INFLUENCE OF LARD HEAT TREATMENT ON CHANGES IN THE CONTENT OF CHOLESTEROL AND FORMATION OF CHOLESTEROL OXIDATION PRODUCTS

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The aim of this study has been to determine the influence of high temperature (150°C) and layer thickness of lard used upon changes in the content of cholesterol and formation of cholesterol oxidation products (COPs). It was found that heat treatment in a thin layer of lard was characterised by a more intensive degradation and thermal oxidation of sterols in comparison to heating a thick layer of lard. A decline of cholesterol in the analysed lard has been noted along progressing heat treatment which equaled to 7.6% for thin lard layer (3 mm) and 4.3% for thick lard layer (10 mm). The content of cholesterol oxidation products during heat treatment, which lasted from 0 to 120 min, was very differentiated and in the case of a thin lard layer ranged from 1.5 to 10.4 µg/g, and in the case of a thick lard layer – from 1.5 to 8.9 µg/g.

INTRODUCTION

Sterols are alicyclic steroid alcohols, containing secondary alcohol group. The group of these compounds is characterised by a tetracyclic ring structure, containing sterane core with hydroxide group at the C_3 position and a lateral chain at the C_{17} position.

Sterols are a significant element of the nonsaponifying fat fractions. They are insoluble in water and extracted with the use of grease solvents. They appear mainly in a crystallic form and are characterised by high melting temperatures.

Sterols, due to their origin, have been classified into the following groups: (i) zoosterols, animal origin *e.g.* cholesterol (Figure 1); (ii) phytosterols, plant origin, *e.g.* sitosterol (β-sitosterol); and micosterols, microbiological origin, *e.g.* ergosterol.

Cholesterol is the main sterol present in fats of animal origin, which is present in grocery products in a free and estrified state mainly with fatty acids [Toivo *et al.,* 2001]. The main source of cholesterol in a human diet today are: meat, eggs, ham and sausages, dairy products, and fats of animal origin.

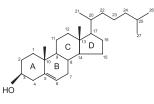Sterols present in foods undergo oxidation process as a result of many factors (high temperature, oxygen, presence of free radicals and peroxides, dyes *etc.*), [Johnsson *et al.,* 2003; Baggio & Bragognolo, 2006]. The process of sterols oxidation is a multi-level one. It is believed that sterol hydroxyperoxides are precursors to sterol autooxidation, which undergo reduction to oxysterols. The main products of oxidation are hydroxy-, keto- and epoxy-derivatives which form derivatives of sterol triol as a result of hydratation. The processes of sterol oxidation might, therefore, take place along the whole food chain from "field to table" – from storage and manufacturing of products through their processing, distribution and preparation for consumption. One of the most popular cooking methods in Poland is frying and deep-frying. During this process, the medium (fat) reaches temperature from 150 to 200°C, which leads to changes in the product *e.g.* formation of brown skin, protein coagulation and also fat autooxidation. In the past years lard and other animal fats have been used as a frying medium for a number of food products [Marikkar *et al.* 2003], nowadays they are mainly used in domestic cooking.

Until today only three means of sterols oxidation have been discovered, namely: autooxidation, photoxidation and enzymatic oxidation. The most common phenomenon concerning the oxidation of sterols occurring in the case of grocery products is the autooxidation of sterols (Figure 2). Initially, the autooxidation reaction is initiated by the formation of a peroxy radical situated by the carbon in the position at the C_7 ring of the B sterol, hydrogen by the methylene group is detached and oxygen molecule is attached. Then the peroxy radical reacts with the hydrogen from the methylene group and a new peroxy radical, as well as 7-hydroperoxide sterol is being formed. Hydroperoxide may undergo the following reactions: (i) epoxidation in the presence of sterols hyperoxide or trig-

FIGURE 1. Cholesterol structure (5α-cholesten-3β-ol).

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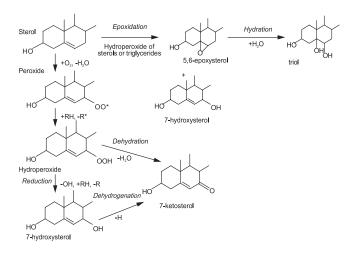
FIGURE 2. Autooxidation of sterols [Soupas, 2006].

lycerides, which leads to the formation of 7-hydroxysterol and 5,6-epoxysterol. After the hydration, the 5,6-epoxycholesterol transforms into triol; (ii) reduction to the 7-hydroxysterol, which after dehydrogenation forms 7-ketosterol; (iii) and dehydration to 7-ketosterol.

