UNIVERSIDADE ESTADUAL DE CAMPINAS

FACULDADE DE ENGENHARIA DE ALIMENTOS

DEGRADAÇÃO DO COLESTEROL SUBMETIDO AO AQUECIMENTO NA PRESENÇA DE ÁCIDOS GRAXOS E ANTIOXIDANTES: ESTUDO EM SISTEMAS-MODELO

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CAMPINAS
2011

FICHA CATALOGRÁFICA ELABORADA PELA BIBLIOTECA DA FEA – UNICAMP

Nogueira, Gislaine Chrystina
N689d Degradação do colesterol submetido ao aquecimento na presença de ácidos graxos e antioxidantes: estudo em sistemas-modelo / Gislaine Chrystina Nogueira. -- Campinas, SP: [s.n], 2011.

Orientador: Neura Bragagnolo
Tese (doutorado) – Universidade Estadual de Campinas. Faculdade de Engenharia de Alimentos.

1. Óxidos de colesterol. 2. Termo-oxidação. 3. Antioxidantes. 4. Ácidos graxos. 5. Sistemas-modelo I. Neura Bragagnolo. II. Universidade Estadual de Campinas. Faculdade de Engenharia de Alimentos. III. Título.

cars/bibfea

Título em inglês: Cholesterol thermal degradation in the presence of oxidants and fatty acids: model-system study
Palavras-chave em inglês (Keywords): Cholesterol oxides, Thermal oxidation, Antioxidants, Fatty acids, Model-system
Titulação: Doutor em Ciência de Alimentos
Banca examinadora: Neura Bragagnolo
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Programa de Pós Graduação em Ciência de Alimentos

Este exemplar corresponde à redação final da tese defendida em 03/03/2011 por Gislaine Chystina Nogueira de Faria aprovado pela comissão julgadora em 03/03/2011.

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in memorian

Aos meus pais, Gilson e Eliane, e irmãos, Gisele e Gilson, pela oportunidade de mostrar que toda semente plantada e cuidada com carinho gera frutos;
Ao meu eterno e amado companheiro, Henrique, pela paciência, pelo amor e por nunca me deixar desistir;
Á minha querida Dark pela incansável companhia por todos esses anos.

AGRADEÇO

À Profa. Dra. Neura Bragagnolo pela alegria com que me recebeu e orientou, acreditando no meu potencial e contribuindo enormemente para mais este importante passo na minha carreira profissional e principalmente pela sua amizade.

À Faculdade de Engenharia de Alimentos pela oportunidade de avanço profissional e científico.

À FAPESP pela bolsa de estudos e pelo apoio financeiro ao projeto.

Ao revisor da FAPESP pelas palavras de estímulo e credibilidade em todos os pareceres de relatório.

À banca examinadora por todas as correções e sugestões que enriqueceram meu trabalho e principalmente meu aprendizado.

À amiga e parceira Lilian por todas as “teorias”, contribuições, co-autorias e amizade.

À amiga Adelia por todas as discussões científicas, espirituais e amizade.

À amiga Renata Cury pela amizade e companhia de todas as quartas-feiras...

Aos professores Dra. Anita J. Marsaioli, Dr. Renato Atílio Jorge, Dr. Claudio Borsarelli pelo tempo dedicado e ajuda para transpor os inúmeros obstáculos.

Às professoras Dra. Adriana Z. Mercadante e Dra. Florinda O. Bobbio pela amizade e inspiração.

A todos os colegas do laboratório pela amável oportunidade de conhecer, conviver e experimentar.

"A mente que se abre a uma nova idéia jamais voltará ao seu tamanho original"

(Albert Einstein)

Sumário

RESUMO GERAL

Uma das rotas de degradação do colesterol, quando submetido ao aquecimento em presença de oxigênio, é a formação de óxidos de colesterol (COP). Estes compostos têm sido associados a uma série de efeitos biológicos e doenças degenerativas e sua formação pode ser influenciada pela presença de outras substâncias, como antioxidantes e ácidos graxos. A influência dos antioxidantes está associada ao seu mecanismo de ação, que podem atuar nas fases de iniciação ou de propagação da oxidação lipídica. Já a influência dos ácidos graxos está associada com o grau de insaturação na molécula, em função da formação de radicais livres. Com o objetivo de verificar o comportamento do colesterol durante o aquecimento frente a influencia dessas substâncias, sistemas modelo contendo colesterol e β-caroteno, TBHQ, vitamina E, ácido esteárico (saturado) ou ácido linolênico (com 3 insaturações), foram estudados. Os sistemas foram submetidos a 140, 180 e 220 °C, na presença de oxigênio, até que pelo menos 75% do colesterol fossem degradados. Os teores de colesterol, 7-hidroperoxicolesterol e COP foram avaliados ao longo do período de aquecimento. A degradação do colesterol foi observada em todas as temperaturas investigadas, ocorrendo em velocidade diretamente proporcional ao aumento da temperatura, totalizando aproximadamente 8 minutos a 220 °C, 20 minutos a 180 °C e 1 hora a 140 °C. De acordo com os resultados obtidos, a formação de COP atinge um máximo e a seguir sofre degradação, exceto na temperatura de 140 °C, em que o máximo da formação de COP só foi observado nos sistemas adicionados de ácidos graxos. A presença de ácidos graxos acelerou a formação de COP, entretanto, os sistemas contendo ácido linolênico apresentaram menores teores totais em todas as temperaturas estudadas e os sistemas contendo ácido esteárico apresentaram menores teores apenas a 220 °C, em comparação ao colesterol puro. Com exceção do TBHQ, a 140 °C, todos os antioxidantes estudados reduziram e/ou retardaram a formação de COP durante o aquecimento.

GENERAL SUMMARY

One of the pathways of cholesterol degradation, when it is submitted to heat, is the production of cholesterol oxidation products (COP). Those compounds have been associated to several biological effects and degenerative diseases and their formation can be influenced by other substances, such as antioxidants and fatty acids. The influence of the antioxidants is related to their mechanisms, which can act in the initiation and or propagation phases of the lipid oxidation. The influence of the fatty acids is associated to their degree of unsaturation, because of the free radical formation. In order to verify the cholesterol behavior during heating with the influence of those substances, model systems containing cholesterol and β-carotene, TBHQ, vitamin E, stearic acid or linolenic acid, were studied. The model systems were heated at 140, 180 and 220 °C until at least 75% of cholesterol was degraded. The amount of cholesterol, COP and 7-hydroperoxycholesterol – an intermediate compound of COP formation – was evaluated during heating process. Cholesterol degradation was observed at all studied temperatures, occurring at a velocity directly proportional to the temperature increase, totalizing approximately 8 min at 220 °C, 20 min at 180 °C and 1 h at 140 °C. According to the obtained results, COP are formed reaching a maximum content and after that are degraded, but at 140 °C, the maximum was reached only at the systems containing fatty acids. The presence of fatty acids accelerated COP formation; however, the systems containing linolenic acid showed lower total content at all studied temperatures, on the other hand, the systems containing stearic acid showed lower content only at 220 °C, when compared to the pure cholesterol system. Except TBHQ, at 140 °C, all antioxidants were able to reduce and/or delay the maximum amount of total COP during heating.

INTRODUÇÃO GERAL

O colesterol é um importante componente das membranas celulares e um dos precursores da vitamina D. É encontrado em produtos de origem animal e sua estrutura molecular consiste no anel do ciclopentano peridrofenantreno, unido a uma cadeia lateral alifática, totalizando 27 carbonos e um grupo hidroxila no carbono 3 (Figura 1). Entre os carbonos 5 e 6 existe uma insaturação, o que deixa o carbono 7 disponível para a formação de radical livre e consequentemente, o colesterol susceptível à oxidação [1].

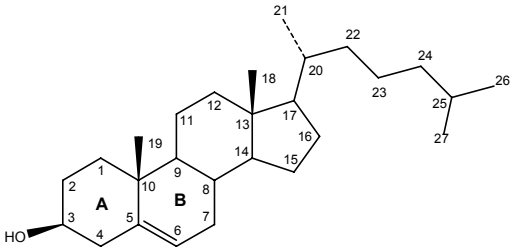

Figura 1. Estrutura molecular do colesterol

Durante muitos anos, acreditou-se que o colesterol, apesar de seu importante papel biológico, era um vilão, pois estava associado a doenças cardiovasculares. Entretanto, a comunidade científica descobriu que os produtos oriundos da sua oxidação desempenhavam um risco ainda maior à saúde humana devido aos seus efeitos biológicos, tais como: citogenicidade, carcinogenicidade mutagenicidade, modulação de membranas celulares, degeneração macular, entre outros [2-5].

Mais de 70 tipos de óxidos de colesterol já foram identificados. Os principais produtos da oxidação do colesterol (COP) são formados pelo ataque do oxigênio ao carbono 7 com a formação de um hidroperóxido (7-hidroperoxicolesterol) que pode ser convertido em 7-hidróxicolesterol por aquecimento, ou em 7-cetocolesterol por meio de desidratação. Além desses compostos, o hidroperóxido pode formar um radical peroxila que, em presença de outra molécula de colesterol, dá origem aos derivados do epoxicolesterol. Assim, os óxidos de colesterol

normalmente encontrados em sistemas modelo como os estudados neste trabalho são 7-cetocolesterol, 7α- e 7β-hidroxicolesterol, α- e β-epoxicolesterol [1, 5-8].

Por se tratar de um processo de reação em cadeia, os fatores que normalmente promovem a formação de radicais livres, tais como temperatura, presença de metais, luz, etc. irão acelerar a oxidação. Considerando que a maioria dos produtos de origem animal sofre tratamento térmico antes do consumo, há uma preocupação no sentido de conhecer e quantificar a formação destes compostos [9, 10].

Os métodos para determinação de colesterol e COP já estão bem estabelecidos e a maioria deles baseia-se no uso de cromatografia líquida de alta eficiência, com separação por fase normal em coluna de cianopropil. No presente trabalho, foi possível identificar, na mesma corrida cromatográfica, o 7-hidroperoxicolesterol, que é o intermediário da formação dos principais COP encontrados em alimentos [11].

Durante o aquecimento, o colesterol sofre degradação, em velocidade diretamente proporcional à temperatura empregada, ou seja, quanto maior a temperatura, menor o tempo necessário para degradar a mesma quantidade de colesterol. Uma pequena parte do colesterol degradado forma COP (aproximadamente 10%) e a parte restante forma, segundo a literatura, produtos voláteis ou polímeros que ainda não foram identificados [12]. Além disso, cada COP também pode sofrer degradação, em maior ou menor grau, pela exposição à altas temperaturas. Embora os COP estejam presentes em quantidades relativamente pequenas nos alimentos, o consumo crônico torna-se um risco à saúde. O efeito da temperatura sobre o colesterol está descrito no primeiro capítulo deste trabalho que trata, principalmente, de um condensado dos inúmeros trabalhos publicados na literatura que se referem à medida de COP em alimentos ou sistemas-modelo quando expostos ao aquecimento [9].

Para entender o que realmente acontece com o colesterol durante o aquecimento, o emprego de sistemas modelo tem sido bastante útil, pois consegue isolar fatores como a influência do meio de reação. Vários estudos têm sido feitos no sentido de esclarecer os possíveis efeitos de outros compostos,

presentes em alimentos ou não, na oxidação do colesterol, tais como antioxidantes e ácidos graxos [13-16].

É fato que compostos como ácidos graxos interferem na extensão do processo oxidativo, principalmente em função de seu grau de insaturação. Encontram-se na literatura teorias que propõem um aumento da oxidação do colesterol na presença de um ácido graxo insaturado em virtude da formação de radicais livres oriundos de sua própria autoxidação. Por outro lado, preconiza-se que os ácidos graxos saturados exerçam um efeito protetor ou nenhum efeito sobre o colesterol contra a oxidação, dependendo da temperatura [17, 18].

A ação antioxidante de alguns compostos também influencia a oxidação do colesterol, pois o processo é passível de ser interrompido ou retardado pela ação de compostos que reduzem a formação dos radicais livres, seja por doação de próton, como os tocoferóis e o TBHQ, ou pelo sequestro do radical livre, que é um dos mecanismos de ação do β-caroteno, por exemplo [19-22].

Este trabalho teve por objetivo verificar o comportamento do colesterol durante o aquecimento frente a influencia dessas substâncias, através do estudo de sistemas modelo contendo colesterol e β-caroteno, TBHQ, vitamina E, ácido esteárico (saturado) ou ácido linolênico (com 3 insaturações). Os sistemas foram submetidos a 140, 180 e 220 °C, na presença de oxigênio, até que pelo menos 75% do colesterol fossem degradados. Os teores de colesterol e COP, bem como os teores de 7-hidroperoxicolesterol foram avaliados ao longo do período de aquecimento.

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CAPÍTULO 1

REVISÃO BIBLIOGRÁFICA

CHOLESTEROL DEGRADATION IN FOODS: EFFECTS OF TEMPERATURE

Nogueira, G.C. and N. Bragagnolo,

Capítulo publicado *in* *Lipids: categories, biological functions and metabolism, nutrition and health*, P.L. Gilmore, Editor. 2010, Nova Science Publishers, Inc: Hauppauge. p. 181-198.

RESUMO

O colesterol pode ser degradadado sob várias circunstâncias, sendo a exposição ao calor uma das causas mais comuns. O aquecimento na presença de oxigênio pode contribuir para a formação de compostos que comprometem a saúde humana, como os produtos da oxidação do colesterol (COP). Os alimentos ricos em colesterol são suscetíveis a este problema porque raramente são consumidos crus ou imediatamente após o processamento. Durante o armazenamento e o processamento térmico de alimentos, os COP podem apresentar aumento ou diminuição de suas concentrações dependendo do binômio tempo *x* temperatura a que são submetidos. Além disso, outros compostos, normalmente presentes nos alimentos, como os ácidos graxos, podem acelerar o processo de oxidação do colesterol. Para entender melhor os mecanismos da degradação e da oxidação do colesterol, muitos autores propuseram o uso de sistemas-modelo com menor complexidade. Esta revisão oferece uma discussão sobre como a temperatura pode influenciar a degradação e a oxidação do colesterol.

ABSTRACT

Cholesterol can degrade under several circumstances, being the temperature exposition one of the most common causes. One of the regards about cholesterol degradation is that the presence of oxygen can lead to the formation of health hazardous compounds such as cholesterol oxidation products (COP). Cholesterol rich foods are susceptible to this problem because they are almost never consumed raw or immediately. During food storage or heat processing those COP can increase or decrease depending on the temperature and exposition time. Besides those factors, other compounds, usually present in food, can be able to accelerate the oxidation process, such as fatty acids. In order to better understand the mechanisms of cholesterol degradation and oxidation, many authors have proposed the use of model-system with lower complexity composition. This review offers a discussion about how the temperature can influence the cholesterol degradation and oxidation.

INTRODUCTION

One of the most common causes of lipid degradation in food is temperature, because it accelerates the oxidation process yielding compounds that can be related to loss of quality, such as flavor and texture modification, and hazard health. Cholesterol can suffer the thermal degradation, and even the reduction of cholesterol seems to be pleasant nowadays, the products originated from this degradation are more dangerous to human health than the cholesterol itself. Since the cholesterol is an unsaturated lipid, it can undergo oxidation and produces the cholesterol oxidation products (COP) by the mechanisms of chain reaction similarly to the observed for unsaturated fatty acids. COP are related to several biological effects, such as atherogenesis, cytotoxicity, mutagenesis and carcinogenesis, although there are no established safety levels for COP ingestion, these compounds can be absorbed from the diet, constituting a risk for human health due to chronic exposure [1-4].

The great majority of the products from animal origin, which present significant cholesterol contents in its composition, is consumed preferentially after some thermal treatment. The temperatures used in the preparation, either domestic or industrial, can start the oxidation process and moreover, cholesterol oxides can be formed during the storage, even under low temperatures.

Evidently, the higher the temperature and the used time of exposition, the greater will be the degradation of the cholesterol and its oxidation. However, there is no proportional relation between the degradation and the formation of cholesterol oxides, since other products of degradation can be formed and the reaction

depends on the initial concentration of the cholesterol, hence, generally, the higher the cholesterol concentration, the greater the amount of the formed oxide will be. Moreover, the bands of employed temperatures can be associated to the physical state of the cholesterol whose melting point is around 140 °C.

The COP can also suffer degradation with the temperature and change to unknown compounds. Thus, foods produced by light thermal treatments can present bigger oxide amounts than the most drastic treatments.

Foods as milk, eggs and meats of several types can present different composition of COP probably due to the complexity of the food matrix that can present great variety in its composition, mainly in lipid fraction and proteins among others compounds that can influence the oxidation process. In such a way, the use of model-systems with lower complexity composition has assisted in the understanding of the cholesterol oxidation mechanisms.

Cholesterol Oxidation

The cholesterol is a crystalline solid very stable under room temperature, without light and metal contact [5]. However, even under low temperature C_7 allylic and C_{25} tertiary, radicals which are primary formed on solid cholesterol, react with oxygen yielding peroxyl radicals [6]. The C_7 and 7-peroxyl radicals are the only stable radicals that survive under room temperature and they form the first detectable oxides, 7-hydroperoxides [5]. Between room temperature and cholesterol melting point (142.7 °C), cholesterol can produce other hydroperoxides,

alcohols, ketones, carboxylic acids beyond others [5]. Above the melting point, the oxidation velocity is very high, but at 200-300 °C, degradation occurs more than oxidation [7].

The oxidation process occurs initially in the B ring instauration, and according to the free radical mechanism happens in three stages: initiation, propagation and termination, the same as in unsaturated fatty acids. Heat treatment is one of the factors that produces the free radical formation in initiation phase. Those free radicals form cholesterolperoxyl radicals in the presence of triplet oxygen ($^3O_2$) and then react with another cholesterol molecule and yield free radical and hydroperoxycholesterol, establishing the chain reaction.

The thermal decomposition of 7-hydroperoxides (Figure 1) yields the corresponding epimeric 7-hydroxides (7α- and 7β-OH) and 7-ketocholesterol, which compose the highly characteristic pattern of cholesterol autoxidation products so frequently encountered [5].

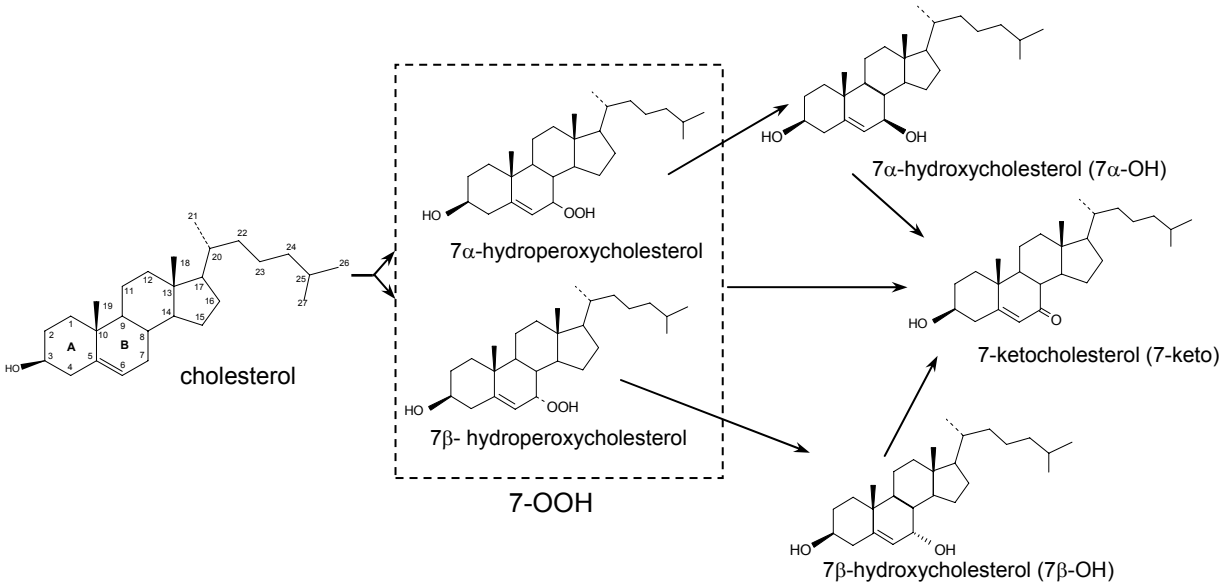

Figure 1. Formation of epimeric 7-hydroxycholesterol and 7-ketocholesterol.

The epoxidation of the double bound of C_5 is a bi-molecular reaction and occurs by the 7-hydroperoxycholesterol already formed to another cholesterol molecule (Figure 2), yielding the isomeric 5,6α- and 5,6β-epoxycholesterol (α- and β-epoxides), both can give cholestanetriol (triol) by hydration [5]. The formation of epoxides can also occur through other oxygen reactive species such as O_3, H_2O_2 and HO· [8].

