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Formation and Inhibition of Cholesterol Oxidation Products during Marinating of Pig Feet

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ABSTRACT: Cholesterol oxidation products (COPs), formed during the heating of cholesterol-rich foods, have been shown to cause cancer and coronary heart disease. The objectives of this study were to develop a GC-MS method for the determination of COPs in pig feet meat, skin, and juice during marinating and to study the formation and inhibition of COPs as affected by the incorporation of soy sauce and sugar. Results showed that an HP-5MS column could provide an adequate separation of cholesterol, 5α-cholestane (internal standard), and seven COPs, including 7α-OH, 7β-OH, 5,6β-OH, 5,6α-OH, triol, 25-OH, and 7-keto, within 15 min with a temperature-programming method. Most COPs in pig feet meat were generated at a larger amount than in pig feet skin and marinating juice over a 24 h heating period at about 100 °C. The Maillard browning index rose with increasing heating time, whereas the pH showed a slight change in marinated juice. Both reducing sugar and free amino acid contributed to the formation of Maillard reaction products. The incorporation of soy sauce and crystal sugar into fresh juice was effective in inhibiting COPs formation in pig feet, skin, and juice over a 30 min preheating period.

KEYWORDS: *pig feet, cholesterol oxidation products, GC-MS, marinating*

INTRODUCTION

Cholesterol oxidation products (COPs), formed through the oxidation of cholesterol, can be found in many cooked cholesterol-rich foods such as egg and meat products.[1,2] It has been well documented that the intake of COPs in excess can be detrimental to human health. For instance, COPs may induce cell toxicity through inhibition of HMG-CoA reductase, an enzyme responsible for cholesterol synthesis, which in turn increases cell membrane permeability and then leads to cell necrosis.[3] In addition, COPs may accumulate by the arterial wall to form "fatty streaks" due to scavenging by macrophage and formation of foam cells, resulting in atherosclerosis and coronary heart disease.[4,5] Therefore, it is pivotal to determine the variety and amount of COPs formed in food products during heating.

More than 80 COPs generated by photooxidation, enzymes, and processing have been characterized.[6] However, according to the literature, only 8 COPs, including 25-hydroxycholesterol (25-OH), cholestanetriol (triol), 5,6α-epoxide (5,6α-EP), 5,6β-epoxide (5,6β-EP), 7α-hydroxycholesterol (7α-OH), 7β-hydroxycholesterol (7β-OH), 7-ketocholesterol (7-keto), and cholesta-3,5-dien-7-one, are commonly present in foods.[7] Accordingly, only low amounts of COPs are distributed naturally in fresh foods such as eggs, as they may be produced by metabolites originated enzymatically by hen metabolism or evidence that lipid oxidation had already occurred.[8] However, after drastic processing conditions such as frying, roasting, and storage, a high amount of COPs can be produced.[9−11] Also, the presence of different varieties of food components may prevent or promote cholesterol oxidation. Using polyunsaturated fatty acid as an example, linoleic acid was shown to accelerate cholesterol oxidation amid formation of a large amount of free radicals during heating or storage of food products,[12] yet, the incorporation of soy sauce or tea leaf into raw juice was effective in retarding COP formation during marinating of eggs, which may be attributed to the presence of compounds possessing antioxidant activity such as phenolic acids, flavonoids, or Maillard reaction products.[2] Obviously, the incorporation of antioxidant compounds should be the most efficient way to prevent cholesterol oxidation during processing of food products.

Numerous papers have been published in terms of analysis of COPs in various food products. Nevertheless, to date no standard method of COP analysis has been established, mainly because the instability of the intermediate product formed during cholesterol oxidation can be further converted to other unknown products during analysis. Thus, the development of a reliable assay in determining COPs in food commodities is an urgent problem to overcome. COPs are often extracted by low-polarity solvents, followed by cold or hot saponification to remove unwanted triacylglycerol, free fatty acid, and water-soluble impurities.[2] Alternatively, solid phase extraction was used instead of saponification to remove impurities,[1] with the former being shown to be superior to the latter as both the COP degradation and unwanted reaction were minimized.[13] Nonetheless, lower recoveries of 5,6α-EP and 5,6β-EP were reported in eggs and pork when using solid-phase extraction as a certain amount of COPs is retained in the solid phase.[1] Both high-performance liquid chromatography (HPLC) and gas chromatography (GC) coupled with mass spectrometry (MS) are routinely employed for qualitative and quantitative analyses of COPs in food products. However, both methods have advantages and limitations. Compared to HPLC, GC requires a lengthy derivatization procedure during extraction to enhance the volatility and thermal stability of COPs.[2] Nonetheless, GC

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could provide a better separation power and higher sensitivity than HPLC,[5] and this is why GC-MS is still a preferred method of COP analysis in food commodities.

Pig feet, especially marinated pig feet, represent a popular food commodity in Taiwan. Marinating is often carried out by mixing pig feet with soy sauce and various ingredients such as sucrose and spice and heating at about 100 °C for an extended period of time to impart a characteristic flavor, color, and tenderness to pig feet. As adult pig meat was found to contain 44 and 49 mg of cholesterol in 100 g of pork loin and 100 g of fresh ham,[14] respectively, it is quite possible that a high amount of COPs can be produced during marinating of pig feet. The objectives of this study were to develop a GC-MS method for the determination of COPs in marinated pig feet and to study the formation and inhibition of COPs as affected by the incorporation of soy sauce and sugar into raw (unheated) juice.

