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Detection of Cholesterol Oxidation Products (COPs) in Raw and Chilled Storage of Chicken Sausages Formulated with Chicken Fat and Red Palm mid Fraction

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Abstract: The purpose of this paper is to determine the effect of different lipid sources (animal and plant) during chilled storage on the formation of cholesterol and cholesterol oxidation products in sausages formulated with chicken fat and red palm mid fraction. The commercial sample, as represented by the chicken sausage and the mechanical deboned meat sausage was analyzed as a comparison. The sausages were produced in a plant scale for two batches, vacuum packed and stored at -4°C or chilled condition. At time intervals of week 0, 1, 2 and 3, the sausages were analyzed using gas chromatography with flame ionization detector for determining cholesterol and cholesterol oxidations products, which were 25-hyroxycholesterol, α-epoxycholesterol, β-epoxycholesterol and 7-ketocholesterol. This study showed the variable amount of compounds analyzed throughout the period of analysis, 25-hydroxycholesterol detected in PMF at week 3 (0.77 ppm). The significantly high amount of cholesterol was detected in MDM (239.99 ppm) at week 3. The different type of sausages formulated with chicken fat and palm mid fraction which were animal and plant fats, respectively did not show any significant changes towards the formation of cholesterol and COPs throughout the storage period in chilled condition. It is recommended for future works to prolong the period of storage to obtain concrete result at the end of analysis, analyze the compounds using gas chromatography with mass spectrometry to improve the detection limit and to expand the reference standard of cholesterol oxidation products to be used as the compounds may varies.

Key words: Cholesterol · Cholesterol oxidized products · Sausages

INTRODUCTION

Lipids are organic compounds that are found in living organisms and these lipids are soluble in nonpolar solvents [1]. Lipids include all types of fats, oil, phospholipids, sterols and waxes [2] can undergo alterations during the storage of food with consequent losses in nutritional value. Basically, cholesterol present in animal origin foods will undergo autoxidation during processing as well as during storage which can produce toxic products. Cholesterol oxidation products (COPs) are similar to cholesterol, which contain an additional functional group, for instance hydroxyl, ketone or an epoxide group in the sterol nucleus and/or on the side chain of the molecule. Oxidation of lipids and sterol (cholesterol) follows the same oxidation patterns such as autoxidation, photoxidation and enzymic oxidation that will produce hydroperoxides [3].

The hyroperoxides derived from oxidation of unsaturated fatty acids play a significant role to facilitate cholesterol oxidation at Δ-5 double bond, which is more sensitive to oxidation [3]. In addition, the foods with high cholesterol contents such as egg, whole milk, meat, sea food can have COPs that are present in our diet. However, the fresh foods contain low levels of COP and the levels may go up during processing and storage (time and condition) [4]. Generally, heat, pH, light, oxygen, water activity and the presence of unsaturated fatty acids are

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the major factors that influence COP formation during food processing or storage [4, 5]. COPs have been shown to promote the formation of fatty plaques in the arteries, toxic to living cells, mutagenic and possibly carcinogenic affects in both in vivo and in vitro studies. COPs also cause cell membrane damage and inhibition of cholesterol biosynthesis. Several investigations have demonstrated that 25-OH cholesterol and triol are toxic agents causing atherosclerosis. The presence of α-epoxy cholesterol and β-epoxy cholesterol has been reported to be carcinogenic [6, 7].

The consumption of pre-cooked foods of animal origin is becoming more popular in the world due to the low cooking time. However, the intake of cholesterol oxide containing foods has bad affects on health. Thus, it is important to identify and quantify these products in foods in the market [8, 9]. From the nutritional point of view, sausages are an important source of proteins of high biological value [10]. However, this traditional processed meat product show some negative aspects as a consequence of their high animal fat content, including cholesterol and COPs. The relatively high cholesterol level is one of the enhancing factors for some diseases like coronary heart diseases [11].

Although the people have commonly consumed various process meat products for many years, little information is available on the occurrence of cholesterol and COPs in commercially manufactured sausages. Few integrated studies can be found in the literature on cholesterol and, COPs, virtually none verifying the effect of storage on these products. Thus, the objectives of this study are to investigate the chilled storage sausages and the type of fats, particularly chicken fat and red palm mid fraction on the formation of cholesterol and COPs, in comparison with commercialized chicken sausage in the market or MDM chicken sausage.

