Food Chemistry 171 (2015) 233–240

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Cholesterol transformations during heat treatment

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ARTICLE INFO

Article history:
Received 28 August 2013
Received in revised form 18 August 2014
Accepted 27 August 2014
Available online 7 September 2014

Keywords:
Cholesterol degradation
Oxysterols
Polymers
Volatile compounds
Cholestadienes
Fragmented cholesterol molecules

ABSTRACT

The aim of the study was to characterise products of cholesterol standard changes during thermal processing. Cholesterol was heated at 120 °C, 150 °C, 180 °C and 220 °C from 30 to 180 min. The highest losses of cholesterol content were found during thermal processing at 220 °C, whereas the highest content of cholesterol oxidation products was observed at temperature of 150 °C. The production of volatile compounds was stimulated by the increase of temperature. Treatment of cholesterol at higher temperatures i.e. 180 °C and 220 °C led to the formation of polymers and other products e.g. cholestadienes and fragmented cholesterol molecules. Further studies are required to identify the structure of cholesterol oligomers and to establish volatile compounds, which are markers of cholesterol transformations, mainly oxidation.

1. Introduction

Cholesterol is the most known sterol present in food. This steroid alcohol due to unsaturation in the ring B is susceptible to oxidation. Cholesterol in foods is present mainly in free or esterified form. During thermal processing and storage cholesterol and other sterols undergo changes like: degradation, oxidation and polymerisation. Thermo-oxidation degradation of phytosterol was investigated by Rudzińska, Przybylski, and Wąsowicz (2009). They showed that not only oxidation products but also volatile compounds and polymers of phytosterol are formed during thermal oxidation. There is a lack of publications about cholesterol alteration during thermal processing, that present such holistic approach as Rudzińska et al. (2009) proposed.

Heat induced cholesterol degradation was widely investigated. Park and Addis (1986) reported, that cholesterol losses during lard processing at 155 °C lasting 250 h was 50%, which is consistent with the notion of a linear loss of cholesterol during heating. During lard heating at the same temperature lasting 2 h cholesterol degraded about 4.3–7.6%, depending on the thickness of the heated fat layer (Derewiaka & Obiedziński, 2010a).

Oxidation of cholesterol during heating was investigated by many researchers. The pioneer in this field was Smith (1981, chap. 4). This process is similar to hydrocarbons oxidation. Autoxidation of sterols is free radical chain reaction, which leads to the formation of a variety of oxides (Lethonen, Lampi, Ollilainen, Struijs, & Piironen, 2011). The oxidation of sterol is strongly related to the temperature of processing and other factors like moisture, acidity index etc. Cholesterol oxidation products have been proven to have an adverse effects on human body e.g. cytotoxic, apoptotic and pro-inflammatory and also some investigations have shown that take part in a atherosclerotic and neurodegenerative process (Otaegui-Arrazola, Menéndez-Carreño, Ansorena, & Astiasarán, 2010). They have been found at low concentrations in foodstuffs e.g. meat, fish, eggs and milk and their products (Calderón-Santiago, Peralbo-Molina, Priego-Capote, & Luque de Castro, 2012; Derewiaka & Obiedziński, 2010b; Orczewska-Dudek, Bederska-Łojewska, Pieszka, & Pietras, 2012; Ubhayasekera, Jayasinghe, Ekanayake, & Dutta, 2012).

Polymerisation of sterols was mainly described in studies involving phytosterols. Struijs, Lampi, Ollilainen, and Piironen (2010) presented formation of stigmasterol polymers after heating at 180 °C. Similar investigation was published in the same year by Rudzińska, Przybylski, Zhao, & Curtis (2010) describing thermal oxidation of sitosterol and production of dimers, trimers and tetramers. Lethonen, Lampi, Agalga, Struijs, and Piironen (2011) indicated that formation of sterol oligomers (e.g. pentamers and hexamers) was induced by the presence of acyl moiety and its

Abbreviations: NMR, nuclear magnetic resonance; IR, infrared spectroscopy; SEC, size exclusion chromatography; APCI, atmospheric pressure chemical ionisation; MS, mass spectrometry.

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http://dx.doi.org/10.1016/j.foodchem.2014.08.117
0308-8146/

unsaturation. The characteristics of sitosterol oligomers produced during thermal processing were presented by Sosińska, Przybylski, Hazendonk, Zhao, and Curtis (2013). They applied spectroscopic methods such NMR, IR, Raman and SEC/APCI/MS to establish sitosterol dimers structure.

There are only a few studies describing formation of volatile compounds due to thermal-oxidation of sterols. In Van Lier, de Costa, & Smith, 1975 determined at least fourteen volatile compounds that were produced during decomposition of cholesterol. The amount of volatiles, formed during heating of phytosterol in different temperatures, was discussed by Rudzińska et al. (2009). They found mainly hydrocarbons, ketones, aledehydes and acids in the profile of volatile fraction formed during heating.

The aim of the study was to evaluate the intensity of cholesterol degradation, oxidation, polymerisation and formation of volatile compounds during thermal treatment at temperatures typical for food processing.

