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Analysis and formation of polycyclic aromatic hydrocarbons and cholesterol oxidation products in thin slices of dried pork during processing

Yu-Ting Hung [a], Yu-Tsung Lee [b], Baskaran Stephen Inbaraj [a], Kandi Sridhar [a], Bing-Huei Chen [a,c,*]

[a] Department of Food Science, Fu Jen Catholic University, New Taipei City 242, Taiwan
[b] Research Center for Chinese Herbal Medicine and Research Center for Food and Cosmetic Safety, Chang Gung University of Science and Technology, Taoyuan City 333, Taiwan
[c] Department of Nutrition, China Medical University, Taichung 404, Taiwan

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ABSTRACT

This study aims to determine toxic compounds polycyclic aromatic hydrocarbons (PAHs) and cholesterol oxidation products (COPs) in thin slices of dried pork as affected by different flavorings and roasting temperature treatments through employing a QuEChERS method coupled with gas chromatograph–tandem mass spectrometer (GC-MS/MS) and gas chromatograph–mass spectrometer (GC-MS), respectively. By employing this method, high accuracy and precision was attained for freeze-dried pork hind leg sample. Following addition of 8 different flavorings with roasting temperature at 120, 160, and 200 °C, the levels of total COPs and PAHs in thin slices of dried pork followed a temperature-dependent increase during roasting, which was further confirmed by principle component analysis. High level of soy sauce or sugar inhibited COP formation, while the low-level minimized PAH formation in thin slices of dried pork during roasting. Sugar was more effective in inhibiting COP formation while soy sauce was more efficient in reducing PAH formation.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs), composed of only carbon and hydrogen atoms with two or more aromatic rings, can be formed through incomplete combustion or pyrolysis of coal, oil, gas and wood. According to a report issued by European Food Safety Authority (2008) and European Commission (2011), a total of 24 PAHs have been listed as the most frequently occurring PAHs in food and environment. Of the various PAHs, benzo[*a*]pyrene (BaP) was classified into Group 1 carcinogens (carcinogenic to humans), cyclopenta[*c*,*d*]pyrene (CPP), dibenzo[*a*,*h*]anthracene (DBahA) and dibenzo[*a*,*l*]pyrene (DBalP) classified into Group 2A carcinogens (probable human carcinogen), naphthalene (NaP), benzo[*a*]anthracene (BaA), chrysene (CHR), 5-methylchrysene (MCH), benzo[*b*]fluoranthene (BbFA), benzo[*j*]fluoranthene (BjFA), indeno[1,2,3-*c*,*d*]pyrene (IP), dibenzo[*a*,*i*]pyrene (DBaiP), and dibenzo[*a*,*h*]pyrene (DBahP) classified into Group 2B carcinogens (possible human carcinogen), acenaphthene (AcP), fluorene (Flu), phenanthrene (Phe), anthracene (Ant), pyrene (Pyr), benzo[*c*]fluorene (BcF), dibenzo[*a*,*e*]pyrene (DBaeP) and benzo[*ghi*]perylene (BghiP) classified into Group 3 (not carcinogenic to human) based on a report issued by the International Agency for Research on Cancer (IARC, 2012).

Human exposure to PAHs has been reported to be mainly from air pollution and processed foods, especially edible oil and meat products (Alomirah et al., 2011). In a study dealing with vegetable oils in canned foods, a BaP level of > 0.0020 and 0.0019 mg/L were reported in sunflower oil of 15% canned vegetable samples and olive oil from canned tuna fish, while the highest level of 0.0113 mg/L was shown in oil from canned mushroom (Moret, Purcaro, & Conte, 2005). In a later study, Chen, Kao, Chen, Huang, and Chen (2013) demonstrated that the longer the sugar smoking time, the more the formation of PAHs in meat. Following sugar smoking for 6 min, red meat could produce the highest level of total PAHs (0.0339–0.1255 mg/L), followed by poultry meat (0.0191–0.0282 mg/L) and fish meat (0.0091–0.0318 mg/L) with the highly toxic BaP being undetected.

Cholesterol oxidation products (COPs), formed from cholesterol in the presence of heat, light, free radical, enzyme or metal ion, can be generated in high level in cholesterol-rich foods such as eggs and animal

* Corresponding author.
E-mail address: 002622@mail.fju.edu.tw (B.-H. Chen).

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meats (Lee, Chien, & Chen, 2008). The mechanism of cholesterol oxidation is similar to lipid oxidation, with autoxidation, enzymatic oxidation and photooxidation dominating based on the formation route of various types of COPs reported elsewhere (Chen, Lu, Chien, & Chen, 2010). Of the various COPs, the epoxy-containing COPs (5,6α-epoxycholesterol, 5,6α-EP or 5,6β-epoxycholesterol, 5,6β-EP) were reported to possess high mutagenicity (Sevanian & Peterson, 1986), while triol was shown to induce carcinogenicity through over expression of COX-2 and subsequent increased production of PGE2 (Lo, 2005). It has been well documented that many factors such as heating condition, cooking method, light and the presence of oxygen or oxidizing agent can alter both variety and amount of COPs formed in foods (Hsu & Chen, 2020; Hsu, Inbaraj, & Chen, 2020). For instance, Derewiaka and Molińska (2015) studied the effect of temperature (120–220 °C) and time length (30–180 min) on COP formation and reported that the highest level of total COPs was generated at 150 °C for 120 min with 7-keto dominating, while the cholesterol degradation occurred with temperature ≥ 180 °C.

The analysis of PAHs or COPs in meat and meat products has been difficult due to presence of trace amount (ppb for PAHs and ppm for COPs) and complex matrix in meat. Many methods have been developed to determine PAHs or COPs in meat products, including extraction by solvent, purification by solid-phase extraction, and identification and quantitation by GC-MS or HPLC-MS (Gosetti et al., 2011; Lee, Chien, & Chen, 2008). However, most extraction and purification methods are time consuming. Recently, the QuEChERS method has been developed to extract and purify COPs or PAHs in various meat products (Chiu, Kao, & Chen, 2018; Kao, Chen, Chen, Huang, & Chen, 2012). Its application in the determination of pesticides, animal drugs, mycotoxins, acrylamides, environmental hormones and bisphenols in various kinds of food products have been well documented (Rejczak & Tuzimski, 2015). The major advantages of QuEChERS method include simplicity, fastness and cost-effectiveness involving minimum pretreatment steps (minimizes both time and experimental error), less solvent consumption and attainment of excellent recoveries (Musarurwa, Chimuka, Pakade, & Tavengwa, 2019). However, the application of QuEChERS method coupled with GC-MS/MS and GC-MS for determination of PAHs and COPs in thin slices of dried pork remains unexplored. Also, there is a paucity of data regarding their formation as affected by flavorings containing sugar and soy sauce as well as the other ingredients at different roasting temperatures. Thus, in this study we intend to evaluate current QuEChERS methods coupled with GC-MS/MS and GC-MS for determination of PAHs and COPs, respectively, in thin slices of dried pork, a popular meat commodity in Asian countries especially Taiwan and China, as affected by 8 flavorings containing different levels of sugar (0, 4, 8, 16%) and soy sauce (0, 4 and 8%) and 3 roasting temperatures (120, 160 and 200 °C).

2. Materials and methods

2.1. Preparation of thin slices of dried pork

Pork hind leg samples were purchased from 10 different commercial vendors in a local market of New Taipei City, Taiwan. After removal of fascia and fur, the pork hind leg samples were homogenized into minced meat, divided into several portions of about 100 g each, vacuum packed and stored in a refrigerator at 4 °C for further use. Then each portion was seasoned with different flavorings including salt (1.5 g/100 g; 1.5%), soybean oil (5 g/ 100 g; 5%), black pepper (1 g/100 g; 1%), almond slices (1 g/100 g; 1%) as well as various proportions of sugar (0, 4, 8 or 16 g/100 g; 0, 4, 8 or 16%) and soy sauce (0, 4 or 8 g/100 g; 0, 4 or 8%). In the subsequent sections the proportion of all the flavorings will be expressed as percentage. Fig. 1 illustrates 8 different flavoring treatments along with their composition used in this study. The proportions of salt, soybean oil, black pepper and almond slices as shown above was fixed, while both sugar and soy sauce were added in 8 different proportions: 8% sugar plus 8% soy sauce and the other ingredients (standard flavoring, STF), 8% sugar plus 4% soy sauce and the other ingredients (F1), 4% sugar plus 8% soy sauce and the other ingredients (F2), 16% sugar plus 4% soy sauce and the other ingredients (F3), 4% sugar plus 4% soy sauce and the other ingredients (F4), 16% sugar plus 8% soy sauce and the other ingredients (F5), the STF without soy sauce (F6), and the STF without sugar (F7). The STF, composed of 8% sugar, 8% soy sauce, 1.5% salt, 5% soybean oil, 1% black pepper, and 1% almond slices, was selected as it is often used in many factories for processing thin slices of dried pork with a roasting temperature 160 °C in Taiwan. As a decrease in COP formation in thin pork slices was observed following a rise in both sugar and soy sauce levels (sugar more effective than soy sauce) in our study, the flavorings F3 and F5 containing one higher level of sugar at 16% was also included in addition to 4% and 8% sugar. After mixing thoroughly, the flavored minced meat was flattened into thin slices (1 mm thin slices) by a KW-RP01 model rolling pin from Holar Industrial Inc. (Taipei, Taiwan), followed by drying in a cold air dryer (RO-340 model, Firstek Scientific Co. (New Taipei City, Taiwan) at 50 °C for 2 h one side and then turning over for the other side (2 h) for a total of 4 h (0.1 mm thin slices after drying). Then the air-dried pork slices were further heated in an oven (SM-803 T+3S+3B model, Sinmag Equipment Corp., Taipei, Taiwan) with temperature at 120, 160 or 200 °C for 2 min one side and then turned over for the other side (2 min)