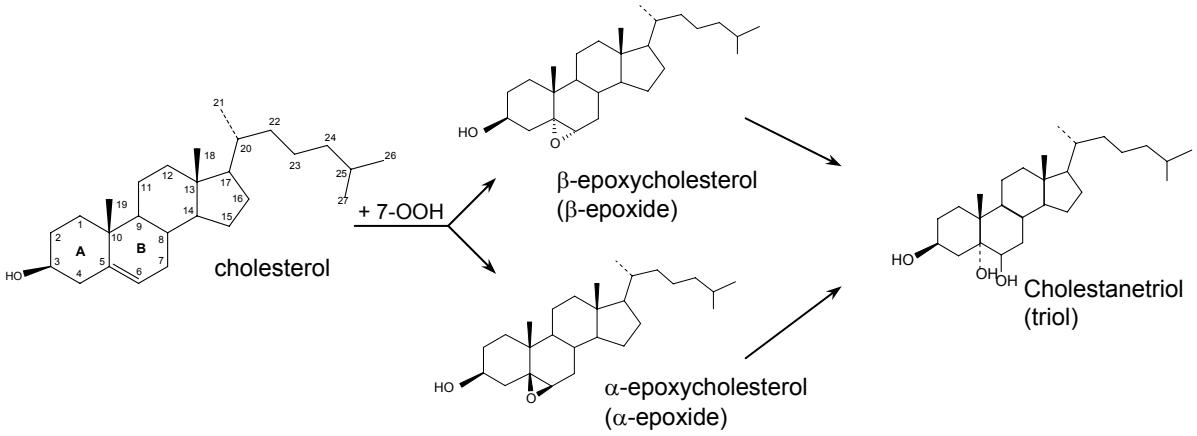

Figure 2. Epoxidation of cholesterol.

Degradation x oxidation

COP formation are not proportional to the cholesterol degradation during heat treatment, probably due to the formation of other products of higher or lower molecular weight such as polymers and volatiles, respectively and other products still unknown, called just degradation products [7, 9-11]. However, it is possible that COP can also be degraded vanishing in the end of the heat treatment [7]. Furthermore, the oxidation depends on the medium residual cholesterol [12].

Some samples of tallow heated at 135 and 180 °C presented increasing of cholesterol loss proportionally to the increase of temperature, however the same was not observed for COP formation [7], this means that part of cholesterol can degrade without yielding COP or COP can be degraded before measurement. Sum of COP found when solid cholesterol was heated at 140 °C was different of the total degraded cholesterol [10].

After heat at 150 °C, dried 7-ketocholesterol presented loss 17%, however, in the presence of cholesterol the loss was 36% [10], it shows that COP degradation occurs and it is speeded by the presence of cholesterol. Therefore, it is reasonable enough to consider that the difference between the degraded cholesterol and COP formed also increases due to COP degradation.

Lard with two levels of cholesterol concentration, 950 e 140 mg/100g, were heated at 180 °C for 240 and 160h, the loss of cholesterol observed in both tests was 19 e 70%, respectively [12], which is that lard with lower cholesterol amount presented higher degradation although it was heated for less time, however, the difference observed for COP formed were not equally related, with a slightly difference between the total amount for each test. The authors also observed that in the sample with smaller amount of cholesterol, the major COP was 7-ketocholesterol, while sample with the higher concentration of cholesterol presented 7β-OH in the same amount as 7-keto.

The influence of temperature and exposition time on cholesterol autoxidation in food

All heat treatment applies time x temperature in order to achieve the desired process. The conditions employed depend on the type of process and used matrix, but generally, as higher the temperature of heat treatment as lower is the exposition time but on the other hand, as lower the temperature is, as higher will be the shelf-life during storage. With respect of cholesterol and COP formation, as higher the reached temperature, higher the cholesterol degradation and COP formation. Nevertheless, as the exposition time increases under high temperatures, COP can undergo degradation as well as the cholesterol [7].

The effect of temperature on cholesterol oxidation can be separated in three ranges: low temperatures (till 40 °C), heating until cholesterol melting point (140 °C) and temperatures above cholesterol melting point (higher than 140 ºC). In the first range, storage and transportation under room temperature, refrigerated or frozen conditions are considered, noticing that in some regions during some season the room temperature can reach more than 40 °C. The temperatures under 140 °C are reached in some domestic cooking process, such as boiling, microwave or grilling and the cholesterol behave, under this circumstances, is susceptible to interactions with other compounds such as fatty acids and/or natural colorants, vitamins, amino acids among others that make part of the food [13]. Rarely the temperature above cholesterol melting point is reached in food processing, even when those treatments occur with higher temperatures, such as spray drier, in

common basis the temperature observed in food during heat treatments never reaches more than 100 °C.

At temperatures above 140 °C, when cholesterol is liquid, the autoxidation occurs very fast as well as the degradation of COP, however this behavior can be observed only in model-systems.

1. Storage

The formation of COP at temperatures below 40 °C was studied, mainly in cholesterol rich foods stored under frozen, cold or room temperature. In a general way, there is slow COP formation at this range of temperature and factors such as initial contents of those compounds and exposition to light, metals and oxygen for long periods are more effective to oxidation than the temperature. Nevertheless some products can present increasing on the COP amount exclusively during the storage period instead of during the heat treatment, maybe because of the induction period, which means the necessary time for the oxidation reaches a critical phase, in this case the existence of detectable amounts of COP [14].

Some kinds of cheese and butter were stored at 4 and -24 °C and presented small amounts of COP [15]. Only 7α-OH, 7β-OH and 7-keto were found in one single sample of fresh cheese, in a total amount of 0.1 μg/g. In parmesan cheese stored at -24 °C for 25 months less than 0.1 μg of 7-keto/g was found and the amount observed in butter oil were higher, 0.28 μg de 7-keto/g, with amounts bellow the quantification limit for 7α-OH, 7β-OH, triol and 25-OH, probably due to

the heat treatment used before the storage period and to the higher initial cholesterol content in those products. Cheddar cheese kept under cold storage (4 °C) up to 7 months was practically stable about cholesterol degradation and COP accumulation. The highest amount of total COP was 6 μg/g, with major incidence of 7β-OH [16]. On the other hand, butter stored at -26, 4 and 16 °C for 6 months presented increasing of 1.4, 4.2 and 5.2 times in total COP content respectively [16]. Similar behavior was reported by Hiesberger and Luf [17], with total COP of 23.6 for butter stored at 4 °C and 39.6 μg/g for 20 °C, however those samples were exposed to different light conditions. The formation of 3.3 μg of 7-keto/g in cream cheese stored at 37 °C for a year under illumination was also reported [18].

Read-to-eat infant foods prepared with UHT milk and stored for 9 months at 25 °C did not show significant variation on COP amount (max. 0.21 μg/g) during the period [19]. However, powder milk stored for 6 month presented increasing of 110 times in total COP content [20]. When whole milk powder (total COP 1.1 μg/g) was stored for a year at 32 °C the amount of COP increased five times, whilst at 55 °C the total amount reached 4.6 μg/g, both were kept under N_2 atmosphere. However, the samples kept without controlled atmosphere presented 34 μg/g at 32 °C and 121 μg/g at 55 °C and in all cases 7-keto was the major COP formed. Thus, the atmosphere has an important role in the prevention of COP formation, because it was possible to control the oxidation process even with higher temperature conditions.

Meat and its derivative products can be one of the most important COP sources on a diet [21]. One of the most important factors is the protein denaturation

by cooking, which can lead to the release of catalytically-active iron from metallo-proteins (mainly myoglobin) [9].

Chicken breast raw and grilled were stored at -18 °C for 90 days presented increasing formation of 7α- and 7β-OH during the storage period, from under detection limit (0.02 μg/g) to 12 μg/g and 24.5μg/g, respectively, whilst 7-keto increased from 2.6 to 8 μg/g for 60 storage days and then was reduced (5.15 μg/g) at the end [22]. Galvin et al. [23] analyzed cooked chicken breast and thigh stored at 4°C, they observed increasing in total COP from 0.14 to 1.43 μg/g for breast and from 0.23 to 4.20 μg/g for thigh after 12 days, although before the storage the breasts samples showed only 25-OH (0.1 μg/g) and in the end, besides the increasing of 15 times in the amount of this oxide, 0.4 μg/g of 7-keto was also detected. The thigh samples presented initially 0.6 μg/g of 25-OH and in the end 7-keto has contributed to 17% of total amount. Vore [14] reported similar values for raw and grilled beef and observed a significant increasing on 7-keto content during the 4 days storage at 4°C, from 0.1 to 0.4 μg/g in raw and from 0.1 to 4.8 μg/g in grilled meat.

Frozen storage of fish gave forth more intense cholesterol oxidation than heat treatment. Sardines and hakes kept in freezer (-18 °C) for 120 days had almost 6 and 9 times more COP than samples before storage, respectively [24-26]. In all cases, the extent of cholesterol oxidation during storage was higher than the observed by grilling; it means that the storage period can be more important than heat treatment over cholesterol oxidation.

Due to the high cholesterol content they have, eggs can present significant amount of COP, mainly when this product is submitted to a dry process prior to storage time. A gradual increase in COP amount was observed in powder egg stored for 12 months at 25 °C in the dark, from 23 to 227 μg/g, and β-epoxide (77 μg/g) was the major COP at the end of the period [27]. In addition, the authors did not observed new COP formation during the storage period. According to Nourooz-Zadeh and Appelqvist [28] samples of fresh and spray dried eggs presented COP formation after 6 months of storage at 4 °C. Remarkably, the COP found at higher amounts were β-epoxide (2.7 to 12 μg/g) and the epimeric 7α- and 7β-OH (0.7 to 8.9 μg/g and 0.2 to 9.4 μg/g, respectively); while the lowest concentration were found to 7-keto (0.6 to 2.9 μg/g). However, Mazalli and Bragagnolo [27] found higher values (75 μg/g of total COP) after storage for 3 months at 25 °C, and β-epoxide corresponding to 60% of total COP content. On the other hand, Obara et al. [29] found lower amounts: 0.31 μg/g before and 0.51 μg/g after storage at 20 °C in the same period with α-epoxide corresponding to 40% of total. However, the initial amount of total COP observed on Mazalli and Bragagnolo [27] samples were much higher than those reported by Obara et al [29]. Although the authors did not mention the used spray drier method, it is possible that this is the cause of such difference, since direct heat method produces higher amounts of nitrogen oxides (NOx) which favors COP formation [30].

Egg powders can be used as ingredients to other food products which can also be submitted to heat treatments yielding an increasing of total COP in the final product. As an example, egg pasta prepared with powder egg obtained by spray

drier presented the double content of COP than those prepared with fresh eggs [31].

Egg yolks from raw fresh eggs stored at 25 °C presented increasing of almost 2.5 times the amount of COP, while at 5 °C the difference reached 2 times initial values [32]. The authors reported the formation of 7α- and 7β-OH and 7-keto, with no detection of epoxycholesterol, differently of those observed in whole spray dried eggs indicating that the formation of epoxides may be related to the heating process of spray drier and low water activity.

The formation of COP in high lipid content products that are not submitted to heat treatment such as mayonnaise was also evaluated during storage at 4 and 25 °C [33]. The authors have only detected 7-keto prior to the storage, which has increased during the 165 days. The presence of 25-OH, 7α- and 7β-OH was observed in the samples kept at 25 °C with 135 days of storage, while the samples kept at 4 °C presented those compounds only at the end of the storage. The total amount of COP (sum of 7-keto, 25-OH, 7α- and 7β-OH) varied between 2 μg/g in the beginning and 20 and 30 μg/g to mayonnaise samples stored at 4 and 25 °C, respectively.

2. Heat Treatment

Above 40 °C the cholesterol oxidation is more intense, and the extent of degradation can vary according to the reached temperatures, exposition time and the kind of heat treatment method used. In such a way, temperatures reached in

cooking process as boiling, electric or conventional and microwave oven, grilling, spray drier and deep frying play an important role on cholesterol oxidation [9, 13, 34, 35]. Foods prepared within this range of temperature are mainly meat – that can vary on type (pork, beef and poultry), fish or sea food, milk and dairy products and eggs.

The majority of published data measures the cholesterol oxidation as the total COP, which commonly refers to the sum of the principal COP found in food, such as 7-keto, 7α- and 7β-OH, 25-OH and α- and β-epoxides and sometimes triol [35]. Generally, 7-keto is preferential formed and since it is the most incident oxide found in heat treated food has been used as a marker of cholesterol oxidation [36-39]. However, 7-keto can also degraded [36] and quantification of this compound can underestimate the real extent of cholesterol oxidation.

Total COP content found in beef cooked in electric oven at 135 °C for 10 minutes increased 73% compared to the raw meat, those COP from C_7 (7-keto, 7α- and 7β-OH) increased 21% and the sum of 20α- and 25-OH increased 40% [40]. At lower temperature, although longer, beef boiled at 85 °C for 30 minutes presented an increasing of 30% of total COP, with C_7 COP raising 29% and a little amount of triol (0.06 μg/g) formation [41, 42]. The presence of water during the cooking process may have favored the formation of triol. On the other hand, a fast cooking at microwave oven (900 W for 3 minutes) promoted formation of COP 6 times higher than original sample of beef hamburger [43]. Besides the increasing in 7-keto amount (from 0.23 to 0.34 μg/g), the authors reported the formation of α-epoxide, 7α- and 7β-OH, with 5 times increasing on C_7 COP concentration.

The increase of processing temperature can reach the internal temperature of the product up to 100 °C, yielding higher amounts of COP. After deep frying (150-160 °C) the amount of COP in beef hamburgers increased from 5.5 to 6.7 μg/g, however individual amounts of each COP varied after cooking process, occurring an increasing of 7α- and 7β-OH, triol formation and 7-keto, α- and β-epoxide decreasing [44]. The 7β-OH turned into the major COP after heat treatment (31% of the total amount), although the increase of 7α-OH has been of 142% [44]. Similar values were found in beef hamburgers grilled at 180 °C, however, only β-epoxide increased 16%, while 7β-OH and α-epoxide decreased 19 and 8%, respectively, and the others remained the same [45]. The pan frying of hamburger without any oil produced increasing of 85 % in COP formation, while with the use of olive oil, corn oil and partly hydrogenated plan oil less increase were observed, with 53, 6 and 15%, respectively [45]. When beef hamburger was deep fried in olive oil for 6 minutes at 180 °C COP formation increased 48% (7α-OH, 7β-OH and triol), and 7-keto, initially present in the sample, was 60% decreased [43]. On the other hand, retail beef burgers grilled at 165 °C for 8 minutes did not show any COP formation, as well as the meat balls roasted at 250 °C for 20 minutes [46]. It is possible that some additives used in industrialized meat products could be responsible for the protection of the cholesterol against its oxidation.

Chicken breast showed increasing of 130% in total COP after boiling at 90 °C for 20 minutes [47]. In raw sample were detected side chain COP (22*R*-, 24*S*-, 22*S*-OH) probably of metabolically or animal feed source. After heating, formation

of 25-OH (4.4 μg/g), 7-keto (1 μg/g), 7α- (4 μg/g) and 7β-OH (6.4 μg/g) were observed, besides β-epoxide was detected bellow quantifying limit (<9.8 μg/g) and 22*R*-OH increased 65% [47]. With microwave oven cooking (900 W for 3 minutes) chicken patty presented 2 and 3 times increasing of 7-keto and 7β-OH initially found and also formation of α-epoxide (0.15 μg/g) and 7α-OH (12.7 μg/g) [43]. The major oxide found was 7α-OH (51% of total COP) [43]. Chicken breast and thigh roasted at 160 °C for 40 minutes, presented as major COP the 25-OH, the thigh has showed higher total COP (20α-OH, 25-OH and 7-keto) amount compared to the breast sample, 0.6 and 0.1 μg/g, respectively [23]. However the authors did not quantified other COP, which could explain the small amount of total COP found.

Retail chicken hamburgers fried with olive oil at 180 °C for 6 minutes presented increasing from 4.01 to 10.75μg/g in total COP (7α-, 7β- and 25-OH, 7-keto, α-epoxide and triol) amount, the major COP found was 7α-OH [43]. On the other hand, Mariutti et al. [47] found only three oxides (22*R*-, 24*S*-OH and 7-keto) within the researched 8 oxides (22*R*-, 22*S*-, 24*S*-, 25-, 7α-, 7β-OH, β-epoxide, 7-keto) in chicken breast fried with soybean oil for 20 minutes (180 °C), 22*R*-OH was found in higher amount (87%) and total COP after heat treatment was found to be 16.8 μg/g. Comparing the result with raw chicken breast, in which total COP was 17.3 μg/g, frying process resulted decrease of those values, suggested by the authors as thermal degradation or migration of COP to the frying oil [47]. Roasted chicken breast (220 °C for 20 minutes) presented total COP 60% higher (27.1 μg/g) than the amount found in raw sample, the major oxide formed was 7-keto (35% of total COP) and a slightly 8% decrease of 22*R*-OH was observed [47].

When compared to fried chicken breast (180 °C), the authors observed more intense oxidation, with formation of 7α- and 7β-OH and 22*S*-OH besides the oxides observed before and 7.5% degradation of 22*R*-OH [47]. Conchilo et al. [48] reported 5 times increasing in total COP for chicken breast roasted at 220 °C for 20 minutes (11.54 μg/g of lipid), 7-keto represent only 20% and β-epoxide almost 40% of total COP, however 22*R*-, 22*S*- or 24*S*-OH were not analyzed. Chicken breast grilled at 170 °C for 4 minutes showed formation of 7α- (5.5 μg/g) and 7β-OH (28 μg/g), which were not detected in the sample prior to heat treatment, and also the double amount of 7-keto initially present (5 μg/g) [22].

Turkey meat cooked in boiling water at 85 °C for 30 minutes presented increasing of 35% in total COP amount (from 2,65 to 3,58 μg/g) [41, 42], the C_7 COP increased 38%, α-epoxide maintaining the same level and the small amount of 20α-OH and triol detected in the raw sample was not detected anymore after cooking [41, 42].

In pork meat an increasing of 105% in total COP amount was observed after cooked in boiling water at 85 °C for 30 minutes [41, 42] with 86% increasing in C_7 COP, higher proportion of 7-keto (57%). Moreover, the authors observed formation of 0.49 μg/g of 20α-OH and 0.47 μg/g of triol. When roasted in electric oven for 10 minutes at 135 °C, pork meat showed increasing of 76% in total COP, with 81% increasing of C_7, 100% increasing of $C_{20\text{-}25}$, and 58% increasing of epoxides [40]. On the other hand, heat treatment did not lead to COP formation in retail pork sausages grilled at 165 °C for 30 minutes [46].

With greater amounts of polyunsaturated fatty acids (PUFA), which are more susceptible to oxidation and because of that more free radicals can be formed, fishes can show a more intense cholesterol oxidation [49, 50]. Sardines grilled at 175 °C for 4 minutes (core temperature 75 °C) presented increasing of 114% in total COP, and the major COP found in those samples was19-OH before and after heat treatment, corresponding to 72 and 63% of total, respectively [25]. The formation of 25-OH reached almost 6 times the value found in the raw fish, compared to the others COP. Similar values were found in Atlantic hake fillet grilled at 165 °C for 4 minutes (raw 14.4 μg/g and grilled 35.4 μg/g), however the content of 25-OH increased only 2 times compared to the raw sample [24]. Total COP found in salmon fried at 180 °C for 4 minutes with soybean and olive oil and roasted at 220 °C for 30 minutes were much smaller, 0.58, 0.60 e 0.63 μg/g, respectively, although heat treatments had lead to a significant increase comparing to the raw samples [51]. The variation in the level of COP found among authors can be attributed to the type of fish, but also to the retail conditions, the hakes were bought frozen, the sardine were bought fresh but were transported for almost 20 h, while the salmon has apparently been bought fresh and analyzed immediately and to quantification method (HPLC x GC). Moreover, different COP were formed in salmon considering the oil used, with soybean oil total COP were 50% higher than olive oil, and triol>7-keto>7β-OH; and with olive oil 7-keto>triol and 7β-OH was not detected [51].

In milk the formation of COP occurs under powerful process conditions such as the use of very high temperatures for long time or prolonged storage under high

temperatures [52]. On the other hand, nor even severe process such spray drier by direct heat lead to an increase in COP right after the heat treatment [35, 53]. COP formation start to appear during storage time, showing that the heat treatment can initiate the oxidation process after an induction period [20, 52]. Thus, comparison of heating process like those is possible only when the samples are at the end of the same storage conditions and period, although other factors can influence the COP formation.

Between indirect and low or high NO_x direct heat, total COP found in whole milk powder was 80, 350 and 540 μg/g of fat, respectively [53]. The major COP found was 7-keto (35 μg/g of fat), followed by β-epoxide (30 μg/g of fat) and side chain oxides and triol were not detected. Smaller values of 7-keto (1.1-3.2 μg/g of fat) were found in retail whole milk powder immediately after heat process [54] and total COP 37.2 μg/g of fat after 1 year storage [35].

The increasing of 50, 60 and 70% in total COP was observed in cheese heated at 70, 105 and 134 °C for 1.5, 3.5 and 5 minutes, respectively. The major oxide found was 7-keto (0.1 to 0.17 μg/g), and 7α- and 7β-OH were detected under quantification limit (<0.1 μg/g) [15]. Melted cheeses presented higher amounts of COP with comparative lower temperatures (64 and 98 °C). The cheese heated at 64 °C for 1 minute and at 98 °C for 3 e 4.5 minutes showed 100, 240 e 410% COP increase, respectively.

