A total of 100 raw eggs were divided into 10 groups with 10 each. Then five groups of different marinated juices were prepared: (1) 300 g of soy sauce (10%) and 2700 g of water (90%); (2) 30 g of soy sauce (1%) and 2970 g of water (99%); (3) 300 g of black tea leaf (10%) and 2700 g of water (90%); (4) 30 g of black tea leaf (1%) and 2970 g of water (99%); and (5) 3000 g of water (100%). Likewise, each eggshell was broken slightly and 10 eggs were poured into an electric fryer containing 3 kg of fresh marinated juice as described above. The cooker was preheated for 40 min until the internal temperature of the eggs reached 100 °C, followed by marinating at 70 °C for 48 h. Similarly, black tea leaves were removed after 1 h of heating to prevent the production of an astringent flavor in the tea-leaf eggs. Duplicate experiments were performed for a total of 10 treatments. After 48 h of heating, samples of eggs and marinated juices were collected for COP analysis using the same method as above.

Table 1. Ratio of Mass to Charge (*m*/*z*) Used for the Detection of Seven COPs and Internal Standard 5α-Cholestane by SIM Mode

COP	group[a]	*m*/*z*[b]
5α-cholestane	1	217.3, 218.3, 372.4, 456.4, 457.4, 458.4
7α-OH	1	217.3, 218.3, 372.4, 456.4, 457.4, 458.4
7β-OH	1	217.3, 218.3, 372.4, 456.4, 457.4, 458.4
5,6β-EP	2	366.4, 384.4, 441.4,442.4, 459.4, 474.4
5,6α-EP	2	366.4, 384.4, 441.4,442.4, 459.4, 474.4
triol	3	321.3, 403.4, 404.4, 456.4, 457.4
25-OH	4	131.1, 367.4, 457.4, 472.4, 473.4, 474.4
7-keto	4	131.1, 367.4, 457.4, 472.4, 473.4, 474.4

[a] Group 1, 0−13.45 min; group 2, 13.45−14.00 min; group 3, 14.00−15.00 min; group 4, 15.00−25.00 min. [b] Underlined values represent the major ions used to detect COPs.

Extraction and Purification of COPs in Eggs and Juices. A method based on that of Lee et al. (*3*) was modified to determine COPs in marinated eggs and juices. A 1 g sample of egg powder was mixed with 30 mL of chloroform/methanol (2:1, v/v) in a flask, after which the mixture was shaken for 30 min and filtered through a glass filter paper, followed by the addition of 0.5 g of anhydrous sodium sulfate to remove residual moisture, evaporation to dryness under vacuum, dilution to 5 mL with hexane, and filtration through a 0.45 μm membrane filter for purification. A 5 mL extract was poured into a silica cartridge (sorbent weight, 500 mg; column volume, 6 mL; surface area, 562 m^2/g; average pore size, 60 Å; average particle size, 45 μm; Agilent Technology), which was preactivated with 6 mL of ethyl acetate and 10 mL of hexane. Then the impurities were successively eluted with 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, COPs were eluted with 5 mL of acetone, evaporated to dryness under nitrogen, and dissolved in 1 mL of pyridine for derivatization. The extraction and purification step for the marinated juice sample is the same as for the egg with the exception that 30 mL of juice was mixed with 30 mL of hexane in the beginning, after which the mixture was shaken for 30 min, followed by sonication for 10 min to remove air bubbles, collection of the hexane layer, evaporation to dryness, and dilution to 5 mL with hexane for subsequent purification using a silica gel cartridge. For derivatization, 50 μL of marinated egg or juice sample was poured into a 250 μL vial insert, followed by the addition of 20 μL of 0.5 ppm of the internal standard 5α-cholestane and 50 μL of derivatizing agent and reaction in the dark for 1 h. Next, 1 μL of sample was collected and injected for GC-MS analysis.

Separation and Quantitation of COPs in Marinated Egg and Juice. An Agilent Technologies HP-5MS capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness, 5% diphenylpolysiloxane, and 95% dimethylpolysiloxane, ultra low bleed) was used to separate the various COPs in marinated eggs and juices with helium as carrier gas and flow rate = 1.0 mL/min, injector temperature = 290 °C, split ratio = 5:1, column temperature = 210 °C initially, maintained for 4 min, increased to 295 °C at 50 °C/min, held for 6 min, raised to 310 °C at 50 °C/min, and maintained for 13 min. The GC-MS interface temperature was 300 °C with electron multiplier voltage = 70 eV and ion voltage = 1360 V. Detection was carried out using SIM mode. The various COPs were identified according to elution order by dividing into four groups (**Table 1**): 0−13.45 min for the first group with the fragmentation ions *m*/*z* 217.3, 218.3, and 372.4 used to detect 5α-chlestane and *m*/*z* 456.4, 457.4, and 458.4 to detect 7α-OH and 7β-OH; 13.45−14.00 min for the second group with the fragmentation ions *m*/*z* 366.4, 384.4, 441.4, 442.4, 459.4, and 474.4 to detect 5,6α-EP and 5,6β-EP; 14.00−15.00 min for the third group with the fragmentation ions *m*/*z* 321.3, 403.4, 404.4, 456.4, and

457.4 to detect triol; 15.00−25:00 min for the fourth group with the fragmentation ion m/z 131.1 to detect 25-OH and m/z 367.4, 457.4, 472.4, 473.4, and 474.4 to detect 7-keto. Perfluorotributylamine was used for auto tune with m/z at 69, 219, and 502. In addition, the various COPs were identified by comparing retention times and mass spectra of unknown peaks with reference standards using the NIST mass spectra database and cochromatography with added standards.