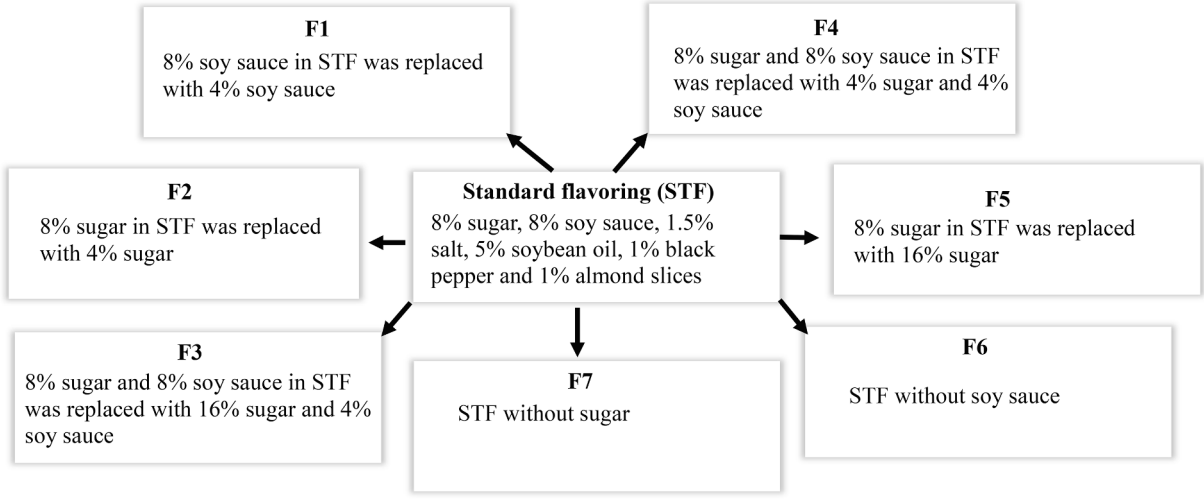

Fig. 1. Different flavouring treatments along with their composition used in this study.

for a total of 4 min to obtain the final product of thin slices of dried pork. The entire processing steps involved in the preparation of thin slices of dried pork from pork hind leg and their products with different flavorings and roasting temperatures are shown in Fig. S1 (supplementary information).

2.2. Chemical reagents

A total of 23 PAH standards, including NaP, acenaphthylene (AcPy), AcP, Flu, Phe, Ant, fluoranthene (FL), Pyr, BcF, BaA, CPP, CHR, MCH, BbFA, BjFA, BaP, IP, DBahA, BghiP, DBalP, DBaeP, DBaiP and DBahP were obtained from Sigma Co. (St. Louis, MO, USA). Cholesterol and 5 COP standards including 7-ketocholesterol (7-keto), 5,6α-EP, 5,6β-EP, 25-hydroxycholesterol (25-OH) and triol, as well as internal standard 5α-cholestane were also procured from Sigma Co., while both 7α-hydroxycholesterol (7α-OH) and 7β-hydroxycholesterol (7β-OH) standards were obtained from Steraloids Co (Wilton, NH, USA).

The HPLC-grade acetonitrile and analytical grade acetone were from Merck Co. (Darmstadt, Germany), pyridine from J.T. Baker Co (Phillipsburg, NJ, USA) and both toluene and hexane from Sigma Co. Deionized water was made using a Milli-Q water purification system (Millipore Co, Bedford, MA, USA). The COP derivatization agent Tri-Sil TBT, composed of *N*-trimethylsilyimidazole (TMSI), N,O-bis trimethylsilyl acetamide (BSA) and trimethylchlorosilane (TMCS) at a ratio of 3:3:2, was from Thermo Fisher Scientific Co (San Jose, CA, USA). The QuEChERS extraction kit containing extraction and purification powders was from Yo-Ho Co (New Taipei City, Taiwan). Two DB-5MS capillary columns with film thickness 0.25 μm used for separation of COPs (30 m × 0.25 mm ID) and PAHs (15 m × 0.25 mm ID) were from Agilent Technologies Co. (Palo Alto, CA, USA).

2.3. Extraction and purification of PAHs and COPs in freeze-dried pork and thin slices of dried pork

A QuEChERS method based on Kao, Chen, Chen, Huang, and Chen (2012) was used to extract and purify PAHs from freeze-dried pork and thin slices of dried pork. Although this study mainly focusses on determining PAH or COP contents in thin pork slices as affected by different flavoring and roasting temperature, their levels in freeze-dried pork were also determined as it was used as a blank sample matrix for matrix effect determination and method validation. Accordingly, from the analytical point of view, it is important to evaluate the reported QuEChERS method coupled with GC-MS/MS and GC-MS for determination of PAHs and COPs in thin pork slices, respectively. Initially, a 5-g homogenized sample was mixed with a ceramic homogenizer in a 50-mL centrifuge tube, followed by adding 10 mL of deionized water and shaking for one min. Then, 10 mL of acetonitrile was added, shaken for one min, the extraction powder (1 g $MgSO_4$; 1 g CH_3COONa, 1 g) added, shaken again for one min and centrifuged at 4000 *g* for 5 min (15 °C). The supernatant (6 mL) was then collected and poured into a 15-mL centrifuge tube containing the purification powder (300 mg PSA; 900 mg $MgSO_4$; 300 mg C18EC), followed by shaking for one min, centrifuging at 4000 *g* for 5 min (15 °C), collecting the supernatant (1 mL) and evaporating to dryness under nitrogen. Finally, the residue was dissolved in one mL hexane, filtered through a 0.22-μm Nylon membrane filter and injected for GC-MS/MS analysis. For extraction and purification of COPs, the same QuEChERS method as used above for PAHs was adopted with the exception that acetonitrile solvent was replaced with acetone and 1 g $MgSO_4$ in extraction powder with 4 g $MgSO_4$ as well as the final residue dissolved in pyridine instead of hexane for GC-MS analysis (Chiu, Kao, & Chen, 2018).

2.4. Evaluation of matrix effect

The evaluation of matrix effect for PAHs and COPs was based on a method described by Chang, Zhang, Wang, and Chen (2019). Initially, a standard calibration curve was developed by preparing five concentrations of each PAH standard (10, 30, 50, 70 and 100 ng/mL) in hexane or six concentrations (0.625, 1.25, 2.5, 5, 10 and 20 μg/mL) of each COP in pyridine and injecting into GC-MS/MS or GC-MS. Likewise, the same number of concentrations of each PAH or COP standard were prepared and added to freeze-dried pork hind leg extract (blank sample matrix) for GC-MS/MS or GC-MS analysis to prepare matrix-matched calibration curves. Then the matrix effect of PAHs and COPs was determined by using the formula as shown below (Chang, Zhang, Wang, & Chen, 2019):

$$\text{Matrix effect } (\%) = \frac{A_{matrix}}{A_{solvent}} \times 100$$

where, A_{matrix} and $A_{solvent}$ are the peak areas of each PAH or COP in freeze-dried pork hind leg extract and standard solution, respectively.

2.5. Method validation of PAHs and COPs

Both limit of detection (LOD) and limit of quantitation (LOQ) for 23 PAHs were determined by preparing a series of 16 concentrations (0.03, 0.05, 0.1, 0.3, 0.5, 0.7, 0.9, 1.0, 3.0, 5.0, 7.0, 9.0, 10, 30, 50 and 70 ng/mL) for each PAH and adding to blank sample matrix for extraction, purification and GC-MS/MS analysis. Similarly, both LOD and LOQ for COPs was determined by preparing a series of 14 concentrations (2.5, 5, 10, 20, 30, 40, 50, 100, 150, 200, 300, 400, 500 and 600 μg/mL) for each COP and adding to blank sample matrix for extraction, purification and GC-MS analysis. The LOD of PAHs or COPs was then determined based on the signal/noise ratio (S/N) $\geq$ 3, while the LOQ was based on S/N $\geq$ 10.

The recovery of PAHs or COPs was determined by adding separately two standard solutions of PAHs (10 and 50 ng/g) or COPs (1 and 5 μg/g) to blank sample matrix, followed by extraction, purification, and GC-MS/MS or GC-MS analysis for obtaining the amount of various PAHs or COPs in meat samples. Then the recovery of PAHs or COPs was obtained by using the formula as shown below:

$$\text{Recovery } (\%) = \frac{PAH/COP_{found} - PAH/COP_{original}}{PAH/COP_{amount\ added}}$$

where, PAH/COP_{found} and $PAH/COP_{original}$ are the amounts of PAH/COP determined after adding the respective standards and that originally present in the sample respectively, while $PAH/COP_{amount\ added}$ is the amount of standard added to the sample matrix.

