

## Impact of Cooking, Storage, and Reheating Conditions on the Formation of Cholesterol Oxidation Products in Pork Loin

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### Abstract

This study investigates the effect of cooking, storage, and reheating conditions on the formation of cholesterol oxidation products (COPs) in pork loin. Samples of pork loin procured 24 h postmortem were initially processed and assessed for total fat and cholesterol content. The cooking methods evaluated were pan roasting, steaming, oven grilling, and microwaving. Cooked pork loin samples were stored at 4°C and reheated after 3 and 6 d of storage using the original method of preparation or alternately, microwaving. Fat content increased significantly with cooking as a result of the loss in moisture but cholesterol content remained unchanged. Pan roasting and microwave cooking caused a significantly higher production of COPs, as with the process of reheating using microwave, pan roasting, and oven grilling methods. The major COPs found in pork loin were cholestanetriol, 20-hydroxycholesterol, and 25-hydroxycholesterol, whose concentrations varied according to the different cooking and reheating methods used. Moreover, the aerobic storage of cooked pork loin under a refrigerated condition also increased the formation of cholesterol oxides on reheating.

**Keywords:** pork loin, cooking, refrigerated storage, reheating, cholesterol oxidation products

*Received August 4, 2015; Revised November 22, 2015; Accepted November 23, 2015*

### Introduction

Cooking is essential prior to the consumption of meat as it improves taste, flavor, and digestibility, kills microorganisms, and extends shelf life (Bognar, 1998; Rodriguez-Estrada *et al.*, 1997). However, the negative effects of cooking include lipid oxidation and the generation of aromatic polycyclic hydrocarbons (Rodriguez-Estrada *et al.*, 1997). Lipid oxidation affects the taste, flavor, appearance, nutritional value, and safety of food (Mohamed *et al.*, 2008), resulting in the deterioration of consumable products and an undesirable odor. The oxidation reaction in meat depends on the methods of cooking, temperature,

and time (Broncano *et al.*, 2009). More significant qualitative changes are observed when food is cooked at a higher temperature (Hoac *et al.*, 2006), and these changes also depend on the preparation time at different temperatures. Excessive oxidation of meat lipids produces potential precursors of highly reactive aldehydes in food, which is a source of oxidative stress (Bou *et al.*, 2009; Luna *et al.*, 2010) that has been linked to atherosclerosis, inflammation, arthritis, Alzheimer's, and Parkinson's diseases (Yin and Poter, 2005). Lipid oxidation in meat during cooking involves cholesterol, and the formation of cholesterol oxidation products (COPs) is related to cooking temperature and time (Paniangvait *et al.*, 1995).

Cholesterol is a compound of biological importance but its oxidation products have been proven to be cytotoxic, mutagenic, and carcinogenic (Ryan *et al.*, 2005) and COPs are also considered as a primary factor in causing atherosclerosis (Leonarduzzi *et al.*, 2005). Cooking, dehydration, and deep frying are some of the main causes of cholesterol

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oxidation in foodstuff of animal origin (Savage *et al.*, 2002). The extent of cholesterol oxidation in food is influenced by the food matrix composition, presence of pro- and anti-oxidants, as well as food processing and storage conditions (Guardiola *et al.*, 2002). Lee *et al.* (2006) studied the effects of various cooking methods, along with storage (4°C) and re-heating conditions, on cholesterol and the formation of COPs in beef loin, and generally observed a significant reduction in cholesterol with an increase in COPs after food processing. Hu *et al.* (2014) compared the effects of cooking methods on pork lipid digestibility and the formation of COPs, detecting a significantly higher level of COPs in microwave-treated samples. In a recent study, Freitas *et al.* (2015) reported the reduction in cholesterol content, accompanied by an increase in COPs (especially 7-ketocholesterol) in fish fillets cooked using different methods.

The objective of this study was to determine the effect of cooking, refrigerated storage, and reheating conditions on cholesterol and COPs formation in pork loin.