For quantitation, the standard curves of 7α-OH, 7β-OH, triol, and 25-OH were each prepared using five concentrations of 3.0, 1.5, 1.2, 0.6, and 0.3 μg/mL, whereas 7-keto was prepared using 15, 7.5, 6.0, 3.0, and 1.5 μg/mL. Likewise, both 5,6α-EP and 5,6β-EP standard curves were prepared using 6.0, 3.0, 1.5, 1.2, and 0.6 μg/mL for the former and 2.0, 1.0, 0.5, 0.4, and 0.2 μg/mL for the latter. Then the internal standard 5α-cholestane was mixed with each COP standard for a fixed concentration at 0.5 μg/mL. After GC-MS analysis, the linear regression equations and correlation coefficients (R^2) were automatically calculated from the standard curves, which were obtained by plotting concentration ratio against its area ratio. The recovery of each COP was determined by adding 1 mL of a mixed standard solution containing 70 μg/mL each to egg and juice samples separately and then subjecting it to derivatization for GC-MS analysis using the same procedure as described above. The recovery was then obtained on the basis of the ratio of each COP standard after and before GC-MS analysis: (spiked amount − original amount/spiked amount) × 100%. The various COPs in egg and juice samples were determined using a formula as described in a previous study (*3*).

Determination of Total Phenolic Compound in Egg and Juice Samples. A method based on that of Turkmen et al. (*14*) was modified to determine total phenolic compound in egg and juice samples based on the gallic acid standard curve, obtained by preparing six concentrations of 5, 10, 50, 100, 150, and 200 μg/mL of gallic acid standard, in which a portion of 50 μL each was collected and mixed with 200 μL of Folin−Ciocalteu reagent, followed by homogenization at room temperature for 5 min, the addition of 1 mL of 15% sodium carbonate solution, reaction at room temperature for 60 min, and measurement of the absorbance at 750 nm.

For sample determination, 1 g of marinated egg was mixed with 10 mL of deionized water and the mixture was shaken for 30 min. After centrifugation at 6000*g* for 10 min at 4 °C, the supernatant was collected and diluted to 20 mL. Then the solution was filtered through a 0.45 μm membrane filter, and 50 μL was collected for absorbance measurement at 750 nm. For marinated juice, a 50 μL sample was collected for absorbance measurement at 750 nm. The total phenolic compounds in both egg and juice samples were calculated on the basis of the regression equation of the gallic acid standard curve.

Determination of Total Flavonoids in Egg and Juice Samples. A method based on that of Luximon-Ramma et al. (*15*) was modified to determine total flavonoids in egg and juice samples using the catechin standard curve, obtained by preparing six concentrations of 1, 5, 10, 25, 50, and 100 μg/mL of catechin standard, in which a portion of 500 μL each was collected and mixed with 75 μL of 5% sodium nitrite solution, after which the mixture was allowed to stand at room temperature for 5 min, followed by the addition of 150 μL of 10% aluminum chloride solution and 500 μL of 1 M sodium hydroxide solution after 5 min and measurement of the absorbance at 510 nm.

For sample determination, 1 g of marinated egg was mixed with 10 mL of deionized water, after which the solution was shaken for 30 min and centrifuged at 6000*g* for 10 min at 4 °C. Then the supernatant was collected and diluted to 20 mL, followed by filtration through a 0.45 μm membrane filter and collection of 500 μL for absorbance measurement at 510 nm. For marinated juice, a 500 μL sample was collected for direct measurement of absorbance at 510 nm. The total flavonoids in both egg and juice samples were calculated on the basis of the catechin standard curve.

Determination of Browning Index in Egg and Juice Samples. A method described by Chawla et al. (*16*) was used to determine the browning index in both egg and juice samples. Briefly, 1 g of egg sample was mixed with 10 mL of deionized water, after which the solution was shaken for 30 min and then centrifuged at 6000*g* for 10 min at 4 °C. The supernatant was collected and diluted to 10 mL, and the solution was filtered through a 0.45 μm membrane filter for absorbance measurement at 420 nm. For the juice sample, 1 mL was collected for absorbance measurement at 420 nm.

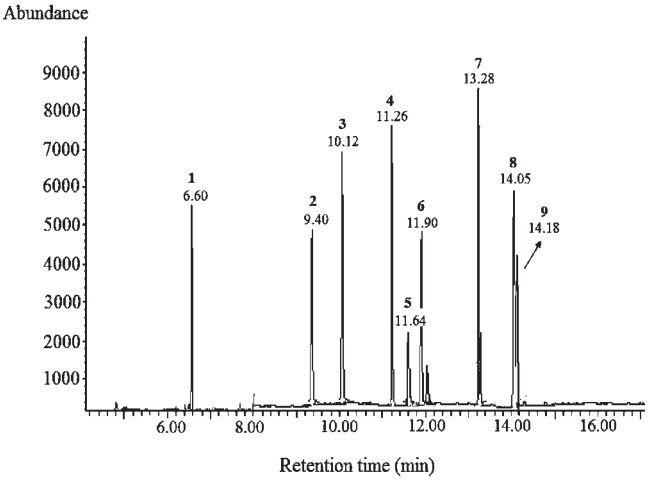

Figure 1. GC-MS-SIM chromatogram of cholesterol and seven COP standards plus one internal standard, 5α-cholestane, by using the conditions described by Lee et al. (*3*). Peaks: 1, 5α-cholestane (internal standard); 2, 7α-OH; 3, cholesterol; 4, 7β-OH; 5, 5,6β-EP; 6, 5,6α-EP; 7, triol; 8, 25-OH; 9, 7-keto.