For the precision study, the intra-day variability was determined by adding 10 ng/g of PAH or 1 μg/g of COP standard to blank sample matrix, followed by extraction, purification and GC-MS/MS or GC-MS analysis for obtaining the amount of various PAHs or COPs in meat samples. The intra-day variability was based on triplicate analyses in morning, afternoon and evening on the same day for a total of 9 analyses. Likewise, the inter-day variability was determined in the same way with the exception that triplicate analyses was performed one day for three consecutive days for a total of 9 analyses. Then, the coefficient of variation (CV) was determined by calculating the standard deviation and substituting in the formula, CV= (standard deviation/average) ×100.

2.6. Separation, identification and quantification of PAHs or COPs

A DB-5MS capillary column (15 m × 0.25 mm ID, film thickness 0.25 μm) was used to separate 23 PAHs within 78 min in the splitless mode with helium carrier gas at 1.25 mL/min, MS-interface temperature at 280 °C and injector temperature at 320 °C. The following temperature programming condition was used: initial temperature at 80 °C, maintained for one min, raised to 200 °C at 5 °C/min, maintained for 10 min, raised to 220 °C at 5 °C/min, maintained for 5 min, raised to 230 °C at 1 °C/min, maintained for 10 min, and raised to 320 °C at 10 °C/min, maintained for 10 min. A triple quadrupole tandem mass spectrometer

(QqQ) (model 7890 B and 7000 C) from Agilent was used with electrospray ionization and the operation parameters of 23 PAH standards detected by multiple reaction monitoring (MRM) mode are shown in Table S1.

For COP separation and identification, a DB-5MS capillary column (30 m × 0.25 mm ID, film thickness 0.25 μm) was used in the splitless mode to separate 7 COPs and internal standard (5α-cholestane) within 14 min with helium carrier gas at 1 mL/min, injector temperature at 280 °C, MS-interface temperature at 300 °C and the temperature programming as shown below: initial temperature at 250 °C, raised to 290 °C at 10 °C/min, maintained for 5 min, raised to 291 °C at 0.1 °C/min, and maintained for one min. A GC-MS instrument (Model 7890 and 5975) from Agilent was used. After calibrating with perfluorotributylamine (PFTBA) standard with mass-to-charge (m/z) ratio at 69, 219, and 50, the various COPs in samples were detected by selected ion monitoring (SIM) mode based on the elution order and the characteristic m/z ratio (Table S2, supplementary information).

For PAH quantitation, 5 concentrations (10, 30, 50, 70 and 100 ng/mL) of 21 PAH standards were prepared in hexane separately, added to blank sample matrix for GC-MS/MS analysis and the matrix-matched calibration curves developed by plotting concentration against peak area of quantitative ion and the linear regression equations along with coefficient of determination (R^2) were calculated. It is worth pointing out that PAHs were quantified using the matrix-matched calibration curves instead of standard calibration curves mainly because the latter could result in quantitation of 4 priority PAHs (BaP, CHR, BaA and BbFA) exceeding the maximum safety limit (0.01 mg/L for 4 PAHs) regulated by European Commission (2011).

For COP quantitation, 6 concentrations (0.625, 1.25, 2.5, 5, 10 and 20 μg/mL) of 7 COP standards dissolved in pyridine were prepared separately. Then 40-μL of each standard solution was collected into a 2-mL vial containing 250-μL inner tube, followed by adding 20 μL 5α-cholestane (2 μg/mL) and 40 μL derivatization agent (Tri-Sil TBT) for reaction at room temperature in the dark for 1 h and subsequent injection into GC-MS for analysis. The standard calibration curve of each COP was prepared by plotting concentration ratio (COP standard *versus* internal standard) against area ratio (COP standard *versus* internal standard) and both linear regression equations and R^2 were determined. The contents of PAHs or COPs in meat samples were calculated using a formula as described in our previous studies (Hsu & Chen, 2020; Hsu, Inbaraj, & Chen, 2020).

$$\text{Amount of PAH (ng/g)} = (A_s - b) \times \left(\frac{1}{a}\right) \times EV \times DF \times \left(\frac{1}{\text{recovery}}\right) \times \left(\frac{1}{\text{sample weight}}\right)$$

$$\text{Amount of COP } (\mu g/g) = \left(\frac{A_s}{A_i} - b\right) \times \left(\frac{1}{a}\right) \times C_i \times EV \times DF \times \left(\frac{1}{\text{recovery}}\right) \times \left(\frac{1}{\text{sample weight}}\right)$$

where, A_i and C_i are the peak area and concentration of COP internal standard (5α-cholestane) respectively, while a and b are the slope and intercept of standard curve. EV is the extraction volume, DF is the dilution factor and A_s is the peak area of PAH or COP in sample.

2.7. Statistical analysis

All the data were obtained in triplicate and analyzed by using the statistical analysis system (SAS, 2014) with MANOVA (multivariate analysis of variance) and Duncan's multiple range test for elucidating statistical significance in comparison ($p < 0.05$). Principal component analysis (PCA) was performed to analyze the relative contribution of flavorings and temperature on generation of PAHs and COPs by using Origin® 2019b version 9.65 (OriginLab Corporation, Northampton, MA, USA).

3. Results and discussion

3.1. Evaluation of PAH analysis by QuEChERS and GC-MS

In many published reports the solid-phase extraction method was frequently used for extraction and purification of PAHs in meat products, while the QuEChERS method was less often used. Forsberg, Wilson, and Anderson (2011) compared the effect of 5 QuEChERS extraction powders on the recovery of 15 PAHs in fish meat and reported that a high recovery (90%) was attained by using the extraction powder containing $MgSO_4$ (6 g) and $NaC_2H_3O_2$ (1.5 g). Recently Taiwan Food and Drug Administration (TFDA, 2018) demonstrated that with the extraction powder containing 4 g $MgSO_4$ and 1 g $NaC_2H_3O_2$ and the purification powder containing PSA-C18 EC-$MgSO_4$ (1:1:3), the highest accuracy and precision of PAHs in meat products was obtained by GC-MS/MS. Thus, in this study we used extraction powder (4 g $MgSO_4$ and 1 g $NaC_2H_3O_2$) and purification powder containing PSA-C18 EC-$MgSO_4$ (1:1:3) for PAH determination in thin slices of dried pork by GC-MS/MS.

It has been well established that GC-MS/MS is superior to GC-MS in terms of sensitivity and selectivity (Varlet, Serot, Monteau, Le Bizec, & Prost, 2007). Fig. 2A shows the GC-MS/MS chromatogram of PAHs detected by MRM mode. A total of 23 PAH standards was adequately separated within 77 min (Fig. 2A and B). Table S3 shows the matrix effect of 21 PAHs in freeze-dried pork hind leg by GC-MS/MS to be from 1.18 to 1.80, implying a signal enhancement occured for all the 21 PAHs. However, the matrix effect of NaP and Pyr was not determined as the former was detected in hexane and the latter in blank samples (freeze-dried pork hind leg). Thus, in this study NaP was not quantified in meat sample while Pyr was quantified based on the standard curve instead of matrix-matched calibration curve. In many studies the signal enhancement was observed with GC-MS/MS analysis (Liu, 2015). However, with LC-MS/MS analysis, the signal can be enhanced or suppressed depending on ion source. For instance, with electrospray ionization the signal can be suppressed, while with atmospheric pressure chemical ionization the signal can be enhanced (Chang, Zhang, Wang, & Chen, 2019).

Of the various PAHs, the LOD of AcPy, Flu, AcP, Phe, Pyr, BcF, BaA, CPP, CHR, DBaeP and DBaiP were 0.1 ng/mL, while that of BbFA, BjFA, BaP, IP, DBahA, benzo[*g,h,i*]pyrene and DBahP was 0.3 ng/mL. For the other two PAHs, the LOD of MCH and DBalP was 1 and 0.03 ng/mL, respectively. Compared to LOD, the LOQ of all the 22 PAHs were three times higher with the exception of DBalP at 0.1 ng/mL. Similar LOD and LOQ of PAHs in meat products as determined by GC-MS/MS was reported (Veyrand et al., 2007).

Table S4 shows the recovery data of 22 PAH standards and standards added to freeze-dried pork hind leg by GC-MS/MS. The average recovery of 22 PAH standards ranged from 81.2 to 98.3 % and from 76.5 to 90.7 % in freeze-dried pork hind leg. This result conforms to a regulation set up by Taiwan Food and Drug Administration (TFDA, 2013), stipulating that with the added standard concentrations at 0.01 and 0.05 mg/L, the recovery should be from 60 to 125 % and from 70 to 120 %, respectively. This outcome also revealed that a high accuracy method was attained in our study. In a previous study, Kao, Chen, Chen, Huang, and Chen (2012) also used QuEChERS and GC-MS to determine 16 PAHs in poultry meat and the recovery was from 71.7 to 107 %.