MATERIALS AND METHODS

Materials. A total of 14 kg of pig feet, 16 L of soy sauce, and 3 kg of crystal sugar were purchased from a local supermarket in Taipei, Taiwan, separately. Standards of cholesterol and several COPs, including 5,6β-EP, 5,6α-EP, triol, 25-OH, 7-keto, and internal standard 5α-cholestane, were from Sigma (St. Louis, MO) with purity from 95 to 99%. Both 7α-OH and 7β-OH were from Steraloids (Wilton, NH) with a purity >99%. The COP derivatization agent (BSA + TMCS + TMSI, 3:2:3) was from Supelco (Bellefonte, PA). Both potassium sodium tartrate and 3,5-dinitrosalicyclic acid were from Sigma. Glucose was from Merck (Darmstadt, Germany). Both sodium hydroxide and phenolphthalein were from Riedel-de Haën Co. Formaldehyde (37% solution) was from J. T. Baker Co. (Phillipsburg, NJ). The HPLC grade solvents including methanol, diethyl ether, hexane, chloroform, acetone, and ethyl acetate were from Mallinckrodt Co. (Paris, KY). Deionized water was obtained using a Milli-Q water purification system from Millipore Co. (Bedford, MA).

Instrumentation. A GC-MS (model 5973) instrument used for separation of COPs was from Agilent Technologies. The freeze-dryer (FD24) was from Taiwan Chin-Ming Co. (Taipei, Taiwan). The Sorvall RC5C high-speed centrifuge was from DuPont Co. (Wilmington, DE). The spectrophotometer (model CE3021) was from Cecil Co. (Cambridge, U.K.). The sonicator (DC 400) was from Chuan-Hua Co. (Taipei, Taiwan). The rotary evaporator (N-1) was from Eyela Co. (Tokyo, Japan). The silica cartridge (sorbent weight = 500 mg, surface area = 562 m^2/g, average pore size = 60 Å, average particle size = 45 μm, column volume = 6 mL) was from Agilent Technologies.

Methods. *Marinating of Pig Feet.* Initially 12 groups of unheated juice with 2 kg each containing 200 g (10%) of soy sauce, 20 g (1%) of crystal sugar, and 1780 g (89%) of water were prepared. This is the standard formula used for marinating pig feet in most restaurants in Taiwan. Similarly, two groups of pure water with 2 kg each were also prepared. Then, each group containing unheated juice or pure water was poured into a 10 L electric cooker containing 1 kg of pig feet, after which the mixture was preheated at about 100 °C for 30 min to obtain cooked pig feet. During cooking, the cooker was covered with a lid on the top to prevent moisture loss. Next, the cooked pig feet were transferred to another cooker containing the same preheated juice (2 kg) in a 90 °C water bath and marinated for 0, 2, 4, 8, 12, or 24 h. Pig feet preheated in pure water at 100 °C for 30 min were used as control treatment. Duplicate experiments were performed for each marinating time, and heated juice was replenished with fresh juice every 2 h to maintain the juice at a constant level (2 kg). After marinating, 100 g of pig feet meat, 100 g of pig feet skin, and 500 mL of pig feet juice were collected separately, and both portions of meat and skin were freeze-dried, followed by grinding into powder and collection of 1 g of meat and skin separately for COP analysis by GC-MS. For marinated juice, 30 mL was collected for COP analysis.

Moisture Determination. All of the pig feet meat and skin samples were freeze-dried prior to moisture determination. Two grams of pig feet meat and skin was collected separately onto a crucible predried to constant weight, followed by drying in a 105 °C oven for 12−16 h, cooling in a desiccator, and then weighing. Next, drying was continued in a 105 °C oven for 1 h until constant weight followed by cooling. The percentage of moisture was calculated on the basis of the ratio of sample weight after drying relative to that before drying.

Analysis of Free COPs. A method based on that of Lee et al.[1] was modified to extract cholesterol and COPs from pig feet samples. One gram of freeze-dried pig feet meat and skin were collected separately for COP analysis. Each sample was poured into a 60 mL extraction tube and mixed with 40 mL of chloroform/hexane (2:1, v/v), after which the mixture was shaken at room temperature (25 °C) for 30 min and filtered through a glass filter paper containing 0.5 g of anhydrous sodium sulfate to remove residual moisture. After evaporation of the solvent, the residue was dissolved in 5 mL of hexane and filtered through a 0.45 μm membrane filter for subsequent purification using a silica cartridge. Five milliliters of COP extract was poured into the cartridge, which was previously activated with 5 mL of ethyl acetate followed by 10 mL of hexane. The impurities were eluted successively 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), followed by eluting COPs with 10 mL of acetone. For extraction of COPs from marinated juice, 30 mL was collected and mixed with 30 mL of hexane, after which the mixture was shaken for 30 min and then sonicated for 15 min to remove air bubbles. This procedure was repeated twice, and the hexane layer was collected, evaporated to dryness, and dissolved in 5 mL of hexane for purification using a silica gel cartridge as mentioned above. The COP eluates were evaporated to dryness, dissolved in 1 mL of pyridine, and filtered through a 0.2 μm membrane filter for derivatization by pouring 20 μL of COP eluate into a 250 μL vial insert, followed by adding 20 μL of Sylon BTZ and 10 μL of internal standard 5α-cholestane (0.5 μg/mL) to allow the derivatization reaction to proceed in the dark for 1 h. Then 1 μL was collected for GC-MS analysis with selected ion monitoring (SIM) mode.