Determination of pH in Juice Samples. A 15 mL juice sample was collected and poured into a beaker for pH determination with a pH-meter (model SP-701, San-Tai Co., Taipei, Taiwan)

Statistical Analysis. All of the experiments were conducted in duplicate, and the data were analyzed using SAS (*17*). The data were also subjected to ANOVA analysis and Duncan's multiple-range test for comparison of significant difference ($P < 0.05$).

RESULTS AND DISCUSSION

Evaluation of Analysis of Free COPs in Eggs and Juices. Initially we tried to find a better solvent system than that used by Lee et al. (*3*) for enhancement of extraction efficiency, and thereby various solvents including hexane, methanol, ethyl acetate, acetone, and chloroform were tested in combination or alone for comparison of free COP extraction efficiency in egg and juice samples. But, after numerous studies, the solvent mixture of chloroform/methanol (2:1, v/v) employed by Lee et al. (*3*) was still shown to be the most appropriate as interference with the subsequent quantitation by GC-MS was minimized amid the presence of a lower amount of impurities in eggs. Following extraction, the purification of free COPs by employing a silica cartridge was found to be superior to that by a C18 cartridge after GC-MS analysis, with the recoveries of the former being 98.3, 96.2, 92.0, 85.9, 94.8, 92.7, and 96.4% for 7α-OH, 7β-OH, 5,6β-EP, 5,6α-EP, triol, 25-OH, and 7-keto in tea-leaf eggs, respectively, whereas in marinated juice the recoveries were 100.1, 97.4, 88.2, 83.1, 97.4, 95.6, and 98.1%.

Figure 1 shows the GC-MS chromatogram of cholesterol and 7 COP standards plus one internal standard, 5α-cholestane, by employing the separation conditions described by Lee et al. (*3*). However, with this method both 25-OH and 7-keto were overlapped. Thus, by modifying the temperature programming condition, an adequate resolution of nine standards, including 5α-cholestane (internal standard), 7α-OH, cholesterol, 7β-OH, 5,6β-EP, 5,6α-EP, triol, 25-OH, and 7-keto, was attained, with the retention times being 9.86, 12.13, 12.44, 13.26, 13.65, 13.86, 14.75, 15.39, and 15.68 min, respectively (**Figure 2**). Compared to some other published reports (*9*, *18*−*20*), the separation time of our method was reduced substantially while a high recovery was maintained. Moreover, in our pre-experiment 19-OH was used as internal standard for COP quantitation; however, it was found to be overlapped with COPs on the GC chromatogram, and

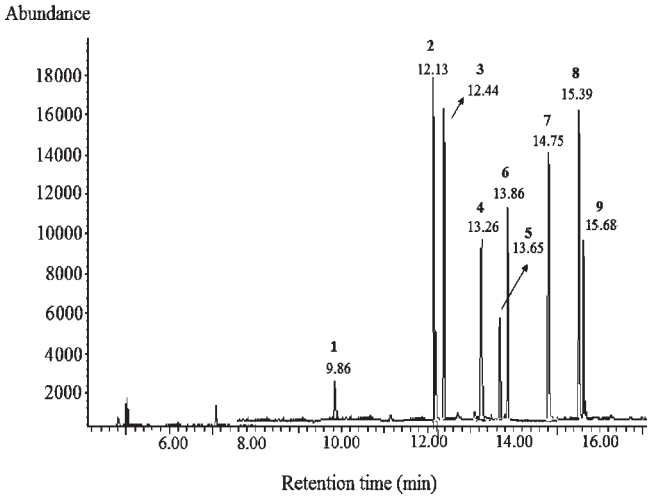

Figure 2. GC-MS-SIM chromatogram of cholesterol and seven COP standards plus one internal standard, 5α-cholestane, by using the modified temperature programming condition. Peaks: 1, 5α-cholestane (internal standard); 2, 7α-OH; 3, cholesterol; 4, 7β-OH; 5, 5,6β-EP; 6, 5,6α-EP; 7, triol; 8, 25-OH; 9, 7-keto.

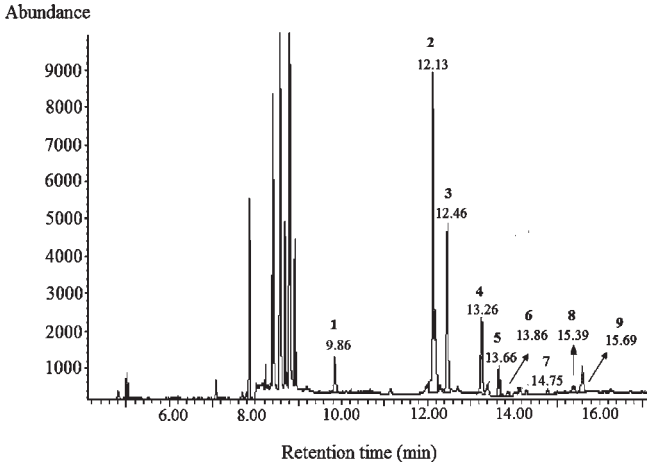

Figure 3. GC-MS-SIM chromatogram of tea-leaf egg extract when purified using a silica cartridge. Peaks: 1, 5α-cholestane (internal standard); 2, 7α-OH; 3, cholesterol; 4, 7β-OH; 5, 5,6β-EP; 6, 5,6α-EP; 7, triol; 8, 25-OH; 9, 7-keto.