## Material and Methods

### Reagents and solutions

Cholesterol, COPs standards [7-ketocholesterol (7-keto), 6-ketocholesterol (6-keto), 7 $\alpha$ -hydroxy-cholesterol (7 $\alpha$ -OH), 7 $\beta$ -hydroxycholesterol (7 $\beta$ -OH), 5,6 $\alpha$ -epoxycholesterol (5,6 $\alpha$ -EP), 5,6 $\beta$ -epoxycholesterol (5,6 $\beta$ -EP), 25-hydroxycholesterol (25-OH), 20-hydroxycholesterol (20-OH), and cholestanetriol (triol)], butylated hydroxytoluene (BHT), pyridine, and silicic acid (100 mesh) were purchased from Sigma-Aldrich Co., LLC (Korea). Bis-(trimethylsilyl)-trifluoroacetamide (BSTFA) + 1% trimethylchlorosilane (TMCS) was obtained from Supelco (Bellefonte, USA). HPLC grade hexane, ethyl acetate, acetone, methanol, and chloroform, Celite 545, and calcium phosphate (CaHPO<sub>4</sub>·2H<sub>2</sub>O) were purchased from Fisher Scientific Co. (USA).

### Sample preparation and cooking

Pork loin, after 24 h postmortem, was purchased from a local market, trimmed of its surface adipose tissues, and sliced to an approximate 2 cm thickness. The total fat and cholesterol level were analyzed during the preliminary test. Cooking methods used were pan roasting (PR), steaming (ST), oven grilling (OG), and microwaving (MW). The initial surface temperature of the samples was 4°C and the samples was cooked to an internal temperature of 70±2°C via PR (170°C for 20 min), ST (using electric steamer), OG

(150°C for 1 h), and MW (2,450 MHz for 10 min). The cooked samples were stored at 4°C for 6 d under aerobic conditions, and subsequently reheated after 3 and 6 d, using the same set of cooking methods. However, a different set of times was used for the reheating process: PR (5 min), ST (10 min), OG (10 min), and MW (1 min). The raw and cooked samples were then analyzed for the amounts of COPs at 0, 3, and 6 d of storage.

### Determination of total cholesterol

The pork fat (0.5 g) was diluted in 10 mL of freshly prepared methanolic potassium hydroxide solution (1 M) and 1 g of sea sand was added. The mixture was heated for 25 min and the supernatant was transferred into a 25 mL volumetric flask with a pipette. The residue was boiled with 6 mL of isopropanol under reflux for 5 min, and the solution was collected, cooled, and diluted to the mark with isopropanol. The turbid solutions were filtered through a Whatman No. 1 filter paper (Whatman Inc., NJ). The clear aliquot was then used for the cholesterol assay according to the test kit instructions (Cat. No 139050, Boehringer Mannheim, Germany). The blank sample was prepared by mixing 0.4 mL of the extracted sample solution and 5 mL of solution 4 (cholesterol reagent mixture). Meanwhile, the sample solution was obtained by mixing 2.5 mL of the extracted sample solution and 0.02 mL of solution 3 (enzyme mixture). The prepared blank and sample solutions were sealed with paraffin film and incubated at 37-40°C for 60 min. The absorbance values of the blank (A1) and sample (A2) solutions were determined using a UV spectrophotometer (UV1601, Shimadzu Co., Japan) at 405 nm. Cholesterol contents (mg/100 g) were calculated using the following equation:

$$\text{Cholesterol content (mg/100 g)} = [0.711 \times (A2 - A1) / \text{sample weight (g)}] \times 100 \times 25$$