The various COPs and cholesterol were separated using an Agilent Technologies HP-5MS column (30 m × 0.25 mm i.d., 0.25 μm film thickness, 5% diphenylpolysiloxane, and 95% dimethylpolysiloxane, ultralow bleed) with injector temperature 280 °C, split ratio 5:1, and carrier gas He at a flow rate of 0.7 mL/min. The column temperature was programmed as follows: 220 °C initially, raised to 280 °C at 50 °C/min, to 289 °C at 4 °C/min, maintained for 9 min, increased to 295 °C at 1 °C/min, and maintained for 10 min. The GC-MS interface temperature was 310 °C, with electron multiplier voltage of 70 eV and ion voltage of 1360 V. Detection was performed using SIM mode according to elution order and the following characteristic m/z ions: internal standard 5α-cholestane with m/z 217.3, 218.3, and 372.4 and retention time 5−8.3 min; 7α-OH with m/z 217.3, 218.3, 372.4, 456.4, 457.4, and 458.4 and retention time 8.3−9.6 min; cholesterol with m/z 329.4, 353.5, 368.4, 458.5, and 459.5 and retention time 9.6−10.69 min; 7β-OH with m/z 217.3, 218.3, 372.4, 456.4, 457.4, and 458.4 and retention time 10.69−11.5 min; 5,6β-EP and 5,6α-EP with m/z 366.4, 384.4, 441.4, 442.4, 459.4, and 474.4 and retention time 11.5−13 min; triol with m/z 321.3, 403.4, 404.4, 456.4, and 457.4 and retention time 13−14 min; 25-OH and 7-keto with m/z 131.1, 367.4, 457.4, 472.4, 473.4, and 474.4 and retention time 14−18 min. Perfluorotributylamine was used for autotune with m/z at 69, 219, and 502. In addition, cholesterol and various COPs were identified by comparing retention times and mass spectra of unknown peaks with reference standards, as well as by adding standards to sample for cochromatography.

An internal standard, 5α-cholestane, was used for quantitation. Seven concentrations of 0.2, 0.6, 1.5, 2.5, 5, 10, and 20 μg/mL were prepared for 7α-OH, 7β-OH, 5,6β-EP, and 5,6α-EP separately, whereas six concentrations of 0.2, 0.6, 1.5, 2.5, 5, and 10 μg/mL were prepared for 25-OH and 7-keto. Then 20 μL from each concentration was collected and mixed with 10 μL of 5α-cholestane (0.5 μg/mL) in a 250 μL vial insert, followed by adding 20 μL of derivatizing agent Sylon BTZ, reacting in the dark for 1 h, and injecting 1 μL for GC-MS analysis. The standard curves of each COP were obtained by plotting concentration ratio (standard versus internal

standard) against its area ratio, with the regression equations and correlation coefficient (R^2) being automatically calculated. The various COPs were then determined using a formula as described in a previous study.[2]

For determination of the limit of detection (LOD) and limit of quantitation (LOQ), three concentrations of 50, 100, and 300 ng/g were prepared for each COP, followed by injecting into GC three times to obtain three standard curves by plotting concentration against area for each concentration. LOD and LOQ were calculated using the following formulas: LOD = 3.3(σ/S) and LOQ = 10(σ/S), where S is the average slope of three standard curves and σ is the standard deviation of the intercept of three standard curves. Recovery was determined by adding 1 mL of COP standards (125 μg/mL each) to 1 g of pig feet meat, 1 g of pig feet skin, and 30 mL of juice separately, followed by solvent extraction, silica cartridge purification, and GC-MS analysis using the same procedure as described above. Duplicate analyses were performed, and the recovery was obtained on the basis of the ratio of the amount after GC-MS (spiked amount minus original amount) relative to the amount spiked before GC-MS. For reproducibility determination, 1 g of pig feet meat was extracted, purified, and derivatized following the same procedure shown above and the extract injected into GC three times each in the morning, afternoon, and evening for a total of nine replicates in a day for the intraday variability, whereas, for the interday variability, it was injected three times a day for a total of nine replicates within 3 days. The difference in COP contents within a day and between days was measured in terms of the coefficient of variation (CV, %).

Determination of Reducing Sugar in Pig Feet Meat and Skin as well as Juice. Initially, 0.25 g of dinitrosalicylic acid (DNS) and 75 g of potassium sodium tartrate were mixed and dissolved in 50 mL of NaOH (2 M) to prepare DNS reagent. Then six concentrations of 0.1, 0.25, 0.5, 1, 2.5, and 5 mg/mL of glucose standard were prepared, after which 0.7 mL of each concentration was collected and mixed with 7 mL of DNS reagent, followed by heating in 100 °C water for 10 min, cooling to room temperature, and measuring absorbance at 570 nm. The calibration curve of glucose standard solution was thus prepared with the regression equation $y = 0.165x - 0.0258$ and correlation coefficient (R^2) 0.9996. For sample determination, 2 g of freeze-dried pig feet meat and skin was mixed with 20 mL of distilled water separately, after which the mixture was shaken for 30 min and centrifuged at 4000g for 20 min. The supernatant was collected and the residue extracted repeatedly, and both filtrates were combined and diluted to 50 mL for reducing sugar measurement. For marinated juice, 5 mL was collected and diluted to 50 mL for reducing sugar determination. Each sample (0.7 mL) was poured into a 15 mL tube and mixed with 7 mL of DNS reagent, followed by heating in 100 °C water for 10 min and measuring absorbance at 570 nm to obtain reducing sugar content based on the glucose standard curve. In addition, 0.7 mL of distilled water was used as blank control.

Determination of Amino Nitrogen in Pig Feet Meat and Skin as well as Juice. Two grams of dried pig feet meat or skin was mixed with 20 mL of deionized water separately, after which the mixture was shaken for 30 min and allowed to settle for 30 min before filtration. The residue was extracted again, and both filtrates were pooled and diluted to 50 mL as sample solution. For marinated juice, 25 mL was collected directly for amino nitrogen determination. A 25 mL sample solution was mixed with a 20 mL of a neutral formaldehyde solution and 20 mL of distilled water, whereas another 25 mL of sample solution was mixed with 40 mL of distilled water as blank control. A few drops of phenolphthalein was added to both sample solution as indicator and titrated with 0.05 mol/L NaOH until red color formed. The amino nitrogen content was calculated using the formula

$$\text{amino nitrogen (mg/g)} = (a - b) \times F \times 0.0007 \times D/25 \times 1/S \times 10^3$$

where a = titration volume (mL) of 0.05 mol/L NaOH for sample solution, b= titration volume (mL) of 0.05 mol/L NaOH for blank control, F = 0.05 mol/L (NaOH factor), D = sample solution (mL) after dilution, and S = sample weight (g).