2. Materials and methods

2.1. Materials

Cholesterol and 5α-cholestane standards were purchased from Sigma–Aldrich (St. Louis, MO, USA) and 19-hydroxycholesterol from Steraloids (Newport, RI, USA). Solvents (acetone, hexane, methanol, diethyl ether, potassium hydroxide, tetrahydrofuran) were purchased from POCH (Gliwice, Poland), while 1,2-dichlorometane from Aldrich (Dorset, England). A sylilation agents BSTFA (N,O-Bis(trimethylsilyl) trifluoroacetamide) with 1% TMCS (trimethylchlorosilane) and anhydrous pyridine were purchased from Sigma-Aldrich (St. Louis, MO, USA). Cholesterol oxidation standards: 7β-hydroxycholesterol, 5α,6α-epoxycholesterol, 5β,6β-epoxycholesterol, 5α-cholestane-3β, 5β,6β-triol (triol), 7-ketocholesterol, 25-hydroxycholesterol came from Sigma-Aldrich Co. (Poznań, Poland). SPE DSC-Si Silica tubes (1 g/6 mL) and SPME Fibre divinylbenzene/carboxene/.

polydimtheylsiloxane (DVB/CAR/PDMS) (2 cm) were purchased from Supelco (Bellefonte, PA, USA).

2.2. Sample heat treatment

Cholesterol standard (10–20 mg) was placed in a glass ampoule of 20 mL capacity and the glass neck of the ampoule was closed over the burner flame. Closed ampoules were heated at 120, 150, 180 and 220 °C for 30, 60, 120 and 180 min. Heating procedures were done in triplicate. A control sample was a sample of non-heated cholesterol standard.

2.3. Determination of cholesterol, oxysterols and cholestadienes

Cholesterol, oxysterols and cholestadienes were separated on SPE cartridges. 2 mg of heated cholesterol sample, diluted in hexane, was applied on SPE column (conditioned with 2 mL of hexane). Column was washed with 4 mL of hexane:diethyl ether (75:25; v/v), cholesterol was eluted with 15 mL of hexane: diethyl ether (60:40; v/v) (I fraction) whereas oxysterols and cholestadienes with 10 mL of acetone (II fraction). To the first fraction 0.2 mL of 5α-cholestane solution (10 mg/25 mL of chloroform) was added and to the second fraction 0.2 mL of 19-hydroxycholesterol solution (8.5 mg/25 mL of chloroform) and 0.2 mL of α-cholestane solution (10 mg/25 mL of chloroform) was added. Fractions were evaporated under a stream of nitrogen. Samples were dissolved in 2 mL of hexane and saponified with addition of 0.5 mL of 2 N potassium methoxide for 1 h at room temperature. Afterwards, solvents were removed under a stream of nitrogen and samples were derivatized for 24 h at room temperature due to addition of 100 μL of anhydrous pyridine and 100 μL of sylilation agents. Cholesterol, oxysterols and cholestadienes were analysed on gas chromatograph equipped with a mass spectrometer (GCMS-QP2010S) Shimadzu Corporation (Shim-Pol A. M. Borzymowski, Poland) using. DB5ms (30 m × 0.25 mm × 0.25 μm) capillary column Phenomenex (Torrance, CA, USA). Helium was used as a carrier gas at a flow rate of 0.61 mL/min. The injector temperature was set at 250 °C, and the column temperature was programmed as follows: 200 °C for 1 min, subsequent increase to 250 °C at the rate of 15 °C/min, then to 310 °C at the rate of 3 °C/min for 6 min. The interface temperature for GC–MS was 260 °C. Temperature of ion source was 250 °C and ionisation energy was 70 V. The split ratio was 50:1. The total ion monitoring (TIC) was used to detect sterols, oxysterols and cholestadienes (m/z ranged 100–600). The internal standard 5α-cholestane was used to quantify cholesterol and cholestadienes, 19-hydroxycholesterol to quantify oxysterols. Regression coefficient of oxysterols curves were between $R^2 = 0.94$ for 5β,6β-epoxycholesterol to $R^2 = 0.997$ for triol. Cholesterol and cholestadienes content was expressed as equivalents of 5α-cholestane in mg per g of heated cholesterol standard, where as oxysterols content was expressed as mg of 19-hydroxycholesterol per g of heated cholesterol standard. Identification of compounds was made on the basis of mass spectral libraries (NIST 47, NIST 147 and Wiley 175) as well as data from literature and by comparison of their retention times with authentic standards, including cholesterol, 7β-hydroxycholesterol, 5α,6α-epoxycholesterol, 5β,6β-epoxycholesterol, triol, 7-ketocholesterol, 25-hydroxycholesterol. Three replicates per each sample were analysed.

2.4. Determination of fragmented cholesterol molecules

Fragmented cholesterol molecules were separated on SPE cartridges. 2 mg of heated cholesterol sample, diluted in hexane, was applied on SPE column (conditioned with 2 mL of hexane). Column was washed firstly with 4 mL of hexane: diethyl ether (75:25; v/v) and 15 mL of hexane: diethyl ether (60:40; v/v) was used to collect sterol (I fraction) and than fragmented cholesterol molecules were eluted with 10 mL of acetone (II fraction). To the second fraction 0.2 mL of 5α-cholestane solution (10 mg/25 mL of chloroform) was added Fraction was evaporated under a stream of nitrogen. Samples were dissolved in 2 mL of hexane and saponified with addition of 0.5 mL of 2 N potassium methoxide for 1 h at room temperature. Afterwards, solvents were removed under a stream of nitrogen and samples were derivatized for 24 h at room temperature due to addition of 100 μL of anhydrous pyridine and 100 μL of sylilation agents. Fragmented cholesterol molecules were analysed on gas chromatograph equipped with a mass spectrometer (GCMS-QP2010S) Shimadzu Corporation (Shim-Pol A. M. Borzymowski, Poland) using. DB5ms (30 m × 0.25 mm × 0.25 μm) capillary column Phenomenex (Torrance, CA, USA). Helium was used as a carrier gas at a flow rate of 0.61 mL/min. The injector temperature was set at 250 °C, and the column temperature was programmed as follows: 200 °C for 1 min, subsequent increase to 250 °C at the rate of 15 °C/min, then to 310 °C at the rate of 3 °C/min for 6 min. The interface temperature for GC–MS was 260 °C. Temperature of ion source was 250 °C and ionisation energy was 70 V. The split ratio was 50:1. The total ion monitoring (TIC) was used to detect fragmented cholesterol molecules (m/z ranged 100–600). The internal standard 5α-cholestane was used to semi-quantify fragmented cholesterol molecules. Fragmented sterol molecules content was expressed as equivalents of α-cholestane in mg per g of heated cholesterol standard. Identification of compounds was made on the basis of mass spectral libraries (NIST

