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Effect of Storage on Fatty Acid Methyl Ester (FAME) and Cholesterol Oxidation Products (COPs) in Different Type of Sausages

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Abstract: Cholesterol oxidation products (COPs) have been shown potentially atherogenic, mutagenic and carcinogenic effects towards human. Storage of food will increase the production of COPs. The objective of this research is to investigate the effect of cold storage (chilled storage, 4°C and frozen storage, -20°C) on fatty acids content and formation of cholesterol oxides in three different lipid sources of sausage products containing chicken fat (CF), Super Olein (SO) and Red Palm Stearin (RPS).Three types of chicken sausages with different lipid sources: CF, SO and RPS were prepared and stored in chiller for zero, first, second and third weeks and stored frozen for zero, fourth and eight weeks. Fatty Acids and COPs in samples were extracted and analyzed using gas chromatography with flame ionization detector (FID). The reduction of unsaturated fatty acids, cholesterol content and formation of cholesterol oxides were found during throughout the storage life. The Red Palm Stearin (RPS) samples showed significant increment of the total saturated fatty acid compared to Super Olein (SO) and chicken fat (CF). It was concluded the saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA) increased in comparison to polyunsaturated fatty acids (PUFA) in all samples during chilled storage and the amount of COPs were in the following order; CF>SO>RPS. It implies that sausages formulated with SO and RPS are effective in reducing COPs formation. The recommendations are to wider the range of COPs reference standards.

Key words: Cholesterol Oxidation Products (COPs) · Fatty Acid Methyl Ester (FAME) · Gas Chromatography (GC)

INTRODUCTION

COPs have been shown to have a variety of potentially atherogenic (tending to promote the formation of fatty plaques in the arteries), cytotoxic (toxic to living cells), mutagenic and possibly carcinogenic affects in both *in vivo* and *in vitro* studies. Several investigations have demonstrated that 25-hydroxycholesterol (25-OH) and triol (cholestane-3β,5α,6β-triol) are toxic agents which contribute towards atherosclerosis. Alpha-epoxycholesterol (α-epoxycholesterol) and beta-epoxycholesterol (β epoxycholesterol) have been reported to be carcinogenic [1, 2].

Sausage is a comminuted processed meat product that contains high cholesterol, total lipid and saturated fatty acids. These lipid components, especially in the unsaturated form may easily deteriorate into oxidation that can generate the cholesterol oxides and alteration in fatty acids [3].

Lipid oxidation is one of the major reactions, which can occur during the storage of food in conditions such as heat, presence of light, metals, natural sensitisers and oxygen. This lipid oxidation will affect the fatty acid composition and cholesterol, with the formation of compounds that will be harmful to human health, such as cholesterol oxides [4].

The objective of this research is to study the effect of cold storage on the changes the fatty acid composition and formation of cholesterol oxides in three different lipid sources from sausage products containing chicken fat (CF), Super Olein (SO) and Red Palm Stearin (RPS) and to investigate the effectiveness of

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animal fat replacer (Super Olein and Red Palm Stearin) instead of chicken fat in order to reduce the cholesterol oxidation product.

MATERIALS AND METHODS

Sample Preparation: Three types of chicken sausages with different lipid sources such as CF, SO and RPS were prepared and stored for zero, one, two and three weeks at chilled temperature (4°C) and for zero four and eight weeks at frozen temperature (-20°C). FAME and COPs were analyzed in samples for all type sausages for comparison.

Material: Fatty acids standard mixture (Supelco 37) was purchased from Sigma Chemical Company. Cholesterol and cholesterol oxidized standards such as 5α-cholestane, 25-hydroxycholesterol (25-OH), 7-ketocholesterol (7-keto), α-epoxycholesterol and β-epoxycholesterol were purchased from the Sigma Chemical Company. The purities of the standards ranged from 95% to 99%. Gas Chromatography grade such as n-hexane was purchased from Merck and Fisher chemical company. Analytical grade solvents such as n-hexane, 2-propanol, ethanol with 95% in purity, potassium hydroxide pellet and sodium methoxide with 30% methanol were purchased from Merck and Fisher chemical company. Trimitylsilyl-imidazole (TMSI) reagent was purchased from Sigma Aldrich.