Table S5 shows the precision data of 22 PAH standards analyzed by GC-MS/MS. The CV of repeatability was from 5.35 to 11.81 %, while that of intermediate precision was from 6.47 to 13.39 %. Similarly, the CV of repeatability of 22 PAH standards added to freeze-dried pork hind leg was from 3.70 to 12.63 %, while that of intermediate precision was from 7.47 to 14.08 % (Table S6). All the repeatability and intermediate precision data meets the regulation of TFDA (2013), stating that the CV of the former should be < 30% for the analyte concentration ≥

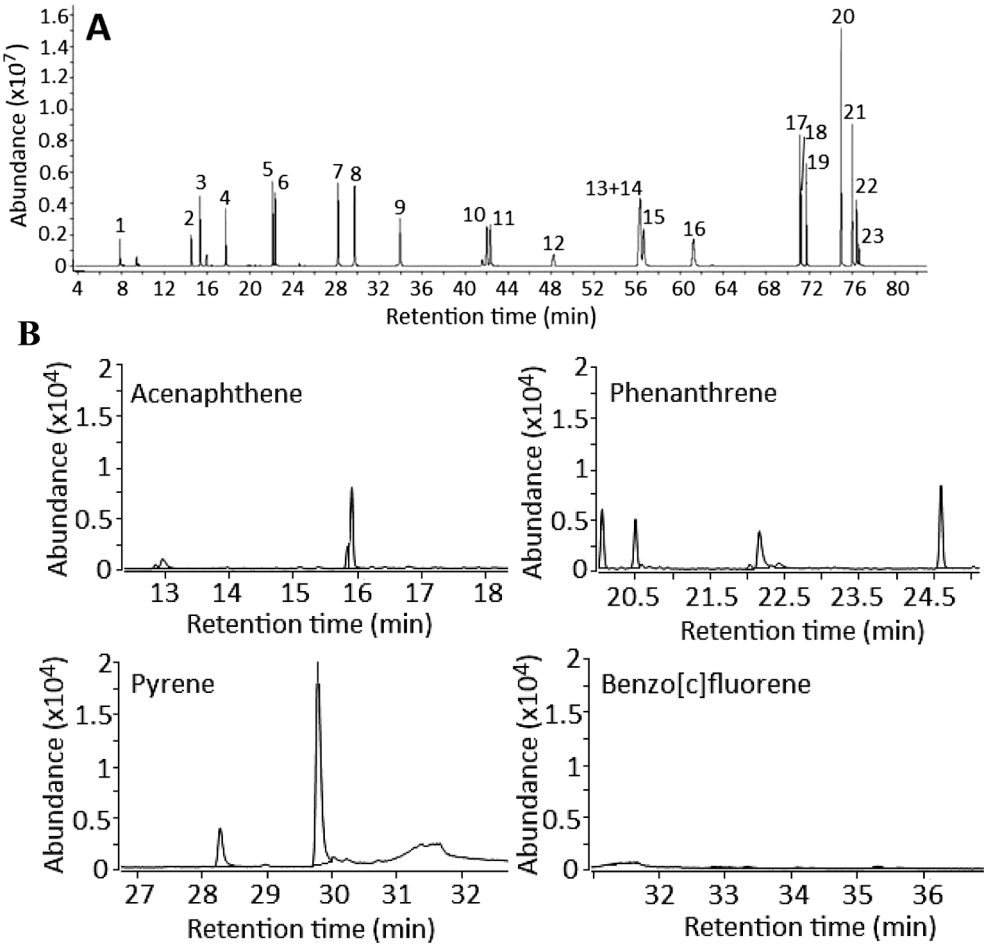

Fig. 2. GC-MS/MS chromatogram of PAHs detected by multiple reaction monitoring (MRM) mode in 23 PAH standard mixtures (A) and selected PAHs in roasted thin slices of dried pork at 160 °C with standard flavoring (STF) (B). Peaks: 1, naphthalene; 2, acenaphthylene; 3, acenaphthene; 4, fluorene; 5, phenanthrene; 6, anthracene; 7, fluoranthene; 8, pyrene; 9, benzo[*c*]fluorene; 10, benzo[*a*]anthracene; 11, chrysene; 12, methyl chrysene; 13, benzo[*b*]fluoranthene; 14, benzo[*j*]fluoranthene; 15, cyclopenta[*c*,*d*]pyrene; 16, benzo[*a*]pyrene; 17, indeno [1,2,3-*cd*]pyrene; 18, dibenzo[*a*,*h*]anthracene; 19, benzo[*g*,*h*,*i*]perylene; 20, dibenzo [*a*,*l*]pyrene; 21, dibenzo[*a*,*e*]pyrene; 22, dibenzo[*a*,*i*]pyrene; 23, dibenzo[*a*,*h*]pyrene. STF: 8% sugar, 8% soy sauce, 1.5% salt, 5% oil, 1% black pepper and 1% almond slices. PAHs = polycyclic aromatic hydrocarbons and GC-MS/MS = gas chromatograph–tandem mass spectrometer.

0.001–0.01 mg/L, while the CV of the latter should be < 32% for the same analyte concentration. This outcome also implied that a high precision was attained for the method employed in our study.

3.2. PAH contents in thin slices of dried pork

Table 1 shows PAH contents (ng/g) in raw pork and thin slices of dried pork added with the STF and flavoring without soy sauce or sugar. Only three PAHs including AcP (0.450 ng/g), Ant (0.332 ng/g) and Pyr (1.513 ng/g) were detected in raw pork. However, following the STF treatment and heating at 120, 160 and 200 °C for 4 min, some more PAHs such as BcF, AcPy or IP were generated. A temperature-dependent increase was also observed for the variety and total PAH contents in thin slices of dried pork, with the highest level shown at 200 °C (6.504 ng/g), followed by 160 °C (5.213 ng/g) and 120 °C (4.447 ng/g). A similar trend was also shown for the treatment of flavoring without soy sauce or sugar. By comparison, at the same roasting temperature, STF treatment resulted in the highest level of total PAHs, followed by the flavoring treatment without soy sauce or sugar. This result indicated that the addition of both soy sauce (8%) and sugar (8%) to the flavoring may promote PAH formation in thin slices of dried pork during roasting, while the absence of soy sauce or sugar in the flavoring may reduce the formation of total PAHs. Compared to STF, this outcome also implied that the addition of soy sauce (8%) or sugar (8%) may minimize PAH formation.

The formation of PAHs in thin slices of dried pork is probably due to formation of lipid degradation products during roasting. In a previous report Chen and Chen (2001) studied the formation mechanism of PAHs in model lipids and food lipids and postulated that the lipid degradation products containing conjugated double bonds may react with dienophile compounds to form PAHs through Diels-Alder reaction. In another study Shukla and Koshi (2011) illustrated that PAHs can be formed through hydrogen abstraction/acetylene addition, phenyl addition/cyclization and methyl addition/cyclization. In addition, the benzene ring-containing compounds from lipid degradation may further react with C1-C4 compounds from hydrogen abstraction/acetylene addition through Diels-Alder reaction for PAH formation (Chen & Chen, 2001). It is worth pointing out that the highly toxic BaP remained undetected in thin slices of dried pork probably because of short roasting time. In a similar study Chung, Yettella, Kim, Kwon, Kim, and Min (2011) studied the effect of grilling and roasting on the levels of PAHs in beef and pork and reported that following grilling at 200 °C, both pork loin and pork chop with sauce generated a higher level of total PAHs than beef loin and steak with sauce, respectively. Based on a report by Shukla and Koshi (2012), indene and benzene ring may undergo hydrogen abstraction/acetylene addition to generate BcF and NaP, respectively, followed by formation of AcP and Phe, and leading to Pyr formation. Alternatively, AcP may also undergo hydrogen abstraction to produce AcPy during roasting at 200 °C, while Pyr may be oxidized to generate IP, a PAH composed of 6 rings. Kaneko, Kumazawa, and Nishimura (2013) further pointed out that the benzene-containing compounds in soy sauce may increase from 3.37 mg/L to 40.4 mg/L after heating. As the benzene-containing compounds can be a precursor for PAH formation (Chen & Chen, 2001), the addition of high level of soy sauce (8%) as flavoring may accelerate PAH formation in thin slices of dried pork during heating. Furthermore, the formation of 5-hydroxymethylfurfural (5-HMF) from sucrose degradation during heating may result in PAH formation through Diels-Alder reaction (Settle et al., 2017). This may

Table 1
PAH contents (ng/g) in raw pork and thin slices of dried pork with standard flavoring and flavoring without soy sauce or sugar.[1]