During heating of butter, Seckin and Metin [55] observed increase amount of COP with the increasing on the exposition time (10, 15 and 20 minutes) and temperature (180, 200, 220 and 230 °C). When exposed for 10 and 20 minutes, at all

temperatures, the greater COP concentration was presented by 7-keto (de 3.13 to 7.07 μg/g), except at 230 °C for 20 minutes, which β-epoxide was the higher COP amount (7.92 μg/g). With 15 minutes of exposition, at 180, 200 and 220 °C, β-epoxide was the major oxide ranged between 3.13 and 5.08 μg/g. Higher value for β-epoxide (4.74 μg/g) was found in butter heated for 10 minutes at 170 °C and 7-keto (38% of total) was the most incident [38]. When heated at 180 °C for 10 and 20 minutes, butter presented 14.63 and 27.30 μg/g of total COP, respectively and, again, 7-keto represented 35 and 32% of total COP, respectively. On the other hand, less than 0.2 μg/g oxides were detected in butter heated at 175°C for up to 15 minutes, and for 30 minutes, 1.61 μg/g of COP were detected [15]. Only at 210 °C for 30 minutes the authors found COP content comparable to the former study (19.21 μg/g) and, in this case, 7-keto (5.68 μg/g) was not the major COP, but 7β-OH, with 6.49 μg/g.

The obtaining of dried egg by spray drier provides at least three factors that should be concerning to COP formation: heating air system (direct or indirect heat), inlet and outlet temperatures. When the air is heated indirectly, the formation of COP is lower than when it is directly heated, probably die to the formation of nitrogen oxides which have higher oxidative effect on the product [56, 57]. According to Guardiola et al. [13], there is a critical point at inlet temperatures between 193 and 231 °C, and outlet temperatures, between 128 and 142 °C, from which COP formation increases remarkably. However, as the same as happens to milk powder, the conditions and period of storage offers more influence on COP formation than the heat treatment itself [27, 58, 59].

Probably because of its higher cholesterol concentration, the COP amount found in powder egg is around 1000 times higher than in other food. Retail samples of whole powder eggs presented 41.21 mg/g of total COP, and β-epoxide was the major COP representing 43% of total [60] and 0.03 mg/g total COP, with 7α-OH representing 23% [31]. Freshly spray dried eggs, with inlet temperatures between 120 and 170 °C and outlet temperature between 52 and 77 °C presented total COP for whole egg and egg yolk 0.31 and 0.34 mg/g, respectively, and α-epoxide corresponded to 42 and 32% of total amount [29]. On the other hand, despite of the more severe conditions (inlet 180 to 231 °C and outlet 120 to 142 °C), 6 times lower COP were reported [13]. Although there was reported strong correlation between temperatures of spray drier [58], and heating method used [30] and COP formation on egg powder production.

Fried and boiled eggs presented higher amounts of COP [32]. The amount of COP found in boiled (97 °C for 5 min) egg yolk was 2.17 times higher than the amount found in raw egg yolk, while fried egg yolk (190 °C for 4 min in soybean oil) presented 3.5 more COP than the raw sample. In both cases, only were detected 7α-, 7-β-OH and 7-keto.

Because different internal temperatures can be reached depending on the cooking process and meat cut [61] there are variation on the amount of total COP, as well as on each COP individually. The formation of COP can be associated to the membrane rupture during the mince process and the heat treatment can make the interaction between unsaturated fatty acids and cholesterol easier [23].

Moreover, the influence of matrix composition on the cholesterol oxidation occurs mostly because of the fatty acids profile [41].

It is very hard to know which parameters such as exposure time or temperature or both combined, can cause more impact, due to the heat transfer occurred in each process. When different cooking methods of beef patties were compared, the use of microwave crispy method showed higher 7-keto amount than other methods. However, raw samples presented higher amounts of 7-keto than cooked samples [36]. Suggesting that heat treatment can degrade 7-keto and microwave method has been less aggressive than the others.

Generally, the higher the temperature and time of heating, the higher the amount of COP formed; however, it is important to consider that COP may undergo further breakdown. Hence, aggressive cooking methods, which apply high temperatures for long time, may present lower content of COP despite the oxidation extent.

The media used during cooking process, such as fat or oil, can interfere on cholesterol oxidation, since the temperature reached on the surface of the food could be higher than core temperature, increasing oxidation. Moreover, the fatty acids profile of the used fat or oil can provide more free radicals during the process, leading to more oxidation; for instance oils with high amount of PUFA.

As seen, the COP formation study in stored or thermal processed foods is very complex, because of cooking method used, with different combination of time and temperature and the initial oxidation extent, which have great influence on the results. Furthermore, the interaction occurred between cholesterol, COP, fatty

acids, proteins and other compounds in food turns the conclusion about this theme a very difficult challenge.

Model-Systems

In order to elucidate the effects of temperature on the cholesterol degradation and oxidation, many authors have been dedicated for the last decades to elaborate some systems that simulate the cholesterol behavior against some regular food compound. Model-systems can be consisted of pure and dry cholesterol, mixed with inert or edible oil and also part of lipid fraction extracted from the food. In those systems, the difference between cholesterol degradation and oxidation is more evident.

In most of the studies that shows pure cholesterol heated, organic solutions of cholesterol are prepared and the organic solvents are eliminated prior to controlled heating process. Thus, cholesterol as is in crystalline solid state is very stable at room temperature up to 100 °C [5, 10] and the velocity of its degradation depends on the initial amount of cholesterol in the system. When heated at 100°C, 50 mg of cholesterol was practically stable for 24 h [34]. However, above 120°C, the degradation was higher and 40% of cholesterol has degraded in 24 h; at 150 °C 60% of cholesterol has degraded at the same period. The complete cholesterol degradation occurred after 6 h after heating at 200°C [34]. Model-systems containing 1 mg of cholesterol presented 76% degradation with 30 minutes at 150 °C, 72 and 86% at 180°C for 20 and 40 minutes, respectively (Kim & Nawar, 1993). With lower cholesterol concentration model-systems (0.1mg) 90% has degraded

with 10 minutes at 200 °C and 30 minutes at 175 °C; with 30 minutes of heating at 150 °C approximately 65% of cholesterol degraded and 20%, at 125 °C [62].

COP formation also depends on the initial cholesterol concentration. In the 50 mg cholesterol model, the amount of 7-keto was 0.4% of cholesterol after 12 h at 150 °C, from which it started to decrease [34]. Nevertheless, systems with 0.1 mg of cholesterol presented continuous increase of 7-keto for 30 minutes at 150 °C up to 2% of initial cholesterol content, while under higher temperatures, the decrease of 7-keto was observed after 10 minutes exposure [62]. Systems with 1 mg of cholesterol 7-keto reached 13% of initial cholesterol amount after 2 h heating at 140 °C [10].

The increase in cholesterol degradation above 140 ºC can be attributed to its state change after the melting point have been achieved [10]. Apparently, it is possible that the state change can be only an improved way to obtain a more fluid model-system to accomplish the oxidation reaction, thus, liquid inert media added to the model can help the oxidation process in the same way. At 140 °C, 100 mg of cholesterol dissolved in parafin oil presented 35% of total COP (epoxides, 7-keto, 7-OH, triol and 7-hydroperoxides) and 6% of 7-keto after 120 minutes of heat with 80% of cholesterol degradation [63].

Through kinetic results Chien et al [64] observed that cholesterol degrades faster at 150 °C than at 140 °C [63] and also that the epoxidation and free radical formation by hydroperoxides are the main route for cholesterol oxidation [11].

In order to elucidate the manner that cholesterol interact with the media Kim and Nawar [65] evaluated the dried cholesterol behavior against triacylglycerols (TAG) at 130 °C and observed that the cholesterol oxidizes more with TAG than

alone. According to the authors, a reliable explanation could be that TAG oxidizes first, yielding the free radical and peroxydes formation leading to a more extent cholesterol oxidation. Furthermore, TAG melting points are lower than cholesterol, so the liquid mixture can favor the cholesterol oxidation even under temperatures beneath its melting point or still by the influence of other functional groups.

Mixtures containing cholesterol and TAG (estearate, oleate and linoleate) heated at 180 °C for 1 h, yielded the formation of 7-keto, 7-OH and epoxides with total amount of 20% of the initial cholesterol similar to those found for pure cholesterol system, with cholesterol degradation ranging from 71 to 83%. However, the mixture of cholesterol with phospholipid presented lower degradation (53%) and lower COP formation (2,8% of initial cholesterol amount) [66].

According to Osada *et al.* [50] there is a degradation cholesterol scale considering the unsaturation fatty acid degree in which the saturated fatty acid can be protective to the cholesterol and the unsaturated accelerate the degradation. Nevertheless, cholesterol has degraded more in the presence of monounsaturated fatty acid (MUFA) and less with di-unsaturated (DUFA) [66]. MUFA was less degraded than DUFA in the presence of cholesterol [67]. In the presence of MUFA and DUFA, the relation of epoxides/C_7 oxides increased 20% compared to pure cholesterol and 10% to the saturated fatty acid mixture [66].

Mixtures of cholesterol with different oil (with different unsaturation grade) were heated at 110 °C for 22 h in a conventional oven presenting significant changes on total COP amount [68], the amount of 7-keto (32.4 μg/g) found in the mixture of cholesterol with fish oil (higher content of long chain PUFA) were

significantly higher than those found in mixture of sunflower oil (26.4 μg/g), palm oil (16 μg/g) and flax oil (6.5 μg/g). However, the mixture containing fish oil was the only one that presented 7-keto (6 μg/g) prior to the heat treatment, it is possible that the initial amount of this oxide has greater influence to the oxidation than the fatty acid profile. On the other hand, among the vegetable oils, the formation of COP can be associated to the saturated and unsaturated fatty acids (n3<saturated<n6) [68]. Xu *et al.* [62] found higher values for 7-keto in mixtures of cholesterol with rice oil (200 μg/g), compared to those obtained in mixtures with olive oil (100 μg/g), rapeseed oil (125 μg/g) and soybean oil (25 μg/g) after 30 minutes heating at 150 °C and suggested that the differences were due to antioxidants found naturally in the used oils.

Lard with two levels of cholesterol (950 and 140 mg/100g) was heated at 180 °C and the loss of cholesterol was 15 and 75%, respectively, during the heat treatment [67], and also an increasing in COP formation (7α-OH, 7β-OH, α-epoxide, β-epoxide, triol, 7-keto), but samples with higher amount of cholesterol produced higher amount of every COP. The total COP content was 490 μg/g for higher cholesterol level and 315 μg/g for lower level. The formation of triol was observed in small amount e reported only for the sample with lower concentration of cholesterol, 10 μg/g after 60 h heating. According to the authors, the formation of triol occurred only after enough amounts of free fatty acids accumulation to cause epoxide ring hydrolysis.

CONCLUSION

In a general way, the initial quality of the raw material, such as the initial amount of COP, lipid oxidation stage, time and temperature and more than that, storage conditions are extremely important to COP formation in food. More studies are necessary to determine the process and storage conditions where this formation is minimized, especially concerning food safety.

The identification of other compounds, higher molecular weight, such as polymers, as well as the volatile compounds during cholesterol heat treatment can offer valuable data to elucidate the complex mechanism which cholesterol is oxidized.

Model-systems can make easier the understanding on how cholesterol behaves against different parameters such as temperature and reaction media. The gradual increase on the complexity of those models, such as the incorporation of other components that could be into the food, like for example amino acids, can lead to the discovery of new routes of cholesterol degradation and COP formation. However, the results obtained for model-systems were not well correlated to those obtained for regular foods.

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CAPÍTULO 2

Synthesis of 7-hydroperoxycholesterol and its separation, identification and quantification in cholesterol heated model systems

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Artigo publicado no *Journal of Agricultural and Food Chemistry, **2010,** 58,* 10226-10230

RESUMO

O 7-hidroperoxicolesterol (7-OOH) é considerado um composto intermediário no mecanismo da oxidação do colesterol sendo o primeiro produto formado quando o colesterol é atacado pelo oxigênio triplete. Entretanto, existe uma limitação nos estudos dos mecanismos de oxidação do colesterol em função da falta do 7-OOH como padrão analítico, provavelmente por causa da sua baixa estabilidade. Para verificar a formação dos hidroperóxidos em sistemas-modelo aquecidos a 140, 180 e 220 °C, 7α-hidroperoxicolesterol (7α-OOH) foi sintetizado por fotoxidação do colesterol seguida de rearranjo em clorofórmio à temperatura ambiente. A estrutura foi confirmada por ressonância magnética nuclear de C^{13} (RMN C^{13}) e pelos espectros de massas obtidos por LC-APCI- MS. Os resultados demonstraram que o 7-OOH é o primeiro composto formado à 140 °C. Entretanto, a concentração de 7-OOH depende da temperatura e do tempo de exposição, sendo que quanto maior o tempo, maior a concentração de 7-OOH na temperatura mais baixa, e quanto menor o tempo, menor a concentração de 7-OOH nas temperaturas mais altas (180 e 220 °C). A partir do 7-OOH, o mecanismo conhecido para a oxidação do colesterol em três fases (iniciação, propagação e terminação) pôde ser confirmado, uma vez que à temperaturas inferiores a 142 °C, quando o colesterol está em seu estado sólido, o estágio de oxidação encontra-se na iniciação onde a formação do hidroperóxido é predominante.

Palavras-chave: LC-APCI- MS, óxidos de colesterol, degradação térmica, mecanismos de fragmentação

ABSTRACT

7-hydroperoxycholesterol (7-OOH) is considered to be an intermediate compound of the cholesterol oxidation path as the first product formed when cholesterol is oxidized by triplet oxygen. However, there is a limitation on cholesterol mechanisms studies because of lack of 7-OOH analytical standard availability due to its low stability. In order to verify the formation of hydroperoxides in cholesterol model systems heated at 140, 180 and 220 °C, 7α-hydroperoxycholesterol (7α-OOH) was synthesized by cholesterol photoxidation followed by rearrangement at room temperature in chloroform. Its structure was confirmed based on ^{13}C NMR and mass spectra obtained by APCI-LC-MS. The results demonstrated that 7-OOH is the first compound formed when the temperature is lower (140 °C). However, the concentration of the 7-OOH depends on the temperature and time of exposure, being the higher the time, the higher the amount of 7-OOH at lower temperatures, and the lower the time, the lower the amount of 7-OOH at higher temperatures (180 and 220 °C). By the formation of 7-OOH, the known cholesterol oxidation mechanism in three phases (initiation, propagation and termination) could be confirmed, once at lower temperatures the stage of cholesterol oxidation is at initiation at which hydroperoxide formation predominates.

Keywords: LC-APCI- MS, cholesterol oxides, thermal degradation, fragmentation mechanism

INTRODUCTION

Cholesterol can undergo oxidation when exposed to high temperatures, light, dehydration, storage, radiation and combinations of these conditions *(1-3)*. The resulting cholesterol oxidation products (COP) have been reported to exhibit a wide range of adverse biological effects on animals, such as atherogenesis, cytotoxicity, mutagenesis and carcinogenesis *(4)*.

The mechanism of cholesterol oxidation is similar to that known for unsaturated lipids, which is divided in three phases: initiation, propagation and termination *(5)*. The 7-hydroperoxycholesterol (7-OOH) is considered to be the first intermediate compound in the cholesterol oxidation path, as it is formed directly from cholesterol by oxidation promoted by triplet oxygen *(3, 5)*. Thus, the identification of this intermediate plays a key role in quantitative studies of cholesterol and COPs by high-performance liquid chromatography (HPLC). However, such studies have been limited because of lack of 7-OOH analytical standard availability due to its low stability *(6-8)*.

The most commonly used HPLC method for COPs quantification employs CN column and mixture of *n*-hexane and isopropyl alcohol as mobile phase, allowing the separation of cholesterol and 11 COPs *(6, 9, 10)*; however the quantification of 7-OOH by this method has not been previously reported. Quantification of 7-OOH has been currently based on chromophoric formation by Würster dye method *(6, 7)* after thin layer chromatography (TLC) or preparative HPLC separation; however, these methods present low sensitivity or accuracy.

In the last decade, liquid chromatography mass spectrometry (LC-MS) using atmospheric pressure ionization (i.e., atmospheric pressure chemical ionization, APCI; or electrospray ionization, ESI) has emerged as a powerful analytical technique for identification and quantification of several COPs *(11-15)*. However, the use of this technique for the identification and quantification of 7-OOH has also been limited due to its low stability, so that APCI-MS data for such compound has not been previously reported.

In this work the synthesis and the characterization of 7α-hydroperoxycholesterol (7α-OOH) by ^{13}C nuclear magnetic resonance (NMR) and atmospheric pressure chemical ionization mass spectrometry (APCI-MS) is described. The synthesized compound was also used as standard for the quantification of 7-OOH in cholesterol heated model systems by high-performance liquid chromatography (HPLC) in order to verify the formation of this compound under high temperature conditions.

MATERIALS AND METHODS

Synthesis of 7α-OOH

Cholesterol (Sigma-Aldrich, St. Louis, MO, 95% purity) and 1% Rose Bengal (Sigma-Aldrich, St. Louis, MO) were mixed with 10 mL of pyridine (MERCK, Darmstadt, Germany, analytical grade) and submitted to photoxidation by sodium light exposure (400W) and oxygen insufflation under continuous stirring and controlled temperature (5 °C), as described by Beckwith and co-workers *(16)*.

The synthesized compound was purified by flash column chromatography (silica gel, 4:1 and 1:1 *n*-hexane:ethyl acetate v/v). Twenty fractions of 15 mL each were collected and analyzed by TLC using silica gel G/aluminium pre-coated plates (250 μm, Whatman, Freiburg, Germany). The development of TLC was carried out in a glass tank lined with filter paper and equilibrated for 30 min with 5 mL of *n*-hexane/ethyl acetate (4:1 v/v). Approximately 10 μL of each extract was spotted on the plate. The chromatogram was developed over a distance of 3 cm at room temperature after which the plate was dried and the color development of cholesterol, Rose Bengal and the reaction product was carried out with an acid solution of anis aldehyde. Reaction product presented three bands, one at the same R_f of cholesterol, another at the same R_f of Rose Bengal and the third one that should be 5α-hydroperoxycholesterol (5α-OOH). Fractions 11 to 17 (*n*-hexane:ethyl acetate 1:1 v/v), which presented the single band at the same R_f that supposed to have 5α-OOH, were grouped and concentrated under gently nitrogen stream to afford pure 5α-OOH, which was dissolved in chloroform and kept at room temperature for 68 h to rearrange and form 7α-OOH. All solvents were analytical grade.

Structure confirmation by ^{13}C NMR

The synthetic 7α-OOH was solubilized in deuterated chloroform ($CDCl_3$) (Cambridge Isotope Laboratories Inc, Andover, MA) and characterized by ^{13}C nuclear magnetic resonance (NMR) in a spectrometer INOVA-500 (B_0=11T) (Varian, Palo Alto, CA, USA) operating at 125,696 MHz for ^{13}C and equipped with

5 mm probe for direct and indirect detection, selective pulse and *Sun* workstation. Spectra were obtained at 24.6 °C with 45 ° pulses, using broad band decoupling (WALTZ sequence), spectral width of 30 kHz, and acquisition time of 1.3 s, delay time of 1.5 s. The chemical shifts were obtained in ppm using tetramethylsyloxane (TMS) and deuterated chloroform ($CDCl_3$) as reference standard. The difference between methylic, methylenic, methynic and not bounded carbons was established by DEPT spectra (90 ° and 135 °, which CH_3/CH = positive sign, CH_2 = negative sign and C_0 = no sign).

Cholesterol and COP quantification by HPLC

A Shimadzu (Kyoto, Japan) liquid chromatograph equipped with UV (SPD-10 AVVP) and RI (RID 10A) detectors was used. The analytical column was a Nova Pack CN HP (Waters, Milford, MA), 300 x 3.9 mm i.d., containing 4 μm particles of packing material; injection loop of 20 μL; oven temperature was 32 °C. A mixture of hexanes (min. 63% of *n*-hexane) and propan-2-ol was used as mobile phase (97:3 v/v) at a flow rate of 1 mL/min *(9)*. Cholesterol and 7α-, 7β-OH, 7-keto and 7-OOH were quantified using the UV detector at 210 nm. The α- and β-epoxycholesterol were quantified using RI detector at 32°C. Identification of cholesterol and COPs was made by comparison of the retention times of peaks in samples with those of reference standards and spiking. The compound identities were further confirmed by LC-APCI-MS.

The 7-OOH was quantified as the sum of 7α- and 7β-OOH using a calibration curve plotted with 6 points with a concentration range from 0.7 to 182 μg/mL of synthesized 7α-OOH. Cholesterol, 7α- and 7β-hydroxycholesterol (7α-OH and 7β-OH), 7-ketocholesterol (7-keto), α- and β-epoxycholesterol (α- and β-epoxy) were quantified by external calibration, with curves ranging from 0.2 to 6 mg/mL for cholesterol, and from 0.5 to 100 μg/mL, for each COP.