FAME Analysis: Lipid extraction was performed according to [5] and converted to fatty acid methyl esters according to [6]. FAME was analyzed using gas chromatography (Agilent technologies 7890A) with flame ionization detector. The GC conditions were as follows: injection port temperature was 250°C; flame ionization detector temperature was 280°C. Oven temperature program was set at an initial temperature of 130°C for 1 min, then heated to 170 °C at 6.5 °C/min, raised to 215°C at 2.75°C/min and finally held at 215 °C for 12 min and raised to 230°C at 40°C/ min and held for 3 min. The carrier gas was helium. The column flow rate was 1.9 ml/min. The sample size injected for each analysis was 1 ìl. Compounds were identified by comparison with the retention times of known standards (Supelco™ 37 component FAME) [7] with some modifications.

Determination of COPs: Cholesterol and cholesterol oxides were saponified [8]. It was derivatised [9]. The COPs were analyzed using a gas chromatography (Agilent technologies 7890A), ZB-5 column was used, FID injector, Helium was used as carrier gas at an inlet pressure of 18 psi (1 psi = 6894.76 Pa) while nitrogen was employed as make-up gas with a flow rate of 30 ml/min; 0.4 µl of the derivatised sample was injected in splitless mode (splitless time: 1min). The initial oven temperature was 60°C for 1 min, programmed at the rate of 50°C/min to 260°C, held at 260°C for 5 min, then raised at 1 °C/min to 280 °C and maintained at this temperature for 10 min. The injector and FID temperatures were set at 260 °C and 310 °C, respectively.

Statistical Analysis: A one-way analysis of variance (ANOVA) was applied on the data. The means of the different storage periods and sample types (CF, SO and RPS) were compared using the Duncan multiple range test, with $P<0.05$. The software used was Statistical Analysis Software (SAS) version 9.2 Enterprise Guide 4.

RESULTS AND DISCUSSION

Effect of Chilled Storage on FAME and COPs Content in Chicken Sausages: There were no significant difference ($P<0.05$) in the amount of saturated fatty acid (SFA) in all types of sausages after three weeks of chilled storage (Table 1). This showed that saturated fatty acid was stable in samples due to the high melting points and van der Waals interactions between the molecules [10]. There were also a slight contrast in values between total monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) for all the three types of samples. Total MUFA showed an increasing amount of concentration from zero to third weeks, whereas total PUFA indicated a decreasing amount through shelf life studies. This study was an indicator of oxidation [11]. The MUFA content increased throughout storage which may be due to the oxidation of PUFA breaking down. Chicken sausages containing SO and RPS contained vitamin E which may act as antioxidant, with the total PUFA content decreasing significantly. The decreased PUFA content in the sample may be caused by the loss of vitamin E in the process of sausages production, or exposed to the light during production of sausages; this vitamin E cannot protect unsaturated fatty acids from oxidation. Another possibility that could be considered from this result was the ability of PUFA to oxidize rapidly [12] especially for processed meat products in chilled storage within three weeks than frozen storage. However, the fat treatments stored chilled, were effective in delaying of the breakdown of PUFA at 0 and first week respectively. Total *cis* fatty acid in three types of samples were significantly decreased ($P<0.05$) from zero to third

Table 1: Total fatty acids composition (ppm) of chilled and raw three types of chicken sausages at 0 week to 3 week of storage at 4°C