PAHs[2]	Raw pork	STF[3]			F6[3]			F7[3]		
		120 °C	160 °C	200 °C	120 °C	160 °C	200 °C	120 °C	160 °C	200 °C
Acenaphthylene	trace[4]	trace	trace	0.335 ± 0.015^{A}	trace	trace	trace	trace	trace	trace
Acenaphthene	0.450 ± 0.080^{E}	0.548 ± 0.090^{E}	0.706 ± 0.015^{B}	0.721 ± 0.050^{B}	0.560 ± 0.090^{D}	0.581 ± 0.080^{D}	0.801 ± 0.080^{A}	0.567 ± 0.100^{D}	0.566 ± 0.050^{D}	0.678 ± 0.090^{C}
Fluorene	nd[5]	trace	trace	trace	nd	trace	trace	trace	trace	trace
Phenanthrene	nd	0.350 ± 0.060^{B}	0.427 ± 0.025^{A}	0.430 ± 0.070^{A}	trace	trace	0.470 ± 0.030 ^{A}	trace	trace	0.395 ± 0.080^{B}
Anthracene	0.332 ± 0.030^{A}	trace	trace	trace	trace	trace	trace	trace	trace	trace
Fluoranthene	nd	trace	trace	trace	trace	trace	trace	trace	trace	trace
Pyrene	1.513 ± 0.060^{D}	3.243 ± 0.300^{B}	3.770 ± 0.270^{A}	4.356 ± 0.350^{A}	2.035 ± 0.450^{D}	3.317 ± 0.050^{B}	3.769 ± 0.040^{B}	1.911 ± 0.210^{D}	3.084 ± 0.200^{C}	3.385 ± 0.160^{B}
Benzo[*c*]fluorene	trace	0.306 ± 0.003^{A}	0.310 ± 0.003^{A}	0.328 ± 0.040^{A}	trace	trace	trace	trace	trace	trace
Benzo[*a*]anthracene	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Cyclopenta[*c,d*] pyrene	trace	trace	trace	trace	trace	trace	trace	trace	trace	trace
Chrysene	trace	trace	trace	trace	trace	trace	trace	trace	trace	trace
Benzo[*a*]pyrene	trace	nd	nd	nd	nd	nd	nd	nd	nd	nd
Indeno[1,2,3-*c,d*] pyrene	trace	trace	trace	0.314 ± 0.030^{A}	trace	trace	trace	trace	trace	trace
Total	2.295^{H}	4.447^{D}	5.213^{B}	6.504^{A}	2.595^{G}	3.898^{E}	5.040^{C}	2.478^{G}	3.650^{F}	4.458^{D}

[1] Mean of triplicate analyses ± standard error. [2]Polycyclic aromatic hydrocarbons (PAHs) such as 5-methylchrysene, benzo[*b*]fluoranthene and benzo[*j*]fluoranthene found in traces as well as undetected PAHs such as dibenzo[*a,h*]anthracene, benzo[*g,h,i*]perylene, dibenzo[*a,l*]pyrene, dibenzo[*a,e*]pyrene, dibenzo[*a,i*]pyrene and dibenzo[*a,h*]pyrene are not shown in this table. [3]Flavorings: standard flavoring (STF) = 8% sugar, 8% soy sauce, 1.5% salt, 5% oil, 1% black pepper and 1% almond slices; F6 = 8% sugar, 1.5% salt, 5% oil, 1% black pepper and 1% almond slices; F7 = 8% soy sauce, 1.5% salt, 5% oil, 1% black pepper and 1% almond slices. [4]trace = PAHs levels are higher than or equal to limit of detection (LOD), but below limit of quantitation (LOQ), or the negative data obtained due to PAH levels being lower than the background values of calibration curves; [5]nd = not detected (below LOD). Mean values bearing different capital letters (A-H) in the same row are significantly different ($p < 0.05$; Duncan's multiple range test) at different temperatures compared to that in raw pork.

explain why the level of total PAHs was higher for STF (8% soy sauce and 8% sugar) and F5 (8% soy sauce and 16% sugar) treatments. Also, the level of total PAHs was lower for the F7 treatment (without sugar) than that for F6 (without soy sauce), indicating that soy sauce (8%) should be more effective in inhibiting PAH formation than sugar (8%), which may be attributed to the presence of isoflavone in soy sauce (Hsu & Chen, 2020). This phenomenon may also explain why two more PAHs, BcF and IP, were generated in thin slices of dried pork with the STF when compared to the flavoring without soy sauce or sugar at 200 °C (Table 1).

3.3. PAH contents (ng/g) in thin slices of dried pork as affected by different flavorings and processing conditions

The effect of different flavorings and processing condition on PAH levels in thin slices of dried pork is shown in Table 2. Following heating at 120 °C, a total of 4 PAHs including AcP, Phe, Pyr and BcF were formed for all the 6 flavoring treatments. More specifically, with F5, the highest level of total PAHs (5.520 ng/g) was shown with Pyr present in the largest amount (3.957 ng/g), followed by AcP (0.662 ng/g), Phe (0.539 ng/g) and BcF (0.362 ng/g). Conversely, the lowest level of total PAHs (2.482 ng/g) was observed for the flavoring treatment (F4), with Pyr present in the highest content (1.505 ng/g), followed by AcP (0.381 ng/g), Phe (0.300 ng/g) and BcF (0.296 ng/g). A similar trend was observed for the other 4 flavoring treatments: STF, F1, F2, as well as F3. By comparison at the same level of sugar, a low level of soy sauce (4%) could generate a low amount of total PAHs. Likewise, at the same level of soy sauce, a low level (4%) of sugar could produce a small amount of total PAHs. This outcome revealed that the addition of low level of sugar (4%) or soy sauce (4%) may prevent PAH formation, with the latter being more effective than the former, probably because of presence of isoflavone in the latter. Collectively, the lower the level of sugar or soy sauce, the less the formation of PAHs in thin slices of dried pork during roasting.

Like 120 °C, a similar tendency was observed at 160 °C and 200 °C with the flavoring (F5) generating the highest level of total PAHs, followed by F3, STF, F2, F1, and F4. However, compared to the treatment at 120 °C and 160 °C, some more PAHs including AcPy and IP were produced at 200 °C for the 3 flavoring treatments including STF, F3 as well as F5. This phenomenon further demonstrated that the addition of high level of sugar (8% or 16%) or soy sauce (8%) to the flavoring could promote formation of PAHs with 6 rings such as IP. Also, the formation of AcPy is probably due to oxidation of AcP. Nevertheless, the highly toxic BaP remained undetected in thin slices of dried pork treated with 6 different flavorings, probably because of short roasting time. As mentioned above, with heating temperature > 170 °C, sugar can be dehydrated to form 5-HMF for subsequent indene formation through Diels-Alder reaction, leading to FL formation through hydrogen abstraction/acetylene addition. In several previous studies Duedahl-Olesen, Navaratnam, Jewula, and Jensen (2015) reported that both BaA and CHR were present at a much higher level in heavy roast coffee than in medium roast coffee due to caramelization occurred during roasting. In another study Chen, Kao, Chen, Huang, and Chen (2013) studied the PAH formation in sugar-smoked meat and reported that the longer the smoking time, the more the formation of PAHs. Also, the level of total PAHs was higher in red meat than in poultry meat. However, the highly toxic BaP remained undetected in sugar-smoked meat, revealing that sugar-smoking is safer than the other traditional smoking methods such as wood-smoking. Similarly, Kao, Chen, Chen, Huang, and Chen (2012) studied the effect of marinating and frying on PAH formation in poultry meat and reported that PAHs with 2–4 rings were more rapidly formed, in which NaP dominated. However, a large amount of total PAHs (79.7 ng/g) in duck meat was produced after 15-min frying. Apparently, the variety and amount of PAHs formed in meat products can be dependent upon meat variety, cooking method, heating temperature and time.

3.4. Evaluation of COPs analysis by QuEChERS and GC-MS

A method based on Chiu, Kao, & Chen, 2018 was used to extract and purify COPs in thin slices of dried pork by QuEChERS for subsequent

Table 2
PAH contents (ng/g) in thin slices of dried pork as affected by different flavorings and processing conditions.[1]