47, NIST 147 and Wiley 175) as well as data from literature. Three replicates per each sample were analysed.

2.5. Separation of volatile compounds

The glass ampoule with heated cholesterol standard was placed in Schott bottle (250 mL) and 1 μL of internal standard (1,2-dichlorobenzene, 0.01% solution in methanol) was added, after shaking of the bottle the glass ampoule was smashed. Volatile compounds were collected on a SPME DVB/CAR/PDMS fibre for 5 min at room temperature (the fibre was inserted into the bottle through the hole in the cap of the bottle, RSD of the method counted for 1,2-dichlorobenzene was 3.9%) and carry into the GC–MS injector. ZB FFAP (30 m × 0.25 mm × 0.25 μm) capillary column Phenomenex (Torrance, CA, USA) was used to separate volatile compounds. Helium was used as a carrier gas at a flow rate of 1.0 mL/min. The injector temperature was set at 220 °C, and the column temperature was programmed as follows: 40 °C for 2 min, subsequent increase to 220 °C at the rate of 5 °C/min for 2 min. The solvent delay during GC–MS analysis was 1 min. The interface temperature for GC–MS was 230 °C. Temperature of ion source was 240 °C, ionisation energy was 70 V. The total ion monitoring (TIC) was used to monitor volatile compounds (m/z ranged 40–500). The internal standard 1,2-dichlorobenzene was used to semi-quantify volatile compounds, and their content was expressed as equivalents of 1,2-dichlorobenzene in mg per g of heated cholesterol standard. Identification of volatile compounds was made on the basis of mass spectral libraries (NIST 47, NIST 147 and Wiley 175). Three replicates per each sample were analysed.

2.6. Oligomers analysis

Gel permeation chromatography (GPC) on phenogel (100 Å, 5 μm, 300 × 7.8 mm, Phenomenex, Torrance, CA) column was used to determine the profile of polymers. For this purpose, sample solution in THF of 20 μL was injected on the column kept at room temperature. Oligomers were separated in isocratic elution mode with THF at a flow rate of 0.8 mL/min. Components were detected with evaporative light scattering detector ELSD (Gdańsk Innovation Centre-Ltd.), operated at 30 °C with air at a pressure of 0.7 bar. Polymers were quantified by external standard method where cholesterol was the calibration standard. A standard curve was prepared for the five concentrations of cholesterol in THF from 0.05 to 0.25 mg/mL, and regression coefficient of the curve was $R^2 = 0.998$. The amounts of oligomers represented as equivalents of cholesterol in mg per 1 g of heated cholesterol. Identification of monomers and oligomers of cholesterol was made on the base of comparing retention times of sitosterol and 3,3′-disitosteryl ether and their cholesterol equivalents. Disteryl ethers were prepared as described in Sosińska et al. (2013). Three replicates per each sample were analysed.

3. Results and discussion

3.1. Cholesterol content

Heat treatment of the standard led to decrease of cholesterol (PubChem CID: 5997) content (Table 1). With increasing temperature and time of heat processing, cholesterol losses were more intense. In samples heated at 120 °C for 30 min and 180 min, 12.2% and 13.9% losses of cholesterol content were observed, respectively. Decrease of cholesterol concentration was similar after heating at 150 °C and 180 °C. In samples processed at 150 °C for 30 and 180 min it was 51.4% and 86.0%, whereas in samples heated at 180 °C for 30 and 180 min it was 59.3% and 83.3%, respectively. The highest losses of cholesterol were observed during heating at 220 °C for instance after 1/2 and 3 h of processing the losses were between 96.0% and 99.5%. These results shows that thermo-oxidation of cholesterol increased rapidly above the melting point of cholesterol (147–148 °C) and was the most intense at 220 °C. Thermo-oxidation above the melting point leads to a better contact of cholesterol with agents causing oxidation e.g. oxygen, air, water and transition metals.

Kim and Nawar (1993) studied the stability of cholesterol standard at different temperatures. After heating at 120 °C for 60 h the decrease of its content was about 10%. Moreover cholesterol losses after 30 min of heating at 150 °C and after 20 and 40 min at 180 °C were 75.6%, 71.7% and 83.6%, respectively and were higher that cholesterol decrease observed in our study.

Osada, Kodama, Yamada, and Sugano (1993) also studied cholesterol content during heating at 150 °C but for 24 h and found strictly lower losses of cholesterol (65%) than in our study. Chien, Wang, and Chen (1998) presented different result because cholesterol percentage decreased sharply and after 30 min of heating at 150 °C its decomposition was 66.7%. Results obtained in our study are similar because cholesterol losses in samples heated at 150 °C for 30 and 180 min was 51.4% and 86.0%, respectively.