Determination of Browning Index in Pig Feet Meat and Skin as well as Juice. One gram of dried pig feet meat or skin was mixed with 10 mL of deionized water separately, after which the mixture was shaken for 30 min and centrifuged at 6000g for 10 min (4 °C). The supernatant was collected and diluted to 10 mL, followed by filtering through a 0.2 μm membrane filter and collecting 1 mL for absorbance measurement at 420 nm. Similarly, 1 mL of juice was collected and diluted 10 times for direct absorbance measurement at 420 nm.

Statistical Analysis. Duplicate experiments were carried out, and the data were subjected to statistical analysis using SAS software system.[15] Both ANOVA and Duncan's multiple-range test were also used for significance in mean comparison ($p < 0.05$).

RESULTS AND DISCUSSION

Analysis of Free COPs. In the beginning, a method based on that of Lee et al.[1] was used for evaluation of extraction efficiency. However, with a solvent system of chloroform/methanol (2:1, v/v), we found large amounts of impurities were coextracted with COPs. In another study, Busch and King[6] compared the extraction efficiency of three solvent systems, methylene chloride/methanol, hexane/isopropanol, and chloroform/methanol, with chloroform/methanol shown to be the most efficient in extracting COPs. Nevertheless, with this solvent system we found that the extraction time was lengthy and it was difficult to achieve a two-phase separation because of emulsion formation. Therefore, in our study, hexane was used instead of methanol; that is, a solvent system of chloroform/hexane (2:1, v/v) was adopted to extract COPs from pig feet samples.

For purification of COPs, a silica cartridge or NH_2 cartridge was often employed to reduce saponification time and solvent consumption.[2,16,17] Also, it has been reported that saponification can produce artifacts of COPs, which in turn may decrease quantitation accuracy.[18] A high recovery from 83.1 to 118.6% was attained when using a silica cartridge for COP purification in marinated eggs.[1] However, with a NH_2 cartridge, a low recovery of 34% was shown for triol, probably caused by weak adsorption force.[1] In addition to the silica cartridge and NH_2 cartridge, a C18 cartridge was used to purify COPs from lard by Yen et al.,[19] and a high recovery (>90%) was achieved. Nonetheless, the C18 cartridge was found to be inappropriate for COP purification in pig feet samples, which may be caused by differences in food matrix. Thus, in our experiment the silica cartridge was selected for COP purification. Following the extraction and purification procedure as described in the preceding section, the various COPs were subjected to separation, identification, and quantitation by GC-MS. The recoveries of various COPs ranged from 87.5 to 106% in pig feet skin, from 86.5 to 102.5% in pig feet meat, and from 85.5 to 107% in marinating juice (Table 1). Additionally, the CV (%) for the intraday and interday variabilities varied from 0.8 to 5.2 and from 0.9 to 5.7, respectively (Table 2), demonstrating the superiority of this method when compared to several other published methods.

On the basis of the condition described under Methods, 5α-cholestane (internal standard), cholesterol, and 7 COP standards, including 7α-OH, 7β-OH, 5,6β-EP, 5,6α-EP, triol, 25-OH, and 7-keto, were separated within 15 min with retention times being 7.364, 10.096, 9.679, 11.301, 11.793, 12.083, 13.586, 14.520, and 14.746 min, respectively (Figure 1). 5α-Cholestane was shown to be an appropriate internal standard as it did not interfere with separation of the other

Table 1. Recovery of COPs in Pig Feet Skin, Meat, and Juice When Purified Using a Silica Cartridge and Spiked with 125 μg/mL of Each COP Standard

COP	recovery of COPs[a] (%)		
	skin	meat	marinating juice
7α-OH	87.5 ± 2.1	86.5 ± 0.7	98.0 ± 5.7
7β-OH	93.5 ± 0.7	96.0 ± 2.8	97.0 ± 0.7
5,6β-EP	104.0 ± 5.7	101.0 ± 4.2	97.0 ± 1.4
5,6α-EP	100.5 ± 0.7	102.5 ± 10.6	104.0 ± 3.5
triol	98.5 ± 4.9	92.0 ± 4.2	85.5 ± 0.7
25-OH	91.5 ± 0.7	93.0 ± 4.2	103.0 ± 0.7
7-keto	106 ± 1.4	101.5 ± 6.4	107.0 ± 0.7

[a]Mean of duplicate analyses ± standard deviation.

Table 2. Intraday and Interday Variability Data for Seven COP Contents in Pig Feet Meat As Determined by GC

COP	intraday variability		interday variability	
	mean ± SD[a] (ng/g)	CV[b] (%)	mean ± SD[a] (ng/g)	CV[b] (%)
7α-OH	78.2 ± 0.6	0.8	78.7 ± 0.9	1.1
7β-OH	85.4 ± 1.8	2.1	86.0 ± 2.3	2.7
5,6β-EP	676.3 ± 19.2	2.8	684.2 ± 6.3	0.9
5,6α-EP	529.6 ± 14.9	2.8	537.6 ± 17.2	3.2
triol	31.8 ± 1.2	3.8	32.7 ± 0.9	2.8
25-OH	34.5 ± 1.8	5.2	35.2 ± 2.0	5.7
7-keto	120.6 ± 4.3	3.6	138.5 ± 6.2	4.5

[a]Mean of nine replicates (n = 9) ± standard deviation with three replicates each in morning, afternoon, and evening within the same day for intraday variability and three replicates in a day for a total of nine replicates within 3 days for interday variability. [b]Coefficient of variation (%).