PAHs[2]	Flavoring[3]					
	STF	F1	F2	F3	F4	F5
120 °C						
Acenaphthylene	trace[4]	trace	trace	trace	trace	trace
Acenaphthene	0.548 ± 0.090^{B}	0.459 ± 0.036^{C}	0.531 ± 0.070^{B}	0.572 ± 0.068^{B}	0.381 ± 0.040^{D}	0.662 ± 0.040^{A}
Fluorene	trace	trace	trace	trace	trace	trace
Phenanthrene	0.350 ± 0.060^{B}	0.335 ± 0.024^{C}	0.349 ± 0.060^{B}	0.363 ± 0.055^{B}	0.300 ± 0.020^{C}	0.539 ± 0.020^{A}
Anthracene	trace	trace	trace	trace	trace	trace
Fluoranthene	trace	trace	trace	trace	trace	trace
Pyrene	3.243 ± 0.300^{B}	2.583 ± 0.181^{C}	3.031 ± 0.420^{B}	3.490 ± 0.557^{B}	1.505 ± 0.210^{D}	3.957 ± 0.410^{A}
Benzo[*c*] fluorene	0.306 ± 0.003^{A}	0.302 ± 0.030^{A}	0.306 ± 0.021^{A}	0.314 ± 0.064^{A}	0.296 ± 0.002^{A}	0.362 ± 0.014^{A}
Benzo[*a*] anthracene	nd[5]	nd	nd	nd	nd	nd
Cyclopenta[*c*,*d*] pyrene	trace	trace	trace	trace	trace	trace
Chrysene	trace	trace	trace	trace	trace	trace
Benzo[*a*]pyrene	nd	nd	nd	nd	nd	nd
Indeno[1,2,3-*c*, *d*]pyrene	trace	trace	trace	trace	trace	trace
Total PAHs	4.447^{C}	3.679^{E}	4.217^{D}	4.739^{B}	2.482^{F}	5.520^{A}
160 °C						
Acenaphthylene	trace	trace	trace	trace	trace	trace
Acenaphthene	0.706 ± 0.015^{C}	0.616 ± 0.070^{D}	0.690 ± 0.170^{C}	0.747 ± 0.079^{B}	0.605 ± 0.102^{D}	0.876 ± 0.059^{A}
Fluorene	trace	trace	trace	trace	trace	trace
Phenanthrene	0.427 ± 0.025^{B}	0.352 ± 0.014^{C}	0.358 ± 0.040^{C}	0.436 ± 0.062^{B}	0.336 ± 0.058^{C}	0.542 ± 0.041^{A}
Anthracene	trace	trace	trace	trace	trace	trace
Fluoranthene	trace	trace	trace	trace	trace	trace
Pyrene	3.770 ± 0.270^{A}	3.224 ± 0.160^{B}	3.280 ± 0.334^{B}	3.908 ± 0.151^{A}	2.774 ± 0.067^{C}	3.970 ± 0.474^{A}
Benzo[*c*] fluorene	0.310 ± 0.003^{A}	0.307 ± 0.013^{A}	0.309 ± 0.026^{A}	0.324 ± 0.024^{A}	0.316 ± 0.065^{A}	0.383 ± 0.040^{A}
Benzo[*a*] anthracene	nd	nd	nd	nd	nd	nd
Cyclopenta[*c*,*d*] pyrene	trace	trace	trace	trace	trace	trace
Chrysene	trace	trace	trace	trace	trace	trace
Benzo[*a*]pyrene	nd	nd	nd	nd	nd	nd
Indeno[1,2,3-*c*, *d*]pyrene	trace	trace	trace	trace	trace	trace
Total PAHs	5.213^{C}	4.499^{E}	4.637^{D}	5.415^{B}	4.031^{F}	5.771^{A}
200 °C						
Acenaphthylene	0.335 ± 0.020^{A}	trace	trace	0.341 ± 0.005^{A}	trace	0.357 ± 0.012^{A}
Acenaphthene	0.721 ± 0.050^{C}	0.629 ± 0.090^{D}	0.632 ± 0.020^{D}	0.788 ± 0.020^{B}	0.674 ± 0.120^{D}	0.893 ± 0.050^{A}
Fluorene	trace	trace	trace	trace	trace	trace
Phenanthrene	0.430 ± 0.070^{B}	0.332 ± 0.030^{C}	0.345 ± 0.030^{C}	0.438 ± 0.060^{B}	0.311 ± 0.030^{D}	0.586 ± 0.035^{A}
Anthracene	trace	trace	trace	trace	trace	trace
Fluoranthene	trace	trace	trace	0.309 ± 0.025^{A}	trace	0.314 ± 0.009^{A}
Pyrene	4.356 ± 0.350^{C}	3.385 ± 0.159^{E}	3.880 ± 0.480^{D}	4.679 ± 0.410^{B}	3.351 ± 0.340^{E}	5.101 ± 0.170^{A}
Benzo[*c*] fluorene	0.328 ± 0.040^{A}	0.311 ± 0.050^{A}	0.313 ± 0.002^{A}	0.361 ± 0.004^{A}	0.309 ± 0.030^{A}	0.390 ± 0.003^{A}
Benzo[*a*] anthracene	nd	nd	nd	nd	nd	nd
Cyclopenta[*c*,*d*] pyrene	trace	trace	trace	trace	trace	trace
Chrysene	trace	trace	trace	trace	trace	trace
Benzo[*a*]pyrene	nd	nd	nd	nd	nd	nd
Indeno[1,2,3-*c*, *d*]pyrene	0.314 ± 0.030^{A}	0.305 ± 0.030^{A}	0.311 ± 0.030^{A}	0.319 ± 0.030^{A}	0.302 ± 0.030^{A}	0.320 ± 0.033^{A}
Total PAHs	6.504^{C}	4.962^{E}	5.481^{D}	7.235^{B}	4.947^{E}	7.961^{A}

[1] Mean of triplicate analyses ± standard error. [2]Polycyclic aromatic hydrocarbons (PAHs) such as 5-methylchrysene, benzo[*b*]fluoranthene and benzo[*j*]fluoranthene found in traces as well as undetected PAHs such as dibenzo[*a*,*h*]anthracene, benzo[*g*,*h*,*i*]perylene, dibenzo[*a*,*l*]pyrene, dibenzo[*a*,*e*]pyrene, dibenzo[*a*,*i*]pyrene and dibenzo[*a*,*h*]pyrene are not shown in this table. [3]Flavorings: standard flavoring (STF) = 8% sugar, 8% soy sauce, 1.5% salt, 5% oil, 1% black pepper and 1% almond slices; F1 = 8% soy sauce in STF was replaced with 4% soy sauce; F2 = 8% sugar in STF was replaced with 4% sugar; F3 = 8% sugar and 8% soy sauce in STF was replaced with 16% sugar and 4% soy sauce; F4 = 8% sugar and 8% soy sauce in STF was replaced with 4% sugar and 4% soy sauce; F5 = 8% sugar in STF was replaced with 16% sugar. [4]trace = PAHs levels are higher than or equal to limit of detection (LOD), but below limit of quantitation (LOQ), or the negative data obtained due to PAH levels being lower than the background values of calibration curves; [5]nd = not detected (below LOD). Mean values bearing different capital letters (A-F) in the same row are significantly different ($p < 0.05$; Duncan's multiple range test) at different temperatures compared to that in raw pork.

separation, identification and quantification by GC-MS. Fig. 3A shows GC-MS chromatogram of cholesterol, internal standard (5α-cholestene) and 7 COP standards with good separation efficiency within 14 min. The various COPs in thin slices of dried pork were thus identified by employing SIM detection mode as described in the method section. The matrix effect of COPs in freeze-dried pork hind leg by GC-MS is shown in Table S7, with the average value being from 1.37 to 1.76, implying that all the 7 COPs possessed a signal enhancement effect. This outcome is similar to a study by Georgiou, Constantinou, Andreou, Hapeshi, Fatta-Kassinos, and Kapnissi-Christodoulou (2016), reporting the matrix effect of 7 COPs in freeze-dried pork hind leg to be from 0.95 to 1.23 by UPLC-MS/MS analysis. The LOD of 7α-OH, 7β-OH, 5,6β-EP, 5,6α-EP, triol, 25-OH and 7-keto were 2.5, 5, 200, 200, 20, 50 and 200 μg/g respectively, while the LOQ were 10, 15, 600, 600, 100, 150 and 600 μg/g. The recovery of 7 COP standards and standards added to blank sample is shown in Table S8, with the former ranging from 83.09 to 99.86 % and CV from 1.74 to 6.77 %, and the latter from 83.81 to 99.71 % and CV from 2.62 to 4.72 %. This result is similar to a report by Chen, Chien, Inbaraj, and Chen (2012), showing the recovery of COPs in marinated pig feet to be from 86.5 to 102.5 %. Also, the recovery data of COPs obtained in our study meets the regulation issued by TFDA (2013), stating the recovery should be from 70 to 120 % and from 75 to 120 % for the analyte concentration 1 mg/L and 5 mg/L, respectively. Table S9 shows precision data of 7 COP standards and standards added to blank samples, with the CV of repeatability and intermediate precision being 2.13–6.20% and 2.88–8.32% respectively for the former, as well as 2.69–8.63% and 3.11–11.26% for the latter. Like recovery data, the precision data obtained in our study also meets the regulation issued by TFDA (2013), stating that the CV should be < 14% for the analyte concentration ≥ 1 mg/L. Collectively, all the method validation data shown in this study demonstrated that a high accuracy and precision was attained for COP analysis in freeze-dried pork hind leg by QuEChERS coupled with GC-MS.