Compounds	Sausage type	0 week	1 week	2 week	3 week
□ SFA	CF	8.55±0.44^{Ca}	8.67±0.90^{Ca}	8.44±0.56^{Ca}	8.72±0.93^{Ca}
	SO	10.79±1.10^{Ba}	11.34±0.26^{Ba}	10.05±0.72^{Ba}	11.61±0.93^{Ca}
	RPS	14.18±0.26^{Ab}	14.57±0.43^{Ab}	14.48±1.47^{Ab}	17.50±2.17^{Aa}
□ MUFA	CF	13.32±0.73^{Ab}	13.94±0.84^{Bb}	14.93 V 0.98^{Bb}	20.03±3.83^{Aa}
	SO	13.44±2.46^{Ac}	16.19±0.64^{Ab}	17.36±1.30^{Aab}	19.88±2.53^{Aa}
	RPS	15.38±0.31^{Ac}	14.37±0.37^{Bc}	17.11±1.80^{Ab}	20.90±1.47^{Aa}
□ PUFA	CF	5.06±0.59^{Aa}	5.19±0.04^{Ba}	3.35±0.85^{Ab}	3.50±0.56^{Ab}
	SO	5.62±0.60^{Aa}	6.01±0.33^{Aa}	2.69±0.25^{Ab}	2.30± 0.37^{Bb}
	RPS	4.60±0.92^{Aa}	5.18±0.15^{Ba}	3.54±0.85^{Ab}	2.39±0.20^{Bc}
□ *cis*-FA	CF	15.03±1.12^{Aa}	15.52±0.61^{Ca}	11.47±2.03^{Ab}	13.94±2.81^{Aab}
	SO	14.28±3.78^{Ab}	18.97±0.39^{Aa}	12.50±0.94^{Ab}	14.60±1.93^{Ab}
	RPS	16.52±0.68^{Aa}	16.63±0.59^{Ba}	12.15±1.52^{Ab}	12.56±2.69^{Ab}
□ *trans*-FA	CF	1.26±0.17^{Ab}	1.40±0.16^{Bb}	4.78±0.79^{Ba}	5.00±0.90^{Aa}
	SO	1.49±0.56^{Ac}	2.09±0.26^{Ac}	6.03±0.48^{ABa}	3.92±1.61^{Ab}
	RPS	1.77±0.59^{Ac}	2.21±0.06^{Ac}	6.42±0.46^{Aa}	3.93±1.21^{Ab}

Table 2: Cholesterol and cholesterol oxides level (ppm) of chilled and raw three types of chicken sausages at 0 week to 3 week of storage at 4°C

Compounds	Sausage type	0 week	1 week	2 week	3 week
Cholesterol	CF	649.04±271.51^{Aa}	529.32±64.93^{Aa}	557.27± 114.38^{Aa}	287.10±31.09^{Ab}
	SO	508.67±296.24^{Aa}	412.25±73.75^{Bab}	418.66±118.25^{Bab}	205.44±28.64^{Bb}
	RPS	749.56± 293.88^{Aa}	449.31±29.25^{ABb}	466.09±41.13^{ABb}	178.10±33.62^{Bc}
5α-cholestane	CF	6.84±3.97^{Ab}	13.34±2.80^{Aab}	14.91±2.31^{Aa}	12.71±0.74^{Aab}
	SO	6.18±2.21^{Ab}	11.85±1.29^{Aa}	15.69±1.93^{Aa}	13.19±0.67^{Aa}
	RPS	5.86±2.16^{Ab}	13.64±1.31^{Aa}	17.11±1.35^{Aa}	12.93±1.46^{Aa}
α-epoxycholesterol	CF	10.31±5.57^{Aa}	4.68±2.05^{Aa}	1.62±0.38^{Aa}	36.58±14.59^{Aa}
	SO	6.45±4.80^{Aab}	3.05±1.94^{Aab}	2.35±1.59^{Ab}	8.09±2.56^{Aa}
	RPS	8.75±2.20^{Aa}	4.39±6.61^{Aa}	5.88±5.25^{Aa}	5.97±1.01^{Aa}
β-epoxycholesterol	CF	15.09±7.44^{Aa}	6.73 V 1.46^{Ab}	2.85±0.70^{Ab}	18.15±5.50^{Aa}
	SO	6.42±2.82^{Ab}	3.48±1.95^{Ab}	2.57±1.05^{Ab}	15.79±3.99^{Aa}
	RPS	14.81±2.24^{Aa}	3.06±1.73^{Ab}	2.87±1.62^{Ab}	7.62±1.17^{Bab}
25-hydroxycholesterol	CF	4.34±1.16^{Aab}	2.04±0.87^{Ab}	2.68±0.94^{Ab}	5.63±1.67^{Aa}
	SO	2.18±0.69^{Ab}	2.64±0.48^{Aab}	3.31±1.06^{Aab}	5.32±2.64^{Aa}
	RPS	2.31±0.76^{Ab}	2.35±0.61^{Ab}	2.53±0.86^{Ab}	5.93±1.73^{Aa}
7-ketocholesterol	CF	13.86±2.70^{Aa}	1.87±0.34^{Ab}	3.27±2.13^{ABb}	6.23±0.79^{Aa}
	SO	7.93±2.12^{Ba}	2.57±0.16^{Ab}	4.96±2.28^{Aab}	2.91±0.51^{Bab}
	RPS	6.00± 1.52^{Ba}	2.03±0.71^{Ab}	1.18±0.87^{Bb}	2.91±0.51^{Bab}