Barriuso et al. (2012) heated cholesterol standard at 180 °C for 30 and 180 min of cholesterol and reported higher decomposition of cholesterol that in the present study because the decrease was 74.7% and 93.2%, respectively.

In our previous study, we showed that conditions of cholesterol heating determines different rate of cholesterol decomposition (Derewiaka & Obiedziński, 2010a). Lard was heated at 150 °C using thin and thick layer of the fat. After 120 min of processing the loss of cholesterol was 7.6% for the 3 mm layer and 4.3% for the 10 mm layer. The thickness of heated fat layer has influenced the rate of thermal decomposition of cholesterol, which indicates that the ratio between lard surface staying in direct contact with atmospheric air and its capacity. This indicates that differences in oxidation rates of cholesterol found by various research groups stem from various heating conditions i.e. oxygen availability.

Table 1
Cholesterol amount (mg/g of control sample) during heat treatment at different temperatures and times.

Temperature	Time			
	30 min	60 min	120 min	180 min
Control sample	968. 0 ± 74.0			
120 °C	878.0 ± 56.6a	878.0 ± 87.4a	854.5 ± 27.0a	860.6 ± 10.4a
150 °C	485.5 ± 50.4a	357.3 ± 75.7ab	267.2 ± 70.2bc	139.8 ± 40.8c
180 °C	406.9 ± 79.0a	239.0 ± 28.5b	173.3 ± 10.6b	167.5 ± 36.8b
220 °C	40.3 ± 6.6a	21.7 ± 2.1b	7.6 ± 2.5c	4.8 ± 1.8d

Values within a row with different letters are significantly different ($p < 0.05$).

3.2. Formation of cholesterol oxidation products, cholestadienes and fragmented cholesterol molecules

After thermal processing of cholesterol standard oxidation products were found (Table 2) in all samples except for control and heated at 120 °C samples. Rich profile of cholesterol oxidation products was found in samples heated at 180 °C containing eleven oxysterols: 4α and 4β-hydroxycholesterol, 6α and 6β-hydroxycholesterol, 7α and 7β-hydroxycholesterol (PubChem CID: 473141), 25-hydroxycholesterol, 5α,6α- and 5β,6β-epoxycholesterol, triol and 7-ketocholesterol (PubChem CID: 193313). Additionally other compounds were identified in those samples e.g. cholestadienes and fragmented cholesterol molecules.

The highest content of total cholesterol oxides was observed when cholesterol standard was heated at 150 °C. After 120 min of processing 167.7 mg of oxysterols per 1 g of non-heated cholesterol standard was found (mostly epoxycholesterols and 7-ketocholesterol), which means that about 16.7% of non-heated cholesterol has been transformed into cholesterol oxides (Table 4). When the highest temperature was applied, the lower content of oxysterols has been determined. After 30 and 180 min of heating at 180 °C 76.8 and 51.6 mg/g, respectively, of oxysterols were found (hydroxycholesterols, epoxysterols, 7-ketocholesterol and triol), whereas at 220 °C – after the same time periods, 77.6 and 15.3 mg/g, respectively, were determined (mostly 7-ketocholesterol, triol and 25-hydroxycholesterol). Therefore transformation of cholesterol into its oxides ranged between 5.2–7.7% at 180 °C and 1.5–7.8% at 220 °C over time course of heat processing (Table 4).

Free radical reaction of a cholesterol can be initiated through either hydrogen atom transfer from the allylic position or peroxyl radical addition to the double bond. Initial hydrogen atom abstraction by a peroxyl free radical occurs by donation of a hydrogen atom from the C-7, therefore C-7-oxysterols are the most frequently observed and abundant oxysterols. Initially formed hydroperoxides undergo rapid transformations at higher temperatures with formation of more stable hydroxyl- and ketosterols; simultaneously epoxides are formed by addition reaction of a peroxy radical to the 5,6-double bond (Yin, Xu, & Porter, 2011). The highest diversity of oxysterol derivatives was observed in the group of hydroxycholesterols, found in substantial amounts during heating at 180 °C. The profile and content of cholesterol oxides found in heated samples was influenced not only by their formation but also further transformations and degradation.

Yan and White (1990) investigated the influence of heat treatment of lard on the oxidation of cholesterol. They found that total content of cholesterol oxidation products formed after 25 days of heat treatment at 180 °C represented only 31% of the loss of cholesterol. Barriuso et al. (2012) heated cholesterol standard at 180 °C and they found that the total accumulation of cholesterol oxides achieved maximum after 10 min of heating and was 73.8 mg/g of standard. The most abundant oxysterol was 7-ketocholesterol reaching almost 64.3% of total cholesterol oxides, next derivatives from quantitative point of view were 7α- and 7β-hydroxycholesterol, epoxides and triol. In our study not only derivatives described by Yan and White (1990) and Barriuso et al. (2012) were found, but also other hydroxycholesterols such as: 25-hydroxycholesterol, 4α and 4β-hydroxycholesterol, 6α and 6β-hydroxycholesterol.

Other products formed during thermal processing of cholesterol, were also detected and identified on basis of mass spectra libraries and literature. These were cholestadienes (cholesta-3,5-dien-7-one PubChem CID: 11954162 and cholesta-4,6-dien-3-ol PubChem CID: 85700) and also fragmented cholesterol molecules found, after derivatization found as trimethylsilyl derivatives e.g.:

Table 2
Cholesterol oxidation products content [mg/g] formed during thermal processing of cholesterol standard at different temperatures.