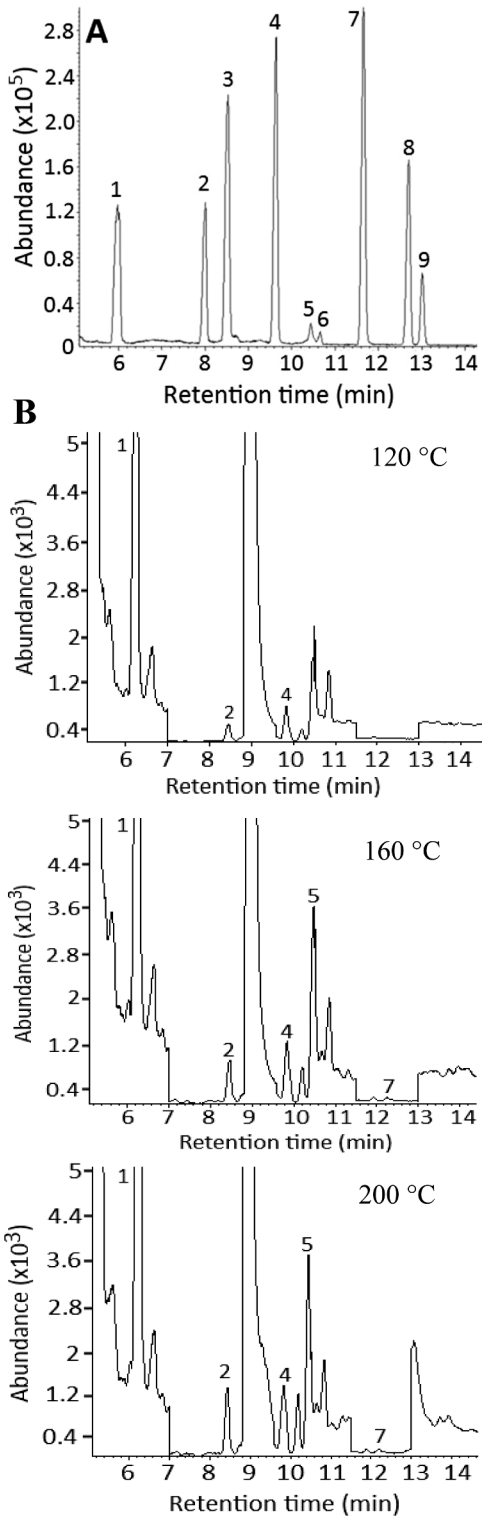

Fig. 3. GC-MS-TIC chromatogram of COP and cholesterol standards (A) as well as GC-MS-SIM chromatograms of COPs in thin slices of dried pork as affected by different roasting temperatures with standard flavoring (B). Standard flavoring (STF): 8% sugar, 8% soy sauce, 1.5% salt, 5% oil, 1% black pepper and 1% almond slices. Peaks: 1, 5α-cholestane (internal standard); 2, 7α-hydroxycholesterol; 3, cholesterol; 4, 7β-hydroxycholesterol; 5, 5,6β-epoxycholesterol; 6, 5,6α-epoxycholesterol; 7, triol; 8, 25-hydroxycholesterol; 9, 7-ketocholesterol. COPs = cholesterol oxidation products, GC-MS-TIC = gas chromatograph–mass spectrometer-total-ion-chromatogram and GC-MS-SIM = gas chromatograph–mass spectrometer-selected ion monitoring chromatogram.

3.5. COP contents in raw pork and thin slices of dried pork with STF and flavoring without soy sauce or sugar

Table 3 shows COP contents (μg/g) in raw pork and thin slices of dried pork with STF and flavoring without soy sauce or sugar (flavorings F6 and F7). Only 7α-OH (0.077 μg/g) and 7β-OH (0.090 μg/g) were determined in raw pork, probably caused by increased surface exposure of cholesterol to air during pork homogenization. The formation of 7α-OH and 7β-OH is probably due to reduction of 7α-hydroperoxycholesterol (7α-OOH) and 7β-hydroperoxycholesterol (7β-OOH), the initial cholesterol oxidation products, respectively (Chen, Lu, Chien, & Chen, 2010). Also, 7β-OH was formed at a higher level than 7α-OH, probably due to a smaller steric hindrance effect of the former (Chen, Lu, Chien, & Chen, 2010). However, with STF and roasting at 120 °C for 4 min, both 7α-OH and 7β-OH in thin slices of dried pork decreased to 0.011 and 0.016 μg/g, respectively, probably due to degradation. In contrast, with STF, two more COPs, 5,6β-EP and triol were generated when roasted at 160 and 200 °C for 4 min respectively, probably due to the accelerated cholesterol oxidation and degradation as the cholesterol melting point is 147–148.5 °C. Also, the total COP contents followed a temperature-dependent increase, which equaled 0.027, 1.217 and 1.269 μg/g at 120, 160 and 200 °C, respectively. The formation of 5,6-β-EP is probably due to cholesterol oxidation in the presence of 7β-OOH, while the triol formation is due to hydration of 5,6β-EP under acidic condition caused by hydrolysis of triglyceride during roasting of air-dried pork (Hsu & Chen, 2020). Similarly, a temperature-dependent rise of total COPs was shown for the treatment of flavoring without soy sauce (F6). However, at 160 or 200 °C, only three COPs (7α-OH, 7β-OH and 5,6β-EP) were formed for the flavoring treatment without soy sauce or sugar, while five COPs (7α-OH, 7β-OH, 5,6β-EP, 5,6α-EP and triol) were generated for the flavoring treatment without sugar (F7). This outcome implied that sugar may be more effective than soy sauce in inhibiting cholesterol oxidation. By comparison at the same temperature (120 or 200 °C), the flavoring treatment without sugar generated the highest level of total COPs, followed by that without soy sauce and STF. Interestingly, with the flavoring treatment at 160 °C, the treatment without soy sauce produced the lowest level of total COPs (0.98 μg/g). It may be postulated that the addition of sugar may minimize COP formation, probably due to formation of diketo compound from sugar degradation for subsequent chelation of prooxidants such as Fe^{+2} or Cu^{+2} (Hsu & Chen, 2020). Nevertheless, the addition of soy sauce (8%) and sugar (8%) in the STF may also minimize formation of total COPs. As mentioned above, the presence of small amount of isoflavone in soy sauce may also be effective in inhibiting cholesterol oxidation. Comparatively, sugar may be more effective than soy sauce in retarding cholesterol oxidation. Similar outcome was reported by Lee, Chien, and Chen (2008), demonstrating that sugar was more effective than soy sauce in inhibiting COP formation in marinated pork during heating.

3.6. COP contents in thin slices of dried pork as affected by different flavorings and roasting temperature

The COP contents in thin slices of dried pork as affected by different flavorings and roasting temperature is also shown in Table 3. Fig. 3B shows GC-MS-SIM chromatograms of COPs in thin slices of dried pork as affected by different roasting temperature at 120 °C, 160 °C and 200 °C with STF. Only two COPs, 7α-OH and 7β-OH, were detected in thin slices of dried pork for all the 6 flavoring treatments (STF and F1-F5), while only minor difference in the level of total COPs was shown among the various treatments. However, after roasting at 160 °C, two more COPs, 5,6β-EP and triol, were generated, with the flavoring containing F7 produced the highest level of total COPs (1.927 μg/g), followed by F4 (1.47 μg/g), F2 (1.385 μg/g), F1 (1.313 μg/g), F3 (1.288 μg/g), STF (1.217 μg/g), F5 (1.105 μg/g) and F6 (0.980 μg/g). As mentioned before, the formation of 5,6β-EP is probably due to cholesterol oxidation in the presence of the initial oxidation product 7β-OOH, while the triol

Table 3
COP contents (μg/g) in raw pork and thin slices of dried pork as affected by different flavorings and roasting temperatures.[1]

Flavoring[2]	COP contents							
	7α-OH	7β-OH	5,6β-EP	5,6α-EP	triol	25-OH	7-keto	total
Raw pork	0.077 ± 0.003	0.090 ± 0.001	nd[3]	nd	nd	nd	nd	0.167^{F}
120 °C								
STF	0.011 ± 0.000^{E}	0.016 ± 0.001^{E}	nd	nd	nd	nd	nd	0.027^{G}
F1	0.011 ± 0.000^{E}	0.016 ± 0.001^{E}	nd	nd	nd	nd	nd	0.027^{G}
F2	0.011 ± 0.000^{E}	0.016 ± 0.001^{E}	nd	nd	nd	nd	nd	0.027^{G}
F3	0.011 ± 0.000^{E}	0.016 ± 0.001^{E}	nd	nd	nd	nd	nd	0.027^{G}
F4	0.012 ± 0.001^{E}	0.020 ± 0.001^{E}	nd	nd	nd	nd	nd	0.032^{G}
F5	0.011 ± 0.000^{E}	0.016 ± 0.001^{E}	nd	nd	nd	nd	nd	0.027^{G}
F6	0.014 ± 0.001^{E}	0.019 ± 0.001^{E}	trace	nd	trace	nd	nd	0.033^{G}
F7	0.013 ± 0.001^{E}	0.018 ± 0.001^{E}	0.275 ± 0.009^{E}	nd	trace	nd	nd	0.306^{F}
160 °C								
STF	0.054 ± 0.001^{D}	0.074 ± 0.001^{D}	0.976 ± 0.050^{C}	trace[4]	0.113 ± 0.000^{B}	trace	nd	1.217^{D}
F1	0.052 ± 0.001^{D}	0.071 ± 0.001^{D}	1.076 ± 0.013^{B}	trace	0.114 ± 0.001^{B}	trace	nd	1.313^{C}
F2	0.055 ± 0.000^{D}	0.076 ± 0.001^{D}	1.139 ± 0.030^{AB}	trace	0.115 ± 0.001^{B}	trace	nd	1.385^{C}
F3	0.052 ± 0.001^{D}	0.070 ± 0.001^{D}	1.051 ± 0.030^{B}	trace	0.113 ± 0.000^{B}	trace	nd	1.288^{D}
F4	0.053 ± 0.001^{D}	0.074 ± 0.001^{D}	1.230 ± 0.040^{A}	trace	0.113 ± 0.000^{B}	trace	nd	1.470^{B}
F5	0.052 ± 0.001^{D}	0.070 ± 0.001^{D}	0.870 ± 0.050^{D}	trace	0.113 ± 0.001^{B}	trace	nd	1.105^{E}
F6	0.068 ± 0.008^{C}	0.099 ± 0.013^{C}	0.813 ± 0.042^{D}	trace	trace	trace	trace	0.980^{E}
F7	0.062 ± 0.001^{C}	0.094 ± 0.002^{C}	1.012 ± 0.024^{B}	0.645 ± 0.033^{A}	0.114 ± 0.001^{B}	trace	trace	1.927^{A}
200 °C								
STF	0.054 ± 0.000^{D}	0.075 ± 0.001^{D}	1.021 ± 0.030^{B}	trace	0.119 ± 0.001^{A}	trace	nd	1.269^{D}
F1	0.054 ± 0.000^{D}	0.077 ± 0.001^{D}	1.085 ± 0.030^{B}	trace	0.119 ± 0.001^{A}	trace	nd	1.335^{C}
F2	0.054 ± 0.000^{D}	0.075 ± 0.001^{D}	1.211 ± 0.025^{A}	trace	0.118 ± 0.001^{A}	trace	nd	1.458^{B}
F3	0.055 ± 0.000^{D}	0.078 ± 0.001^{D}	1.062 ± 0.023^{B}	trace	0.120 ± 0.000^{A}	trace	nd	1.315^{C}
F4	0.053 ± 0.001^{D}	0.072 ± 0.001^{D}	1.268 ± 0.045^{A}	trace	0.120 ± 0.001^{A}	trace	nd	1.513^{B}
F5	0.053 ± 0.000^{D}	0.074 ± 0.001^{D}	0.912 ± 0.014^{C}	trace	0.119 ± 0.001^{A}	trace	nd	1.158^{E}
F6	0.117 ± 0.002^{A}	0.174 ± 0.003^{A}	1.004 ± 0.012^{B}	trace	trace	trace	trace	1.295^{D}
F7	0.076 ± 0.005^{B}	0.106 ± 0.001^{B}	1.063 ± 0.023^{B}	0.665 ± 0.014^{A}	0.120 ± 0.001^{A}	trace	trace	2.030^{A}