### Cholesterol Oxidation Products (COPs)

Cholesterol oxidation products were analyzed according to the method of Lee *et al.* (1996). For the separation of COPs, a solid-phase column was prepared (Park and Addis, 1985; Zubillaga and Maerker, 1991) by mixing silicic acid, Celite 545, and CaHPO<sub>4</sub>·2H<sub>2</sub>O (10:9:1, wt/wt/wt) with 30 mL of chloroform, and the mixture was packed into a glass column (12 mm × 30 cm). The prepared column was repeatedly prewashed with 5 mL of hexane before sample introduction. The total lipids were extracted according to the method of Folch *et al.* (1991). The lipid sample (0.2 g) was dissolved in 2 mL of hexane and ethyl

acetate (100:2, v/v) and introduced into the prewashed column. The sample container was washed twice with 2 mL of the aforementioned hexane/ethyl acetate mixture and the wash solvent was poured into the column. Neutral lipid and cholesterol (phospholipids) were removed by adding 50 mL of solvent I (2:1 of  $\text{CHCl}_3$ : $\text{CH}_3\text{OH}$ , v/v) and 60 mL of solvent II (4:1 of hexane:ethyl acetate, v/v), respectively. Solvent III (40 mL, 10:10:1 of acetone:ethyl acetate:methanol, v/v/v) was used to elute the COPs at a 1 mL/min flow rate. Solvents from the collected solutions were evaporated on a 50°C hot plate, blown with nitrogen gas. The dried extracts were derivatized via heating at 80°C for 1 h in the presence of 200 mL pyridine and 100 mL *bis*-(trimethylsilyl)-trifluoroacetamide + 1% trimethylchlorosilane. The COPs were analyzed using a gas chromatograph (HP 5890 Plus) equipped with an on-column capillary injector and a flame ionization detector. The identification of cholesterol and COPs was based on the comparison between the samples' retention times and those of the standards on co-chromatography and the characteristics of the absorption spectra.

### Statistical analysis

Statistical analysis was performed by one-way analysis of variation (ANOVA) using SAS software (SAS, Release 8.01, SAS Institute Inc., USA), and Duncan's multiple range test was employed to differentiate the significance among the mean values.

## Results and Discussion

### Total fat and cholesterol content

The changes in fat and cholesterol content of pork loin depending on different cooking and reheating conditions are shown in Table 1. The pork loin was cooked (PR, OG, ST, or MW), stored at 4°C for 6 d, and reheated using the same methods. Cooking pork loin significantly increased its total fat content and this may be attributed to the loss of moisture during the cooking process. The highest fat content (6.41%) was observed in PR samples, followed by ST (5.63%), MW (5.26%), and OG (4.83%). However, the cooking process did not significantly alter the cholesterol content of pork loin even though the level of cholesterol varied depending on the different cooking methods used. Cooking induces water loss in food, thus increasing its lipid content; though only a small amount of fat is lost. Badiani *et al.* (2002) reported a remarkable decrease in the moisture content of meat depending on the cooking methods used, leading to different levels of fat and cho-

**Table 1. Impact of cooking method on fat (%) and cholesterol content (mg/100 g) of pork loin**

Cooking methods	Fat	Cholesterol
Raw	4.54±0.01e	48.85±5.95
Pan Roasting (PR)	6.41±0.01a	52.66±2.95
Steaming (ST)	5.63±0.11b	45.11±0.42
Oven Grilling (OG)	4.83±0.01d	48.21±4.50
Microwave (MW)	5.26±0.12c	44.34±1.93

<sup>a-c</sup>Different letters within the same column differ significantly ( $p < 0.05$ ).

lesterol. Serrano *et al.* (2007) suggested a higher moisture loss via microwave cooking, though lower in grilling, owing to the absence of crust formation during microwave heating.