	4α-Hydroxy cholesterol	4β-Hydroxy cholesterol	6α-Hydroxy cholesterol	6β-Hydroxy cholesterol	7α-Hydroxy cholesterol	7β-Hydroxy cholesterol	25-Hydroxy cholesterol	5α,6α-Epoxycholesterol	5β,6β-Epoxycholesterol	Triol	7-Keto cholesterol	Sum
Control	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
120 °C	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
150 °C												
30 min	nd	nd	nd	nd	23.8 ± 2.4	13.5 ± 0.6	nd	13.2 ± 1.2	43.0 ± 5.0	nd	33.5 ± 3.5	129.9 ± 12.9a
60 min	nd	nd	7.5 ± 1.9	nd	nd	7.4 ± 0.5	nd	14.9 ± 1.3	38.6 ± 4.0	nd	50.1 ± 6.9	118.5 ± 14.7a
120 min	nd	nd	10.1 ± 0.9	nd	16.8 ± 2.1	7.7 ± 0.6	nd	25.9 ± 2.5	36.0 ± 3.3	nd	71.2 ± 7.2	167.7 ± 16.6a
180 min	nd	nd	9.6 ± 1.1	1.2 ± 0.1	5.3 ± 0.5	7.2 ± 0.7	6.9 ± 0.4	7.8 ± 0.6	19.8 ± 2.4	1.2 ± 0.1	28.5 ± 3.2	89.7 ± 10.0a
180 °C												
30 min	0.7 ± 0.1	2.4 ± 0.2	7.0 ± 0.9	0.7 ± 0.1	3.5 ± 0.3	6.4 ± 0.4	6.4 ± 0.5	6.0 ± 0.5	20.0 ± 2.1	1.5 ± 0.2	22.4 ± 1.9	76.8 ± 7.2a
60 min	0.9 ± 0.2	2.1 ± 0.2	8.1 ± 1.5	0.6 ± 0.1	4.3 ± 0.3	6.5 ± 0.4	5.8 ± 0.6	5.0 ± 0.4	17.3 ± 3.0	0.9 ± 0.1	21.0 ± 1.3	72.5 ± 8.1a
120 min	0.7 ± 0.1	2.0 ± 0.2	7.5 ± 1.1	0.5 ± 0.1	2.0 ± 0.3	3.4 ± 0.3	5.8 ± 0.4	1.8 ± 0.2	11.5 ± 1.1	1.5 ± 0.2	18.0 ± 2.1	54.2 ± 6.0b
180 min	0.6 ± 0.1	1.3 ± 0.2	7.1 ± 1.3	0.6 ± 0.1	1.7 ± 0.1	2.7 ± 0.3	5.3 ± 0.5	2.1 ± 0.2	11.6 ± 1.4	0.7 ± 0.1	18.5 ± 2.4	51.6 ± 6.5b
220 °C												
30 min	nd	0.2 ± 0.0	7.9 ± 1.3	0.7 ± 0.1	3.8 ± 0.4	8.3 ± 2.0	8.7 ± 0.7	2.5 ± 0.5	10.8 ± 0.6	1.1 ± 0.0	31.6 ± 2.8	77.6 ± 4.9a
60 min	nd	0.1 ± 0.0	3.1 ± 1.3	nd	1.4 ± 0.6	2.0 ± 0.9	22.9 ± 2.0	nd	7.6 ± 0.6	1.5 ± 0.3	10.3 ± 0.9	49.2 ± 8.3b
120 min	nd	nd	nd	nd	0.3 ± 0.1	0.1 ± 0.0	2.7 ± 0.3	nd	1.4 ± 0.1	0.8 ± 0.1	8.3 ± 1.5	13.5 ± 2.0c
180 min	nd	nd	nd	nd	0.4 ± 0.1	0.1 ± 0.0	3.2 ± 0.2	nd	1.9 ± 0.6	1.0 ± 0.1	8.4 ± 1.4	15.3 ± 1.5c

Values of sum of oxysterols within the same temperature of processing with different letters are significantly different ($p < 0.05$). Nd – not detected.

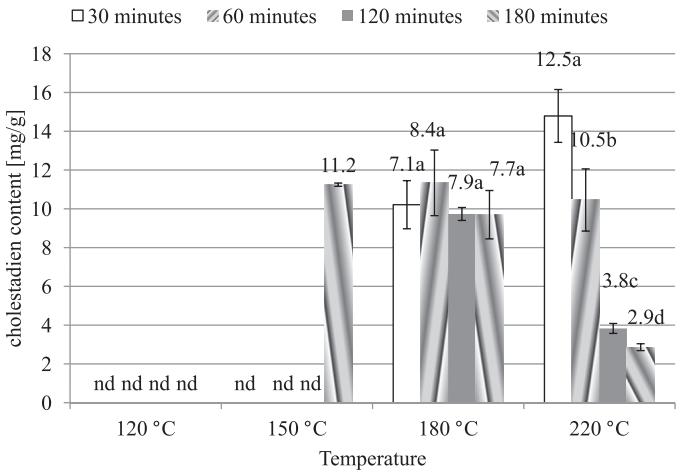

Fig. 1. Cholestadienes content [mg/g] determined during heat treatment of cholesterol at different temperatures. Values within the same temperature of processing with different letters are significantly different ($p < 0.05$). Nd – not detected.