Confirmation of compounds identities by LC-APCI-MS

A Shimadzu HPLC equipped with quaternary pumps (LC-20AD) and a degasser unit (DGU-20A5) connected in series to a photodiode array detector (PDA) (SPD-M20A) and to a Esquire 4000 mass spectrometer (Bruker Daltonics, Bremen, Germany), fitted with an atmospheric pressure chemical ionization source (APCI) and an ion-trap analyzer was used. The HPLC conditions were the same as described above. The MS parameters were set as follows: positive ion mode of analysis; source temperature, 400 °C; corona, 4000 nA; dry gas (N_2) 300 °C, 5 L/min flow, and 65 psi nebulizer gas; scan range from *m/z* 80 to 450. The MS spectrum of each peak was compared with the MS spectrum of the COP standards at the corresponding retention time. MS/MS spectrum of the synthesized 7α-OOH was obtained using the ion *m/z* 401 as precursor. The following conditions were used: Collision-induced dissociation was produced with helium (99.999%, White Martins, RJ, Brazil) at a pressure of 30 psi in the ion trap. Capillary voltage was 3500 V, end-plate offset 500 V, skimmer (I) 10.0 V and skimmer (II) 6.0 V. The

octopole was at 2.5 V, octopole D 2.5 and octopole RF 100.0 (Vpp). The acquisition MS/MS fragmentation amplitude was 1.4 V.

Model systems

In order to verify the COPs formation during heating, pure cholesterol (99%, Sigma-Aldrich, St. Louis, MO) was submitted to heat process in a heating block (Dryblock, Catel, Piracicaba, Brazil) under constant oxygen (99.9999%, White Martins, RJ, Brazil) flow (ca. 10 mL/min). The tubes containing 1 mL of a 1 mg/mL cholesterol solution had the solvent (isopropyl alcohol) evaporated under gently nitrogen stream, and then were disposed into the holes of the heating block set at 140, 180 or 220 °C. The tubes were heated until cholesterol concentration reached at least 25% of its initial content. To determine cholesterol, 7-OOH and COP concentration during the heating process 12 tubes were sampled at different times. After heating time the tubes were immediately chilled to stop any reactions, and their contents were diluted with 1 mL of mobile phase and injected into the HPLC to quantify the remaining cholesterol and COPs. The initial amount of cholesterol was measured by the quantification of cholesterol in the test tube without heating.

RESULTS AND DISCUSSION

Synthesis and identification of 7-OOH

The synthesis produced 7α-OOH (38.6% yield) with 68% of purity. Beckwith and co-workers *(16)* reported that re-crystallization could be used to increase the purity

of this compound; however, such procedure led to decomposition of 7α-OOH in 7-keto and 7α-OH.

The identification of 7α-OOH was made by comparison of ^{13}C NMR data (Figure 1) with previously published data for cholesterol *(17)* and other very closely related-structures *(18)*. The main difference between data of 7α-OOH and 7α-OH is in the chemical shift of C-7, which is 12.1 ppm deshielded in comparison with the corresponding carbon of 7α-OOH. This chemical shift is very similar to that reported for 7-hydroperoxystigmasterol (77.9 ppm) *(18)*.

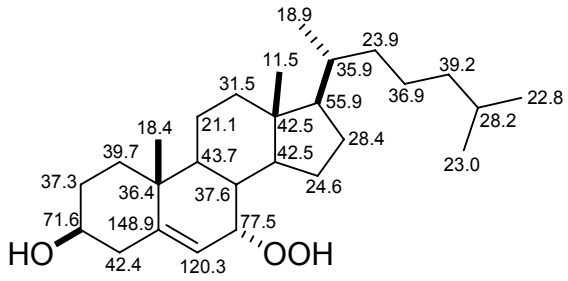

Figure 1. ^{13}C NMR chemical shifts (ppm) of 7α-OOH.

The presence of the hydroperoxy group was easily confirmed by APCI-MS (Figure 2). Loss of 34 mass units directly from the protonated 7α-OOH (*m/z* 419), which is characteristic for peroxide groups *(17)*, as well as the formation of other major product ions are proposed in Figure 3. The formation of the product ion C (*m/z* 383) can be considered useful to distinguish 7-OOH from other COPs, whose mass spectra are very similar due to water elimination resulting in isobaric ions *(9)*.

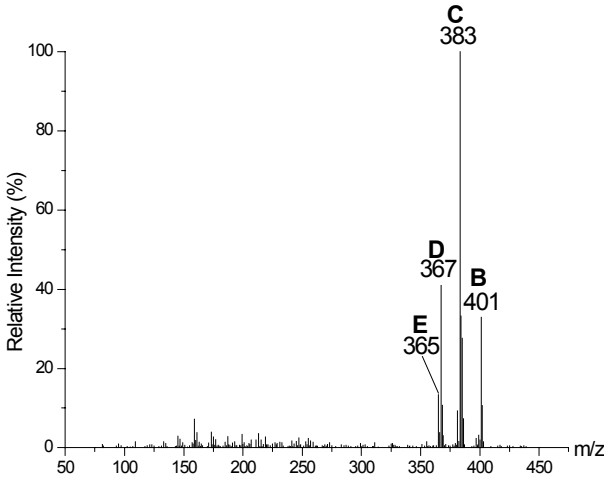

Figure 2. MS espectrum of 7α-OOH. Subtitle: **B**=$[M\text{-}H_2O]^+$, **C**=$[M\text{-}H_2O\text{-}H_2O_2]^+$, **D**=$[M\text{-}2H_2O]^+$, **E**=$[M\text{-}3H_2O]^+$

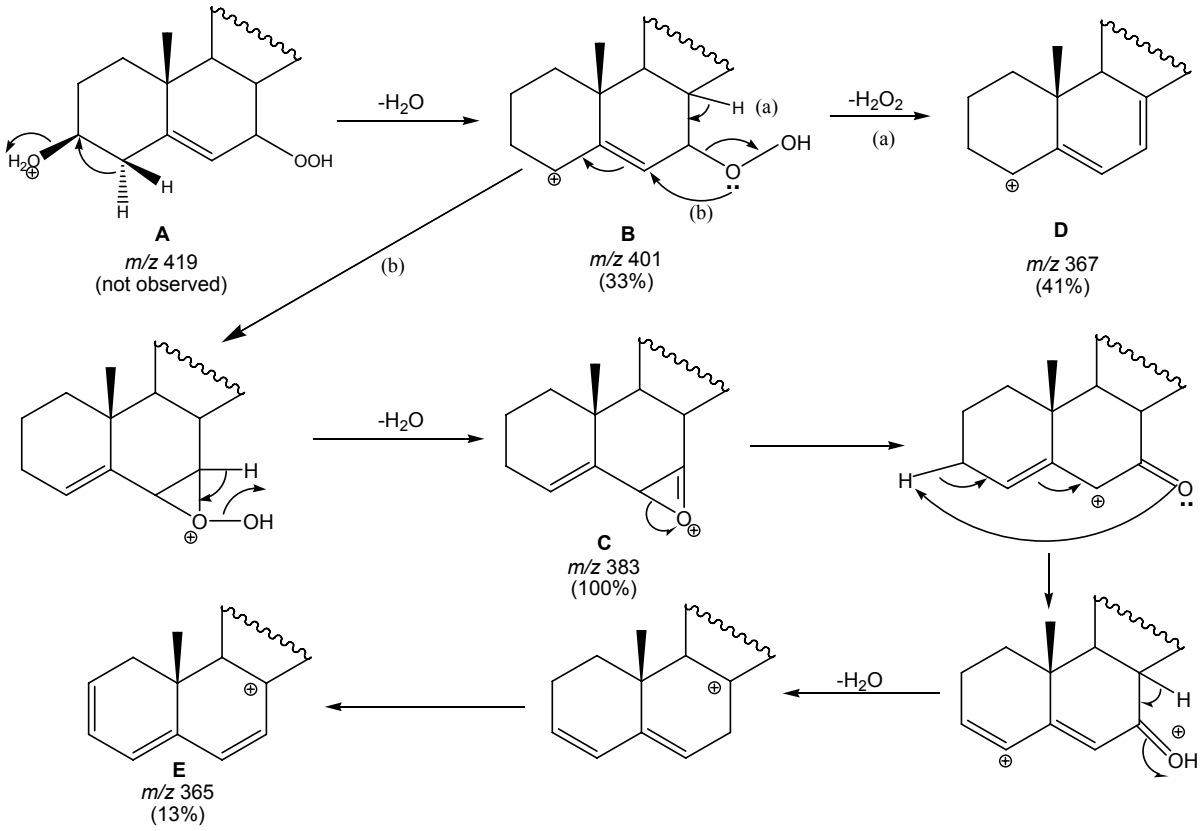

Figure 3. Proposed fragmentation of 7α-OOH during APCI ionization based on results obtained by APCI-MS/MS

The peak relative to 7α-OOH in the model system samples was identified with basis on the comparison of its retention time and mass spectrum with those of the synthesized 7α-OOH standard. The epimeric 7β-OOH was identified following the elution order of the epimeric 7α-OH *(9)*, as shown in Figure 4.

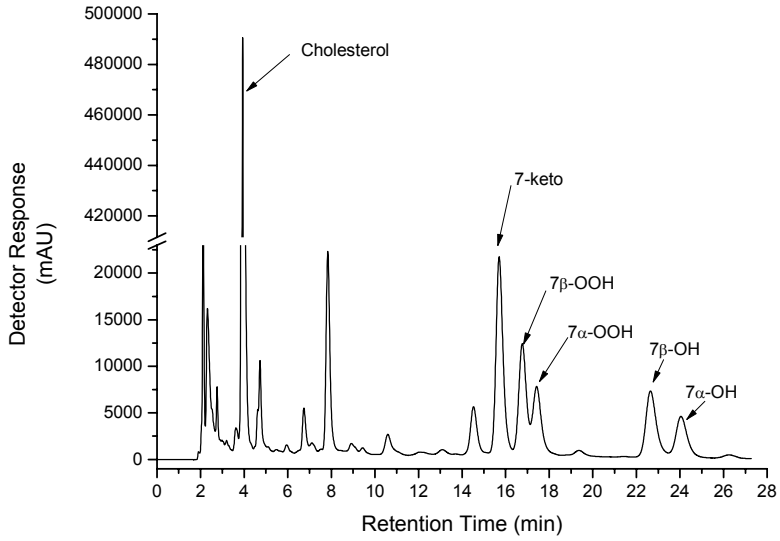

Figure 4. Chromatogram of cholesterol and COP from model system containing pure cholesterol submitted to 220 °C for 45 s obtained by HPLC (UV at 210 nm). Retention times: Cholesterol (3.5 min); 7-keto (14.4 min); 7β-OOH (15.5 min); 7α-OOH (16.1 min); 7β-OH (21.3 min) and 7α-OH (22.7 min).

Cholesterol heating and formation of hydroperoxides and COP

The initial amount of cholesterol was 1.45, 1.42 and 1.6 mg/mL at 140, 180 and 220 °C, respectively, which were at least 75% degraded at the end of the heating process.

During heating the amount of 7-OOH reached a maximum concentration at 45, 87 and 1494 s at 220, 180 and 140 °C, respectively, after which time degradation occurred (Figure 5). The time to reach the maximum concentration of 7-OOH varied at each temperature as well as the amount of 7-OOH formed, possibly because of differences in the cholesterol oxidation rates *(18)*. At the maximum 7-OOH levels (83, 52 and 59 μg/mL), the amount of total COP (sum of 7α-OH, 7β-OH, 7-keto, α- and β-epoxides) was 79, 92 and 110 μg/mL and the cholesterol degradation reached 42, 21, 31% at 140, 180 and 220 °C, respectively.

The formation of 7-OOH is inversely proportional to cholesterol degradation, where the lowest temperature (140 °C) presented higher values than the other temperatures. At 180 °C smaller amounts of 7-OOH and cholesterol degradation percentages were found, while at higher temperatures the amount of 7-OOH increased again, as well as the cholesterol degradation, suggesting that cholesterol degradation can be correlated to 7-OOH formation, independently of temperature. On the other hand, COP formation presented a linear correlation with temperature, being the higher the temperature, the higher COP formation, which is consistent to previously published data *(18)*.

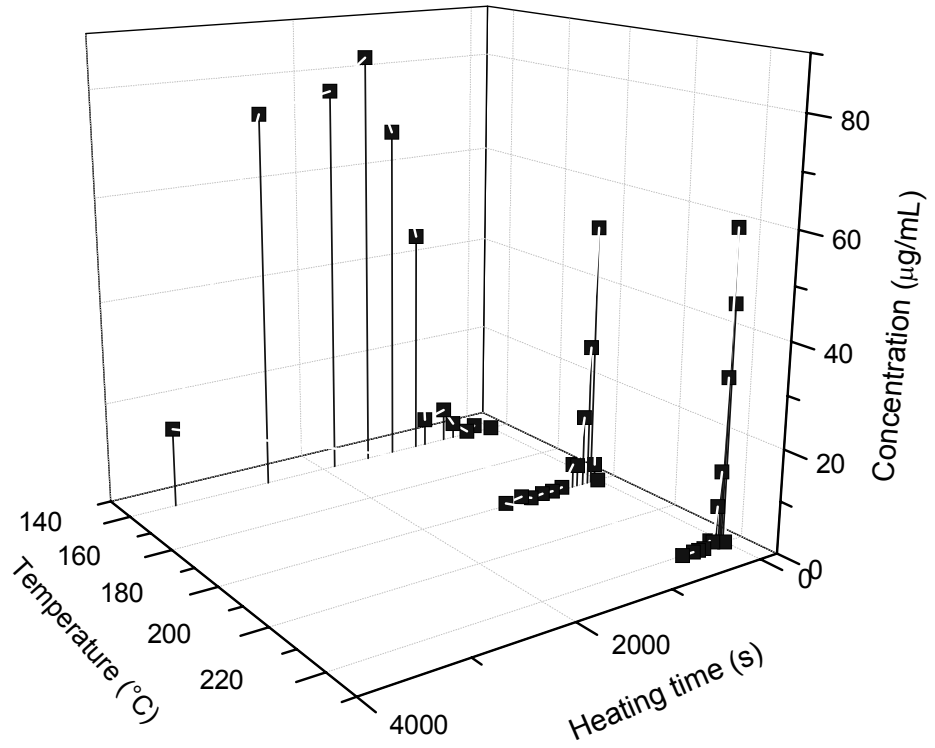

Figure 5. Concentration of 7-OOH during heating at 140, 180 and 220 °C.

The COP detected in the present study were those known to result from cholesterol oxidation such as, 7-keto, 7α- and 7β-OH, α- and β-epoxides (Figure 6). Side-chain derivatives or 3β-,5α-,6β-cholestanetriol were not detected. At 140 °C, only 7-OOH was formed until 212,6 s; between 212,6 and 593,09 s, 7-keto, 7α- and 7β-OH were formed; and after that the formation of α- and β-epoxides began. This observation corroborates with the mechanism in which the formation of epoxides are due to 7-OOH presence by a bimolecular reaction *(5)*. However, this behavior was not observed at higher temperatures, both at 180 and 220 °C, when

at the first sampling time all these 5 cholesterol oxides were detected and quantified. The fast formation of 7-OOH can lead to the fast formation of COP; furthermore, it is expected that the higher the amount of 7-OOH, the higher the amount of COP. Figures 6 shows the differences between 7-OOH content and the sum of COP (total COP). At 140 °C the amount of 7-OOH was quite similar to the total COP formed; however, at higher temperatures the quantity of 7-OOH formed was lower than the amount of COP. Possibly, the amount of 7-OOH was only partially transformed into COP, meaning that temperature must interfere in this balance. Moreover, at 140 °C the formation of 7-OOH was slower than at the higher temperatures and probably its degradation was slower too, explaining the higher concentration of 7-OOH when comparing to the higher temperatures.

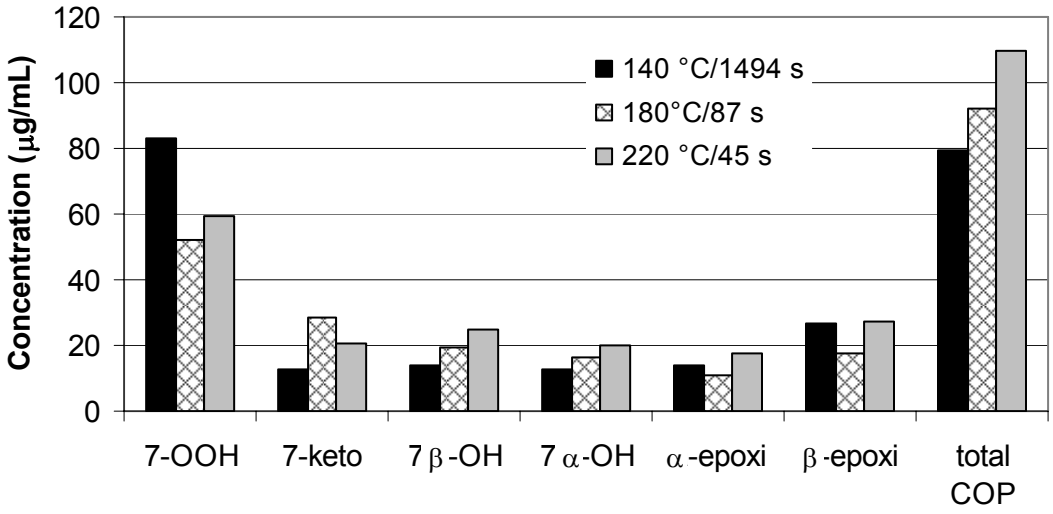

Figure 6. Formation of 7-OOH (maximum) and COPs observed in pure cholesterol model system heated at 140, 180 and 220 °C for 1494, 87 and 45 s, respectively.

The results of cholesterol oxidation at the maximum formation of 7-OOH are shown at Figure 6. The comparison among different heating times is due to the different reaction rates; as can be observed, the cholesterol oxidation occurred more rapidly at higher temperatures. Similar results were found by Kim and Nawar *(18)*. Moreover, degradation of cholesterol at 140 °C occurs slowly, as this temperature is lower than its melting point. At 220 °C, the maximum formation of 7-OOH (59.3 μg/mL) was found to occur at 45 s; after that, fast degradation of 7-OOH was observed. During the same period, 27.2 μg/mL of β-epoxy, 24.7 μg/mL of 7β-OH, 20.8 μg/mL of 7-keto, 19.8 μg/mL of 7α-OH and 17.3 μg/mL of α-epoxy were quantified. The amount of 7-OOH found corresponds to 10% of the remaining concentration of cholesterol; however, the sum of all detected COP at that point corresponds to only 10% of initial cholesterol content. These data indicate that a greater part of degraded cholesterol did not form 7-OOH or other COP, but other polymers or volatile compounds which were not identified in this work. Heating cholesterol at too high temperatures (above 200 °C) may have caused thermal degradation rather than cholesterol oxidation *(19)*. Moreover, the amount of COP detected could be reduced by thermal degradation, since the quantity observed after a given time interval represents the net balance between formation and degradation of each COP *(18)*.

At 180 °C, the maximum formation of 7-OOH (52.2 μg/mL) was observed at 87 s, after that 7-OOH degradation took place. This concentration is similar to that observed at 220 °C. At this temperature the formation of 7-keto, 7β- and 7α-OH, α-

and β-epoxy reached 28.3, 19.5, 16.5, 10.6 and 17.3 μg/mL, respectively at that exposure time. The amount of 7-OOH detected at 180 °C represented 8.3% of the remaining cholesterol and 6.9% of the initial cholesterol. Nawar and co-workers *(12)* reported the amount of COP as representing 21.9% of initial cholesterol; however, the authors heated the cholesterol at 180 °C for one hour.

At 140 °C a greater amount of 7-OOH (82.8 μg/mL) was observed at the maximum formation point (1494 s). At this temperature, the formation of 26.5 μg/mL of β-epoxy, 12.5 μg/mL of 7-keto, 13.9 μg/mL of α-epoxy, 13.7 μg/mL of 7β-OH and 12.7 μg/mL of 7α-OH was observed. Although the formation of 7-OOH was higher than at the other temperatures, once more the amount of 7-OOH formed represents only 5% of the initial and 10% of the remaining cholesterol.

Chien and co-workers used of much higher quantities of cholesterol (100 mg) in a model system heated and detected ca. 0.35 μg of 7-OOH at 150 °C for 90 min *(6)*, and ca. 8 μg of 7-OOH for each g of cholesterol at 140 °C for 120 min *(7)*. However, these authors quantified the 7-OOH by the Würster dye method, which uses preparative HPLC analysis followed by color reaction and UV detection.

In summary, the method used takes the advantage of using mass spectrometry for the identification of 7-OOH. The potential of this technique for the analysis of such compound has not been previously investigated in the literature. By applying this method in cholesterol heated model systems it was possible to verify that 7-OOH is the first compound formed when the temperature is lower (140 °C). However, the concentration of the 7-OOH is depending on the temperature and time exposure, the higher time, the higher the amount of 7-OOH at lower

temperature and the lower time, the lower amount of 7-OOH at higher temperature (180 and 220 °C).

These results corroborate with the three phases of cholesterol oxidation being that at 140 °C the oxidation is still at initiation phase at which the formation of hydroperoxides predominates while at higher temperatures the oxidation is already at propagation phase at which formation of other COPs predominates.