### Cooking methods and COPs formation

Cooking methods significantly influenced the formation of COPs and these results are presented in Table 2. The total amount of COPs varied between 0.67% and 1.15%, depending on the cooking methods used, with the highest level of COPs observed in samples cooked by either PR or MW. Oven grilling produced the least amount of COPs compared to other cooking methods. 20 $\alpha$ -OH, 25-OH, and triol were the major COPs produced when cooking pork loin, while 7 $\beta$ -OH,  $\alpha$ -epoxide, and 7-keto were not detected in the cooked samples. Interestingly, steaming pork loin resulted in a significantly higher production of 20 $\alpha$ -OH and triol compared to other cooking methods. The formation of 25-OH was less affected by the mode of heat treatment as its quantity varied little across the different cooking methods used. It is evident from our results that the level of oxidation is dependent on the cooking method, confirming the results of Dal Bosco *et al.* (2001) who reported a higher concentration of thiobarbituric acid reactive substances in boiled rabbit meat relative to fried and roasted samples. Grau *et al.* (2001) stated that cooking significantly increased oxidation activity as evident from the formation of COPs and TBARS. The increase in total COPs after cooking, as observed during the current study, is a result of cholesterol oxidation and the varying levels of COPs are an impact of the cooking methods used. The variation in heating intensity of different cooking methods resulted in variable destruction of meat structure leading to differences in COPs formation. The more prominent COP formation associated with microwaving confirms the results of Dominguez *et al.* (2014), who, upon cooking foal meat via roasting, grilling, microwaving, and frying, detected the highest level of oxidation in microwaved samples. Baggio and Bragagnolo (2006)

**Table 2. Quantification of cholesterol oxidation products in raw and cooked pork loins at 0 d**

Treatment <sup>1</sup>	Cholesterol oxidation products (β <sup>1</sup> /100 g) <sup>2</sup>						Total amount of COPs/ Cholesterol (%)
	7β-OH	20α-OH**	25-OH**	Triol***	α-epoxide	7-keto	
Raw	n.d.	16.43±6.56 <sup>B</sup>	61.28±30.07	103.54±52.21 <sup>C</sup>	n.d.	n.d.	0.67±0.26 <sup>B</sup>
PR	n.d.	19.88±9.76 <sup>B</sup>	73.59±21.20	193.53±14.73 <sup>B</sup>	n.d.	n.d.	1.15±0.25 <sup>A</sup>
ST	n.d.	32.43±5.29 <sup>A</sup>	83.86±36.48	284.86±8.13 <sup>A</sup>	n.d.	n.d.	0.94±0.20 <sup>AB</sup>
OG	n.d.	11.95±0.75 <sup>B</sup>	110.69±7.73	144.06±7.59 <sup>BC</sup>	n.d.	n.d.	0.67±0.11 <sup>B</sup>
MW	n.d.	20.38±3.40 <sup>B</sup>	89.47±4.73	186.91±29.63 <sup>B</sup>	n.d.	n.d.	1.12±0.21 <sup>A</sup>

<sup>A-C</sup>Means±SE with different superscript in the same column differ significantly.

<sup>1</sup>Pan roasting, PR; steaming, ST; oven grilling, OG; microwaving, MW.

<sup>2</sup>7β-hydroxycholesterol, 7β-OH; 20α-hydroxycholesterol, 20α-OH; 25-hydroxycholesterol, 25-OH; cholestane-3β, 5α, 6β-triol, triol; cholesterol-5α, 6α-epoxide, α-epoxide; 7-ketocholesterol, 7-keto.

observed higher cholesterol oxidation in cooked products compared to raw ingredients, as well as seeing lower oxidation in grilled products. Additionally, Eder *et al.* (2005) also found a higher concentration of COPs in cooked pork compared to fresh meat.