therefore 5α-cholestane was used instead of 19-OH. The standard curve of cholesterol was not prepared as most cholesterol was coeluted with impurities during purification with a silica gel cartridge. More importantly, this study focused on the formation and inhibition of free COPs in marinated tea-leaf eggs and juices. The detection limits (DLs) of seven COP standards as detected by GC-MS based on S/N ≥ 3 were 20, 30, 200, 250, 30, 50, and 50 ng/mL for 7α-OH, 7β-OH, 5,6β-EP, 5,6α-EP, triol, 25-OH, and 7-keto, respectively, whereas the quantitation limits (QL) based on S/N ≥ 10 were 66, 99, 660, 832.5, 99, 165, and 165 ng/mL. The DLs were much lower than those by HPLC with UV detection, as DLs of 140, 120, 120, and 90 ng/mL for 25-OH, 7α-OH, 7β-OH, and 7-keto, respectively, were reported by Baggio and Bragagnolo (*21*).

Figure 3 shows the GC-MS chromatogram of cholesterol and seven free COPs in tea-leaf egg extract using a silica cartridge for purification and SIM for detection. Compared to **Figure 2**, there are more impurities present before 10 min, but these did not interfere with the separation and quantitation of free COPs in egg samples, all of which were eluted after 10 min. In contrast, no free COPs were detected in marinated juices by following the same extraction and purification procedure, which should be caused by the dilution effect as a constant level of 3 L of juice was maintained during marinating. Therefore, for the subsequent experiment regarding the effect of heating time and juice composition on free COP formation, the determination of free COPs in marinated juice was excluded.

Table 2 and **Figure 4** show the effect of heating time on free COP formation in tea-leaf eggs. In precooked eggs (raw eggs boiled for 40 min), 5 COPs, including 7α-OH, 7β-OH, 5,6β-EP, triol, and 7-keto, at levels of 373.9, 418.7, 688.1, 175.9, and 230.0 ng/g, respectively, were detected (**Table 2**) . However, only three COPs, 7α-OH, 7β-OH, and 7-keto, were reported to be present in precooked eggs by Lee et al. (*3*), which should be due to a shorter heating time (30 min) when compared to our present study. Additionally, the method difference may also affect the epoxides' sensitivity. After 12 h of marinating, a plateau of 453.6 and 539.2 ng/g was attained for 7α-OH and 7β-OH, respectively, but a declining trend was followed afterward and reduced to 332.3 and 346.7 ng/g in 48 h (**Table 2**). Similarly, a maximum was reached for 5,6β-EP, triol, and 7-keto after 24 h of marinating, which amounted to 809.2, 220.3, and 316.0 ng/g, respectively, but decreased to 208.6 and 180.5 ng/g for triol and 7-keto in 48 h, with 5,6β-EP being undetected (**Table 2**). Interestingly, both 5,6α-EP and 25-OH remained undetected during precooking and subsequent marinating. Accordingly, the total free COPs followed the same tendency as triol and 7-keto. Similar outcomes were reported by Lee et al. (*3*) and Obara et al. (*9*), as five COPs, 7α-OH, 7β-OH, 5,6α-EP, 5,6β-EP, and 7-keto, were generated in both marinated eggs and spray-dried egg powder. In a study dealing with the effect of storage on COP formation in egg powder, five COPs incuding 7α-OH, 7β-OH, 5,6α-EP, 5,6β-EP, and 7-keto, were also detected, all of which followed a time-dependent increase over a storage period of 6−12 months (*22*). Also, the longer the heating time, the greater the formation of total COPs; the higher the temperature, the greater the variety of COPs. It was postulated that both 7α-OH and 7β-OH were formed through reduction of 7-OOH, the initial oxidation product of cholesterol during heating (*3*). However, both 7α-OH and 7β-OH may undergo dehydrogenation for 7-keto formation after prolonged marinating to 24 h, followed by degradation for 7α-OH, 7β-OH, and 7-keto in 48 h. This phenomenon was also observed in a previous study by Lee et al. (*3*). Comparatively, 7β-OH was produced at a higher level than 7α-OH, which may be attributed to the steric hindrance of 7β-OOH being smaller than that of 7α-OOH (*23*). In addition, the formation rate of 7β-OH was shown to be higher than that of 7α-OH during storage of egg powder, as evidenced by a higher stability of the former (*9*). Likewise, a larger amount of 7β-OH than of 7α-OH was formed in lard during heating at 150 °C for 4 h (*24*). Additionally, the formation rate of 7-keto from 7β-OH was faster than that from 7α-OH (*24*), which may be due to the hydroxyl group at the seventh carbon of the former being more susceptible to reacting with free radicals. Similarly, a peak was reached for 5,6β-EP and triol in 24 h, but the former reduced to a level lower than the quantitation limit, which may be caused by its further conversion to triol through hydrolysis (*3*). For total free COPs, the content attained a peak in 24 h and then declined after extensive heating to 36 and 48 h, which may be accounted for by the leaching of antioxidative compounds from juice to egg during marinating. It is also possible that COPs may undergo degradation or convert to other compounds as discussed above. In another paper Lee et al. (*3*) demonstrated the formation of Maillard reaction products (MRPs) from soy sauce and sugar to be effective against COP formation during marinating. In addition, some other studies have