ACKNOWLEDGMENT

The authors thank Dr. Anita J. Marsaioli from Department of Organic Chemistry – Chemistry Institute – University of Campinas for her technical assistance on the synthesis and characterization of 7α-OOH and Dr. Lilian R.B. Mariutti from Department of Food Science – Faculty of Food Engineer – University of Campinas for her valuable contribution to the cholesterol oxidation discussion.

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CAPÍTULO 3

Antioxidants and fatty acids interaction on cholesterol thermal degradation

RESUMO

O colesterol é suscetível à degradação quando aquecido, sendo que uma das vias dessa degradação é a formação de óxidos de colesterol (COP). Estes compostos têm sido associados a diversos efeitos biológicos e doenças degenerativas. As temperaturas atingidas durante os processos térmicos normalmente empregados, principalmente na superfície dos alimentos, podem chegar a até 230 °C, por exemplo, em spray-dryer, ou fornos convencionais. O efeito da presença de β-caroteno, vitamina E, TBHQ e dos ácidos linolênico e esteárico sobre a degradação e oxidação do colesterol aquecido a 220 °C foi o objeto de estudo neste trabalho. Para isto, seis sistemas modelo contendo aproximadamente 1 mg de colesterol puro e adicionado de 0,1% de cada antioxidante ou 1 mg de cada ácido graxo foram elaborados. Os teores de colesterol, 7-hidroperoxicolesterol e óxidos de colesterol foram avaliados ao longo do período de aquecimento. A degradação do colesterol não foi influenciada pela presença do ácido esteárico e foi acelerada pela presença do ácido linolênico. Observou-se uma pequena tendência a proteção na ação do TBHQ frente à degradação do colesterol, em relação aos demais antioxidantes estudados. Por outro lado, todos os compostos adicionados foram capazes de reduzir a formação COP. A presença de ácido linolênico teve ação antioxidante mais efetiva que os demais compostos adicionados, reduzindo o teor COP totais em aproximadamente 38%.

Palavras-chave: óxidos de colesterol, TBHQ, β-caroteno, vitamina E

ABSTRACT

Cholesterol can be degraded when heated and one path of this degradation is the formation of cholesterol oxidation products (COP). Those compounds have been associated to several biological effects and degenerative diseases. During heating process, such as spray dryer and roast oven, temperatures can reach 230 °C, mainly at the food surface. The behavior of cholesterol, heated at 220 °C, in the presence of β-carotene, TBHQ, vitamin E and linolênic and stearic acids was evaluated in this work. Six model systems were prepared, containing ca. 1 mg of pure cholesterol and added of 0.1% of each antioxidant or 1 mg of each fatty acid. Cholesterol, 7-hydroperoxycholesterol and COP contents were evaluated during heating period. The presence of linolenic acid accelerated cholesterol degradation, which was not influenced by the presence of stearic acid. A small tendency to protect cholesterol degradation was observed for TBHQ, compared to the other antioxidants. Concerning cholesterol oxidation, all added compounds were able to reduce COP formation. Linolenic acid presented the most effective antioxidant activity, since COP content were 38% lower than pure cholesterol.

KEYWORDS: cholesterol oxides, TBHQ, β-carotene, Vitamin E,

Introduction

Heat is one of the most important factors which can cause cholesterol degradation which can lead, among other unknown products, to cholesterol oxidation products (COP) [1-3]. The cholesterol oxides formed can be even worse to human health than the cholesterol itself, since they have been related to several diseases such as atherosclerosis [4, 5] and other biological effects such as carcinogenicity, cytotoxicity, mutagenicity and modulation of cell membrane properties [6]. Thus, the formation of COP should be inhibited or minimized under heat conditions.

The autoxidation of cholesterol and COP formation are similar to the unsaturated fatty acids oxidation [7-9]. Compounds that inhibit fatty acids oxidation may reasonably be expected to inhibit cholesterol oxidation and, also, compounds which accelerates lipids oxidation such as polyunsaturated fatty acids (PUFA) may be expected to favor COP formation [10].

Vitamin E and β-carotene have been used as antioxidant in order to prevent lipid or cholesterol oxidation with positive results, but as feeding diet supplements [11]. Also spices and herbs rich in polyphenolic compounds added to meat prior processing and/or storage have shown good results such as rosemary [12, 13], sage [14] and colorifico – which is rich in carotenoids, with bixin as the major compound [15]. TBHQ has also provided good results on lipid oxidation in cooked ground beef [16]. However, little is known about the effect of the direct addition of those compounds on the cholesterol during heat.

There is no clear information about how the fatty acids degree of unsaturation affects cholesterol oxidation. Nawar and co-workers [17] reported that cholesterol oxidation is accelerated by the presence of triacylglycerols. The presence of oleate provided more cholesterol oxides than the presence of linoleate and stearate when cholesterol was heated at 180 °C for one hour. It should be explained by the oxidation of the fatty acids providing free radicals and peroxides, which can trigger cholesterol oxidation, however linoleate would provide more free radicals than oleate, since it has more double bounds. On the other hand, Xu and co-workers [18] reported that the effect of fatty acids on cholesterol oxidation depends on the extension of heating time.

Considering that high temperature and degree of unsaturation of fatty acids and the presence of anti- and pro-oxidants are important factors on cholesterol oxidation, the effect of TBHQ, vitamin E and β-carotene as well as stearic and linolenic acids were evaluated on cholesterol oxidation in model systems heated at 220 °C by the measurement of COP.

Material and Methods

Chemicals. Cholesterol (95%), vitamin E (97% of α-tocopherol), β-carotene (95%), and linolenic acid (99%) were purchased from Sigma-Aldrich (St. Louis, MO). Stearic acid (99%) and methyl tridecanoate (99%) was purchased from Fluka (Steinheim, Germany). COP standards: 20α-, 22*S*- and 25-hydroxycholesterol, 5,6α- and 5,6β-epoxycholesterol (α- and β-epoxi) and 7-ketocholesterol (7-keto)

were purchased from Sigma (Milford, MA); 22*R*-, 24*S*-, 25*R*- 7β- and 7α-hydroxycholesterol (7α- and 7β-OH) were purchased from Steraloids (Newport, RI) with purity varying from 95 to 98%. *t*-Buthylhydroquinone (TBHQ, 95%) was gently supplied by Plury Química (Diadema, Brazil). All solvents used were HPLC grade.

Model system preparation. Pure cholesterol was dissolved in isopropyl alcohol in order to obtain a 1 mg/mL solution from which 1 mL was taken and poured into glass test tubes. Solutions with 1 mg/mL of each antioxidant and fatty acids were prepared in isopropyl alcohol and hexane, respectively. Tests tubes were added of 10 μL of the antioxidant and/or 1 mL of the fatty acid solution. All solvents were evaporated under gentle nitrogen stream. The tubes were disposed into a heating block (Marconi, Piracicaba, Brazil) set at 220 (219±5) °C under continuous flow (ca. 10 mL/min) of oxygen 99,9999% (White Martins, RJ, Brazil). To determine cholesterol and COP concentration during heat, 12 tubes of each treatment were analyzed at different times until cholesterol reached at least 75% of its initial content. After heating the tubes were immediately chilled to stop any reactions, and their contents were diluted with 1 mL of *n*-hexane + isopropyl alcohol (97:3) forming solution A. The initial amount of cholesterol, COP, antioxidants and fatty acids was measured by the quantification of the test tube without heating.

Cholesterol and COP quantification by HPLC. Solution A was directly injected in a liquid chromatograph (Shimadzu, Kyoto, Japan), equipped with UV (SPD-10 AVVP) and RI (RID 10A); injection loop of 20 μL; oven temperature set at 32 °C. The separation of cholesterol and COP was carried out on a 300 mm x 3.9 mm i.d., 4 μm analytical column Nova Pack CN HP (Waters, Milford, MA) with mobile phase

composed of a mixture of hexanes (min. 63% of *n*-hexane) and propan-2-ol (97:3 v/v) at a flow rate of 1 mL/min [19]. UV detector set at 210 nm was used to quantify cholesterol and 7α-, 7β-hydroxycholesterol, 7-ketocholesterol and 7-hydroperoxycholesterol (sum of 7α- and 7β-hydroperoxycholesterol). The RI detector at 32 °C was used to quantify α- and β-epoxycholesterol. Identification of cholesterol and COP was made by comparison of the retention times of peaks in samples with those of reference standards and co-chromatography. The 7-hydroperoxycholesterol was identified according to Nogueira and co-workers [20].

LC-APCI-MS. Following HPLC-UV-RI quantification, a subset of samples was then injected onto a HPLC-APCI-MS for identity confirmation using a Shimadzu HPLC equipped with quaternary pump system (LC-20AD) and degasser unit (DGU-20A5) connected to an Esquire 4000 mass spectrometer (Bruker Daltonics, Bremen, Germany) with ion-trap analyzer. The HPLC conditions was the same described above and MS parameters was positive ion mode of analysis, source temperature, 400 °C, corona, 4000 nA, dry gas (N_2) at 300 °C, 5 L/min flow and 65 psi.

Antioxidants determination. To determine the antioxidants 400 μL of solution A was taken and solvents were evaporated under nitrogen. Each one was re-dilluted with 200 μL of the appropriated solvent prior the HPLC analysis. The separation was carried out in a 4.6 mm x 15 cm, 5 μm Bondesil C_{18} column (Varian, Palo Alto, CA) for all antioxidants.

β-carotene and TBHQ were quantified in a HPLC with diode array detector (Waters, New Castle, DE) with a quaternary pump system, online degasser,

Rheodyne injection valve (20 μL), external temperature controller set at 35 °C and flow rate 1 mL/min. Identification of β-carotene and TBHQ was obtained by the comparison of retention time of the sample and standard and the quantification was obtained by external standard with peak area calculated at 450 nm. The mobile phase used in separation of β-carotene was methanol + ethyl acetate (1:1 v/v) and calibration curve was made from 0.3 to 10 μg/mL. TBHQ was separated with mobile phase composed by acetic acid 5% + acetonitrile + methanol (50:25:25 v/v) and the calibration curve was made from 0.5 to 12.5 μg/mL.

Vitamin E was quantified in a HPLC (Shimadzu, Kyoto, Japan) equipped with binary pump system, He degasser, diode array detector (DAD) set at 290 nm. The separation was carried out with mobile phase composed by methanol + water (98:2), at flow rate 1.6 mL/min and temperature set at 32 °C. The peaks were identified by the comparison of retention times of the samples and standard (α-tocopherol), confirmed by DAD spectra and the quantification was obtained by external standard ranging from 0.5 to 5 μg/mL.

Fatty acids determination. Fatty acids were quantified in a 400 μL aliquot of the solution A, which had solvent evaporated under nitrogen and was submitted to saponification and sterification according to Kim and Nawar [21]. Methyl esters were extracted with hexane and 2 μL were injected using hot needle technique in a gas chromatograph (Shimadzu, Kyoto, Japan) equipped with split injector (1/50) set at 250 °C and flame ionization detector set at 260°C. Carrier gas was hydrogen at linear velocity of 34 cm/s and nitrogen at 30 mL/min as make up gas.

The column used was a 100 m x 0.25 mm, 0.20 μm CP-SIL 88 (Chromopack,

Middleburg, Netherland). The oven temperature was set beginning at 120 °C kept for 8 min, raising to 160 °C at 20 °C/min, raising to 195 °C at 3 °C/min and kept for 10 min, the temperature was raised to 210 °C at 3 °C/min, and then to 220 °C at 35 °C/min and kept for 3 min, raising again to 240 °C at 20 °C/min and kept for 5 min, in a total of 46 min run. The quantification was carried out by internal standard (tridecanoic acid methyl ester) added prior dilution and injection.

Results and discussion

Cholesterol degradation at 220 °C

According to literature, cholesterol degradation occurs very fast at temperatures above 142.7 °C, which is the cholesterol melting point [2, 22]. In the present work, more than 90% of cholesterol was degraded in about 8 minutes at 220 °C. Figure 1 shows cholesterol degradation related to initial concentration (C/C_0) during heat for model systems containing cholesterol and antioxidants or fatty acids studied.

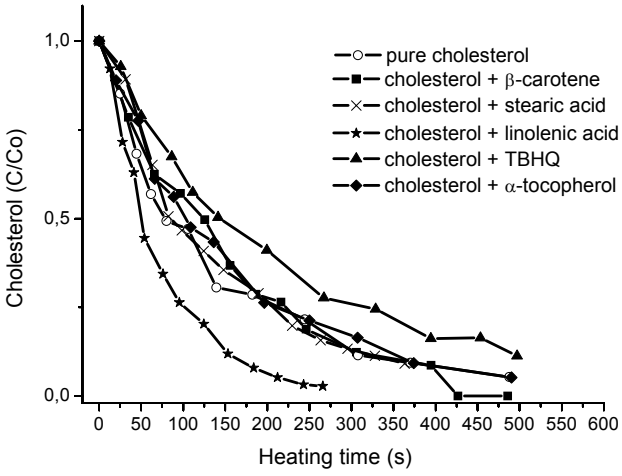

Figure 1. Degradation of cholesterol in model system containing fatty acids or antioxidants during heat at 220°C.

The mechanism of COP formation begins with 7-hydroperoxycholesterol [8, 20] an intermediate compound of COP formation. In the present work 5 COP were detected, 7α-, 7β-hydroxycholesterol, 7-ketocholesterol, α- and β-epoxycholesterol (Figure 2). In the pure cholesterol system all 5 COP was already detected at the first sampling time (25 s), with the amount of 6.8, 9.0, 5.8, 5.3 and 14.3 μg/mL, for 7α-, 7β-hydroxycholesterol, 7-ketocholesterol, α- and β-epoxycholesterol respectively.

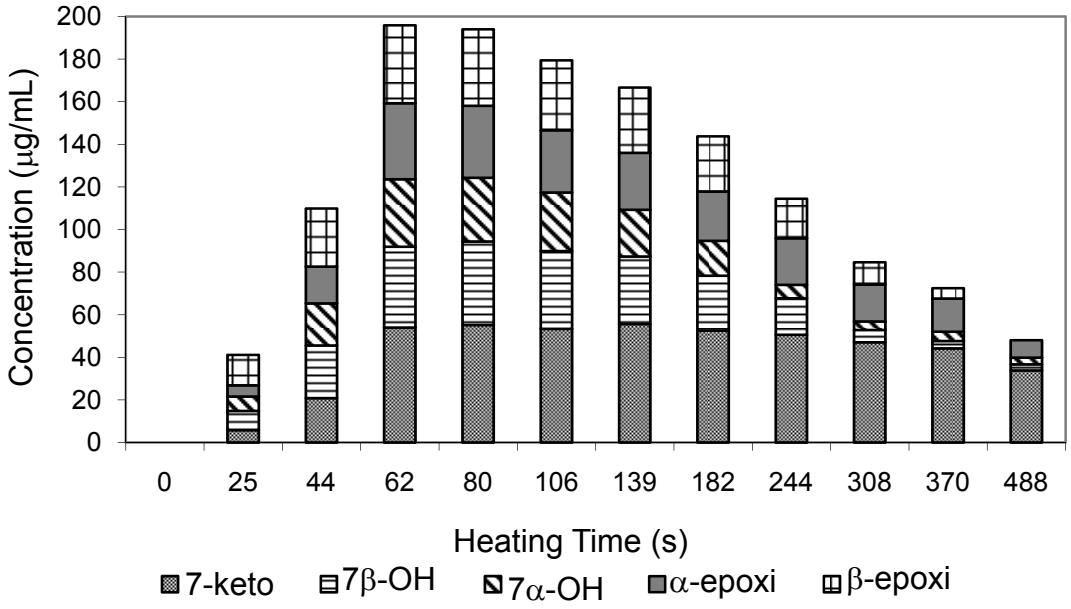

Figure 1. COP formation in pure cholesterol model system heated at 220°C.

The content of total COP reached 196 μg/mL up to 62 s, after that, decreased reaching 48 μg/mL. The 7-ketocholesterol seemed to be the most stable COP, since it represented almost 70% of total COP at the end of heating process and varied from 6 μg/mL at the first 25 s of heating to 55 μg/mL at 80 s. On the other hand, β-epoxi is the most labile, which varied from 14.3 μg/mL in the beginning,

increasing to 36.6 μg/mL and reached 5 μg/mL at the end of heating.

Effect of Antioxidant addition

α-tocopherol was the most resistant compound reaching 23% of its initial content at the end of heating. TBHQ was almost completely consumed, reaching only 4% of its initial content at the end of heating and β-carotene was completely degraded at the first sampling (35 s).

As Figure 1 shows, there was a small tendency of TBHQ to protect cholesterol against degradation. The presence of α-tocopherol and β-carotene did not seem to affect the cholesterol degradation compared to pure cholesterol sample. However, concerning COP formation, the differences between the three antioxidants used and pure cholesterol can be noticed (Figure 2), as demonstrated in Figure 3.

All added antioxidants affect the COP formation. The presence of β-carotene delayed initial COP formation and reduced total COP content by 30% compared to pure cholesterol at maximum point, while the presence of α-tocopherol reduced 11% and TBHQ reduced 12%. As phenolic compounds, TBHQ and α-tocopherol can act as hydrogen donors to stop chain reaction by reducing peroxyl radicals at initiation

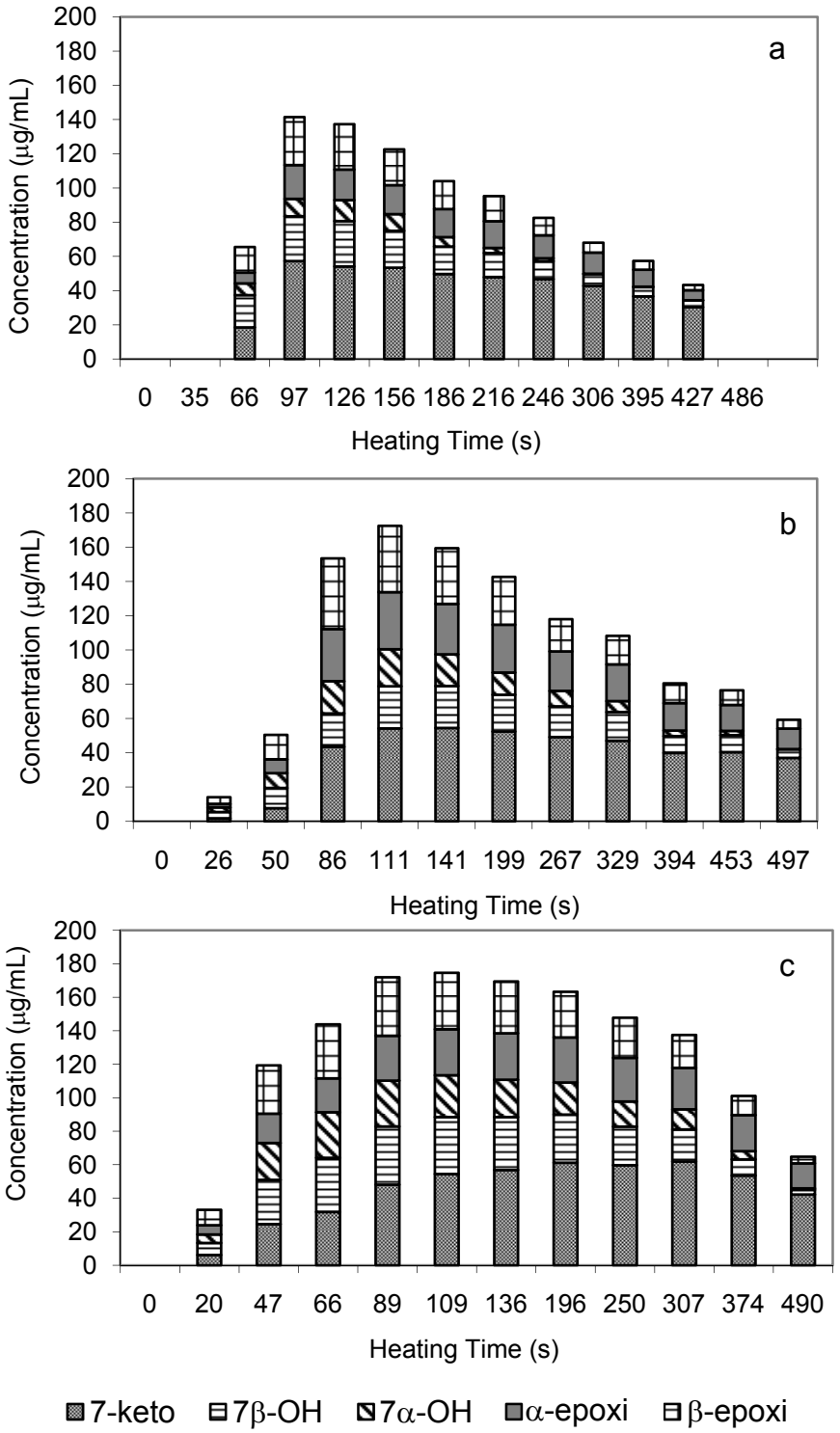

Figure 2. COP formation in model system containing (a) cholesterol+β-carotene; (b) cholesterol + TBHQ and (c) cholesterol + α-tocopherol during heat at 220 °C.

phase. However, the mechanism of chain break reaction provided by β-carotene, which hinders the chain lipoperoxidation on the formation of adducts [23], seems to be more effective. Moreover, the formation of 7-hydroperoxycholesterol observed is much lower in the presence of β-carotene (Figure 4) yielding less formation of COP.