### Storage, reheating, and COPs formation

Cooked pork loin was stored at 4°C for 6 d and samples were reheated after 3 and 6 d using the same cooking procedure or microwave. The COPs formation results upon reheating the cooked pork loin at both intervals are depicted in Table 3. The highest amount of COPs was pro-

duced in pork loin cooked and reheated by either SM (1.07%) or OO (1.06%) at the end of trial. Contrarily, the lowest COPs concentrations were observed in pork loin cooked by raw (0.37%) and PP (0.70%) upon termination. Results of 25-OH showed that the highest value of 25-OH was recorded in OO (111.66 μg/100 g) on the 6<sup>th</sup> day of storage whereas the lowest value by PP (55.85 μg/100 g) at the last day of storage. Findings about 20α-OH values observed variations in the range of 3.07 μg/100 g to 26.07 μg/100 g in different treatments during the storage period. At the end of storage, maximum value was acquired by OO (15.46 μg/100 g) while minimum value

**Table 3. Quantification of cholesterol oxidation products in raw and reheated pork loins during storage**

Treatment <sup>1</sup>	Cholesterol oxidation products <sup>2</sup> (β <sup>1</sup> /100 g) after 3 days storage						Total amount of COPs/ Cholesterol (%)
	7β-OH	20α-OH**	25-OH**	Triol***	α-epoxide	7-keto	
3 d of storage							
Raw	n.d.	5.35±2.51 <sup>C</sup>	65.46±9.92 <sup>BC</sup>	123.85±9.72 <sup>D</sup>	n.d.	n.d.	0.40±0.08 <sup>C</sup>
PP	n.d.	16.29±6.04 <sup>ABC</sup>	52.21±18.76 <sup>C</sup>	646.87±31.24 <sup>A</sup>	n.d.	n.d.	1.36±0.09 <sup>A</sup>
PM	n.d.	21.34±7.54 <sup>AB</sup>	99.76±7.42 <sup>AB</sup>	335.11±24.44 <sup>C</sup>	n.d.	n.d.	0.87±0.07 <sup>B</sup>
SS	n.d.	26.07±9.46 <sup>A</sup>	111.15±15.32 <sup>A</sup>	348.00±182.06 <sup>C</sup>	n.d.	n.d.	1.07±0.45 <sup>B</sup>
SM	n.d.	7.58±3.39 <sup>C</sup>	124.54±16.62 <sup>A</sup>	398.38±70.03 <sup>BC</sup>	n.d.	n.d.	1.18±0.19 <sup>AB</sup>
OO	n.d.	7.80±3.08 <sup>C</sup>	120.89±47.78 <sup>A</sup>	526.82±26.17 <sup>AB</sup>	n.d.	n.d.	1.37±0.23 <sup>A</sup>
OM	n.d.	5.89±3.06 <sup>C</sup>	97.59±6.31 <sup>AB</sup>	352.25±149.49 <sup>C</sup>	n.d.	n.d.	0.96±0.35 <sup>B</sup>
MM	n.d.	14.61±6.84 <sup>BC</sup>	104.28±20.67 <sup>AB</sup>	355.70±18.98 <sup>C</sup>	n.d.	n.d.	1.07±0.11 <sup>B</sup>
6 d of storage							
Raw	n.d. <sup>3</sup>	5.10±2.45 <sup>D</sup>	77.98±17.91 <sup>BC</sup>	95.90±30.90 <sup>B</sup>	n.d.	n.d. <sup>B</sup>	0.37±0.13 <sup>C</sup>
PP	n.d.	3.07±1.81 <sup>D</sup>	55.85±15.14 <sup>C</sup>	309.56±91.18 <sup>A</sup>	n.d.	n.d. <sup>B</sup>	0.70±0.18 <sup>B</sup>
PM	n.d.	11.48±0.01 <sup>B</sup>	61.93±10.72 <sup>C</sup>	304.20±2.12 <sup>A</sup>	n.d.	107.35±2.07 <sup>A</sup>	0.92±0.05 <sup>AB</sup>
SS	n.d.	8.42±0.82 <sup>C</sup>	89.54±14.60 <sup>AB</sup>	283.00±101.72 <sup>A</sup>	n.d.	n.d. <sup>B</sup>	0.84±0.25 <sup>AB</sup>
SM	n.d.	4.11±0.53 <sup>D</sup>	102.92±11.22 <sup>AB</sup>	374.04±12.93 <sup>A</sup>	n.d.	n.d. <sup>B</sup>	1.07±0.05 <sup>A</sup>
OO	n.d.	15.46±0.45 <sup>A</sup>	111.66±24.86 <sup>A</sup>	378.173±22.69 <sup>A</sup>	n.d.	n.d. <sup>B</sup>	1.06±0.16 <sup>A</sup>
OM	n.d.	12.35±1.13 <sup>B</sup>	77.87±4.38 <sup>BC</sup>	285.27±60.03 <sup>A</sup>	n.d.	n.d. <sup>B</sup>	0.79±0.17 <sup>AB</sup>
MM	n.d.	n.d. <sup>E</sup>	110.98±5.11 <sup>A</sup>	317.64±20.70 <sup>A</sup>	n.d.	n.d. <sup>B</sup>	0.97±0.07 <sup>AB</sup>