Table 2. Effect of Heating Time on the Contents (Nanograms per Gram) of COPs in Tea-Leaf Eggs

	time[a]				
COP	0 h	12 h	24 h	36 h	48 h
7α-OH	373.9 ± 3.5 d	453.6 ± 1.2 a	444.4 ± 4.2 b	392.5 ± 2.6 c	332.3 ± 0.8 e
7β-OH	418.7 ± 18.7 c	539.2 ± 16.2 a	482.1 ± 2.2 b	414.8 ± 2.9 c	346.7 ± 6.9 d
5,6β-EP	688.1 ± 1.8 c	777.0 ± 1.3 b	809.2 ± 14.2 a	683.2 ± 2.0 c	nd
5,6α-EP	nd	nd	nd	nd	nd
triol	175.9 ± 3.8 b	186.7 ± 5.2 b	220.3 ± 0.3 a	206.8 ± 0.7 a	208.6 ± 13.4 a
25-OH	nd	nd	nd	nd	nd
7-keto	230.0 ± 14.3 c	291.4 ± 11.5 b	316.0 ± 1.4 a	241.6 ± 6.2 c	180.5 ± 1.4 d
total COPs	1886.7 ± 3.0 c	2248.0 ± 19.2 a	2272.2 ± 21.5 a	1939.0 ± 16.3 b	1068.2 ± 9.7 d

[a] Mean of duplicate analyses ± standard deviation (three injections each). Values within a row with different letters are significantly different ($p < 0.05$). nd, not detected.

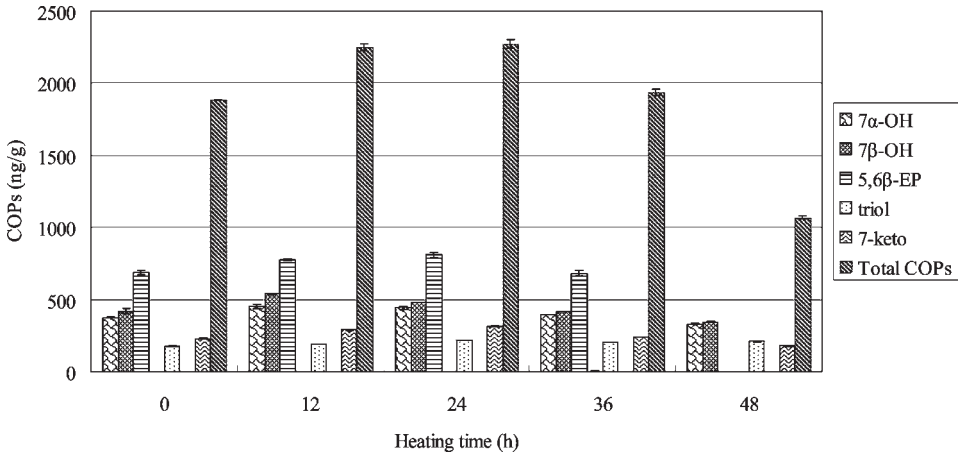

Figure 4. Effect of heating time on the contents (ng/g) of COPs in tea-leaf eggs.

Table 3. Changes of pH and Maillard Browning Reaction (MR) Index as well as Total Flavonoid and Phenolic in Tea-Leaf Egg and Juice When Heated for Various Lengths of Time

	time[a]				
	0 h	12 h	24 h	36 h	48 h
tea-leaf egg					
MR index[b]	0.1 ± 0.0	0.1 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.4 ± 0.1
total phenolics	10.1 ± 0.1 d	13.6 ± 0.1 c	15.2 ± 0.2 b	17.4 ± 0.2 a	17.0 ± 0.2 a
total flavonoids	1.1 ± 0.0 d	1.6 ± 0.0 c	1.7 ± 0.0 b	2.3 ± 0.2 a	2.4 ± 0.2 a
juice					
pH	5.50	5.85	6.13	6.13	6.35
MR index[b]	1.2 ± 0.1 (2.5)[c]	1.3 ± 0.1 (2.5)	1.4 ± 0.1 (2.4)	1.5 ± 0.1 (2.5)	1.6 ± 0.2 (2.6)
total phenolics	38.3 ± 0.0 a	34.8 ± 0.5 b	30.5 ± 0.1 c	25.2 ± 0.1 d	24.1 ± 0.3 e
total flavonoids	27.4 ± 0.1 a	24.9 ± 0.1 b	22.9 ± 0.1 c	21.2 ± 0.0 d	19.2 ± 0.2 e

[a] Mean of duplicate analyses ± standard deviation. Values within a row with different letters are significantly different ($p < 0.05$). [b] Sample diluted 10-fold and absorbance measured at 420 nm. [c] Values in parentheses represent absorbance measured at 420 nm for undiluted sample.

pointed out that MRPs were efficient in inhibiting COP formation by quenching free radicals and chelating ferrous ions (*25*).