[1] Mean of triplicate analyses ± standard error. [2]Flavorings: standard flavoring (STF) = 8% sugar, 8% soy sauce, 1.5% salt, 5% oil, 1% black pepper and 1% almond slices; F1 = 8% soy sauce in STF was replaced with 4% soy sauce; F2 = 8% sugar in STF was replaced with 4% sugar; F3 = 8% sugar and 8% soy sauce in STF was replaced with 16% sugar and 4% soy sauce; F4 = 8% sugar and 8% soy sauce in STF was replaced with 4% sugar and 4% soy sauce; F5 = 8% sugar in STF was replaced with 16% sugar; F6 = 8% sugar, 1.5% salt, 5% oil, 1% black pepper and 1% almond slices; F7 = 8% soy sauce, 1.5% salt, 5% oil, 1% black pepper and 1% almond slices. [3]nd = not detected (below LOD). [4]trace = COP levels are higher than or equal to limit of detection (LOD), but below limit of quantitation (LOQ), or the negative data obtained due to PAH levels being lower than the background values of calibration curves. Mean values with different capital letters (A-G) in the same column are significantly different ($p < 0.05$; Duncan's multiple range test) with different flavorings.

formation is probably caused by hydration of 5,6β-EP under acidic condition. By comparison, the addition of high level of sugar (8%) without soy sauce to the flavoring possessed the most pronounced effect in inhibiting cholesterol oxidation, while the addition of 8% soy sauce without sugar (F7) was the least effective in minimizing cholesterol oxidation. Like 160 °C, the same trend was observed for the roasting temperature at 200 °C, with F7 in the flavoring generated the highest level of total COPs (2.030 μg/g), while 16% sugar and 8% soy sauce produced the lowest level of COPs (1.158 μg/g). Of the various COPs, 5,6β-EP was formed in the highest amount, followed by triol, 7β-OH and 7α-OH. As explained above, compared to 7α-OH, 7β-OH was more susceptible to formation probably due to a smaller steric hindrance effect. Also, 5,6β-EP was formed at a higher level than 5,6α-EP, which can be due to a higher stability of the former (Lee, Chien, & Chen, 2008). In a previous study Conchillo, Ansorena, and Astiasarán (2005) pointed out that the amount of total COPs was very low in raw chicken (2.88 μg/g). However, the level of total COPs increased by 4–7 folds with 5,6β-EP, 7β-OH and 7-keto dominating. Eder, Grünthal, Kluge, Hirche, Spilke, and Brandsch (2005) also reported that the major COPs in heat-processed broiler chickens included 7α-OH, 7β-OH, 5,6β-EP and 7-keto. Comparatively, 7-keto and 25-OH remained undetected in thin slices of dried pork during roasting in our study, probably due to short roasting time (4 min), as both can only be formed under drastic condition.

3.7. Principal component analysis

As shown in Fig. S2, a total of two components including PC 1 with 42.98% and PC 2 with 20.59% illustrated a total of 63.57% variation in PAH and COP formation as affected by different flavorings (STF and F1–F7) and roasting temperatures (T-120, T-160 and T-200). Evidently, with the exception of F6 for COP formation as well as F6 and F7 for PAH formation (group 5), PAHs (group 1) were well-separated from those of COPs (group 3) in the score plot with PAHs formation being higher than COPs for all the 8 flavorings (Fig. S2A). For roasting temperature, both PAHs and COPs formation appeared in two groups with their levels at 200 °C corresponded to group 2 while that at both 120 and 160 °C to group 4, suggesting that a temperature-dependent increase in PAH or COP formation during roasting (Fig. S2A). In addition, the relationship between PCs and original variables of different flavoring and roasting temperature treatments is shown as loading plots in Fig. S2B. Most of the PAH and COP variables for different flavoring treatments were shown to point towards the same direction of PC1, while for roasting temperatures, they were spread in the directions of both PC1 and PC2, implying that both PC1 and PC2 were strongly influenced by different flavorings and roasting temperatures. Also, the smaller the degree of angle between the treatments, the higher the correlation in the formation of COPs or PAHs. Accordingly, from Fig. S2B, the projected lines corresponding to flavorings F1–F5 (set 1 containing both sugar and soy sauce) as well as F6 and F7 (set 2 containing only sugar or soy sauce) showed a small degree of angle between them for the formation of PAHs, with the angle between two sets diverging to a larger extent. Likewise, the projected lines of flavorings F1–F5 for COPs formation converged by a small angle revealing a higher correlation between these flavorings. However, the projected lines of F6 and F7 deviated largely from F1–F5. On the other hand, among different roasting temperatures, the projected lines of T120 and T160 for PAH formation as well as T160 and T200 for COP formation deviated only by a small angle. However, they diverged respectively to a larger extent with T200 and T120, in accordance with the results observed in the score plot discussed above. Both the score plot and loading plot were merged to obtain a biplot as shown in

Fig. S2C, illustrating an overall grouping and correlation for PAH or COP formation as affected by different flavorings and roasting temperature. All the PCA data were in agreement with the results discussed in sections 3.3 and 3.6 for PAH and COP formation as affected by different flavorings and roasting temperature. Similar finding was reported by Kao, Chen, Chen, Huang, and Chen (2012) for studying formation of PAHs in meat products as affected by roasting. Overall, the PCA analysis revealed that regardless of flavoring type, PAHs were formed at a higher level than COPs, with roasting at 120 and 160 °C generating a lower PAH/COP content than that at 200 °C.

4. Conclusion

In conclusion, a high accuracy and precision method was attained for analysis of COPs and PAHs in thin slices of dried pork by QuEChERS coupled with GC-MS and GC-MS/MS, respectively. A temperature-dependent increase was shown for the total amount of both COPs and PAHs in thin slices of dried pork during roasting at 120, 160, and 200 °C. The addition of high level of sugar (8 or 16%) and soy sauce (8%) to the flavoring may reduce COP formation, while the low level (4% sugar and 4% soy sauce) may minimize PAH formation. Sugar was more effective in inhibiting COP formation, while soy sauce was more efficient in retarding PAH formation. Nevertheless, the highly toxic BaP remained undetected in thin slices of dried pork, while the various COPs were only present in small amounts. The PCA showed that regardless of flavoring type, PAHs were formed at a higher level than COPs, while roasting at 120 and 160 °C, a lower PAH/COP content was generated than at 200 °C. Taken together, based on the experimental data obtained, the consumption of thin slice of dried pork may pose no risk to human health.

CRediT authorship contribution statement

Yu-Ting Hung: Methodology, Investigation, Formal analysis. **Yu-Tsung Lee:** Methodology, Investigation, Data curation. **Baskaran Stephen Inbaraj:** Data curation, Validation, Writing - original draft, Writing - review & editing. **Kandi Sridhar:** Formal analysis, Software, Writing - original draft, Writing - review & editing. **Bing-Huei Chen:** Conceptualization, Supervision, Resources, Funding acquisition, Project administration, Writing - original draft, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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