<sup>A-D</sup>Means±SE with different superscript in the same column differ significantly.

<sup>1</sup>PP: Pan roasting (cooking) + pan roasting (reheating), PM: Pan roasting (cooking) + microwaving (reheating), SS: Steaming (cooking) + steaming (reheating), SM: Steaming (cooking) + microwaving (reheating), OO: Oven grilling (cooking) + oven grilling (reheating), OM: Oven grilling (cooking) + microwaving (reheating), MM: Microwave (cooking) + microwave (reheating).

<sup>2</sup>7β-hydroxycholesterol, 7β-OH; 20α-hydroxycholesterol, 20α-OH; 25-hydroxycholesterol, 25-OH; cholestane-3β, 5α, 6β-triol, triol; cholesterol-5α, 6α-epoxide, α-epoxide; 7-ketocholesterol, 7-keto.

was observed in double pan roasted samples i.e., PP (3.07  $\mu\text{g}/100\text{ g}$ ). The significantly higher levels of triol for all the cooking and reheating methods were observed. As depicted in the Table 3, OO showed the maximum value (378.17  $\mu\text{g}/100\text{ g}$ ) of triol followed by SM (374.04). On the contrary, the lowest triol concentration was recorded in raw sample (95.90  $\mu\text{g}/100\text{ g}$ ) at the end of trial. PR and reheating by MW resulted in the formation of 7-keto but it was not detected in any other cooking or reheating methods. Moreover, 7 $\beta$ -OH and  $\alpha$ -epoxide were also not detected in all the test samples.

The increase in COP formation during storage is in line with the results from Monahan *et al.* (1992), who reported a significant increase in the level of cholesterol oxidation of cooked pork products during storage. In a previous study, Lee *et al.* (2006) observed an increase in COP formation after cooking a stored beef loin. Ferioli *et al.* (2008) reported that the COP content in raw and cooked minced beef increased by six times after 2 wk of storage at 4°C, with the COP content being higher in cooked meat than in raw samples. Lee *et al.* (2001) suggested the vacuum storage of cooked meat products as they had also observed an elevated level of COPs in cooked meat post storage. Conchillo *et al.* (2005) reported a significantly higher COP concentration in aerobically stored raw, grilled, and roasted meat compared to their vacuum-packed alternatives. With pan roasting and oven grilling, high cooking temperatures are typically used and this causes a greater extent of cholesterol oxidation, affirming the results of Min *et al.* (2015), which reported that higher cooking temperatures (>150°C) lead to an increased production of COPs. Based on our study, microwave heating significantly increases COP formation and this agrees with El-Alim *et al.* (1999), who observed a significantly higher level of COP in microwave-cooked patties after 7 d of storage at 4°C. In general, the findings from the current investigation confirm previous results on the increased formation of COPs in meat that undergoes the cooking, storage, and reheating processes.

### Acknowledgements

This work was carried out with the support of Cooperative Research Program for Agriculture Science & Technology Development (Project No. PJ011617), Rural Development Administration, Republic of Korea and Institute of Green Bio Science and Technology, Seoul National University.

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