In our experiment the browning index followed a time-dependent increase in both eggs and juices during marinating (**Table 3**), indicating the browning reaction occurring between reducing sugar and amino acid during marinating should be responsible for this effect. Also, the browning index in marinated juice was higher than in marinated egg, which is expected as the former is mainly composed of soy sauce and black tea leaf, whereas the latter contains protein and reducing sugar (*26*). Like the browning index, the pH value in juice also followed a time-dependent rise during marinating. It may be inferred that the liberation of basic compounds such as theobromine and theophylline from black tea leaf during marinating may result in a pH rise from 5.5 to 6.35 over a heating period of 48 h (**Table 3**). Similar outcomes were reported in several previous studies, as a rise in pH from 7.6 to 10.1 in green tea occurred during heating mainly because of theaflavin formation through oxidation of epigallocatechin (*27*). Additionally, the release of metal ions such as calcium may also contribute to a pH increase in juice during marinating. To further demonstrate this phenomenon, a mixture containing only pure water and eggs was heated for 48 h, and a pH change from 7.6 before heating to 8.35 after heating was found. Furthermore, the formation of MRPs would result in a rise in pH in juice during marinating (**Table 3**). That is, the higher the amount of MRPs formed, the higher the pH value in marinated juice.

In addition to pH change, the presence of phenolic compounds and flavonoids in black tea leaf may play a significant role in reducing free COP formation during marinating because of their strong antioxidant activity (*28*). Prior to marinating, eggs were

Table 4. Effect of Different Ingredients in Marinated Juice on the Formation of COPs (Nanograms per Gram) in Tea-Leaf Eggs after Heating for 48 h

	ingredient[a]					
COP	100% water	1% soy sauce	1% tea leaf	10% soy sauce	10% tea leaf	standard formula[b]
7α-OH	425.3 ± 3.5 a	384.3 ± 0.6 b	344.3 ± 16.7 c	388.6 ± 6.8 b	319.5 ± 12.0 d	332.3 ± 0.8 cd
7β-OH	484.4 ± 18.2 a	438.2 ± 1.4 b	371.9 ± 2.0 d	407.5 ± 1.6 c	358.5 ± 2.0 de	346.7 ± 6.9 e
5,6β-EP	811.3 ± 13.3 a	743.9 ± 18.2 b	763.1 ± 7.1 b	nd	nd	nd
5,6α-EP	nd	nd	nd	nd	nd	nd
triol	196.8 ± 3.5 ab	211.1 ± 1.2 a	191.8 ± 0.2 b	201.7 ± 0.4 ab	150.2 ± 1.1 c	208.6 ± 13.4 a
25-OH	nd	nd	nd	nd	nd	nd
7-keto	247.8 ± 1.1 a	192.7 ± 0.5 c	195.3 ± 4.5 bc	200.8 ± 3.4 b	178.2 ± 3.4 d	180.5 ± 1.4 d
total COPs	2165.9 ± 3.5 a	1969.8 ± 18.3 b	1867.1 ± 30.4 c	1198.6 ± 4.6 d	1006.6 ± 14.6 f	1068.2 ± 21.0 e

[a] Mean of duplicate analyses ± standard deviation (three injections each). Values within a row with different letters are significantly different ($p < 0.05$). nd, not detected [b] 10% soy sauce, 1% tea leaf, and 89% water marinated together for 48 h.

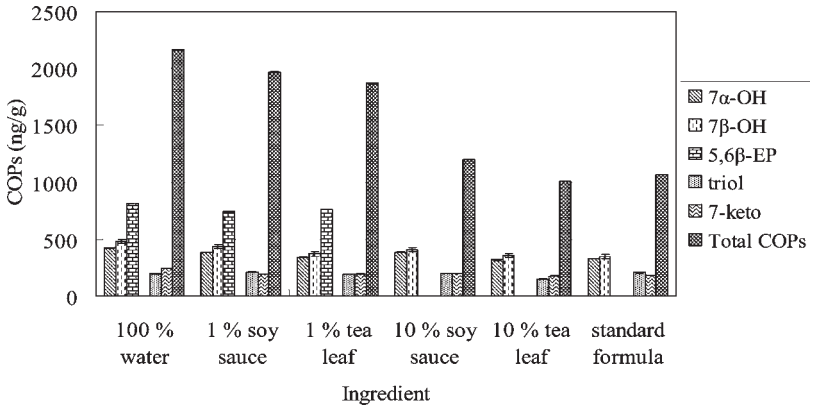

Figure 5. Effect of different ingredients in marinated juice on the formation of COPs (ng/g) in tea-leaf eggs after heating for 48 h.

found to contain total phenolics and total flavonoids at 10.1 and 1.1 mg/g, respectively, followed by a time-dependent increase afterward, with a plateau of 17.4 and 2.3 mg/g being attained in 36 h (**Table 3**). However, there was no significant difference in total flavonoids and total phenolics between 36 and 48 h. For juice, the total phenolics and total flavonoids were 38.3 and 27.4 mg/g, respectively, prior to marinating, whereas both showed a time-dependent decline thereafter, with levels being reduced to 24.1 and 19.2 mg/g in 48 h, respectively (**Table 3**). It may be postulated that the decrease in total phenolics and total flavonoids should be due to leaching from juice to egg during marinating. Additionally, both total phenolics and total flavonoids may undergo degradation after prolonged marinating. This phenomenon was also observed by Su et al. (*28*), as the contents of catechins and theaflavins decreased following a rise in heating time at an elevated temperature, with the degradation rate of theaflavins being faster than that of catechins in green tea. Moreover, both catechins and theaflavins were more stable under acidic condition than under neutral condition, as evidenced by an insignificant change for both at pH ≤5.0 when stored at room temperature for 18 h, but a pronounced destruction of 95% theaflavins and 65% catechins was found when incubated in sodium phosphate buffer of pH 7.4 for 6 h (*28*). In our study a slight pH rise in marinated juice over a 48 h heating period should explain why both total phenolics and total flavonoids showed a time-dependent decrease response. This result suggests that both phenolics and flavonoids in black tea leaf as well as the formation of MRPs during marinating should be an imperative factor in reducing total free COP formation in tea-leaf eggs. Nonetheless, some other factors such as COP degradation or conversion to other compounds during egg marinating cannot be ignored.