Table 3: Total fatty acids composition (ppm) of frozen and raw three types of chicken sausages at 0 week, 4 week and 8 week of storage at -20°C

Total fatty acids	Sausage type	0 week	4 week	8 week
□ SFA	CF	8.55±0.44^{Cb}	7.05±1.86^{Bb}	13.62±1.34^{Ba}
	SO	10.79±1.10^{Bc}	17.87±1.36^{Ab}	23.57±0.82^{Aa}
	RPS	14.18±0.26^{Ab}	16.78±0.95^{Ab}	24.07±4.70^{Aa}
□ MUFA	CF	13.32±0.73^{Aa}	15.92±1.41^{Ab}	12.20±1.90^{ABb}
	SO	13.44±2.46^{Aa}	14.80±2.04^{ABa}	14.46±5.30^{Aa}
	RPS	15.38±0.31^{Aa}	13.48±1.46^{Ba}	8.16±2.53^{Bb}
□ PUFA	CF	5.06±0.59^{Ab}	8.33±0.34^{Aa}	3.68±0.50^{Ac}
	SO	5.62±0.60^{Ab}	7.39±1.01^{Ba}	4.57±0.90^{Ab}
	RPS	4.60±0.92^{Ab}	6.45±0.40^{Ca}	3.76±1.70^{Ab}
□ *Cis*-FA	CF	15.03±1.12^{Aa}	11.61±1.75^{Ab}	3.84±0.53^{Bc}
	SO	14.28±3.78^{Aa}	6.09±1.97^{Bb}	4.66±0.96^{ABb}
	RPS	16.52±0.68^{Aa}	6.54±0.39^{Bb}	5.54±1.44^{Ab}
□ *Trans*-FA	CF	1.26±0.17^{Ac}	3.21±0.53^{Ab}	7.33±0.47^{Aa}
	SO	1.49±0.56^{Ab}	3.15±0.87^{Aa}	1.69±0.34^{Bb}
	RPS	1.77±0.59^{Aa}	3.30±2.27^{Aa}	1.70 ±0.50^{Ba}

Table 4: Cholesterol and cholesterol oxides level (ppm) of frozen and raw three types of chicken sausages at 0 week, 4 week and 8 week of storage at -20°C

Compounds	Sausage type	0 week	4 week	8 week
Cholesterol	CF	649.88±271.51^{Aa}	339.67±31.56^{Ab}	172.78±4.20^{Ab}
	SO	508.67±296.24^{Aa}	296.69±24.97^{ABab}	151±14.23^{Bb}
	RPS	749.56±293.88^{Aa}	250.21±31.45^{Aa}	143.80±8.20^{Bb}
5α-cholestane	CF	6.84±7.97^{Aa}	9.44±1.01^{Ba}	3.98±0.34^{Aa}
	SO	6.18±6.21^{Aa}	10.74±1.187^{Aa}	4.55±0.91^{Ab}
	RPS	5.86±6.16^{Aab}	12.37±2.42^{Aa}	4.27±0.80^{Ab}
α-epoxycholesterol	CF	10.31±5.75^{Aa}	8.44±1.66^{Aa}	6.18±4.39^{Aab}
	SO	6.45±4.80^{Aa}	7.60±2.44^{Aa}	5.22±0.89^{Ba}
	RPS	8.75±2.20^{Aa}	15.76±11.22^{Aa}	14.48±8.71^{Aa}
β-epoxycholesterol	CF	15.08±7.44^{Aa}	11.37±3.86^{Aa}	10.11±2.63^{a}
	SO	6.42±4.82^{Aa}	10.42±1.57^{Aa}	5.83±1.51^{a}
	RPS	14.81±10.24^{Aa}	10.76±6.68^{Aa}	NA
25-hydroxycholesterol	CF	4.34±2.16^{Aa}	3.63±2.02^{ABa}	3.47 ±2.00^{a}
	SO	2.18±0.69^{Ab}	5.73±1.14^{Aab}	9.41±5.44^{a}
	RPS	2.31±0.76^{Ab}	2.38±1.23^{Bb}	5.42±3.13^{a}
7-ketocholesterol	CF	13.86±2.70^{Aa}	11.56±14.02^{Aa}	NA
	SO	7.93±3.12^{Ba}	2.87±1.81^{Ab}	NA
	RPS	6.00±3.51^{Ba}	4.70±4.02^{Aa}	NA

week. This might be due to the degradation of *cis* fatty acids to other compounds within three weeks in chilled storage. Meanwhile, total *trans* fatty acids increased significantly (P<0.05) for all samples after three weeks in chilled storage for all the samples.