Photooxidation forms another mechanism of sterol oxidation. As a result of this reaction the sterol 5-hydroperoxide is formed, which subsequently transforms into more stable 6- and 7-hydroperoxide. Exposure to light causes transformations of 5,6-epoxysterol and 7-hydroxysterol to 6-ketosterol and 7-ketosterol [Rontani *et al.,* 2007; Zhang, 2005].

Enzymatic cholesterol oxidation in food products is activated by the following enzymes from the oxidoreductase class: monooxygenase, dioxygenase, dehydrogenase and oxidase. As a result of the activity of the above-mentioned enzymes the following COPs are created: 7α-hydroxycholesterol, 25-hydroxycholesterol, 20α-hydroxycholesterol, (25R)-26-hydroxycholesterol, 22R-hydroxycholesterol [Ubhayasekera, 2004].

As shown by a number of research [Guardiola *et al.,* 1996; Chang *et al.,* 1997; Wilczak & Kulasek, 2004; Ryan *et al.,* 2005; Zhang, 2005; Baggio & Bragagnolo, 2006], the COPs contribute to disorders of the circulatory system *e.g.* arteriosclerosis, and are characterised, among others, by mutagenic and cancerogenic activities. From a dietary point of view the content of those compounds in grocery products should be reduced. During the heat treatment COPs are absorbed by products. Therefore, the content of COPs in grocery products should be monitored and their formation prevented during the process of food production and preparation.

The aim of this study was to determine the amount of cholesterol oxidation products formed during heat treatment of lard. The results of the research can be helpful in evaluating COP's intake in a human diet.

MATERIAL AND METHODS

Lard samples and heat treatment

Lard samples were purchased in November 2006 on the Warsaw market. Lard has been heated at 150°C using two layers of fat: 3 ± 0.5 and 10 ± 0.5 mm thick. Samples have been placed on Petri dishes 50 mm in diameter after weighing and inserted into a laboratory dehydrator. They were next heated by convection and with the access of atmospheric oxygen. After the temperature of heating has stabilized, the samples were taken out one by one in the following time intervals: 5, 20, 40, 60, 80, 100 and 120 min. Each of the heat treatment processes have been repeated five times.

Chemicals

Internal standards: 5α-cholestane was purchased from Sigma–Aldrich, Poland, 19-5-cholesten-3-beta,19-diol was purchased from Steraloids, UK. Organic solvents: hexane and methanol (analytical grade) were purchased from POCH, Poland.

Silylation reagent BSTFA (N,O-bis(trimethylsilyl)trifluoroacetamide) with 1% of TMCS (trimethylchlorosilane) was purchased in Sigma-Aldrich, Poland, pirydine was purchased from Riedel-de Haën, Germany.

Instrumentation

A gas chromatograph equipped with a mass spectrometer (GC-MS) type GCMS-QP2010S came from Shimadzu Corporation (Shim-Pol A.M. Borzymowski, Poland).

Extraction of cholesterol and COPs from fat matrices

A portion of 100-200 mg of lard was dissolved in 2 mL of hexane and 100 µL of internal standards: 19-hydroxycholesterol (19-OH) (1 ppm) and 5α-cholestane (4 ppm) were added. The mixture was saponified by adding 0.5 mL of a sodium hydroxide solution in methanol (2 N) at a room temperature, 1-2 h. Next, 200 µL of hexane layer was transported into a 1.5-mL vial insert and after evaporation to dryness under nitrogen, the residue was dissolved in 100 µL of pyridine and 100 µL of BSTFA with 1% TMCS, and left to remain in the dark for 24 h for complete derivatization. Then, 1 mL of hexane was added and 1 µL of the mixture was collected for GC-MS analysis. Tree replicates per each sample were analysed. The results obtained were elaborated statistically using Statgraphics Plus 4.1 programme. To identify the significance of differences between the mean values of cholesterol and COPs content in particular lard samples, Tukey's test was used at a significance level of $\alpha = 0.05$.