Effect of Marinated Juice Composition on COP Formation in Tea-Leaf Eggs after Heating. The formation of free COPs in tea-leaf eggs as affected by different ingredients in marinated juice after 48 h of heating is shown in **Table 4** and **Figure 5**. Compared to the control treatment (100% water), the amounts of 7α-OH, 7β-OH, 5,6β-EP, and 7-keto in tea-leaf eggs were reduced by 41.0, 46.2, 67.4, and 55.1 ng/g, respectively, in the presence of 1% soy sauce after 48 h of heating, whereas both 5,6α-EP and 25-OH remained undetected. Conversely, triol showed an increase by 14.3 ng/g, which may be due to hydrolysis of 5,6β-EP or 5,6α-EP under acidic condition of marinated juice during heating. Nevertheless, the total free COPs still dropped by 196.1 ng/g. Likewise, with 10% soy sauce, reductions of 36.7, 76.9, and 47.0 ng/g were found for 7α-OH, 7β-OH, and 7-keto, respectively, when compared to the control treatment, whereas 5,6α-EP, 5,6β-EP, and 25-OH remained undetected, triol showed an insignificant change, and total free COPs diminished by 967.3 ng/g (**Table 4**). This outcome clearly revealed a high level of soy sauce to be more effective against COP formation. In another study, Lee et al. (*3*) pointed out that soy sauce is a rich source of MRPs, and the higher the level of soy sauce, the better the inhibition of COP formation. This finding was further confirmed by Kuda and Yano (*29*), as a marked scavenging capacity of DPPH free radicals and ferrous reducing power was shown at an elevated level of MRPs in fish muscle. To monitor the formation of MRPs during marinating, changes in the browning index in marinated eggs and juices during heating were investigated (**Table 5**). In tea-leaf eggs, a higher browning index (0.3) was induced for marinated juice containing 10% soy sauce and 90% water than that with 1% soy sauce and 99% water, indicating a higher level of MRPs was produced for the former. Likewise, the browning index in marinated juice with 10% soy sauce and 90% water was 1.4, but reduced to 0.2 with 1% soy sauce and 99% water. Obviously, the increase in MRPs in tea-leaf eggs can be due partly to the release and penetration of MRPs from marinated juice during marinating.

Table 5. Changes of pH and Maillard Browning Reaction (MR) Index as well as Total Flavonoids and Phenolics in Tea-Leaf Egg and Juice When Heated with Different Ingredients for 48 h

	ingredient[a]					
	100% water	1% soy sauce	1% black tea leaf	10% soy sauce	10% black tea leaf	standard formula[d]
tea-leaf egg						
MR index[b]	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.3 ± 0.0	0.6 ± 0.0	0.4 ± 0.0
total phenolics	3.7 ± 0.0 f	4.8 ± 0.1 e	6.7 ± 0.4 d	8.1 ± 0.1 c	15.7 ± 0.3 b	17.0 ± 0.2 a
total flavonoids	0.1 ± 0.0 f	0.2 ± 0.0 e	0.7 ± 0.0 d	0.8 ± 0.0 c	3.5 ± 0.0 a	2.4 ± 0.0 b
juice						
pH	8.35	8.24	7.17	6.59	6.10	6.35
MR index[b]	0.0 ± 0.0 (0.0)[c]	0.2 ± 0.0 (0.5)	0.8 ± 0.0 (2.4)	1.4 ± 0.0 (2.4)	1.6 ± 0.1 (2.5)	1.7 ± 0.0 (2.5)
total phenolics		3.6 ± 0.0 e	12.7 ± 0.0 d	15.2 ± 0.0 c	21.4 ± 0.2 b	24.1 ± 0.3 a
total flavonoids		1.9 ± 0.1 e	6.7 ± 0.2 d	7.4 ± 0.1 c	14.1 ± 1.1 b	19.2 ± 0.2 a

[a] Mean of duplicate analyses ± standard deviation. Values within a row with different letters are significantly different ($p < 0.05$). [b] Sample diluted 10-fold and absorbance measured at 420 nm. [c] Values in parentheses represent absorbance measured at 420 nm for undiluted sample. [d] The standard formula is composed of 10% soy sauce, 1% tea leaf, and 89% water.

As mentioned before, the formation of MRPs can cause a slight rise in pH in marinated juice containing 10% soy sauce, 1% black tea leaf, and 89% water during marinating for 48 h, but this trend can be varied when different ingredients are incorporated into marinating juice, as evidenced by a pH of 6.59 in marinated juice containing 10% soy sauce and 90% water and a pH of 8.24 in juice containing 1% soy sauce and 99% water (**Table 5**). Apparently, in the absence of black tea leaf, a high level of soy sauce would lead to an increase in pH in marinated juice. Among the various free COPs studied, most free COPs were more stable under acidic condition except triol. However, there was no significant difference in triol content between 1 and 10% soy sauce, implying the stability of triol was maintained at pH 6.59. In a study dealing with comparison of antioxidant activity by marinating chicken meat in spice-based sauce at 204 °C for 40 min, the antioxidant activity was higher with marinated sauce than that without marinated sauce (*30*), which should be due to the presence of polyphenols in the former. In our study, the total phenolics and total flavonoids were 8.1 and 0.8 mg/g, respectively, in tea-leaf eggs marinated with 10% soy sauce and 90% water, but reduced to 4.8 and 0.2 mg/g with 1% soy sauce and 99% water, all of which were higher than the control treatment (**Table 5**). A similar trend was observed in marinated juice, with total phenolics and total flavonoids being 15.2 and 7.4 mg/g, respectively, for 10% soy sauce and 90% water and 3.6 and 1.9 mg/g for 1% soy sauce and 99% water, whereas both remained undetected for control treatment. This finding demonstrated that the higher the level of total phenolic or total flavonoid, the lower the formation of free COPs in marinated egg and juice.