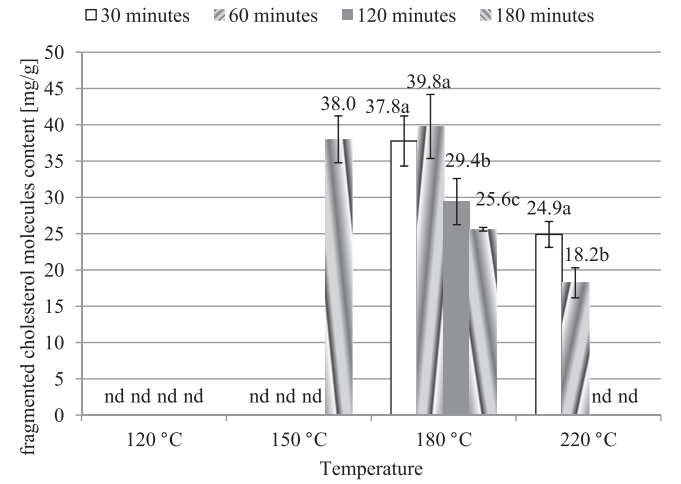

Fig. 2. Content of fragmented cholesterol molecules [mg/g] determined during thermal processing of cholesterol at different temperatures. Values within the same temperature of processing with different letters are significantly different ($p < 0.05$). Nd – not detected.

3β,17,21α-pregn-5-ene, ether of 7α-hydroxypregnenolone and 3β,4β-cholest-5-ene.

Cholestadienes were determined in samples heated at 150 °C (only after 3 h of heating), 180 °C and 220 °C. The highest content of cholestadienes were found in samples heated at 220 °C for 30 and 60 min and at 150 °C for 180 min, 12.5; 10.5 and 11.5 mg/g, respectively (Fig. 1). Cholestadienes are products of cholesterol dehydration and those compounds can be dimerized to form e.g. 3′-bis(3,5-cholestadiene) and 3,3′-bis(4,6-cholestadiene). Unfortunately in our study we were not able to confirm their presence.

The fragmented cholesterol molecules were found in samples heated at 150 °C (only after 3 h of heating) and 180 °C and their content ranged from 25.6–39.8 mg/g (Fig. 2). Their appearance in those samples was due to thermal degradation of cholesterol. Other authors also detected fragmented sterol molecules after thermal processing. Rudzińska et al. (2009) reported, that partially decomposed sterol molecules were found in sitosterol standard samples after thermal oxidation. They reported that amounts of fragmented sterols ranged 153–162 mg/g in samples heated at 60, 120 and 180 °C for 24 h. They reported that the molecular weight of this fraction was between 50 and 200 Da. One year later Rudzińska, Przybylski, Zhao, and Curtis (2010) analysed thermo-oxidation of sitosterol and made an attempt to determine what kind of molecules were formed in fraction containing fragmented sterols and found that fragmented sterols were present also as dimers, trimers and tetramers. Authors explained possible cause of oligomeric compounds presence and suggested that hydroperoxy radicals produced during sterol free radical oxidation are involved in process of oligomers formation.

3.3. Volatile compounds

Total content of volatile compounds formed during heat treatment of cholesterol at different temperatures is presented on Fig. 3. The most abundant group of volatiles were carbonyl compounds e.g. aldehydes (23 different compounds e.g. 3-methylbutanal; 2-methyl-undecanal), ketones (46 compounds e.g. 2-pentanone; 6-methyl-2-heptanone) and alcohols (49 compounds e.g. 3,4-dimethyl-1-pentanol; 4-methyl-1-pentanol, 6-methyl-2-heptanol). Other detected compounds were acids (18), hydrocarbons (35 compounds e.g. 2-methyl-4-decene; 2,6-dimethyl-3-heptene PubChem CID: 44630239) and esters (5 compounds e.g. hexanoic acid, ethenyl ester PubChem CID: 75118). Van Lier et al. (1975) reported that during autoxidation of cholesterol volatile compounds like: carbonyl compounds e.g. alcohols and acids were identified. They speculated that volatile compounds were formed via decomposition of initially formed sterol hydroperoxides (Van Lier et al., 1975). In our study the amount of volatile compounds found after heating of cholesterol was increased at higher temperature. For instance after 1 h of thermal processing of the standard at 120 °C, 150 °C, 180 °C and 220 °C total concentration of volatiles was 0.02, 0.44, 0.98 and 1.40 mg/g of non-heated standard (Fig. 3), respectively, which constitutes 0.002%, 0.07%, 0.10% and 0.14% of cholesterol losses (Table 4). Similar results were presented by Rudzińska et al. (2009) after heating of sitosterol standard at 120 °C and 180 °C for 24 h. The same types of compounds were identified in the fraction of volatiles i.e. hydrocarbons, ketones, aldehydes, acids and others. Total amount of volatiles after 1 h of heating of sitosterol at 120 °C and 180 °C was 0.007 mg/g and 0.0241 mg/g, respectively. Authors presented only contribution of volatile compounds as the part of the sitosterol standard losses only for samples heated after 24 h and they found that it was 0.001% and 0.01%, respectively (Rudzińska et al., 2009).