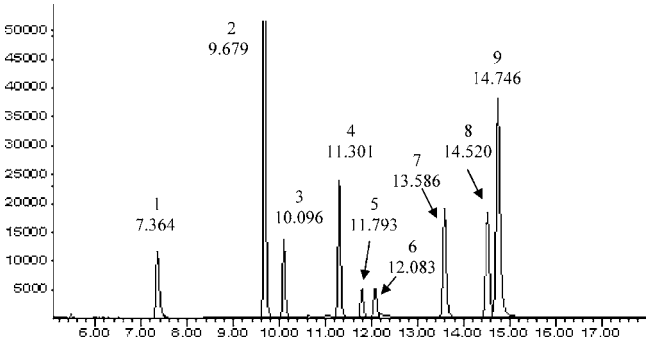

Figure 1. GC-MS chromatogram of cholesterol and seven COP standards plus one internal standard by modifying temperature-programming conditions. Peaks: 1, 5α-cholestane (internal standard); 2, 7α-OH; 3, cholesterol; 4, 7β-OH; 5, 5,6β-EP; 6, 5,6α-EP; 7, triol; 8, 25-OH; 9, 7-keto.

COPs. Also, a higher peak response was found for 5α-cholestane when compared to another internal standard 19-OH. More importantly, compared to some other published studies by Lee et al.[1] and Chen et al.,[2] the retention time was reduced and a better resolution was attained by our method.

For detection of cholesterol and COPs by GC-MS, the SIM mode was used instead of total ion mode to reduce interference by impurities and enhance sensitivity at the same time.[1,2] Accordingly, the most appropriate scanning frequency of ions is 2−3 times/s. However, the sensitivity would decrease if excessive ions are scanned within one group at the same time. Conversely, when only one characteristic ion is selected for scanning, the sensitivity can be enhanced substantially, but peak splitting may occur because of a sharp rise in scanning frequency. Thus, on the basis of elution order, seven groups were selected with three to six characteristic ions in one group being used to detect 5α-cholestane, 7α-OH, cholesterol, 7β-OH, 5,6β-EP, 5,6α-EP, triol, 25-OH, and 7-keto as shown in the preceding section. Although cholesterol could be separated from the other COPs by GC-MS, the quantitation of cholesterol in pig feet samples was not carried out as most cholesterol was found to be coeluted with impurities when using a silica cartridge for purification. This may be accounted for by the presence of a much larger amount of cholesterol when compared to COPs in pig feet samples.

Figure 2 shows the GC-MS-SIM chromatograms of pig feet meat after marinating for 2 h (A) and 24 h (B), with a total of

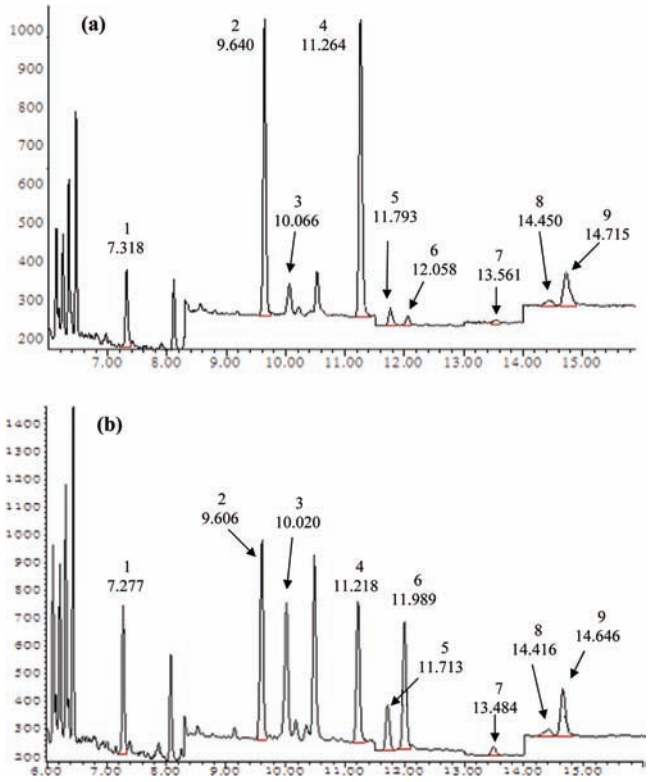

Figure 2. GC-MS-SIM chromatogram of meat extract of pig feet marinated for 2 h (a) and 24 h (b) when purified using a silica cartridge. Peaks: 1, 5α-cholestane (internal standard); 2, 7α-OH; 3, cholesterol; 4, 7β-OH; 5, 5,6β-EP; 6, 5,6α-EP; 7, triol; 8, 25-OH; 9, 7-keto.

seven COPs, including 7α-OH, 7β-OH, 5,6β-EP, 5,6α-EP, triol, 25-OH, and 7-keto, being detected. Apparently with SIM detection mode, a lot of impurities were minimized for COP detection. The linear regression equations for 7α-OH, 7β-OH, 5,6β-EP, 5,6α-EP, triol, 25-OH, and 7-keto were $y = 2.6086x - 1.4698$, $y = 1.8812x - 1.1376$, $y = 0.0559x - 0.0295$, $y = 0.1616x - 0.0959$, $y = 0.5378x - 0.1569$, $y = 1.1318x - 0.3554$, and $y = 0.2947x - 0.0447$, respectively, whereas the correlation coefficients (R^2) ranged from 0.9902 to 0.9986. The LODs for 7α-OH, 7β-OH, 5,6β-EP, 5,6α-EP, triol, 25-OH, and 7-keto were 12, 9, 75, 9.5, 2.5, 3.2, and 2.9 ng/g, respectively, whereas the LOQs were 37, 28.5, 227, 29, 7.7, 9.7, and 8.8 ng/g, respectively. The standard curves, LODs, and LOQs of