GC-MS analysis of cholesterol and COPs

A DB5ms capillary column was used to separate six COPs, including 7β-hydroxycholesterol (7β-OH), 5α,6α-epoxycholesterol (5α,6α-EP), 5β,6β-epoxycholesterol (5β,6β-EP), triol, 7-ketocholesterol, 25-hydroxycholesterol (25-OH) and the internal standards in 31 min with helium as a carrier gas, a flow rate of 0.9 mL/min. The injector temperature was 230°C, and the column temperature was programmed as follows: 50°C in the beginning for 2 min, subsequent increase to 230°C at the rate of 15°C/min, to 310°C at the rate of 3°C/min maintained for 10 min. The interface temperature for GC-MS was 240°C. Temperature of ion source was 220°C, ionization energy was 70V. The selected ion monitoring (SIM) mode was used to detect COPs and total ion monitoring (TIC) mode to detect cholesterol (m/z range 100-600). The elution order of COPs was as follows: 26.0-27.1 min m/z 353, 366 for 19-OH, 27.2–27.6 min m/z 456 for 7β-OH, 27.7–

–28.5 min m/z 474 for 5α,6α-EP and 5β,6β-EP, 29.0–29.7 min m/z 403 for triol, 30.0-31.3 min m/z 131 for 25-OH and 474 for 7-ketocholesterol. During the investigation of cholesterol and COPs their retention times of peaks were compared with retention times of reference standards. The internal standard 19-hydroxycholesterol was used to quantify the amount of COPs and 5α-cholestane to quantify cholesterol.

RESULTS AND DISCUSSION

Pork fat, *i.e.* lard, has been used in the experiment. It contained cholesterol at 0.554±0.011 mg/g of lipids. Exeprimental conditions (150°C, thick and thin fat layer) were designed so as to simulate the heat treatment of lard *e.g.* before meal preparation. Variations in the content of cholesterol together with heating time and thickness of fat layer are shown in Table 1. A successive decline in the content of cholesterol has been detected along with increasing time of heating. It was due to the thermal degradation of cholesterol which equaled to 7.6% for the 3 mm layer and 4.3% for the 10 mm layer. The thickness of heated fat layer has influenced the thermal degradation of cholesterol and was lower for the 10 mm layer from 57 to 68% in comparison to 3 mm layer. The above loss has been determined by the ratio between lard surface staying in direct contact with atmospheric air and its capacity. That ratio increased over 3 times in the case of the thin layer of fat in relation to the thick layer.

The following cholesterol oxidation products have been found in samples being subject to heat treatment: 7β-hydroxycholesterol, 5β,6β-epoxycholesterol, 5α,6-αepoxycholesterol, triol, 25-hydroxycholesterol and 7-ketocholesterol. Figure 3 shows the dynamics of changes in the sum of cholesterol oxidation products depending upon the lard layer thickness used and the time of heat treatment. Heated lard has been characterised by the increased aggregate content of COPs after 100 min of heat treatment for 3 and 10 mm lard layers. The aggregate content of COPs in heated lard of 3 mm layer was higher by 11% to 37% (in the samples heated for 20, 40, 60 and 80 min) than in lard of 10 mm thickness. The high-

TABLE 1. Cholesterol content (mg/g) in a thin (3 mm) and a thick (10 mm) layer of lard during heat treatment (150°C).

Heating time (min)	Cholesterol content (mg/g)	
	Thin (3 mm) layer of lard	Thick (10 mm) layer of lard
0	0.554±0.011[aA]	0.554±0.014[abA]
5	0.556±0.010[aA]	0.556±0.006[abA]
20	0.552±0.016[abA]	0.556±0.013[bA]
40	0.538±0.015[abA]	0.553±0.015[abAB]
60	0.530±0.013[bcA]	0.546±0.019[abAB]
80	0.522±0.014[bcA]	0.542±0.015[abAB]
100	0.518±0.016[bcA]	0.539±0.017[abAB]
120	0.512±0.017[bcA]	0.530±0.018[aAB]

[a,b...] identical quantifications in columns – the lack of significant differences at the level of α≤0.05 in lard samples during heat treatment; [A,B...] identical quantifications in rows – the lack of significant differences at the level of α≤0.05 between different lard samples heated for the same period of time.
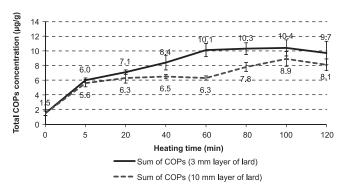

FIGURE 3. Total content of cholesterol oxidation products (µg/g) in a thin (3 mm) and a thick layer (10 mm) of lard during heating (temp. 150°C).

est aggregate COPs content in lard heated in 10 mm and 3 mm thick layer appeared after 100 min of heat treatment and amounted to 10.4 and 8.9 µg/g, respectively.