MATERIALS AND METHODS

Sample: Red palm mid fraction were obtained from Carotino Sdn Bhd (Pasir Gudang, Johor, Malaysia). Chicken, chicken fat and ingredients of sausages were supplied by OmCorp Sdn. Bhd. (Kajang, Selangor, Malaysia).

Chemicals: Fatty acids standard mixtures (Supelco™ 37), was purchased from Sigma Aldrich (M) Sdn Bhd. Reference standard of cholesterol and COPs; 5α-cholestane, 25-hydroxycholesterol (25-OH), 7-ketocholesterol (7-keto), α-epoxycholesterol, β-epoxycholesterol, 5α-cholestane were purchased from Sigma Chemical Company. The purities of the standards ranged from 95% to 99%. Chemicals (hexane and isopropanol) used for chromatography analysis were chromatography grade (Merck) and all other analytical grade solvents were from Sigma Chemical.

Sausages Formulation and Preparation: Two types of chicken sausages formulated with different type of fats, i.e. chicken fat (CF), red palm mid fraction (PMF) which are animal fat and plant lipid, respectively, were prepared in a plant scale by using facilities and ingredients provided by a local meat products manufacturer, OmCorp Sdn. Bhd. (Kajang, Selangor, Malaysia). Two batches of sausages were produced as replications of the analysis. MDM sausage was purchased from two different markets but with similar brands that represent two batches of the sausage.

Five kilogram of each type of sausages (chicken sausages formulated with chicken fat and chicken sausage formulated with red palm mid fraction) were prepared. The sausage was prepared by adding chicken breast meat into a 150 litres bowl cutter. All the ingredients were homogenized between 10 to 15 mins. The sausage batter mixtures were transferred to an automated stuffer and the cellulose casing with 10 mm in diameter was used to stuff the sausage. Then, it was steamed for an hour at 80°C, rinsed with cold water and the casing was peeled. Upon cooling, the batches were vacuum-packed and stored at 4°C.

Treatment of Samples: The sausages were treated by storing it chilled (4°C) and analyzed for COPs at time intervals of 0, 1, 2 and 3 weeks. Prior to that, moisture content of sausages was determined by using moisture analyzer.

Analysis of Cholesterol Oxidation Products (COPs): Saponification of COPs: Cholesterol and cholesterol oxidation products were saponified as described by Saldanha *et al*. [12]. Approximately 2 g of ground sausages were weighed respectively into a 100 ml of conical flask. A total of 10 ml of KOH (1 M) in 95% ethanol was added to the sample at room temperature for 22 hrs in the dark. For the extraction of the unsaponifiable matter, 5 ml of distilled water and 10 ml of hexane were added to the samples and the mixture was shaken by using a vortex. Then, the hexane fraction that was located at the upper layer was separated and transferred into a test tube. The extraction with 10 ml of hexane was repeated for another two times and later dried under a nitrogen flow.

Table 1: Sausage Formulation

Ingredients	Percentage of Ingredients (%)	
	Chicken Fat	Palm Mid Fraction
Chicken Breast Meat	40	40
Chicken Skin (CSF)	5	-
Carotino Palm Mid Fraction (PMF)	-	5
Water	19	19
Emulsion	26	26
Textured Vegetable Protein	3	3
Salt	1.7	1.7
Phosphate	0.3	0.3
Potato Starch	1.2	1.2
Milk Powder	1.2	1.2
Tandori Powder	0.6	0.6
Onion Powder	0.2	0.2
Chili Powder	0.06	0.06
Wonderhot Powder	0.04	0.04
Munich Powder	0.2	0.2

Derivatization of COPs: After drying the solvent under nitrogen gas, 100 µl of Tri-Sil reagent was added and the test tube was kept at 80°C for 60 mins. The solvent was evaporated under a stream of nitrogen gas and the TMS-ether derivatives were dissolved in 1 ml of hexane (chromatography grade). The test tube of samples and reference standards were then sonicated in an ultrasonic bath for 1 min and were centrifuged for 3 mins at 13,000 rpm. The samples and reference standards were stored at -20°C for subsequent analysis by GC within one week after derivatization [13].

Determination of COPs by Gas Chromatography with Flame Ionization Detector (GC-FID): Analysis of COPs as their TMS-ether derivatives was carried out by GC fitted with flame ionization detector (GC-FID). A capillary column of DB-5 (20 mm x 0.1 mm x 0.1 µm) was used to separate the COPs. The method according to Ubhayasekera *et al*. [13] was applied in this study. The column temperature was set at 150°C and held for 5 min, increased to 280°C at the rate 50°C/min, maintained at 280°C for 7.6 min. The temperature then was increased to 300°C at 4°C/min and held for 5.4 mins. The detector was set at 320°C. The heater was put at 290°C with hydrogen flow for 300 ml/min and air flow at 40 ml/min. Identification and purity percentages were performed using TMS ether derivatives of COPs standards.