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Table 3. Effect of Heating Time on the Contents of Free COPs (Nanograms per Gram) in Freeze-Dried Pig Feet Skin, Meat, and Juice

	time[a]						
COP	control[b]	0 h[c]	2 h	4 h	8 h	12 h	24 h
Pig Feet Skin							
7α-OH	87.0 ± 4.2 b	59.8 ± 1.5 e	65.1 ± 1.7 d	67.6 ± 0.2 cd	64.7 ± 0.7 d	70.6 ± 0.7 c	97.3 ± 0.1 a
7β-OH	96.4 ± 3.8 a	61.9 ± 2.4 c	66.8 ± 2.1 bc	66.1 ± 0.4 bc	62.1 ± 1.2 c	70.9 ± 0.8 b	97.3 ± 0.6 a
5,6β-EP	256.8 ± 9.6 d	211.7 ± 17.8 e	283.9 ± 19.6 d	297.9 ± 0.1 d	382.2 ± 24.6 c	519.4 ± 28.1 b	584.7 ± 3.6 a
5,6α-EP	98.3 ± 11.2 e	69.3 ± 3.1 f	83.0 ± 4.3 ef	122.8 ± 2.6 d	214.0 ± 5.6 c	286.0 ± 14.0 b	475.2 ± 3.1 a
triol	25.2 ± 0.7 b	22.0 ± 0.1 d	23.5 ± 0.4 c	26.0 ± 0.3 b	29.4 ± 0.0 a	24.8 ± 0.1 b	29.4 ± 0.2 a
25-OH	35.8 ± 0.7 e	35.1 ± 0.1 e	36.7 ± 0.6 de	38.6 ± 0.3 c	38.5 ± 0.8 cd	41.2 ± 1.3 b	48.3 ± 0.6 a
7-keto	115.1 ± 17.6 b	56.9 ± 5.8 c	75.9 ± 7.3 c	79.0 ± 0.3 c	79.6 ± 2.3 c	108.2 ± 11.9 b	165.6 ± 4.2 a
total COPs	714.5 ± 47.8 B	516.8 ± 30.8 B	634.7 ± 36.0 B	698.0 ± 4.2 B	870.5 ± 35.2 A	1121.2 ± 56.9 A	1497.8 ± 12.2 A
Pig Feet Juice							
7α-OH	72.7 ± 1.0 b	39.7 ± 0.1 d	46.0 ± 0.6 cd	42.2 ± 0.4 cd	41.2 ± 0.0 cd	54.6 ± 0.0 c	135.7 ± 14.1 a
7β-OH	95.5 ± 2.0 b	42.7 ± 0.2 d	50.0 ± 1.0 cd	45.7 ± 0.5 cd	44.8 ± 0.1 cd	61.8 ± 0.2 c	162.8 ± 19.3 a
5,6β-EP	172.1 ± 5.0 b	71.3 ± 1.5 c	103.3 ± 3.9 bc	53.3 ± 3.4 c	51.1 ± 0.7 c	84.6 ± 3.0 c	410.5 ± 79.8 a
5,6α-EP	55.8 ± 0.8 b	40.7 ± 0.5 b	67.9 ± 5.4 b	42.9 ± 0.2 b	42.7 ± 0.3 b	57.3 ± 1.4 b	179.9 ± 28.8 a
triol	20.1 ± 0.0 b	18.9 ± 0.0 d	19.4 ± 0.2 c	19.4 ± 0.1 c	19.7 ± 0.3 bc	20.5 ± 0.1 a	20.7 ± 0.3 a
25-OH	21.0 ± 0.2 cd	21.9 ± 0.2 bc	22.5 ± 0.5 b	19.6 ± 0.3 e	19.3 ± 0.3 e	20.5 ± 0.0 de	24.4 ± 1.1 a
7-keto	124.5 ± 6.0 b	16.6 ± 0.6 c	35.4 ± 3.7 c	31.5 ± 0.1 c	23.3 ± 0.6 c	70.8 ± 1.4 bc	423.4 ± 80.2 a
total COPs	561.8 ± 15.0 B	251.7 ± 3.1 C	344.6 ± 15.3 C	254.7 ± 5.0 C	242.2 ± 2.3 C	370.1 ± 6.1 C	1357.4 ± 223.6 A
Pig Feet Meat							
7α-OH	464.6 ± 27.3 a	140.0 ± 3.8 bc	154.0 ± 3.1 b	117.0 ± 1.8 c	62.7 ± 3.2 d	67.5 ± 1.2 d	86.9 ± 1.9 d
7β-OH	714.1 ± 44.1 a	205.6 ± 8.4 b	226.7 ± 5.8 b	159.4 ± 2.8 c	66.2 ± 1.5 d	66.7 ± 0.1 d	86.9 ± 2.7 d
5,6β-EP	1253.0 ± 89.0 a	414.2 ± 5.7 cd	500.9 ± 33.6 c	445.1 ± 5.1 cd	293.3 ± 14.8 e	354.2 ± 3.3 de	621.3 ± 18.1 b
5,6α-EP	241.1 ± 0.4 b	87.1 ± 0.3 d	118.7 ± 0.9 c	136.7 ± 0.5 c	130.5 ± 3.4 c	138.7 ± 3.8 c	553.8 ± 26.6 a
triol	34.9 ± 1.6 a	28.8 ± 0.5 b	27.7 ± 1.0 b	25.6 ± 0.3 c	23.6 ± 0.2 d	22.9 ± 0.0 d	28.6 ± 0.1 b
25-OH	42.0 ± 1.6 a	30.4 ± 1.0 bc	32.2 ± 1.4 b	29.7 ± 0.2 cd	27.7 ± 0.0 d	28.2 ± 0.2 cd	29.7 ± 0.1 cd
7-keto	1134.0 ± 60.0 a	219.2 ± 2.0 b	268.9 ± 18.5 b	215.7 ± 8.9 b	69.7 ± 0.9 d	111.9 ± 2.6 cd	158.1 ± 5.1 c
total COPs	3883.8 ± 224.0 A	1125.7 ± 21.7 A	1329.2 ± 64.3 A	1129.2 ± 19.6 A	673.8 ± 24.0 B	790.2 ± 11.2 B	1565.5 ± 54.6 A

[a]Mean of duplicate analyses ± standard deviation. Values within a row with the different letters are significantly different ($p < 0.05$). For total COPs only: A–C denote values within a column with the different letters are significantly different ($P < 0.05$). [b]pig feet preheated in pure water at 100 °C for 30 min. [c]pig feet preheated in fresh juice at 100 °C for 30 min.