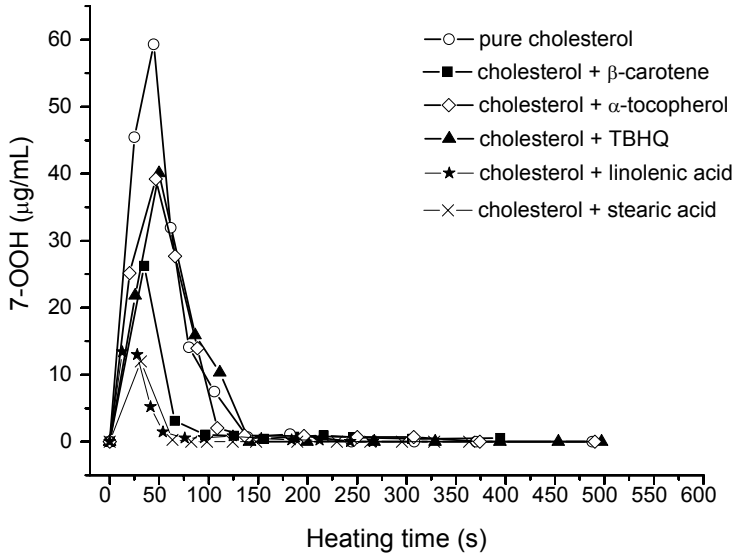

Figure 3. Formation of 7-hydroperoxycholesterol in cholesterol model systems during heat at 220°C

Effect of fatty acids addition

Both fatty acids were degraded during heat. As expected the most stable was stearic acid which reached 25% of its initial content at the end of heating process (approx. 8 min). Linolenic acid reached 22% of its initial content after 41 s, after which was not detected anymore.

The presence of fatty acids, unsaturated or not, may accelerate cholesterol degradation, because the resulting media is more disposed to chemical reactions since cholesterol solubility is increased, increasing the contact surface with oxygen [18, 21, 24]. Moreover, double bonds weaken the C-H bond of methylene in allylic position, therefore polyunsaturated fatty acids (PUFA) are readily susceptible to originate peroxyl radicals which will participate on the propagation phase of lipid oxidation [25]. On the other hand, saturated fatty acids are not susceptible to free radical formation and may play a protective role on cholesterol oxidation [21].

The results obtained in the present study showed that the presence of linolenic acid contributed to cholesterol degradation while the presence of stearic acid did not influenced it (Figure 1). However, reduction of COP formation was observed by the presence of both (Figure 5), compared to pure cholesterol (Figure 2). This means that concerning cholesterol oxidation, at the studied conditions, both fatty acids play a protective role, contradicting the literature [18]. Furthermore, the concentration of COP observed in the presence of linolenic acid is even lower, although cholesterol degradation was higher. It is possible that linolenic acid oxidizes first, competing with cholesterol for the available oxygen. This can be confirmed by the higher formation of COP when TBHQ is added to the model system containing linolenic acid (data not shown). When linolenic acid is protected by the antioxidant, oxygen remains to oxidize cholesterol. Kim and Nawar [24] also reported that cholesterol plays a protective role on unsaturated fatty acid degradation.

The protection of stearic acid could be considered by the same reason of linolenic acid. According to Belitz and co-workers [26], above 60 °C autoxidation

selectivity decreases and the products formed by hydroperoxides homolysis are very reactive and can abstract H-atoms even from saturated fatty acids. Considering the short time exposure to heat, stearic acid was still present at the end, increasing the possibility of competition for the oxygen as much as unsaturated fatty acid.

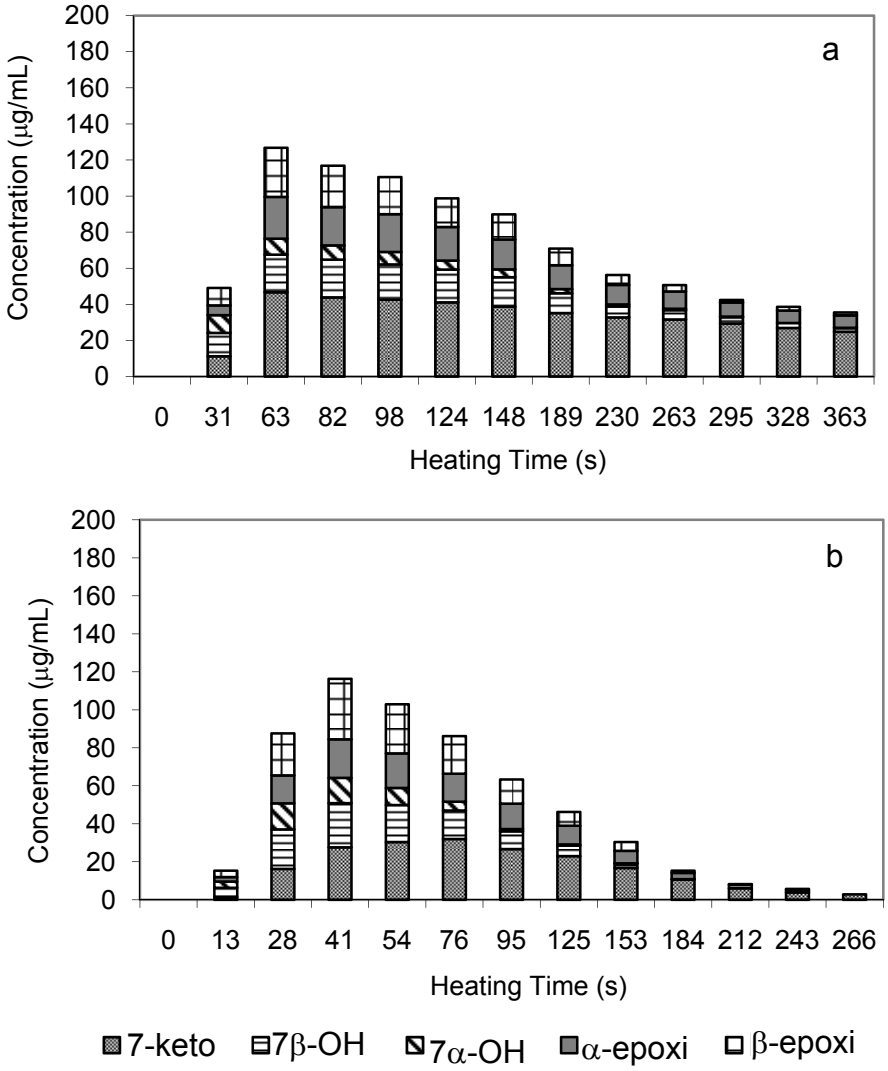

Figure 4. COP formation in model systems containing (a) cholesterol + stearic acid; (b) cholesterol + linolenic acid during heat at 220°C.

Conclusion

The presence of antioxidants commonly used for lipid protection can reduce cholesterol thermal oxidation; however, the mechanism of adduct formation of the β-carotene was more effective than hydrogen transfer of the TBHQ and vitamin E. On the other hand, TBHQ seemed to be more effective than the others on cholesterol degradation protection.

Linolenic acid accelerated cholesterol degradation while stearic acid did not influence it. Nevertheless, both protected cholesterol from oxidation, but linolenic acid was more protective than stearic acid and even than β-carotene.

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The authors thank the São Paulo State (FAPESP) and the National Brazilian Research Foundation (CNPq) for financial support.

CAPÍTULO 4

Cholesterol thermal degradation: effect of fatty acid and antioxidant addition at 180 °C

RESUMO

O colesterol pode degradar-se na presença de oxigênio formando óxidos de colesterol (COP) entre outros compostos ainda não identificados. Considerando os efeitos biológicos dos COP, é importante entender como a presença de ácidos graxos e antioxidantes pode influenciar a degradação do colesterol e formação de óxidos de colesterol durante o aquecimento. Sistemas-modelo, contendo aproximadamente 1 mg de colesterol e 0,1% de TBHQ ou β-caroteno ou vitamina E, ou 1 mg de ácidos esteárico ou linolênico foram aquecidos a 180 °C por aproximadamente 20 minutos. Além do 7-hidroperoxicolesterol (7-OOH), composto intermediário da formação de COP, foram detectados 5 COP ao longo do aquecimento, 7-cetocolesterol, 7α- e 7β-hidroxicolesterol, α- e β-epoxicolesterol, cuja soma (COP total) apresentou aumento até um ponto máximo e em seguida, redução. No sistema contendo colesterol puro, a formação máxima de COP total (147 μg/mL) foi atingida em 4 minutos. Os resultados demonstraram que o α-tocoferol foi capaz de reduzir e retardar a formação de óxidos, uma vez que o máximo de COP total encontrado foi 25% menor e atingido em 10 minutos. Comportamento semelhante foi observado para o β-caroteno, mas com apenas 9% de redução e 4 minutos de atraso. Os sistemas contendo TBHQ e β-caroteno apresentaram maiores teores de 7-OOH em comparação aos demais sistemas. O ácido esteárico acelerou e aumentou a formação COP total, com teor máximo 14% maior, atingido em 2 minutos de aquecimento. A presença de ácido linolênico acelerou a formação máxima de COP (95 s), mas apresentou teor total 18% menor em comparação ao colesterol puro.

Palavras-chave: óxidos de colesterol, TBHQ, β-caroteno, α-tocoferol

ABSTRACT

Cholesterol may degrade under heat in the presence of oxygen yielding cholesterol oxidation products (COP) among other unknown products. Considering COP biological effects, it is important to understand how the presence of fatty acids and antioxidants influence cholesterol degradation and COP formation during heat. Model systems containing ca. 1 mg cholesterol and 0.1% of TBHQ or β-carotene or vitamin E or 1 mg of stearic or linolenic acids were heated at 180 °C for almost 20 minutes. Besides 7-hydroperoxycholesterol (7-OOH), which is the intermediate compound of COP formation, 5 COP were detected, 7-ketocholesterol, 7α- and 7β-hydroxycholesterol, α- and β-epoxycholesterol, and summed (total COP) presented increase until reached a maximum point after which was reduced. For pure cholesterol system maximum total COP (147 μg/mL) was reached at 4 minutes heating. The results showed that α-tocopherol was able to reduce and retard maximum COP formation, since presented 25% less total COP content and reached at 10 minutes. β-carotene presented similar behavior, but only 9% reduction with 4 minutes delay. Systems containing TBHQ and β-carotene presented higher amounts of 7-OOH compared to the others systems. Stearic acid accelerated and increased COP formation, which was 14% higher than pure cholesterol reached at 2 minutes. Linolenic acid accelerated maximum total COP formation (95 s), but presented total COP content 18% lower compared to pure cholesterol.

KEYWORDS: cholesterol oxides, TBHQ, β-carotene, α-tocopherol

Introduction

Cholesterol submitted to heat provides distinct products derived from at least two different paths: degradation, which results in some still unknown compounds but reasonably found to be volatile or polymeric compounds, and oxidation, which is the reaction of triplet oxygen or reactive oxygen species (ROS) yielding cholesterol oxidation products (COP) [1]. The mechanisms involved in cholesterol oxidation are well studied and it is known that the formation of free radical on allylic carbon of the double bound at C5-C6 is the major route of COP formation which begins by the formation of 7-hydroperoxycholesterol that will further turn into 7-ketocholesterol, 7α- and 7β-hydroxycholesterol and also α- and β-epoxycholesterol by reduction, dehydration, dehydrogenation and epoxidation mechanisms [1-3].

COP have been related to the initiation and progression of chronic diseases among them atherosclerosis and neurodegenerative processes [4]. Although COP are produced endogenously, diet can be an exogenous source of these compounds and the amount provided can offer health risk by the continuous intake.

The interactions of other compounds present in food may affect oxidative stability and oxidative pathway of cholesterol [5]. Fatty acids which are in the same lipid fraction can interfere on COP formation especially considering that unsaturated fatty acids (UFA) may provide free radicals by its oxidation accelerating the cholesterol oxidation process [5-8]. On the other hand, UFA can

compete to cholesterol for oxygen for its own oxidation, protecting cholesterol spite of free radicals formation [8]. Nevertheless, saturated fatty acids (SFA) were considered protective of cholesterol oxidation by the scientific community because of the low amounts of COP found in food rich in SFA [9, 10]. However, the performance of these compounds is not quite clear yet because the studies about this matter are usually made by the mixture of cholesterol and different oil or fat which can present other interfering compounds such as phospholipids or tocopherols and, moreover, different amounts and types of UFA and SFA [11-13]. Xu and co-workers [13] reported lower concentrations of COP when cholesterol was mixed with corn oil, olive oil and lard compared to pure cholesterol heated at 180 °C; similar results were obtained by Xu and co-workers [12] who mixed cholesterol with oils obtained from rice, canola, soybean, olive or corn and heated at 175 °C.

The mechanisms of free radical chain reaction which are the same for COP formation and fatty acid oxidation may be minimized or retarded by the addition of antioxidants. In fact, many authors have tested the effects of the addition of antioxidants to food prior to heat treatment with good results [13-16]. However, the effectiveness of the antioxidant activity depends on the mechanism of action of each compound. Antioxidants counteract the oxidative stress in, at least, two different ways: by protecting target lipids from oxidation initiators or by hindrance the propagation phase. In the first case, the prevent antioxidants hinder ROS formation or scavenge species responsible for oxidation initiation, for example carotenoids, such as β-carotene. In the second case, the 'chain breaking'

antioxidants intercept radical oxidation propagators (LOO•) or indirectly participate in stopping radical chain propagation, for example tocopherols and *tert*-butylhydroquinone (TBHQ) [17].

In order to understand the role of tocopherols, TBHQ, β-carotene protection and the interactions of SFA (as stearic acid) and UFA (as linolenic acid) on cholesterol degradation and oxidation during heat, models system containing mixture of cholesterol with those compounds were heated at 180 °C under controlled conditions and oxygen constant supply and the content of cholesterol and COP was measured.

Material and Methods

Chemicals. Cholesterol (95%), vitamin E (97% of α-tocopherol), β-carotene (95%), and linolenic acid (99%) were purchased from Sigma-Aldrich (St. Louis, MO). Stearic acid (99%) and methyl tridecanoate (99%) was purchased from Fluka (Steinheim, Germany). COP standards: 20α-, 22*S*- and 25-hydroxycholesterol, 5,6α- and 5,6β-epoxycholesterol (α- and β-epoxi) and 7-ketocholesterol (7-keto) were purchased from Sigma (Milford, MA); 22*R*-, 24*S*-, 25*R*- 7β- and 7α-hydroxycholesterol (7α- and 7β-OH) were purchased from Steraloids (Newport, RI) with purity varying from 95 to 98%. *t*-Buthylhydroquinone (TBHQ, 95%) was gently supplied by Plury Química (Diadema, Brazil). All solvents used were HPLC grade.

Model system preparation. The preparation of the model system was carried out as described by Nogueira and Bragagnolo [8]; as briefly described: 1 mL of a 1 mg/mL solution of cholesterol in isopropyl alcohol was poured into glass test tubes and mixed with 10 μL of a 1 mg/mL solution antioxidants in isopropopyl alcohol or 1 mL of a 1 mg/mL solution of the fatty acids in hexane. Solvents were removed under nitrogen. The tubes were heated in a heating block (Marconi, Piracicaba, Brazil) set at 180 °C (179±6 °C) under continuous flow (ca. 10 mL/min) of oxygen 6.0 (White Martins, RJ, Brazil). Sampling of 12 tubes was withdrawn at different interval time in order to quantify remaining cholesterol, 7-hydroperoxycholesterol and COP contents. After chilled, 1 mL of the mixture *n*-hexane and isopropyl alcohol (97:3) was added forming solution A and this extract was used for injection into the HPLC to determine cholesterol and COP. The remaining amount of the extract was divided in smaller portions for quantification of antioxidants and fatty acids.

Cholesterol and COP quantification by HPLC. 20 μL of the extract (solution A) was injected in a liquid chromatograph (Shimadzu, Kyoto, Japan) equipped with UV (SPD-10 AVVP) and RI (RID 10A) and oven temperature, 32 °C. The separation of cholesterol and COP was carried out on a 300 mm x 3.9 mm i.d., 4 μm analytical column Nova Pack CN HP (Waters, Milford, MA), using a mixture of hexanes (min. 63% of *n*-hexane) and propan-2-ol (97:3 v/v) as mobile phase at a flow rate of 1 mL/min [18]. Cholesterol and 7α-, 7β-hydroxycholesterol, 7-ketocholesterol and 7-hydroperoxycholesterol (sum of 7α- and 7β-hydroperoxycholesterol) were detected by UV detector set at 210 nm and α- and β-

epoxycholesterol, by RI detector at 32 °C. Identification of cholesterol and COP was made by comparison of the retention times of peaks in samples with those of reference standards and co-chromatography. The 7-hydroperoxycholesterol was identified according to Nogueira and co-workers [19]. Quantification was performed by calibration curves. All peaks were further confirmed by LC-APCI-MS as described by Nogueira and Bragagnolo [20].

Antioxidants determination. The quantification of the antioxidants was carried out as described by Nogueira and Bragagnolo [20] with 400 μL of solution A, as briefly described: the extract had the solvents evaporated under nitrogen and then re-dilluted with 200 μL of the appropriated solvent prior the HPLC analysis. The separation was carried out in a 4.6 mm x 15 cm, 5 μm Bondesil C_{18} column (Varian, Palo Alto, CA) for all antioxidants.

TBHQ and β-carotene were quantified in a HPLC (Waters, New Castle, DE) equipped with quaternary pump system, diode array detector and external controller for temperature (35 °C) and flow rate of 1 mL/min was used. The mobile phase used was methanol + ethyl acetate (1:1) and acetic acid 5% + acetonitrile + methanol (50:25:25) for β-carotene and TBHQ, respectively. Identification was carried out by comparison of the retention time of the standards and the quantification was obtained by external calibration at 450 nm.

Vitamin E was quatified in a HPLC (Shimadzu, Kyoto, Japan) equipped with binary pump system, diode array detector and external controller for temperature (32 °C). Flow rate of 1.6 mL/min of methanol + water (98:2) was used. Identification was carried out by comparison of the retention time of the standards

and quantification was obtained by external calibration at 290 nm.

Fatty acids determination. 400 μL of solution A was submitted to saponification and sterification according to Kim and Nawar [21], after solvent evaporation under nitrogen. The quantification was carried out as described by Nogueira and Bragagnolo [20], briefly: methyl esters were extracted with *n*-hexane and 2 μL were injected using hot needle technique in a gas chromatograph (Shimadzu, Kyoto, Japan) equipped with split injector (1/50) set at 250 °C and flame ionization detector set at 260°C. Carrier gas was hydrogen at linear velocity of 34 cm/s and nitrogen at 30 ml/min as make up gas. The column used was a 100 m x 0.25 mm, 0.20 μm CP-SIL 88 (Chromopack, Middleburg, Netherland).

Results and discussion

Cholesterol degradation at 180 °C

Cholesterol had a fast degradation when heated at 180 °C as 89% of the initial content (1.4 mg/mL) was degraded in about 20 minutes (Figure 1). This result confirms the common sense that cholesterol is very sensible to temperatures above its melting point which is 147.2 °C [1, 22].

During this period cholesterol was partially oxidized since total COP reached only 10% of the initial content at about 4 minutes of heating; the rest of cholesterol may have been transformed into other products such as volatile compounds or polymers [1, 6] which were not characterized in this study.

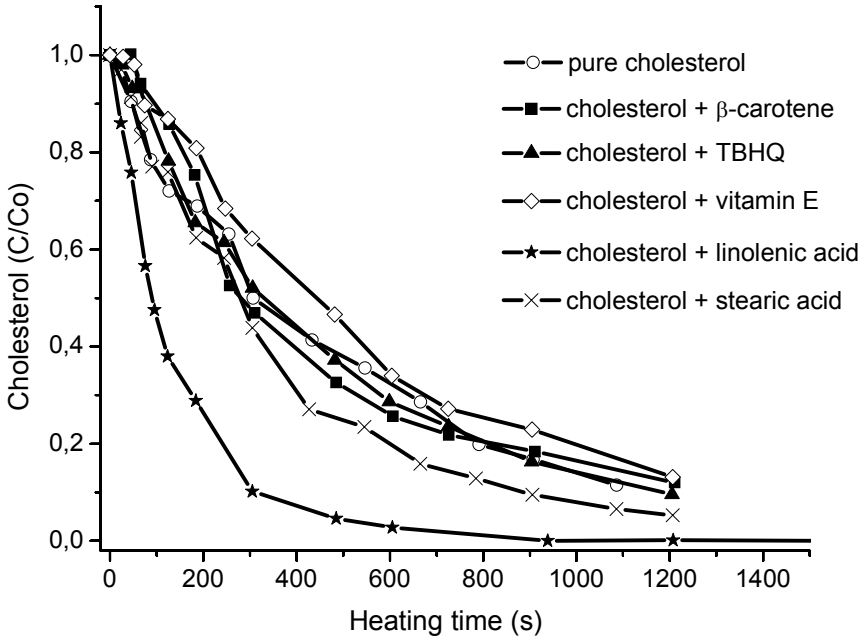

Figure 1. Degradation of cholesterol in model system containing fatty acids or antioxidants during heat at 180°C.