Statistical Analysis: The data were subjected to one way analysis of variance (ANOVA), Duncan Multiple Range Rest, the value of mean and standard deviation by using SAS version 9.2 and Enterprise Guide version 4.

RESULT AND DISCUSSION

Table 2 showed that each compound of COPs as well as cholesterol did not have similar and consistent pattern in term of amount at time interval of weeks analyzed. They were variations in between samples of sausages analyzed; CF, PMF and MDM.

Cholesterol that was determined in MDM or commercialized sausage at week 3 showed the highest value (239.9900±45.8020) when compared to others; CF (0.85084±0.3229) and PMF (0.8899±0.1827). The pattern was similar for the amount of 25-hydroxycholesterol (78.0497±57.9540) in MDM at week 3, despite of the minute amount of it in PMF sausage (0.77457±0.1058) and not detected at all in CF sausage. It is also noted that there was no 25-hyroxycholesterol detected throughout the weeks of analysis for CF sausage. Longer storage period may be needed for the compound to be present at detectable amount. This was in parallel with sample of raw pork chops that the initial COPs were non-detectable limit (limit of detection was 5-10 ng of COPs) and even after 8 days of refrigerated storage, detectable COPs were only present in some sample [14]. This may explain the undetected amount of β-epoxycholesterol at the earlier week of analysis.

In another COP compound analyzed, α-epoxycholesterol was detected in a significantly higher amount at week 0 of analysis for CF sausage (15.8419±10.8775) and PMF sausage (20.0536±15.0335) when compared to week 1 (6.8968±3.9801) and week 2 (1.9939±0.5780). In contrary, MDM (16.4107±4.8293) was significantly higher as analyzed at week 3, in comparison to week 2. This compound, however, increased significantly at week 3 in PMF sausage. It can be due to the antioxidant constituents that present in PMF, mainly the tocopherols, tocotrienols and carotene being used throughout the storage period to scavenge the free radicals formed at the earlier process of sausage production, the manufacturer of red palm mid fraction stated that the fat contained more than 400 ppm of tocopherols and tocotrienols, as well as 406 ppm of carotene.

Meanwhile, the presence of β-epoxycholesterol in all sausages was not detected at week 0. In CF sausage, it has been detected at week 1 (2.1240±2.9140), while in MDM and PMF sausages it was detected at week 3 of analysis, with the amount of 3.5243±4.1897 and 0.7942±0.9196, respectively. The compound however was not detected at the subsequent week of analysis in PMF and MDM sausages. The later presence of this COP

Table 2: Cholesterol and cholesterol oxidation products for different types of sausages treated in chilled storage as analyzed at different time interval

		Week			
Compounds		0	1	2	3
Cholesterol	CF	4.1572±3.2092[a]	1.2752±0.4441[b]	1.6831±0.9796[a]	0.85084±0.3229[b]
	PMF	2.3210±0.7629[a]	1.0184±0.7073[b]	0.9501±0.2274[b]	0.8899±0.1827[b]
	MDM	3.0680±4.3220[b]	0.0000±0.0000[b]	0.8670±1.0450[b]	239.9900±45.8020[a]
α-epoxycholesterol	CF	15.8419±10.8775[a]	2.1240±2.9140[b]	2.8786±1.0290[b]	1.3784±0.4047[b]
	PMF	20.0536±15.0335[a]	6.8968±3.9801[ab]	1.9939±0.5780[b]	12.3604±4.4830[a]
	MDM	6.6184±3.9582[ab]	8.6420±4.9816[ab]	2.1140±2.1656[b]	16.4107±4.8293[a]
β-epoxycholesterol	CF	0.0000±0.0000[b]	0.2343±0.2710[a]	1.0519±0.3687[a]	0.4764±0.7557[a]
	PMF	0.0000±0.0000[b]	0.0000±0.0000[b]	3.5243±4.1897[a]	0.0000±0.0000[b]
	MDM	0.0000±0.0000[b]	0.0000±0.0000[b]	0.7942±0.9196[a]	0.0000±0.0000[a]
25-OHcholesterol	CF	0.0000±0.0000[a]	0.0000±0.0000[a]	0.0000±0.0000[a]	0.0000±0.0000[a]
	PMF	0.0000±0.0000[b]	0.0000±0.0000[b]	0.0000±0.0000[b]	0.77457±0.1058[a]
	MDM	0.8819±1.9720[b]	0.0000±0.0000[b]	0.0000±0.0000[b]	78.0497±57.9540[a]
7-ketocholesterol	CF	3.6602±8.1844[a]	0.0000±0.0000[b]	0.0000±0.0000[b]	0.0000±0.0000[b]
	PMF	0.0000±0.0000[a]	0.0000±0.0000[a]	0.0000±0.0000[a]	0.0000±0.0000[a]
	MDM	2.0921±4.6781[a]	0.0000±0.0000[b]	0.0000±0.0000[b]	0.0000±0.0000[b]