Table 4. Changes of pH and Maillard Browning Reaction (MR) Index in Freeze-Dried Pig Feet Skin, Meat, and Juice When Heated for Various Lengths of Time

		time[a]						
pig feet		control	0 h	2 h	4 h	8 h	12 h	24 h
skin								
	pH							
	MR index	0.02 ± 0.00 e	0.14 ± 0.02 de	0.18 ± 0.07 de	0.25 ± 0.02 d	0.43 ± 0.04 c	0.67 ± 0.06 b	0.92 ± 0.16 a
meat								
	pH							
	MR index	0.10 ± 0.01 e	0.25 ± 0.02 de	0.34 ± 0.07 cd	0.43 ± 0.03 c	0.44 ± 0.03 c	0.63 ± 0.02 b	1.04 ± 0.15 a
juice								
	pH	7.14	5.79	5.95	6.20	6.03	6.15	6.11
	MR index[b]	0.08 ± 0.01 d	0.23 ± 0.00 c	0.23 ± 0.00 c	0.27 ± 0.03 b	0.26 ± 0.01 bc	0.26 ± 0.01 bc	0.36 ± 0.01 a

[a]Values within a row with different letters are significantly different ($p < 0.05$). [b]Sample diluted 10-fold and absorbance measured at 420 nm.

cholesterol were not performed because of difficulty in purification with a silica gel cartridge as indicated above.

Changes of COPs during Marinating of Pig Feet. Table 3 shows the effect of heating time on changes of COPs during marinating of pig feet samples. With control treatment, seven COPs including 7α-OH, 7β-OH, 5,6β-EP, 5,6α-EP, triol, 25-OH, and 7-keto were detected in pig feet meat, skin, and juice. This outcome implied that with heating of pig feet in pure water at 100 °C for 30 min, a substantial amount of COPs was generated, especially for 5,6β-EP and 7-keto. Compared to the marinating treatment for 0 h (preheated in fresh juice at 100 °C for 30 min), a much higher amount of COPs was formed for the control treatment, indicating the soaking of pig feet in marinated juice may be protective against cholesterol oxidation in pig feet meat, skin, and juice. It may be inferred that the composition of marinated juice, especially for soy sauce and sugar, should be an imperative factor in preventing cholesterol oxidation,[1] as evidenced by a higher Maillard browning index for the 0 h marinating treatment, which equaled 0.14, 0.25, and 0.23 for pig feet skin, meat, and juice, respectively (Table 4). It is also possible that the presence of natural antioxidants such as

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Table 5. Effect of Heating Time on the Contents (Milligrams per Gram) of Reducing Sugar and Free Amino Nitrogen in Freeze-Dried Pig Feet Skin, Meat, and Juice

		time[a]						
	pig feet	control	0 h	2 h	4 h	8 h	12 h	24 h
skin								
	reducing sugar	4.4 ± 0.1 a	4.9 ± 0.0 a	4.9 ± 0.2 a	6.3 ± 0.1 a	6.7 ± 1.9 a	6.8 ± 3.5 a	6.2 ± 0.6 a
	free amino acid	trace	trace	3.1 ± 1.9 c	9.0 ± 2.9 ab	10.1 ± 0.6 a	11.8 ± 0.6 a	5.6 ± 0.6 bc
meat								
	reducing sugar	3.9 ± 0.0 c	4.8 ± 0.1 bc	4.7 ± 0.0 bc	6.4 ± 0.3 b	6.3 ± 2.5 bc	7.0 ± 0.0 ab	8.8 ± 0.1 a
	free amino acid	trace	13.1 ± 3.7 c	16.6 ± 3.7 bc	19.7 ± 4.3 bc	24.9 ± 5.7 ab	31.1 ± 5.8 a	26.3 ± 1.2 ab
juice								
	reducing sugar	1.7 ± 0.1 d	6.0 ± 0.1 a	5.6 ± 0.1 a	4.4 ± 0.1 c	4.9 ± 0.0 b	5.0 ± 0.4 b	5.7 ± 0.3 a
	free amino acid	trace	9.6 ± 3.2 b	9.8 ± 1.5 b	11.7 ± 0.3 ab	12.1 ± 0.7 ab	14.0 ± 1.9 ab	15.6 ± 1.2 a

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

α-tocopherol, ubiquinone, ascorbate, and histidine-dipeptides in pig meat may be effective against COPs formation.