3.4. Oligomer formation

Application of size exclusion chromatography with evaporative light scattering detector enabled to distinguish monomers from dimers, trimers and other oligomers. Cholesterol dimers were the most abundant oligomers found in heated samples, similarly to what is observed during thermo-oxidation of phytosterols (Table 3) (Lethonen, Lampi, Agalga, et al., 2011; Menéndez-Carreño, Ansorena, Astiasarán, Piironen, & Lampi 2010; Rudzińska et al., 2009; Rudzińska et al., 2010; Sosińska et al., 2013; Struijs, Lampi, Ollilainen, & Piironen, 2010). The highest total content of oligomers was noted after 3 h of processing at 220 °C and reached 193.4 mg/g of non-heated standard, whereas after 3 h of heating at 180 °C and 150 °C the sum of oligomers was lower reaching 75.6 and 31.5 mg/g, respectively. Tetramers of cholesterol were found only in samples heated at 180 °C after 3 h. Presence of trimers was detected in cholesterol samples processed at 150 °C, 180 °C and 220 °C and it was time dependent (Table 3). The same tendency was reported by Lethonen, Lampi, Agalga, et al. (2011). They heated cholesterol at 100 °C and 140 °C during 4 days, and reported oligomers formation only after heating at higher temperature. After 1 and 4 days of heat treatment at 140 °C they found

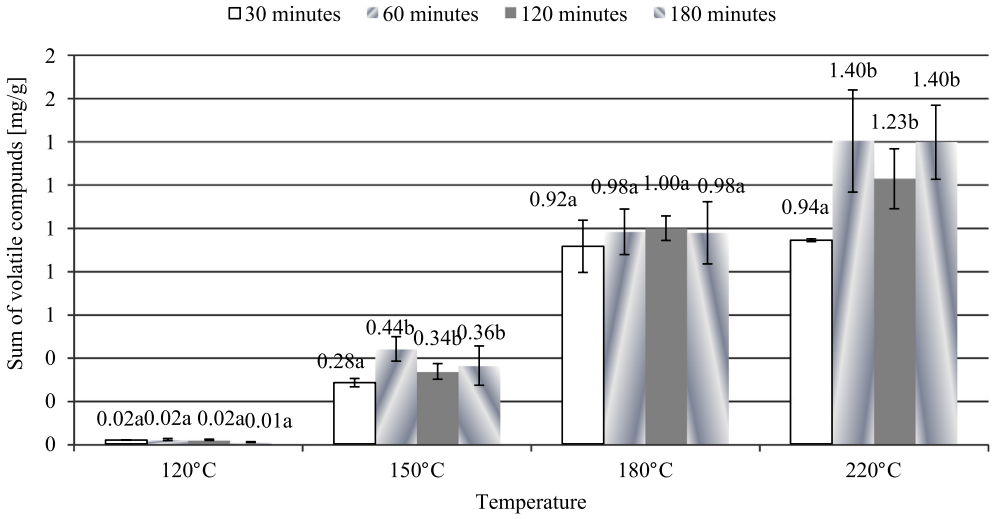

Fig. 3. Total content [mg/g] of volatile compounds determined during heat treatment of cholesterol at different temperatures. Values within the same temperature of processing with different letters are significantly different ($p < 0.05$).

Table 3
Total oligomers, dimmers, trimers and tetramers content [mg/g] formed during thermal processing of cholesterol at different temperatures.

	Tetramers	Trimers	Dimers	Sum of oligomers
120 °C				
30 min	nd	nd	nd	nd
60 min	nd	nd	nd	nd
120 min	nd	nd	nd	nd
180 min	nd	nd	nd	nd
150 °C				
30 min	nd	nd	nd	nd
60 min	nd	nd	7.7 ± 1.0a	7.7 ± 1.0a
120 min	nd	nd	19.5 ± 1.2b	19.5 ± 1.2b
180 min	nd	4.3 ± 1.2	27.2 ± 4.3c	31.5 ± 5.5c
180 °C				
30 min	nd	nd	18.6 ± 2.6a	18.6 ± 2.6a
60 min	nd	4.2 ± 1.6a	24.7 ± 3.3a	28.8 ± 4.2b
120 min	nd	2.8 ± 0.5a	26.2 ± 4.1a	29.0 ± 4.5b
180 min	3.5 ± 0.4	16.4 ± 0.9b	55.6 ± 3.8b	75.6 ± 3.4c
220 °C				
30 min	nd	nd	72.8 ± 17.1a	72.8 ± 17.1a
60 min	nd	nd	184.0 ± 22.2b	184.0 ± 22.2b
120 min	nd	1.8 ± 0.2a	167.5 ± 13.1b	169.3 ± 13.0b
180 min	nd	88.9 ± 12.9b	104.6 ± 19.3a	193.4 ± 32.1b

Values within the same temperature of processing with different letters are significantly different ($p < 0.05$). Nd – not detected.

420 and 596 mg/g of oligomers, respectively. Also Rudzińska et al. (2009) have proven that heat treatment of sitosterol at 180 °C for 24 h led to the formation of tetramers (about 80 mg/g), trimers (180 mg/g) and dimers (200 mg/g). Heat processing of sitosterol at 120 °C resulted in lower production of dimers (220 mg/g) and trimers of sitosterol (160 mg/g). Menéndez-Carreño et al. (2010) accounted 30% of losses of stigmasterol content after heat processing at 180 °C for 3 h by the formation of dimeric and polymeric oxidation products (165 mg/g). Moreover other researchers tried to identify sterol oligomers by means of techniques such as: HPLC–APCI/MS, HPLC–CIS/MS, NMR, IR and Raman spectroscopies. Rudzińska et al. (2010), on basis of APCI/MS data, suggested that the most abundant dimer of sitosterol was 3-, 7′-ether linking an oxidised sitosterol monomer to a second sitosterol molecule in its dehydrated form. Also Stuijs et al. (2010) on basis of spectral data determined by CIS/MS and APCI/MS, along with literature data proposed that dimers were composed of two stigmasterol molecules linked via C–C′ linkage. Sosińska et al. (2013) confirmed using combination of NMR, APCI/MS, IR and Raman spectroscopy, that the most abundant dimeric product of thermo-oxidation of sitosterol was 3β,3β′-sitosteryl ether, and suggests that the C3–C3′ linkage between carbon atoms in ring A and A′ is more likely to form than the C7–C7′ linkage (ring B and B′). Unfortunately the formation of the polymerised form of phytosterol in food products has an adverse effect on their cholesterol-lowering efficiency in fortified food.