Values are means ± standard deviation of the five analysis (two batches analyzed in triplicate); values at different superscript letters are significantly differences ($P<0.05$) between the storage times. Means followed by the same letter do not differ significantly from each other

might be due to the formation of it after storing the samples, which resulted in the transformation of α-epoxycholesterol to its isomer, β-epoxycholesterol. According to Rao *et al*. [15], β-epoxycholesterol or cholesterol-β-epoxide was resistant to change and Conchillo *et al*. [16] reported that β-epoxycholesterol was the most abundant COPs in raw samples and in vacuum-stored grilled chicken, which were in contrary to the result obtained in this study.

The least compound of COPs that can be detected was 7-ketocholesterol which was analyzed at zero time analysis in CF (3.6602±8.1844) and MDM (2.0921±4.6781) sausages. The undetected amount may be due to limitation of the instrument or the unstable structure of the compound whereby it has undergone transformation process, or the compounds were not present in the sample at all. Lercker and Rodriguez-Estrada [17] suggested that no significant differences in the 7-ketocholesterol content were found among the different cooking treatments, but the raw meat studied were already presented at considerably high initial 7-ketocholesterol level (3.5 ppm).

It was reported that the predominant species was reported to be 7-ketocholesterol, accounting for almost half of the total COPs, followed by 7α- and 7β-hydroxycholesterol and both epoxides in order of decreasing concentration [18]. Oxidation reactions are initiated in the highly susceptible membrane bound phospholipids, which contain relative large amounts of polyunsaturated fatty acids. This process particularly affects unsaturated lipids, with poultry meat being one of the most susceptible, because, according to food composition tables, the ratio between unsaturated and saturated fatty acids in chicken is higher than in other meats such as pork, beef and mutton [19].

The inconsistent pattern of cholesterol and COPs content in this study are in contrast with research findings by Baggio and Bragagnolo [20] that showed no COPs were significantly formed during the storage time in any of samples of meat products studied. They believed that the presence of spices and natural condiments in the meat products which inhibit the formation of COPs. Moreover, many spices and herbs have been shown to impart an antioxidant effect in food systems [21]. The antioxidant properties of spices are related to their phenolic contents. It was found that the addition of sage in chicken meat was effective in controlling lipid and cholesterol oxidation [22]. On the other hand, study by Rao *et al*. [15] on water buffalo meat found that COPs increase on cooking and storage. It was also supported by Kowale *et al*. [23] on their study on mutton when cooked and stored at 4°C and -0°C.

The instrument and detection method of cholesterol and COPs conducted by Baggio and Bragagnolo [20] was by using a high performance liquid chromatography (HPLC) with diode array detector which may against analysis done by using a gas chromatography give different result, as the capability and treatment of samples were different when compared. The samples analyzed by HPLC do not need to be derivatized prior to analysis as to make the cholesterol and COPs volatile. Ubhayasekera and colleague, [13] proved that evaluation of COPs would be different between GC-FID and GC-MS. This was again

supported by Hur *et al*. [24] whom stated great discrepancies have been observed in the COPs data obtained from different laboratories for the various matrices analyzed, which may be due to differences in the extraction procedures or the chromatographic methods.

CONCLUSION

The different type of sausages formulated with chicken fat and palm mid fraction which were animal and plant fats respectively did not show any significant contribution towards the formation of cholesterol and cholesterol and COPs throughout the storage period in chilling condition. Some of the compounds were either undetectable or did not present at all. It is recommended for future works to longer the period of storage to obtain concrete result at the end of analysis, analyzing the compounds using gas chromatography with mass spectrometry as to increase the detection limit and expanding the reference standard of cholesterol oxidation products to be used as the compounds may varies and transformed.

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