Effect of Black Tea Leaf on Free COP Formation in Marinated Egg and Juice. The formation of free COPs in marinated egg and juice as affected by black tea leaf is shown in **Table 4** and **Figure 5**. Compared to control treatment (100% water), the levels of 7α-OH, 7β-OH, 5,6β-EP, and 7-keto in eggs were reduced by 81, 112.5, 48.2, and 52.5 ng/g, respectively, after 48 h of heating, whereas both 5,6α-EP and 25-OH remained undetected and triol showed an insignificant change in the presence of 1% black tea leaf. The total free COPs also followed a decline by 298.8 ng/g. In contrast, a sharp degradation of total free COPs by 1159.3 ng/g was found in eggs marinated with 10% black tea leaf, with 7α-OH, 7β-OH, triol, and 7-keto being diminished by 105.8, 125.9, 46.6, and 69.6 ng/g, respectively, whereas 5,6α-EP, 5,6β-EP, and 25-OH remained undetected. This outcome implied that the higher the level of black tea leaf, the lower the formation of free COPs during marinating. As black tea leaf is an abundant source of theaflavins and thearubigins (*28*), both should be mainly responsible for free COP inhibition in marinated tea-leaf eggs because of their antioxidant acitivity.

As described in the preceding section, the formation of MRPs can be affected by the level of black tea leaf incorporated into juice. In tea-leaf eggs, the browning index was much higher (0.6) when marinated with juice containing 10% black tea leaf and 90% water than that with 1% black tea leaf and 99% water, whereas in marinated juice, the browning index was even higher for 10% black tea leaf and 90% water than for 1% black tea leaf and 99% water (**Table 5**). Apparently, a greater amount of MRPs produced in marinated juice may penetrate into eggs during heating, leading to a rise in MRPs in marinated eggs for subsequent inhibition of free COP formation. Like soy sauce, the pH value change showed a similar trend, with a lower pH (6.10) in marinated juice containing 10% tea leaf and 90% water than that (7.17) with 1% tea leaf and 99% water. This finding demonstrated a high correlation between MRPs and pH in reducing free COP formation. In marinated eggs, the total phenolics and total flavonoids were 6.7 and 0.7 mg/g when marinated with juice containing 1% black tea leaf and 99% water, respectively, but rose to 15.7 and 3.5 mg/g with 10% black tea leaf and 90% water, all of which were much higher than the control treatment. Likewise, the total phenolics and total flavonoids were 12.7 and 6.7 mg/g in marinated juice with 1% black tea leaf and 99% water, respectively, but rose to 21.4 and 14.1 mg/g with 10% black tea leaf and 90% water. Obviously a high level of black tea leaf could generate a greater amount of total phenolics and total flavonoids in both marinated juices and eggs and, thereby, reduce the free COP production substantially. Nevertheless, as indicated before, the reduction in COPS may also be caused by their degradation or conversion to other compounds.

All in all, the incorporation of soy sauce or black tea leaf into juice was effective in inhibiting free COP formation in tea-leaf eggs during marinating. Comparatively, a level of 1% black tea leaf was superior to 1% soy sauce in inhibiting total free COP formation, as accounted for by a higher browning index (0.8) for the former. A similar phenomenon was observed by Lee et al. (*3*), as the juice containing 1% soy sauce was more efficient in retarding COP formation than that with 1% sucrose during marinating of eggs at 100 °C for 10 h, with the browning index being higher for the former. Likewise, compared to 10% soy sauce, the free COP reduction in eggs was more pronounced for 10% black tea leaf, with the browning index being 0.3 for the former and 0.6 for the latter, demonstrating the formation of MRPs to be of vital importance in preventing COP formation during marinating. Moreover, the total free COPs generated in eggs was 1006.6 ng/g when marinated with juice containing 10% black tea leaf and 90% water, which was lower than that (1068.2 ng/g) when marinated with the standard formula, revealing that black tea leaf

possessed a greater impact on free COP inhibition than soy sauce. As the marinated juice containing 10% black tea leaf and 90% water showed a higher browning index and total flavonoids than that with the standard juice formula, the formula currently used for marinating tea-leaf eggs may be modified to be more efficient in preventing free COP formation.

In conclusion, five COPs including 7α-OH, 7β-OH, 5,6β-EP, triol, and 7-keto were detected in marinated tea-leaf egg, but not in marinated juice. The browning index in both tea-leaf egg and juice as well as the pH in juice also followed a time-dependent rise during marinating. Both total phenolics and total flavonoids in tea-leaf egg showed the same trend. The incorporation of soy sauce or black tea leaf into marinated juice was effective in inhibiting free COP formation, with the latter being more pronounced than the former, which may be due to the presence of total phenolics and total flavonoids as well as the formation of MRPs. Additionally, some other factors such as COP degradation or conversion to other compounds during egg marinating may also contribute to the free COP reduction.

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