3.5. The quantitative balance of cholesterol changes during thermal processing

Estimation of quantitative balance of cholesterol changes during heating on the basis of our results has been proposed in Table 4. The table summarizes the losses of cholesterol determined after heat treatment at different temperatures and different times and also content of compounds formed during thermo-oxidation expressed as percentage of initial amount of cholesterol. Losses of cholesterol were counted by subtraction from the initial concentration of cholesterol its final concentration. Observed losses of cholesterol were greater than the sum of formed products (oxysterols, volatile compounds, oligomers, cholestadienes and fragmented cholesterol products). For example during thermal processing of cholesterol at 180 °C for 3 h cholesterol losses were about 83.3%, however only 16.3% of its content was accounted as transformed into other products, the remaining 67% of cholesterol must have been degraded further (Table 4). Content of degraded cholesterol was accounted by subtraction of cholesterol losses and sum of products formed during cholesterol transformation. We found that the most abundant groups of components that were determined during cholesterol thermal degradation were oligomers and cholesterol oxidation products, but oligomers were produced at higher temperatures 180 °C and 220 °C (7.56% and 19.34% after 3 h of heating), whereas oxysterols were found mostly at 150 °C (8.97% after 3 h of heating). High percentage of fragmented cholesterol molecules and cholestadienes in the balance of changes of cholesterol was reported. For instance the highest percentage of the fragmented cholesterol molecules and cholestadienes was found after heat treatment at 180 °C for 2 h and it constituted 4.27% and 0.84% of initial amount of cholesterol, respectively. The least abundant group of products formed during heat processing of cholesterol were volatile compounds detected in samples

Table 4
Percentage of cholesterol standard losses, degradation and quantity of changed cholesterol into products during heat treatment at different temperature comparing to it initial concentration.

Compounds	Time	Temperature			
		120 °C	150 °C	180 °C	220 °C
Quantity of cholesterol losses	30 min	12.20%	51.45%	59.31%	95.97%
	60 min	12.20%	64.27%	76.10%	97.83%
	120 min	14.55%	73.28%	82.67%	99.24%
	180 min	13.94%	86.02%	83.25%	99.52%
Cholesterol oxidation products	30 min	nd	12.93%	7.68%	7.76%
	60 min	nd	11.85%	7.25%	4.92%
	120 min	nd	16.77%	5.42%	1.35%
	180 min	nd	8.97%	5.16%	1.53%
Volatile compounds	30 min	0.002%	0.02%	0.09%	0.09%
	60 min	0.002%	0.07%	0.10%	0.14%
	120 min	0.002%	0.06%	0.10%	0.13%
	180 min	0.001%	0.04%	0.10%	0.14%
Oligomers	30 min	nd	nd	1.86%	7.28%
	60 min	nd	0.77%	2.88%	18.40%
	120 min	nd	1.95%	2.90%	16.93%
	180 min	nd	3.15%	7.56%	19.34%
Cholestadienes	30 min	nd	nd	0.71%	1.25%
	60 min	nd	nd	0.84%	1.05%
	120 min	nd	nd	0.79%	0.38%
	180 min	nd	0.81%	0.77%	0.29%
Fragmented cholesterol products	30 min	nd	nd	4.09%	2.72%
	60 min	nd	nd	4.27%	1.82%
	120 min	nd	nd	3.12%	nd
	180 min	nd	4.11%	2.76%	nd
Sum of products formed during cholesterol transformation	30 min	0.002%	12.95%	14.43%	19.10%
	60 min	0.002%	12.69%	15.34%	26.33%
	120 min	0.002%	18.78%	12.33%	18.79%
	180 min	0.001%	17.08%	16.35%	21.30%
Quantity of degraded cholesterol	30 min	12.20%	38.50%	44.88%	76.87%
	60 min	12.20%	51.58%	60.76%	78.73%
	120 min	14.55%	54.50%	70.34%	72.91%
	180 min	13.94%	68.94%	66.90%	78.22%

Nd – not detected.

heated at 150 °C, 180 °C and 220 °C. Their percentage in cholesterol quantitative balance ranged 0.02–0.14%.

4. Conclusions

Most publications describing changes of cholesterol during thermal processing is concentrated on degradation, oxidation and polymerisation processes. Unfortunately the vast majority of publications concerning a quantitative balance of cholesterol changes during heat processing, do not include formation of volatile compounds, cholestadienes or fragmented cholesterol molecules. Our study proofs that quantity of degraded cholesterol was not equal with its losses. Further research is needed to determine the chemical structure of oligomers formed during heat processing of cholesterol for instance applying variety of techniques parallely. Additionally some of volatile compounds formed during this process could be treated as markers of degradation of cholesterol (e.g. 6-methyl-2-heptanone PubChem CID: 13572 or 3,4-dimethyl-1-pentanol PubChem CID: 110906) and inform consumers and manufacturers about advanced transformations of cholesterol that had taken place during heating and storage of foodstuffs.

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