The COPs formed have constituted only 21% of cholesterol loss in 3 mm lard layer and 31% in 10 mm layer. Percentage losses of cholesterol point to the fact that the formation of cholesterol oxidation products constituted only an indirect link of its thermal degradation. A change in the dynamics of the aggregate content of cholesterol oxidation products present in both thick and thin layers of heated lard can be described using second degree polynomial (Figures 4 and 5).
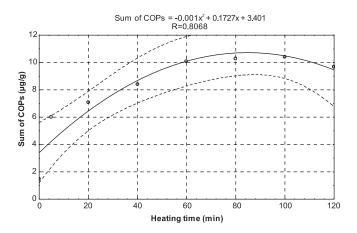

FIGURE 4. Changes of total cholesterol oxidation product content (µg/g) in a thin layer (3 mm) of lard during heating (150°C).

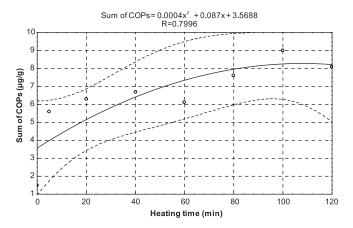FIGURE 5. Changes of total cholesterol oxidation product content (µg/g) in a thick layer (10 mm) of lard during heating (150°C).

The conducted statistical analysis points to the fact that changes in the aggregate COPs content in lard during heat treatment were influenced not only by heating time but also by other factors, like the depth of fat layer. The presented model of cholesterol oxidation products formation (similar to the one taking place in both household and industrial conditions) points to the influence of the thickness of lard layer on the dynamics of cholesterol oxidized derivatives. The results indicate that deep-frying may reduce the intake of COPs in a human diet.

In the conducted test of heating the thick lard layer (10 mm) at 150°C for 2 h the loss of cholesterol content reached 4%. An experiment conducted by Park & Addis [1986] confirmed that losses in cholesterol concentration during heat treatment of lard at 155°C lasting for 250 h amounted to 50%, which is compliant with the thesis about a linear loss of cholesterol content during heat treatment [Hur *et al.,* 2007]. The comparison of the above results shows that initial hours or even minutes of lard heating have led to large losses in cholesterol content. The prolonging time of heat treatment, however, led to the stabilization of cholesterol degradation process. Results of research show [after Obiedziński *et al.,* 1999] that heating lard in higher and higher temperature for longer periods of time leads to the enhanced formation of COPs. A thick layer of lard has been heated in those tests at 100°C, 120°C and 140°C for a period of 1, 2, 6, 24 and 48 h. From 1.8 to 2.2 µg/g of COPs aggregate content has been found in the lard not subjected to heat treatment. The aggregate COPs content present in the lard heated for 2 h in comparison to the initial value increased respectively by 0.7, 1.5 and 3.0 µg/g in the samples heated at 100°C, 120°C and 140°C. The presented research shows that the heat treatment of lard at 150°C has led to a significant increase in aggregate COPs content. Heat treatment lasting for two hours has led to an average COPs increase of 6.9 µg/g in comparison to initial sample. The above observation indicates that the fact that the melting point of free cholesterol (147-148°C) has been breached must have had a significant influence upon the cholesterol oxidation process during the heat treatment of lard at 150°C. Cholesterol in a free state constitutes 95% of the total cholesterol content in lard.

The research concerning standard cholesterol heating conducted by different scientists are divergent. One test has shown that heating at 150°C for 180 min can lead to a 20% loss of cholesterol content [Osada *et al.,* 1993]. Other sources say that heating for 30 min at the same temperature leads to 65% and 70% loss in the initial value of cholesterol [Chien *et al.,* 1998]. A test conducted by Xu *et al.* [2005] proved that environment surrounding sterol constituents is a very important factor influencing processes of degradation and oxidation of cholesterol. It has been proven that heating cholesterol standard in vegetable oil at 150°C for 30 min leads to its far lesser losses than in the case of heating the standard alone. The matrix of vegetable oils has had a protective impact on cholesterol, preventing its degradation by 30-80%. Xu *et al.* [2005] concluded that the amount of 7-ketocholesterol formed during heat treatment of cholesterol in rapeseed oil, soybean oil and olive oil was significantly lower than during heating cholesterol only.

CONCLUSIONS

The process of sterol oxidation is a very complex phenomenon and many factors influence its progress, *i.e.* temperature, time of thermal exposure, thickness of fat layer, availability of oxygen. The dynamics of changes concerning sterol oxidation products under model conditions proves that the process of frying significantly influences the suppression of sterol oxidation process. It needs to be highlighted that heating up the thickest fat layer seems to be the most suitable form of heat treatment because of the process of sterol. It should be conducted in such a way that the surface of fat in contact with atmospheric oxygen remains the smallest.

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