Only oxidation of C7 was observed (Figure 2), which is the main route of cholesterol oxidation pathway providing 7-ketocholesterol, 7α- and 7β-hydroxycholesterol, α- and β-epoxycholesterol. As a sum of 7α- and 7β-hydroperoxycholesterol, 7-hydroperoxycholesterol were also measured.

The presence of oxidation products was observed since the first sampling time (45 s) when 3.5 μg/mL of 7-hydroperoxycholesterol and 17 μg/mL total COP were detected. With 1.5 min of heating 7-hydroperoxycholesterol reached its maximum content (52 μg/mL) and after that it decreased continuously. All COP reached its maximum content at 4 minutes, except for 7α-hydroxycholesterol which presented

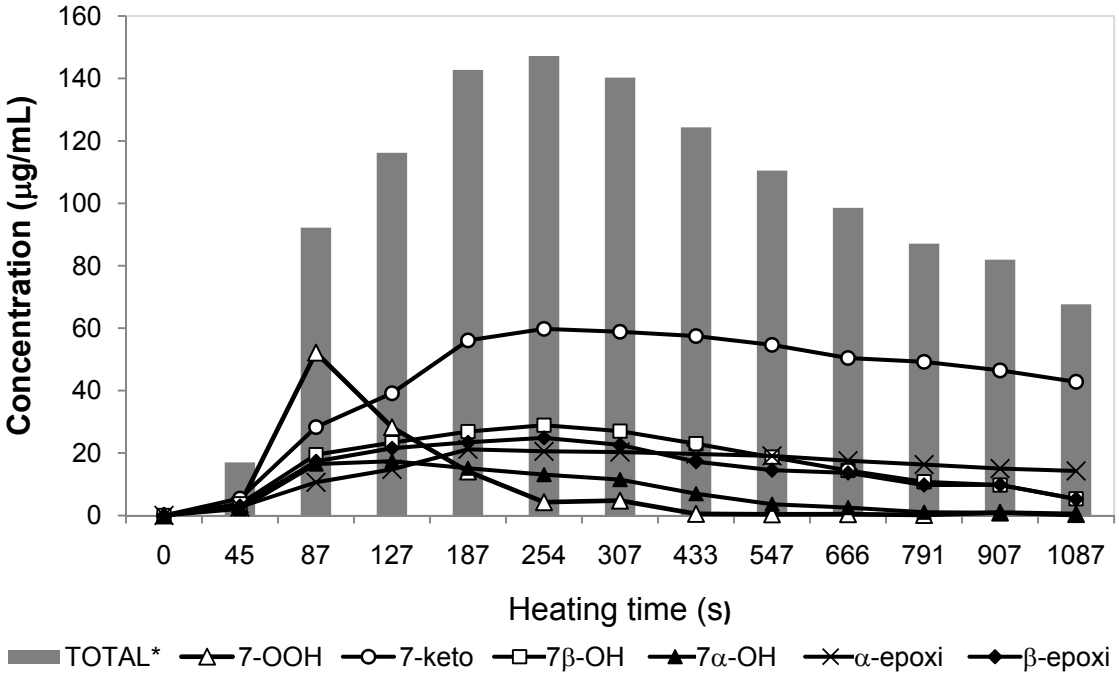

Figure 2. COP formation in pure cholesterol model system heated at 180°C.

maximum concentration (17 μg/mL) at 2 min of heat. The major COP found was 7-ketocholesterol which presented higher content since the beginning, reaching 60 μg/mL at 4 minutes followed by 7β-hydroxycholesterol, β-epoxycholesterol, α-epoxycholesterol and 7α-hydroxycholesterol with 29, 25, 21 and 13 μg/mL, respectively. In fact, literature reports 7-ketocholesterol as the most stable COP and since it is readily formed it had been considered as an oxidation mark [23].

After 4 minutes of heat exposure total COP began to decrease continuously reaching 54% of its maximum amount. However, this diminution was not observed for each COP individually. For instance, 7-ketocholesterol decreased 28% and α-epoxicholesterol, 30%; while β-epoxicholesterol degraded 79%, 7β-

hidroxycholesterol, 82% and 7α-hydroxycholesterol degraded completely. This demonstrates that each COP has a specific thermal stability and the net balance between formation and degradation may not correspond to the real oxidation length. Moreover, it could explain the high differences found between cholesterol degraded and COP formed.

Effect of Antioxidant addition

Substances able to reduce lipid oxidation are expected to diminish cholesterol oxidation. Thus, vitamin E (α-tocopherol) and TBHQ were chosen as proton donors representatives and β-carotene as scavenger of free radical.

Cholesterol degradation was not quite different in the presence of any of these compounds (Figure 1). At this temperature it is expected that these compounds present relatively low stability. For instance, β-carotene presented 100% degradation after 45s of heating, TBHQ presented 94% after 45 s and α-tocopherol, 40% in 8 min and after that was no longer detected.

In spite of the low stability, all antioxidants added were able to retard and even decrease COP formation. Pure cholesterol presented 147 μg/mL of total COP in about 4 minutes (Figure 2), while maximum of total COP (Figure 3) was reached at 5 min in the presence of TBHQ, 8 min with β-carotene and 10 min with α-tocopherol and the values obtained for total COP were 143, 136 and 111 μg/mL, respectively.

Concerning 7-hydroperoxycholesterol formation, which are the first compounds formed when oxygen reacts with cholesterol, the same behavior was observed

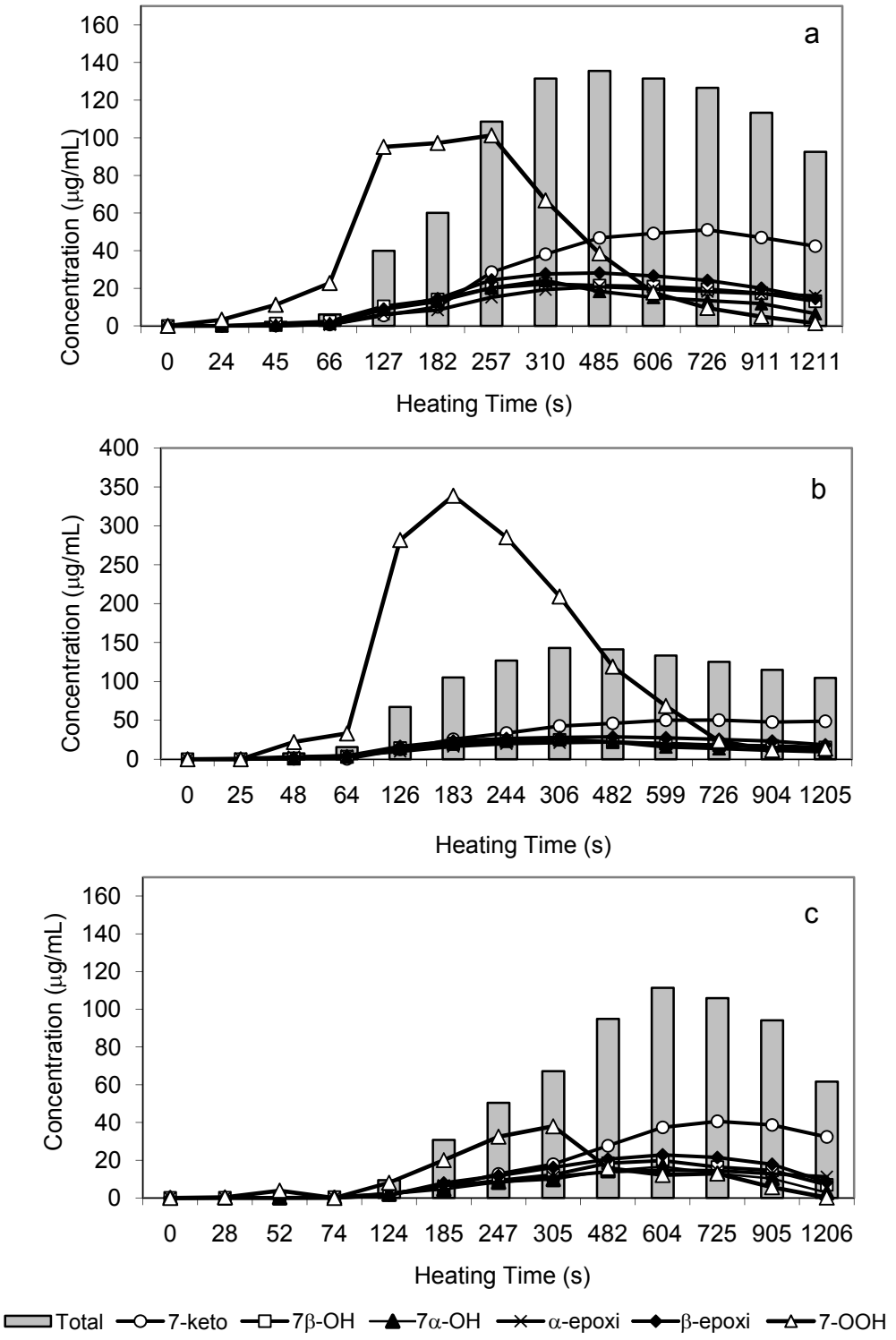

Figure 3. COP formation in model system containing (a) cholesterol+β-carotene; (b) cholesterol + TBHQ and (c) cholesterol + α-tocopherol during heat at 180 °C.

(Figure 3). Only α-tocopherol was able to reduce hydroperoxycholesterol formation, while TBHQ and β-carotene had increased it. TBHQ as well as β-carotene were practically not present after 45 s of heat and α-tocopherol was in the system within 8 min. It seems that during this short presence of TBHQ or β-carotene they were able to reduce 7-hyroperoxycholesterol transformation into COP retarding the process. In fact, higher amount of hydroperoxycholesterol were detected when those compounds were. Thus, it is possible to see here two different ways: protection against hydroperoxycholesterol transformation into COP (propagation phase) and protection against hydroperoxycholesterol formation (initiation phase), the later more effective than the former.

Effect of fatty acids addition

Cholesterol degraded fast when fatty acids were added despite their degree of unsaturation; however, the degradation observed when stearic acid was present was lower than the observed when linolenic acid was added (Figure 1).

The presence of fatty acids may favor oxidation despite their own oxidation because they may solubilize cholesterol facilitating the oxidation reaction by the increasing of contact surface [5, 21, 24].

It is not clear how the degree of unsaturation of the fatty acids influence the cholesterol oxidation. In a first moment, as unsaturated fatty acids are more susceptible to oxidation it may contribute to oxidation by generating more free radicals [25]. On the other hand, the instability of unsaturated fatty acids may induce oxygen as the limiting reagent of the oxidation [20]. The results obtained in

this study corroborate with all these insights of literature. As can be seen in Figure 4, stearic acid favored cholesterol oxidation, accelerating the process and increasing the total COP amount (168 μg/mL), comparing to pure cholesterol system. On the other hand, linolenic acid accelerated even more cholesterol oxidation (maximum total COP reached at 2 minutes); however, its presence reduced the total COP amount in 19% compared to pure cholesterol. Similar results were observed by Nogueira and Bragagnolo [8], who suggested that linolenic acid is oxidated first consuming the oxygen before cholesterol.

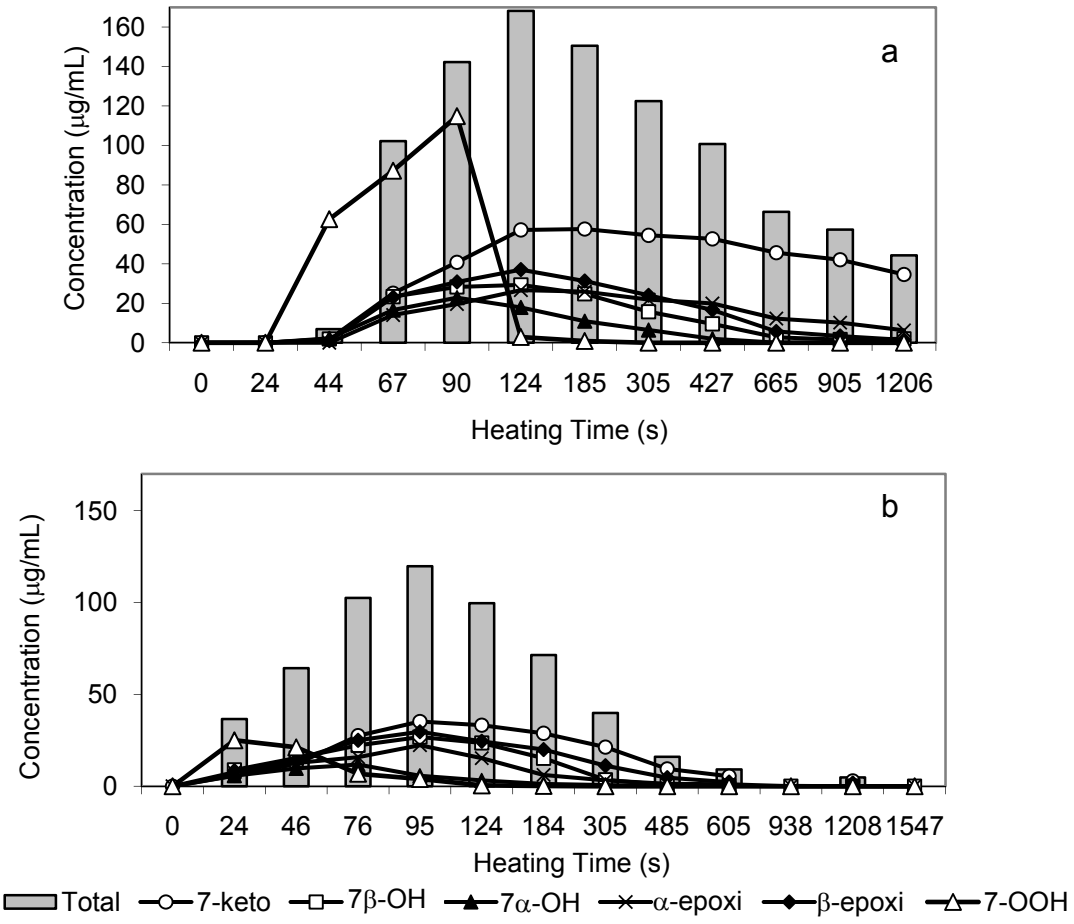

Figure 4. COP formation in model systems containing (a) cholesterol + stearic acid and (b) cholesterol + linolenic acid during heat at 180°C.

Conclusion

The presence of antioxidants commonly used for lipid protection was not able to reduce cholesterol thermal degradation; however, α-tocopherol, which was the most stable during heat, reduced cholesterol oxidation. The presence of TBHQ and β-carotene could retard COP formation by the reduction of 7-hydroperoxycholesterol transformation into COP. Both stearic and linolenic acid accelerated cholesterol degradation and oxidation. However, linolenic acid reduced COP formation by competing with cholesterol for oxygen.

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The authors thank the São Paulo State (FAPESP) and the National Brazilian Research Foundation (CNPq) for financial support.

CAPÍTULO 5

Cholesterol degradation at 140 °C: antioxidant activity and influence of fatty acids degree of unsaturation

RESUMO

Colesterol é suscetível a oxidação assim como outros lipídios insaturados. Devido aos seus efeitos biológicos, a formação dos produtos de oxidação do colesterol (COP) deve ser controlada. O calor, assim como outros compostos normalmente presentes ou adicionados ao alimento antes do processamento térmico, pode influenciar a degradação e a oxidação do colesterol. Com o intuito de entender a interferência desses fatores, determinou-se a formação de COP em sistemas modelo, constituídos de colesterol puro ou adicionado de TBHQ, vitamina E ou β-caroteno ou ácidos esteárico ou linolênico, submetidos ao aquecimento a 140 °C. Foi observado que a presença dos ácidos graxos acelerou a degradação do colesterol; entretanto, o ácido linolênico apresentou pequena proteção do colesterol frente à oxidação enquanto o ácido esteárico aumentou a formação de COP. A vitamina E e o β-caroteno protegeram o colesterol da degradação, enquanto o TBHQ acelerou o decaimento do colesterol. Com relação à oxidação, o TBHQ apresentou redução na formação de COP tanto quanto o β-caroteno; enquanto a presença de vitamina E não resultou em alterações .

Palavras-chave: óxidos de colesterol, TBHQ, β-caroteno, vitamina E, α-tocoferol

ABSTRACT

Cholesterol is prone to oxidation as any unsaturated lipid. Because of their biological effects, cholesterol oxidation products (COP) formation should be controlled. Heat as well as other compounds usually present or added to food prior heating process might influence cholesterol degradation and oxidation. Trying to understand the interference of such factors, COP formation was measured in model systems composed by pure cholesterol ou added of TBHQ, vitamin E or β-carotene or stearic or linolenic acids, heated at 140 °C. The presence of fatty acids accelerated cholesterol degradation; however, linolenic acid showed a small protection over cholesterol oxidation compared to pure cholesterol, while stearic acid increased COP formation. Vitamin E and β-carotene protected cholesterol from degradation, while TBHQ accelerated cholesterol decay. Concerning cholesterol oxidation, TBHQ showed reduction of COP formation as much as β-carotene, nevertheless, the presence of vitamin E did not seem to interfere on this matter.

KEYWORDS: cholesterol oxides, TBHQ, β-carotene, Vitamin E, α-tocopherol

Introduction

Cholesterol is prone to oxidation such as any unsaturated lipid. The triplet oxygen attacks the allylic carbon at position 7 of B ring forming 7-hydroperoxycholesterol which will be transformed into C7 cholesterol oxidation products (COP) or react with another cholesterol molecule producing epoxycholesterols [1]. However, when cholesterol is heated, oxidation is not the only damage suffered, therefore the amount of COP formed during heat is never the same amount of cholesterol degraded, part of this difference may be associated with COP thermal degradation and the other part is probably due to volatile and/or polymers formation [2].

Because of its biological effects, such as carcinogenicity, citotoxicity, mutagenicity and modulation of cell membrane [3], COP formation should be controlled or avoided. Considering the similarity of the mechanisms involved in cholesterol and fatty acid oxidation, substances that are usually employed to reduce fatty acid oxidation may have the same effect on cholesterol.

There are many compounds which are able to prevent lipid oxidation, some of them are synthetically prepared, such as *t*-Buthylhydroquinone (TBHQ), Butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) [4, 5]. However, the constant search for natural products, nowadays, attested several compounds with antioxidant activities such as carotenoids, vitamin E, polyphenols among others [6-9]. Those compounds can be naturally present in food or can be added with other purposes than preservation.

Products which have the capacity of donate protons or scavenge free radical such as tocopherols or carotenoids, respectively, may play an important role on this process [5]. On the other hand, lipid phase has other compounds than cholesterol which may influence the extent of its oxidation during heat, such as fatty acids and their different unsaturation degree [10].

Those are the factors that will be discuss in this work, where cholesterol mixed with TBHQ, vitamin E or β-carotene or mixed with stearic or linolenic acids were heated at 140 °C.

Material and Methods

Chemicals. Cholesterol (95%), vitamin E (97% of α-tocopherol), β-carotene (95%), and linolenic acid (99%) were purchased from Sigma-Aldrich (St. Louis, MO). Stearic acid (99%) and methyl tridecanoate (99%) was purchased from Fluka (Steinheim, Germany). COP standards: 20α-, 22*S*- and 25-hydroxycholesterol, 5,6α- and 5,6β-epoxycholesterol (α- and β-epoxi) and 7-ketocholesterol (7-keto) were purchased from Sigma (Milford, MA); 22*R*-, 24*S*-, 25*R*- 7β- and 7α-hydroxycholesterol (7α- and 7β-OH) were purchased from Steraloids (Newport, RI) with purity varying from 95 to 98%. *t*-Buthylhydroquinone (TBHQ, 95%) was gently supplied by Plury Química (Diadema, Brazil). All solvents used were HPLC grade.

Model system preparation. The preparation of the model system was carried out as described by Nogueira and Bragagnolo [11]. Model systems were heated in a heating block (Marconi, Piracicaba, Brazil) set at 140 °C (141±4 °C).

Cholesterol and COP quantification by HPLC. Determination of cholesterol and COP were carried out as described by Mariutti and co-workers [12], using normal phase HPLC, CN column and *n*-hexane + propan-2-ol as mobile phase at 1 mL/min flow and UV (210 nm) and RI detectors. The 7-hydroperoxycholesterol was identified according to Nogueira and co-workers [13].

Antioxidants determination. The quantification of the antioxidants was carried out as described in Nogueira and Bragagnolo [11], using reversed phase HPLC and C_{18} column with mobile phase composed by methanol + ethyl acetate (1:1) and acetic acid 5% + acetonitrile + methanol (50:25:25) and methanol + water (98:2) for β-carotene, TBHQ and vitamin E, respectively.