During subsequent marinating, most COP contents in pig feet skin increased following a rise in heating time and reached a peak in 24 h, with 5,6β-EP being produced in largest amount, followed by 5,6α-EP, 7-keto, 7β-OH, 7α-OH, 25-OH, and triol (Table 3). The same tendency was observed for most COPs in pig feet juice over a 24 h heating period (Table 3), but in pig feet meat, an inconsistent change for most COPs occurred (Table 3). Comparatively, the total COPs generated in pig feet meat during the heating period of 0−4 h were significantly higher than in pig feet skin and juice. It may be postulated that phospholipids in pig feet meat membrane contained more polyunsaturated fatty acids, which were released because of membrane destruction during heating to promote cholesterol oxidation.[8] In addition, during heating protein denaturation may result in a release of heme ion (Fe^{3+}) or nonheme ion (Fe^{2+}) to accelerate cholesterol oxidation.[20,21] However, after heating for 8 and 12 h, pig feet juice showed lower total COPs than pig feet meat and skin, probably caused by migration of antioxidants from meat and skin into juice. Nevertheless, large amounts of total COPs were produced in pig feet meat, skin, and juice after prolonged heating for 24 h, which may be due to degradation of antioxidants.

More specifically, in pig feet meat, both 7α-OH and 7β-OH showed insignificant change during the initial 2 h heating period and then declined afterwards. The formation of 7α-OH or 7β-OH should be due to reduction of 7-OOH (7α-OOH or 7β-OOH), the initial cholesterol oxidation product formed during heating, whereas the decrease should be caused by subsequent dehydrogenation of 7α-OH or 7β-OH for 7-keto formation after extensive heating.[1,22] Additionally, 7-keto can also be formed through dehydration of 7-OOH, which should explain why a maximum level of keto was produced in pig feet meat when preheated in fresh juice at 100 °C for 30 min. Nonetheless, the decline in 7-keto content after 8 h of heating should be due to its further conversion to cholesta-3,5-dien-7-one. Both 5,6β-EP and 5,6α-EP showed a similar trend but with a plateau reached in 24 h. It has been well documented that the generation of 5,6β-EP and 5,6α-EP can be due to oxidation of cholesterol in the presence of 7β-OOH and 7α-OOH, respectively.[2] Also, 5,6β-EP was formed at a greater amount than 5,6α-EP, which can be attributed to the instability of the former.[2] Theoretically, under acidic condition, both 5,6β-EP and 5,6α-EP should be converted to triol, which showed only a slight change over a 24 h heating period (Table 3), probably caused by a minor rise in pH from 5.79 to 6.11 amid liberation of basic amino acids during heating (Table 4). Likewise, only a slight change was observed for 25-OH, a side-chain oxidation product of cholesterol reported to be more difficult to generate than the other COPs.[1] In a similar study dealing with the effect of heating time on COP formation in ground meat during heating, Lee et al.[1] pointed out that 5,6β-EP was more susceptible to triol formation than 5,6α-EP under acidic condition, but triol may be further converted to cholestan-3β,5α,6-one through dehydrogenation after prolonged heating.[23] In a recent study, Chen et al.[2] further reported that after 48 h of heating of tea-leaf eggs, the degradations of 7α-OH, 7β-OH, and 7-keto were faster, resulting in a substantial decrease in the amount of total COPs. Conversely, in our study the amounts of total COPs after 24 h of heating were much higher than those without heating, which may be accounted for by the difference in food commodity (Table 3).

Effect of Marinated Juice Composition on COP Formation in Pig Feet. Compared to marinated juice, a much larger amount of total COPs was produced in pig feet skin and meat over a 24 h heating period (Table 3), indicating the incorporation of soy sauce and sugar into juice was effective in inhibiting COP formation, possibly caused by formation of Maillard reactions products (MRPs) from amino acid and reducing sugar as mentioned before. Furthermore, soy sauce is rich in browning reaction products such as melanoidin, being reported to possess high antioxidant activity.[24] In addition to melanoidin, basic amino acid in soy sauce should also be a vital factor as the Maillard reaction proceeded more rapidly under basic condition. In a similar study Jayathilaka and Sharma[25] reported that the MRPs produced by glucose−lysine or glucose−glycine were the most efficient in retarding lipid oxidation. To further elucidate the effect of heating time on MRP formation, the browning index measured at 420 nm was used for evaluation (Table 4). A time-dependent increase in MR index was observed for pig feet skin, meat, and juice over a 24 h marinating period, probably because of sucrose hydrolysis into reducing sugar and protein degradation into amino acid during marinating (Table 4). This outcome was further supported by a maximum reducing sugar formation being observed in pig feet skin, meat, and juice after 24 h of heating, but a peak level of free amino acid only shown in pig feet meat and juice (Table 5). The reduction in free amino acid in pig feet skin from 11.8 mg/g for 12 h of heating to 5.6 mg/g for 24 h of heating revealed that caramelization may also occur in skin, contributing to an increment in MR index after prolonged marinating. Nonetheless, as indicated above, the total COPs showed a more pronounced decline for the 0 h marinating

treatment than for the control treatment (Table 3). It may be postulated that the low molecular weight MRPs formed during the period of preheating may possess antioxidant activity.[25] However, after extensive heating for 24 h, the formation of high molecular weight polymerization products from melanoidin may lose antioxidant activity.[26] This may explain why a larger amount of total COPs was produced in pig feet skin, meat, and juice after 24 h of marinating when compared to 0 h of marinating. Additionally, only a slight change in moisture content was shown for pig feet skin, meat, and juice over a 24 h marinating period (data not shown), implying the moisture content change should be an insignificant factor in affecting COP formation.

In conclusion, a GC-MS method was developed to separate internal standard 5α-cholestane, cholesterol, and seven COPs, including 7α-OH, 7β-OH, 5,6β-EP, 5,6α-EP, triol, 25-OH, and 7-keto, within 15 min and using SIM mode for detection. This work is the first of its kind to employ a GC-MS method to detect COPs in pig feet samples. A silica gel cartridge was used for COP purification. After 24 h of marinating, the total COPs were generated in largest amount in pig feet meat, followed by skin and juice. The incorporation of soy sauce and crystal sugar into juice was effective in retarding COP formation in pig feet skin, meat, and juice during preheating for 30 min, probably caused by MRP formation between reducing sugar and amino acid.

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