Values are means±standard deviation of the six analysis (two batches analyzed in triplicate); Values at different letters at the columns (capital letters) are significantly differences (P<0.05) between sample types; Values at different letters at the rows (small letters) are significantly differences (P<0.05) between treatment at storage time; Means followed by the same letter do not differ significantly from one another.

By referring to Table 2, cholesterol and 7-ketocholesterol level decreased significantly from zero to three week of storage for all type of sausages. Decreasing the cholesterol amount in samples may be caused by the generation of numerous oxidation products commonly known as cholesterol oxidation products (COPs) or oxysterols. It is related to the cholesterol structure which was an unsaturated alcohol that contained double bonds which were prone to oxidation [13,14]. However the amount of 25-hydroxycholesterol and β-epoxycholesterol increased during the storage time for all the types of sausage samples.

Effect of Frozen Storage on FAME and COPs Content in Chicken Sausages: The effects of frozen storage on FAME and COPs in samples were presented in Tables 3 and 4, respectively. Saturated fatty acid increased significantly in all types of sausages during the frozen storage. Meanwhile *cis*-fatty acid indicated a contrary result compared to the saturated fatty acid in all type of sausages. CF showed a significant increased (P<0.05) in total *trans* fatty acids values than SO and RPS. This may be due to the nature of chicken fat which contain more unsaturated fatty acids (10-15% of total fatty acids) and a notable amount of PUFA [15-17]. This showed that the plant fat replacers such as SO and RPS can reduce the oxidation of *cis* to *trans* fatty acids. However, the amount of COPs in the frozen storage from 0 to 8 weeks for all types of sausages did not show a significant difference (P>0.05) for all types of sausages. This might be due to the frozen condition which retarded the oxidation process of cholesterol, compared to the amount of COPs formed during chilled storage, as storage temperature may be one of the factor that influence the oxidation process.

Values are means±standard deviation of the six analysis (two batches analyzed in triplicate); Values at different letters at the columns (capital letters) are significantly differences (P<0.05) between sample types; Values at different letters at the rows (small letters) are significantly differences (P<0.05) between treatment at storage time; Means followed by the same letter do not differ significantly from one another.

Values are means±standard deviation of the six analysis (two batches analyzed in triplicate); Values at different letters at the columns (capital letters) are significantly differences (P<0.05) between sample types; Values at different letters at the rows (small letters) are significantly differences (P<0.05) between treatment at storage time; Means followed by the same letter do not differ significantly from one another.

Values are means±standard deviation of the six analysis (two batches analyzed in triplicate); Values at different letters at the columns (capital letters) are significantly differences (P<0.05) between sample types; Values at different letters at the rows (small letters) are significantly differences (P<0.05) between treatment at storage time; Means followed by the same letter do not differ significantly from one another.

Changes of the fatty acid composition and cholesterol content occurred during cold storage. Formation of cholesterol oxides in the chicken sausages formulated were found to be the highest in chicken fat followed by palm Super Olein and Red Palm Stearin. During storage period, it was observed that the concentration of saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA) were higher than polyunsaturated fatty acids (PUFA) in all treated samples. RPS showed the higher SFA content than CF and SO for all types of sausages samples. The cholesterol content decreased significantly over storage period in all treated samples. Study by Xu *et al*., [18] stated that the surrounding fatty acids affected sterol oxidation in a time-dependent manner and their effect was unlikely related to their degree of unsaturation.

CONCLUSION

In conclusion, the fatty acid composition changed during chilling by the increment of saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA) compared to polyunsaturated fatty acids (PUFA) in all samples and the cholesterol oxides of samples became higher in the following order; CF>SO>RPS. It implies that sausages formulated with SO and RPS are effective in order to reduce COPs formation. In future works, it is recommended to wider the reference standard of COPs.

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