Fatty acids determination. Saponification and esterification according to Kim and Nawar [14], after solvent evaporation under nitrogen. The quantification was carried out as described by Nogueira and Bragagnolo [11] using gas chromatography and capillary column.

Results and discussion

Cholesterol degradation at 140 °C

When heated at 140 °C, cholesterol presented 70% of degradation of its initial content (1.5 mg/mL) in about 1 hour of heat (Figure 1). Compared to higher

temperatures [11, 15], the cholesterol degradation at this temperature is slow, probably because its melting point is 142.7 °C taking some time to its completely melt down. During this 1 h period, cholesterol was oxidized forming high amount of 7α- and 7β-hydroperoxycholesterol (7-OOH), that has been detected first at few

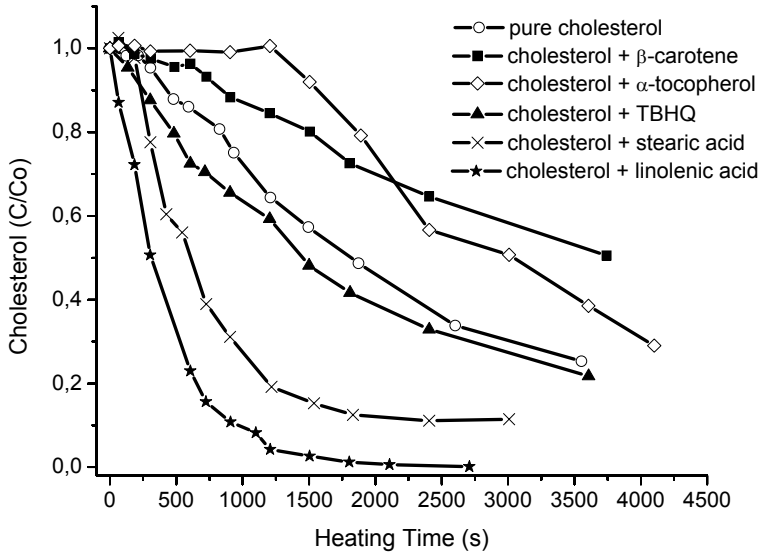

Figure 1. Degradation of cholesterol in model system containing fatty acids or antioxidants during heat at 140°C.

more than 3 minutes, reaching 770 μg/mL in 30 minutes of heat and after that it started to decrease (Figure 2).

Only 5 COP were detected, 7-ketocholesterol, 7α- and 7β-hydroxycholesterol after 5 min of heating and α- and β-epoxycholesterol, after 13 min, confirming the known mechanisms of cholesterol oxidation [16] which begins with 7-hydroperoxycholesterol transformation into C_7 COP by dehydration, dehydrogenation and reduction mechanisms [17] and epoxidation, another main

route, which presents a bimolecular reaction of 7-hydroperoxycholesterol with other cholesterol molecule [16].

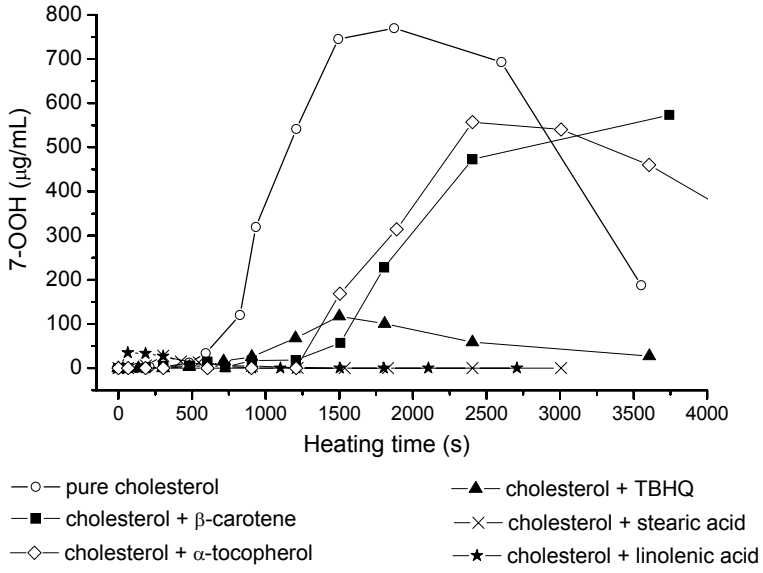

Figure 2. Formation of 7-hydroperoxycholesterol in cholesterol model systems during heat at 140°C

Figure 3 shows the amount of cholesterol oxides found in pure cholesterol model system with total COP (sum of all 5 COP detected) increasing continuously until reached 23% of cholesterol initial content at the end of heating process.

The major COP found was 7-ketocholesterol with concentrations varying from 0.5 to 64.6 μg/mL, followed by β-epoxycholesterol with 7.9 to 31.0 μg/mL, α-epoxycholesterol with 4.6 to 26.3 μg/mL, 7β- and 7α-hydroxycholesterol with 0.7 to 14.9 and 0.3 to 14.1 μg/mL, respectively. In many studies, 7-ketocholesterol has also been found as major COP, and therefore had been used as an oxidation mark [7, 18, 19], its high amounts could be explained by the two pathways of formation,

dehydration of hydroperoxides and dehydrogenation of hydroxides [17], besides its higher stability [20].

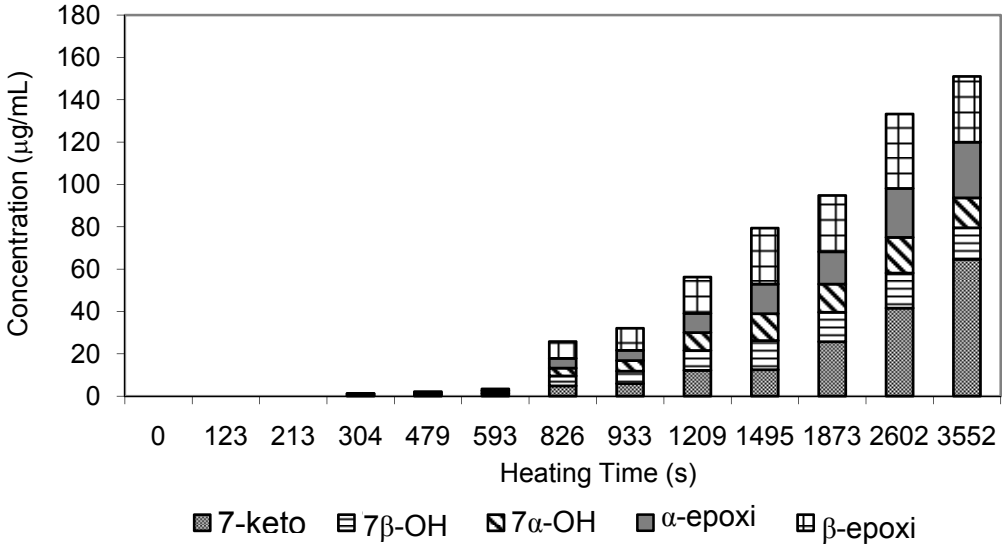

Figure 3. COP formation in pure cholesterol model system heated at 140°C.

Effect of Antioxidant addition

Only β-carotene and α-tocopherol presented delay of the cholesterol degradation (Figure 1). The poor performance of TBHQ can be explained by its own degradation during the first 8 min of heating. Although β-carotene was also thermally degraded within the first 3 min, its presence could accomplish good protection; maybe by its isomers which had been reported with antioxidant activities [21]. α-tocopherol has also been degraded; however, 43% of its initial content remained in the system after the whole heating period.

Concerning oxidation, the presence of α-tocopherol was able to delay COP formation (Figure 4c); however, it presented the same amount of total COP as pure cholesterol in the end of heating. On the other hand, β-carotene reduced COP formation by 10% and also delayed it considerably (Figure 4a).

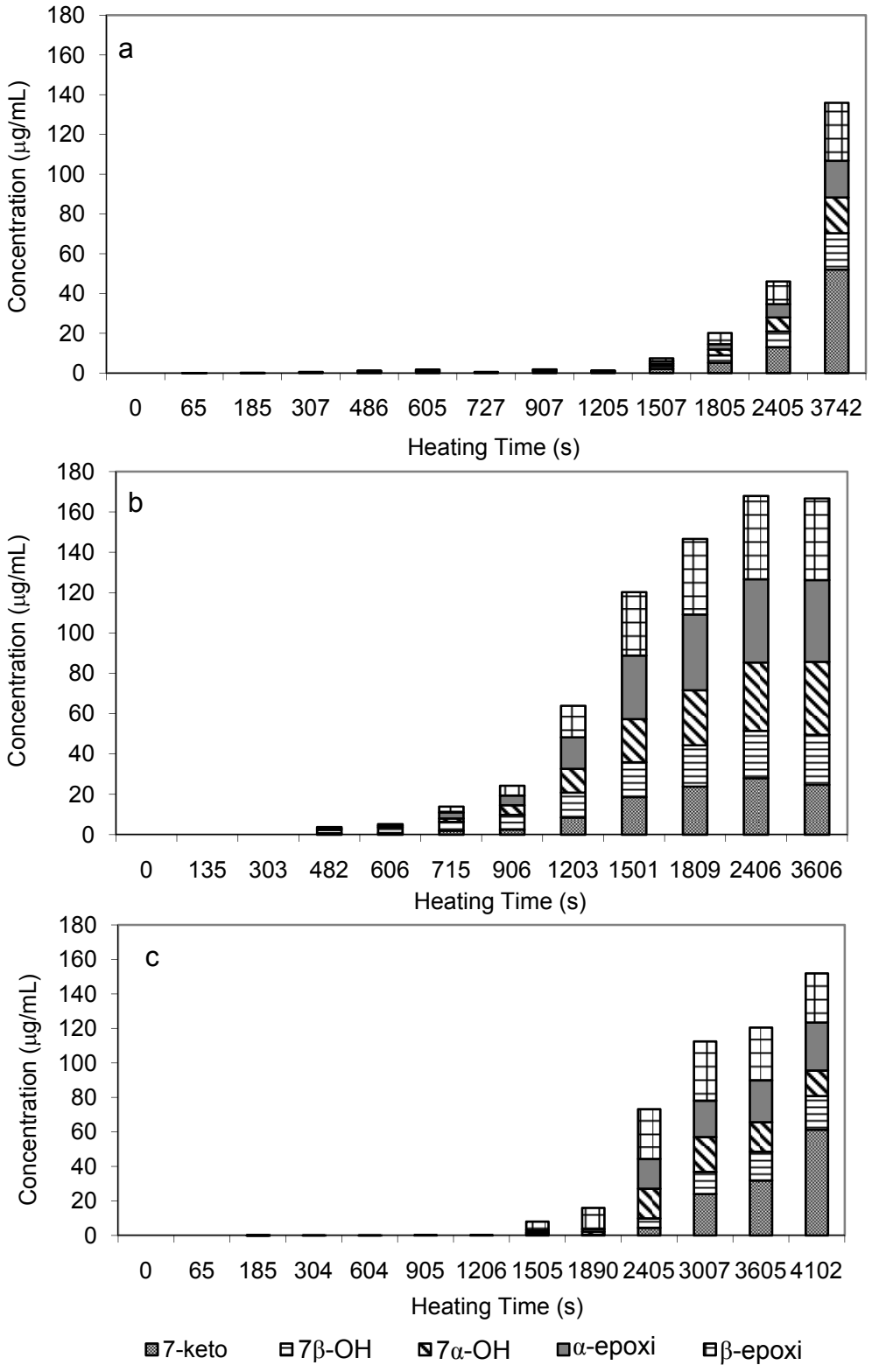

Figure 4. COP formation in model system containing (a) cholesterol+β-carotene; (b) cholesterol + TBHQ and (c) cholesterol + α-tocopherol during heat at 140 °C.

Although TBHQ had presented low protection of cholesterol against degradation, it presented lower formation of 7-hydroperoxycholesterol (Figure 2), hence reduced COP formation by 11% (Figure 4b). Moreover, COP profile in the presence of TBHQ was different, considering that 7-ketocholesterol was not the major COP formed, but epoxycholesterol derivatives. These results suggest that epoxidation route happened preferably.

Effect of fatty acids addition

Cholesterol degradation was accelerated by the presence of fatty acids, probably due to cholesterol solubilization which might favor reactions [10, 22]. Unsaturated fatty acid (linolenic) seemed to contribute even more than saturated fatty acid (stearic) (Figure 1), reinforcing this argument, since the melting point of usaturated fatty acids is lower than the saturated [23].

At Figure 5a can be seen that total COP increased by the presence of stearic acid, 38% higher compared to pure cholesterol at maximum content. The possible reason still stands on the solubilization of cholesterol favoring reaction at this temperature [15].

On the other hand, total COP decreased approximately 11% by the presence of linolenic acid (Figure 5b), as this compound is more susceptible to oxidation due to its three double bonds, it competes with cholesterol for the oxygen [11, 15]. Another explanation may be related to the formation of polymers by the degradation of linolenic acid due to heat [23], which can act as barriers impeding the mobility of the reacting molecules or their combination with oxygen [22]. In fact, linolenic acid was completely degraded after 6 minutes of heating.

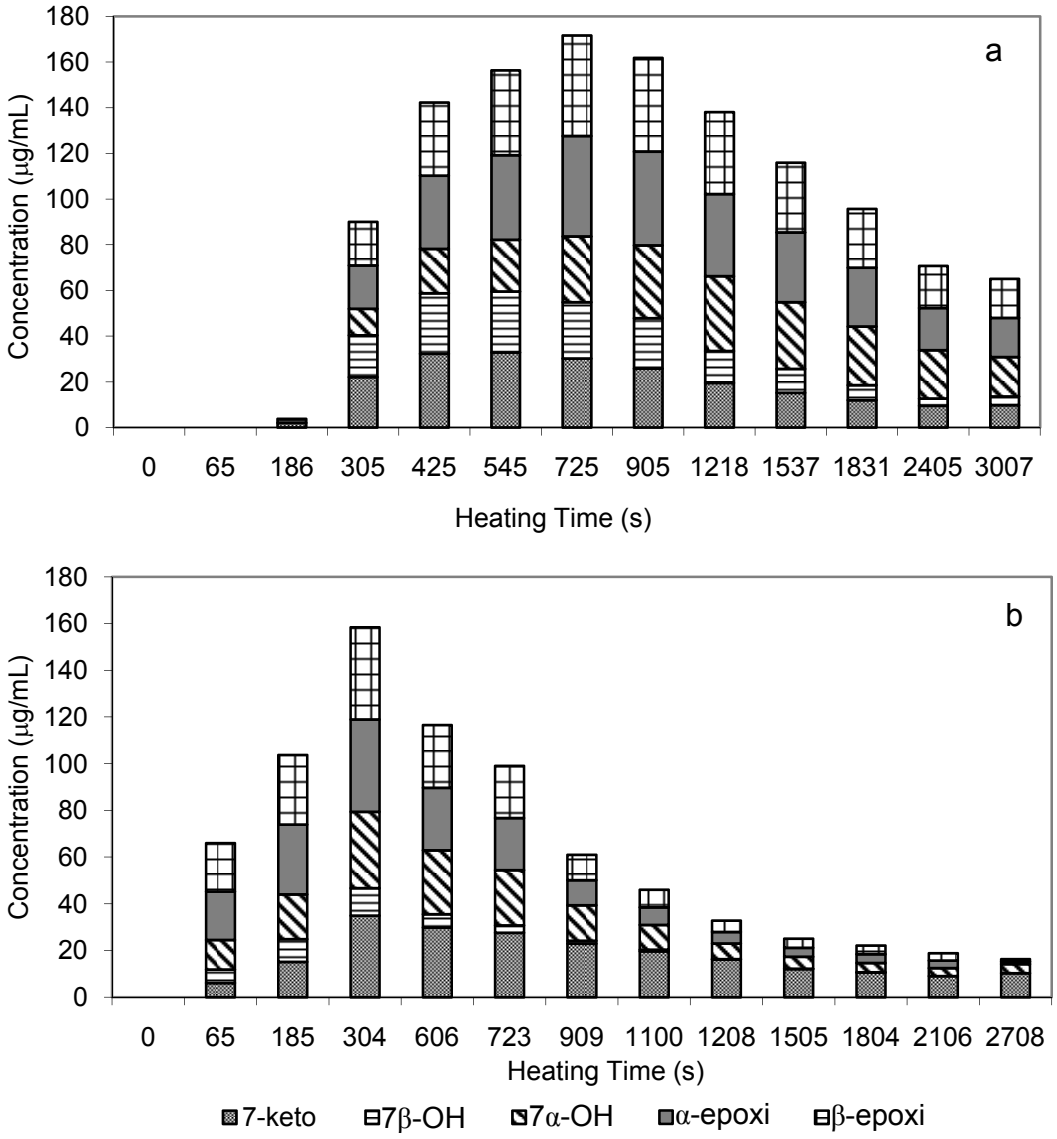

Figure 5. COP formation in model systems containing (a) cholesterol + stearic acid; (b) cholesterol + linolenic acid and during heat at 140°C.

Conclusion

The presence of both fatty acids and TBHQ accelerated cholesterol degradation, while β-carotene and α-tocopherol delayed the process. However, TBHQ, linolenic acid and β-carotene showed reduction on COP formation and despite α-tocopherol delayed cholesterol oxidation, the amount of COP reached the same as pure cholesterol in the end of heating.

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The authors thank the São Paulo State (FAPESP) and the National Brazilian Research Foundation (CNPq) for financial support.

CONCLUSÃO GERAL

O colesterol é degradado quando aquecido à velocidade diretamente proporcional ao aumento de temperatura. Uma das vias de degradação é a autoxidação, com produção de óxidos de colesterol (COP). Nos sistemas modelo estudados foram encontrados apenas os óxidos formados a partir da oxidação do anel B, 7-cetocolesterol, 7α- e 7β-hidroxicolesterol, e α- e β-epoxicolesterol, quando aquecidos. A soma desses óxidos, COP total, apresentou comportamento característico de aumento da concentração até atingir o ponto máximo e posterior redução, que pode ser explicada pela baixa estabilidade dos compostos sob calor.

A autoxidação do colesterol apresenta o mesmo mecanismo da oxidação lipídica, que ocorre em três fases, iniciação, propagação e terminação, em função da produção de radicais livres e formação de radicais hidroperóxidos. A síntese do 7α-hidroperoxicolesterol permitiu a identificação quantificação desses intermediários da reação de oxidação do colesterol simultaneamente à determinação de COP.

A presença de ácido graxo insaturado (ácido linolênico) acelerou a degradação de colesterol e a formação de óxidos, entretanto apresentou teor de COP total menor que os encontrados para o colesterol puro em todas as temperaturas. O aumento da velocidade de degradação e oxidação do colesterol pode estar associado à autoxidação do próprio ácido linolênico que fornece radicais livres em maior quantidade ao meio. Por outro lado, sendo mais reativo, compete pelo oxigênio, reduzindo o teor de COP total.

A presença de ácido graxo saturado (ácido esteárico) acelerou a degradação e oxidação do colesterol, apresentando maiores teores de COP total nos sistemas aquecidos a 140 e 180 °C. Entretanto, a 220 °C, o ácido esteárico não influenciou a degradação do colesterol e, embora tenha acelerado sua oxidação, apresentou menor teor total de COP. Embora a temperatura fosse mais alta, o tempo de exposição ao calor foi muito menor a 220 °C, com isso, o ácido

esteárico ainda estava presente no sistema, em concentração relativamente alta, até o final do aquecimento, podendo sofrer autoxidação e, consequentemente, competir pelo oxigênio tanto quanto o ácido linolênico e assim diminuir a formação de COP. Nas temperaturas menores, provavelmente em função do maior tempo de exposição ao calor, a degradação do ácido esteárico aconteceu antes que pudesse oferecer proteção ao colesterol.

A influência da vitamina E na degradação do colesterol mostrou-se dependente da temperatura, sendo que, a 220 °C não influenciou, a 180 °C apresentou tendência à proteção e a 140 °C protegeu visivelmente o colesterol. Além disso, pode ter atuado na fase de iniciação da oxidação, uma vez que foi capaz de reduzir a formação de hidroperóxidos em todas as temperaturas estudadas, provavelmente, pela doação de prótons, principalmente a 180 °C.

A 220 °C, o TBHQ reduziu ligeiramente a velocidade de degradação do colesterol, mas não apresentou influência a 180 °C e acelerou a 140 °C. Este antioxidante pode ter atuado na fase de iniciação da oxidação do colesterol a 140 e a 220 °C, diminuindo a formação de hidroperóxidos; entretanto, a 180 °C pode ter atuado na fase de propagação, pois apresentou o maior teor de hidroperóxidos e menor teor de COP.

O β-caroteno foi capaz de reduzir a velocidade de degradação do colesterol a 140 °C. Os sistemas contendo β-caroteno apresentaram menores teores de 7-hidroperoxicolesterol e de COP em todas as temperaturas estudadas. A ação antioxidante do β-caroteno, nestes casos, deve-se, principalmente, à formação de adutos (complexos entre β-caroteno e radical livre), impedindo a propagação da oxidação do colesterol. Embora este carotenóide seja bastante sensível a altas temperaturas, alguns produtos da sua degradação também podem apresentar atividade antioxidante.